UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

THAÍS MANZANO PARISOTTO

RELAÇÃO ENTRE OS FATORES IMUNOLÓGICOS, COMPOSIÇÃO BIOQUÍMICA E MICROBIOLÓGICA DO BIOFILME DENTÁRIO, EXPOSIÇÃO A AÇÚCARES E A INCIDÊNCIA DA CÁRIE PRECOCE DA INFÂNCIA

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Aos meus pais, José Antonio e Flávia, por toda educação,

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“A mente que se abre a uma
nova idéia jamais voltará
ao seu tamanho original”

Albert Einstein

RESUMO

A cárie precoce da infância (CPI) é considerada um problema de saúde significativo no Brasil devido a sua alta prevalência. Como o perfil dessa doença na população infantil não é estático, estudos que avaliem o desenvolvimento da cárie, assim como sua etiologia são de grande importância para a prevenção e controle da CPI. Esta tese, constituída por 4 capítulos teve como objetivos: 1.avaliar o comportamento das lesões de manchas brancas ativas (LMB) na dentição decídua em um ano de acompanhamento; 2.explorar a associação entre desenvolvimento de cárie, colonização por bactérias cariogênicas e fatores imunológicos inerentes à saliva; 3.avaliar o poder de predição do fluoreto presente no biofilme dentário no desenvolvimento da CPI, considerando-se a exposição aos açúcares; 4.avaliar a associação entre os polissacarídeos extracelulares insolúveis (PECIs) do biofilme dentário, exposição aos açúcares, microrganismos cariogênicos e a CPI, assim como realizar um *screening* da habilidade das cepas de *Streptococcus mutans (S.mutans)* de produzir glucano *in vitro.* Para a realização desses estudos, pré-escolares (n=179, n=40, n=31, n=65, capítulos 1, 2, 3 e 4 respectivamente) de 3-4 anos, do município de Itatiba-SP foram acompanhados por um ano. Os exames clínicos para diagnóstico de cárie (critério OMS+LMB) foram realizados após verificação de presença/ausência de biofilme visível nos incisivos superiores. Depois disso, as crianças foram divididas nos grupos: livres de cárie e cárie ativos/inativos. O biofilme dentário foi coletado das superfícies lisas livres com auxílio de alças esterilizadas para contagem de microrganismos ou com palitos de madeira para dosagem de flúor e PECIs. A exposição aos açúcares foi avaliada por diário de dieta. As análises imunológicas e da capacidade das cepas de *S.mutans* de produzir glucano foram realizadas por meio ensaios no Luminex^{100} e zimografia, respectivamente. Após análise estatística (α=0,05) verificou-se que a maioria das LMB permaneceram ativas ou remineralizaram após um ano e que as crianças com atividade de cárie no *baseline* apresentaram maior risco de desenvolver novas superfícies cariadas que

aquelas livres de cárie. Além disso, crianças com lactobacilos, menores níveis de IgA salivar anti-GbpB, presença de biofilme visível, maior exposição ao açúcar sólido, menor concentração de flúor e maior concentração de PECIs no biofilme mostraram maior chance de desenvolver CPI comparadas àquelas que não apresentavam essas condições. Não foi verificada correlação entre a habilidade das cepas de *S.mutans* de produzir glucanos e a concentração de PECIs no biofilme. Porém, as cepas que mais produziram glucano foram encontradas em crianças que desenvolveram cárie. Conclui-se que o monitoramento das LMB deve ser o tratamento de escolha no manejo da CPI, visto que a maioria remineralizou/permaneceu ativa, que o sistema imunológico sofreu maturação significativa no período do estudo e que a amplitude de resposta da IgA salivar contra os epítopos de *S. mutans* pode influenciar o grau com que esses microrganismos causam doença. Ainda, considerando-se a exposição à açúcares, a composição bioquímica e microbiológica do biofilme dentário influencia o desenvolvimento da CPI e a capacidade das cepas de *S.mutans* produzirem glucano *in vitro* não pôde refletir a concentração de PECIs no biofilme na população estudada.

Palavras chave: cárie dentária, pré-escolar, dentição primária, imunologia, microbiologia, bioquímica

ABSTRACT

Early childhood caries (ECC) is considered a significant oral health problem in Brazil due to its high prevalence. As caries profile in the pediatric population is not static, studies assessing the development of caries, as well as its etiology are of prime importance for ECC prevention and control. This thesis consists of 4 chapters aiming to: 1.evaluate the behaviour of early caries lesions (ECL) in early childhood; 2.explore the association between caries development, colonization with caries-associated microflora and immunity related to saliva; 3.assess the predictive power of dental plaque fluoride in early childhood caries development regarding sugar exposure conditions; 4.assess the associations between dental plaque extracellular insoluble polysaccharide (IP), sugar exposure, cariogenic microorganisms and ECC; and also to perform a screening of *Streptococcus mutans (S.mutans)* strains' ability to produce glucan *in vitro*. To perfom these studies, preschool children aging 3-4 years, from Itatiba-SP were followed for one year (n=179, n=40, n=31, n=65, chapters 1, 2, 3 and 4 respectively). Clinical examinations for caries diagnosis (WHO criteria+ECL) were performed after recording the presence/absence of visible dental plaque on maxillary incisors. After that, children were divided in groups: caries free and caries active/inactive. Dental plaque was collected from smooth surfaces using sterilized handles for microorganisms' enumeration or wooden sticks for fluoride and IP analyses. Immunological analysis and *S.mutans* ability to produce glucan were performed by Luminex^{100} assays and zimography, repectively. The frequency of sugar exposure was assessed by a diet chart. After statistical analysis (α=0.05) it was shown that most ECL remained active/remineralized after one year and that children with caries activity at baseline had higher risk for developing new carious surfaces than those caries free. In addition, children with lactobacilli, lower levels of salivary IgA anti-GbpB, presence of dental plaque, increased exposure to solid sugar, lower fluoride concentration and higher concentration of IP in dental plaque showed more chances for developing ECC than those who did not show these conditions. There was no correlation between the ability of *S. mutans* to produce glucan and IP

concentration in the plaque. However, the strains with higher glucan production were found in children who developed caries. We conclude that the management of ECL should be conservative, as the majority remineralized/remained active, the secretory immune system is undergoing significant maturation during the period studied, and that the breadth of mucosal IgA response to epitopes of *S.mutans* virulence components may influence the degree to which these cariogenic microorganisms can cause disease. Still, considering sugar exposure, microbiological and biochemical composition of dental plaque influence the development of ECC, and the ability of *S.mutans* strains to produce glucan *in vitro* could not reflect IP concentration in dental plaque in the studied population.

Key words: dental caries, preschool child, primary dentition, immunology, microbiology, biochemistry.

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I – INTRODUÇÃO GERAL

A presença de uma ou mais superfícies dentárias cariadas, sendo elas cavitadas ou não, perdidas ou obturadas em crianças de 0 a 71 meses de idade (Drury *et al.*, 1999, AAPD, 2011-2012) denomina-se cárie precoce da infância (CPI). Essa doença multifatorial e de elevada prevalência no Brasil (53,4 a 69% de acordo com: Ministério da Saúde, 2010, Gradella *et al.*, 2011, Parisotto *et al.*, 2011), é causada por três fatores primários: presença de microrganismos cariogênicos, dieta rica em carboidratos fermentáveis e hospedeiro/superfície dentária susceptível, os quais interagem em um determinado período de tempo (Keyes, 1960, Selwitz, 2007). Considerando-se os resultados dos estudos da última década envolvendo saúde bucal e crianças, qualquer modelo realista que englobe os fatores etiológicos da cárie deve considerar uma perspectiva multinível, além do modelo biológico clássico de Keyes descrito acima. Essa perspectiva multinível deve incluir a família com a qual a criança vive e a comunidade em que essa família está inserida (Fisher-Owens *et al.*, 2007).

A primeira manifestação clínica da CPI é a lesão de mancha branca ativa. Normalmente, essa lesão se localiza no terço cervical dos incisivos decíduos superiores (Ramos-Gomez *et al.*, 2002) onde há o acúmulo de biofilme. Se o diagnóstico precoce da mancha banca ativa não for realizado e se nenhuma medida preventiva for implementada, as lesões podem progredir e se estender até que toda a dentição decídua seja acometida. A destruição dos dentes decíduos pode provocar alterações deletérias na fonação, estética, arcos dentários e mastigação. Essa última é de extrema importância não apenas para o consumo de uma dieta balanceada mas para o aproveitamento adequado do conteúdo nutricional dos alimentos, o que influencia no desenvolvimento geral da criança. Portanto, a CPI compromete a qualidade de vida da criança (Feitosa *et al.*, 2005, Abanto *et al.*, 2011), além de ser um dos preditores de risco mais significativos para o desenvolvimento da cárie nas dentaduras futuras: a mista e a permanente (Peretz *et al.*, 2003, Skeie *et al.*, 2006).

Dentre os fatores etiológicos da CPI, a exposição aos açúcares merece destaque com relação a alterações microbiológicas (Loesche, 1986, Nobre dos Santos *et al.*, 2002, Parisotto *et al.,* 2010) e bioquímicas (Cury *et al.*, 1997, Nobre dos Santos *et al.*, 2002) no biofilme dentário. Os açúcares estão relacionados às diminuições nas concentrações dos íons flúor, cálcio e fósforo, conteúdo inorgânico do biofilme (Nobre dos Santos *et al.*, 2002). Além disso, a sacarose serve de substrato específico para a produção de polissacarídeos extracelulares insolúveis (PECIs) (Loesche, 1986, Aires *et al.*, 2006) que influenciam o conteúdo orgânico. Uma maior concentração de PECIs aumenta a porosidade e viscosidade do biofilme, o que favorece a difusão dos ácidos bacterianos e a aderência de microrganismos cariogênicos (Paes Leme *et al.*, 2006).

No processo carioso, os microrganismos cariogênicos são representados por bactérias capazes de colonizar a superfície dentária e produzir ácidos, em velocidade superior à capacidade de neutralizacão do biofilme, quando o pH encontra-se abaixo do crítico; desse modo, ocorre a dissolução dos tecidos dentários. *Streptococcus mutans* (*S. mutans*) apresentam tais características, uma vez que possuem um conjunto de fatores de virulência que permitem sua adesão e acúmulo no biofilme, além de serem capazes de produzir e tolerar grandes quantidades de ácidos. Nesse sentido, os lactobacilos também merecem destaque.

Considerando-se o processo de aderência e acúmulo de *S. mutans* no biofilme, três grupos de antígenos associados à superfície celular desses microrganismos tem sido muito estudados: adesina antígeno I/II, glicosiltransferases (GtfB, GtfC e GtfD) e proteínas ligantes de glucano (Gbps - GbpA, GbpB, GbpC e GbpD) (Nogueira, 2006). Enquanto as adesinas estão relacionadas com a fase inicial de colonização, pois auxiliam a ligação dos microrganismos à película adquirida, as glicosiltransferases estão relacionadas à síntese de PECIs que favorecem a adesão de *S. mutans* no biofilme já estabelecido. Quanto as proteínas ligantes de glucano, essas estão relacionadas à interação molecular das células de *S. mutans* com a matriz extracelular de

glucanos (PECIs e polissacarídeos extracelulares solúveis). Estudos têm demonstrado que a indução de anticorpos específicos contra alguns desses antígenos pode promover proteção contra o desenvolvimento da cárie dentária em modelos animais (Taubman *et al.*, 1995, Jespersgaard *et al.*, 1999, Koga *et al.*, 2002). Ainda, outros estudos (Smith e Taubman, 1990, Childers *et al.*, 1999) indicam que os antígenos GbpB e Gtfs podem ser importantes para o desenvolvimento da vacina anti-cárie (Smith, 2002).

Na cavidade bucal, as imunoglobulinas responsáveis pela primeira linha de defesa adaptativa contra os antígenos de *S. mutans* são as imunoglobulinas A secretoras (IgAs), as quais estão presentes na saliva e fazem parte do sistema de defesa das superfícies mucosas. O estudo de Nogueira *et al.* (2005) observou que a intensa resposta de anticorpos IgA salivares contra GbpB pode reduzir as chances de infecção inicial por essa bactéria. Além disso, Nogueira *et al.* (2007) verificaram um atraso na resposta imunológica de crianças de baixa idade, as quais apresentavam-se infectadas por *S. mutans* e possuíam baixa resposta ao antígeno GbpB.

Inúmeros estudos mostram que os *S. mutans* estão intimamente relacionados tanto com o desenvolvimento, como com a progressão da cárie na infância (Mattos-Graner *et al.*, 2001, Nobre dos Santos *et al.*, 2002, Vachirarojpisan *et al.*, 2004, Parisotto *et al.*, 2010). Dessa forma, avaliar os níveis IgAs salivares, as quais fazem parte do sistema de defesa das superfícies mucosas contra a invasão de microrganismos, e a capacidade de resposta imunológica das crianças frente aos antígenos de *S. mutans*, como os que estão relacionados ao processo de aderência e acúmulo dessa bactéria no biofilme torna-se muito relevante.

A análise da literatura evidencia que a despeito de existir um extenso número de trabalhos que abordaram a cárie precoce da infância e fatores etiológicos relacionados, um número bastante reduzido considerou a incidência da doença após acompanhamento longitudinal, bem como os fatores imunológicos inerentes ao hospedeiro.

II – CAPÍTULOS

CAPÍTULO 1

Early caries lesions behaviour in early childhood: a 1-year follow-up study

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Short title: Early caries lesions behaviour
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Abstract

Purpose: The aim of this one-year follow-up study was to evaluate the behaviour of early caries lesions (active non-cavitated caries lesions) by surface and type of tooth in early childhood.

Methods: A total of 179 3-and 4-year-old preschoolers took part in this study. Clinical examinations were conducted by one calibrated examiner using mirror, ball-ended probe, gauze for cleaning and drying of teeth and artificial light. The WHO criteria, with an added measurement of early caries lesions (ECL), were employed for the caries examinations. The results were analyzed by descriptive statistics and logistic regression.

Results: After a one year follow up, the studied population developed 1.6±1.64 new carious surfaces. Children with caries activity at baseline showed much higher risks of developing new affected surfaces than caries-free children (OR=17.3,OR= 24.5). The majority of the ECL remained active/unchanged after one year, whereas 35.6% were arrested. About 10% of the ECL became cavitated, were filled or were missing due to caries at follow up. Comparing anterior to posterior teeth, the ECL turned into cavities or fillings more frequently in the posterior region.

Conclusion: Our findings support the conservative management of ECL since after one year, the majority of ECL were kept active or were arrested on smooth surfaces of anterior and posterior primary teeth.

Introduction

In the last few decades, there has been much progress in the field of caries prevention among children, adolescents and adults. Caries prevalence has reduced significantly due to the widespread use of fluoridated toothpastes and tap water, as well as sealant use.[1,2] However, in Brazil as well as in other developing countries, dental caries remain highly prevalent, especially in early childhood.[3,4]

Caries development and progression in primary enamel and dentin show particularities in comparison to permanent teeth, which make them more susceptible to the carious process. The chemical composition of primary teeth associated with inorganic and organic tissue content might explain the different response of primary teeth against demineralization and remineralization. Primary enamel shows a lower calcium content, slightly lower calcium to phosphate ratio, higher water content, and higher organic content[5,6,7] than the permanent enamel. These differences highlight the importance of investigating predictors of future caries in primary teeth.

In this respect, the early identification of caries can be a good strategy for reducing caries development in early childhood, especially when we consider that the consequences of this disease include severe pain, low oral health-related quality of life and loss of school days.[8,9] The first clinical sign of dental caries is represented by early caries lesions (ECL), which can progress to cavitations at the enamel surface if no effective preventive measure is applied.[10]

It is worth mentioning that there are limited longitudinal studies available in the scientific literature regarding the ability of ECL to progress to cavitated lesions,

particularly in children with early childhood caries (0-71 months). Thus, the purpose of this one-year follow-up study was to evaluate the behaviour of early caries lesions (active non-cavitated caries lesions) by surface and type of tooth in early childhood.

Methods

Ethics

The present investigation was approved by the Piracicaba Dental School/University of Campinas Ethics Committee under protocols #015/2006 and #017/2008. In addition, a positive informed written consent was signed by the children's parents.

Sample definition

All 3- to 4-year-old children enrolled in public preschools in the fluoridated (0.5-0.8 ppm F) and urban area of Itatiba-SP, Brazil in 2006 were invited to take part in this one-year follow-up study. Children from public preschools in Itatiba are from low socio-economic backgrounds. These preschoolers were part of a larger longitudinal study, which investigated factors related to the etiology of early childhood caries (e.g., diet, socio-economical factors, and biochemical and microbiological composition of dental plaque). The age ranges of 3 to 4 years at baseline and 4 to 5 years at follow up were chosen because all primary teeth have erupted and no permanent teeth have completely erupted (i.e., they are only starting this process) in these age groups.

After performing a pilot study at baseline, the minimum sample size required to represent the city population was established (n=172) with a 5% standard error, a 95% confidence interval, and a caries prevalence of 0.72. The calculated number was increased by 10%, as this was a one-year longitudinal study. As such, the estimated sample size comprised 189 preschoolers. However, to reduce eventual problems that could contribute to a sample size smaller than the minimum calculated, we decided to invite all 3- to 4-year-old children enrolled in public preschools from fluoridated areas.

At baseline, out of the 546 children invited to take part in the study, 351 participated. The exclusion criteria were the following: 1.) children with syndromes or chronic systemic diseases, 2.) children whose parents did not attend scheduled school meetings at entrance/exit times to understand the study's aims and/or its importance and 3.) children whose parents refused to sign the informed positive consent forms. Some children were excluded from the study due to non-collaboration with the necessary procedures for the clinical examinations.

From baseline to follow up, 172 children were lost due to absence on the day of the examination at follow up and due to non-collaboration with the necessary procedures for the clinical examinations. Thus, the final sample size was 179 preschoolers representing all preschools in Itatiba-SP, Brazil.

As a benefit at baseline, a new toothbrush and a fluoridated dentifrice (1000 ppm), as well as oral health preventive instructions, were provided to all children who took part in this study. Moreover, the full names of all preschoolers with active

caries at baseline were given to the person in charge of the Public Heath Oral Service of Itatiba.

Caries recording system/criteria

The diagnostic criteria used for early childhood caries diagnosis in the present study was the World Health Organization criteria with an additional measurement criteria for early caries lesions (WHO+ECL)[11,12,13] (Table 1). As such, caries were recorded not only when frank cavitations were present, but also when ECL were present. Early caries lesions in smooth surfaces were considered when there was an active rough white spot lesion with a dull, opaque whitish surface without detectable loss of continuity, which were usually adjacent to the soft tissue margins where dental plaque accumulates. For the occlusal surfaces, ECL were recorded for active lesions extending along the walls of the fissure, where increased roughness and opacity were evident. Arrested/remineralized ECL in smooth surfaces were identified when the enamel was shiny whitish, brownish or black, without clinically detectable loss of surface, typically located at some distance from gingival margin. Enamel may feels hard and smooth when the tip of the probe is moved gently across the surface. For the occlusal surfaces, arrested/remineralized ECL were considered when there was intact fissure morphology and lesions extending along the walls of the fissure were shiny whitish[13].

Cavities alone or adjacent to fillings were classified as active when a softened floor was detected and as inactive when the cavity floor was hard and

displayed different degrees of brownish discoloration. Gentle probing was used to check the tooth tissue textures (e.g., rough, hard, and/or soft).

Surfaces were classified as sound when normal enamel translucency was observed after drying the teeth with gauze. As such, children were considered caries- free if they show neither dmfs (decayed, missing and filled surfaces) nor ECL.

The units of evaluation in the clinical examinations were dmfs and dmft (decayed, missing and filled teeth).

Calibration of the examiner

At first, theoretical discussions using clinical photographic slides were held to provide instructions to the examiner about the use of the WHO+ECL criteria. These discussions included explanations about the examinations for early caries lesions. Also, a clinical training session conducted using a gold standard was held with the aim of achieving an acceptable level of agreement before the intra-examiner reliability assessment. The entire time spent on the calibration process (e.g., theoretical discussions, training and calibration exercises) was 30 h. Intra-examiner reliability (Kappa calculation) with regards to all components of the diagnostic criteria was assessed by reexaminations of approximately 10% of children (at baseline and at follow up) with a 1-week-interval period, in order to avoid examiner memorization. Kappa values at baseline and follow up for the teeth and tooth surfaces were 0.75/0.79 and 0.78/0.82, respectively.

Clinical examinations

The clinical examinations were conducted by a single dentist (T.M.P.) in the preschools, rigorously following cross-infection control measures. A focusable flashlight, a mirror and a ball-ended probe were used to favor the identification of carious lesions, to confirm questionable findings and to remove debris to improve visualization. A gauze was used to dry or clean the teeth, enhancing the identification of early caries lesions. During the examinations, the examiner sat behind the child (who was lying on a table), assisted by a scribe.

Statistics

Descriptive statistics such as means, standard deviations and percentages were used to assess caries increments as well as ECL changes after a one-year follow up. Ordinal logistic regression analysis, expressed by odds ratios (OR), was used to indicate the risk for developing caries lesions in the follow up. The analyses were carried out using the SAS 9.1.3 statistical program with a 5% significance level and a 95% confidence interval.

Results

Table 2 shows the mean numbers of surfaces/teeth affected by caries at baseline and at follow up in the studied population. This table also shows the one-year increment of caries, revealing that there was an increase in the dmfs scores at follow up. The risk for future carious lesions development in the primary dentition, considering children with ECL at baseline, is shown in Table 3. From this table it is

clear that children with ECL at baseline had 17.3 more chances to develop more ECL or sustain ECL activity and an estimated risk 24.5 higher to develop cavitations or fillings in the follow up than caries-free children. In addition, changes in ECL are displayed in Figure 1.

Figure 2 shows the distribution of dmfs increments according to surface type: smooth, approximal and occlusal. ECL were predominant in smooth and occlusal surfaces, while cavitation+ECL and filling+ECL+active cavities were predominant in occlusal and approximal surfaces.

Figures 3 and 4 show the changes in ECL by surface type in both anterior and posterior primary teeth. It is clear that ECL that remained active/unchanged or were newly developed during the one year follow-up period correspond to major components of all surface types: smooth, approximal and occlusal in both the anterior and posterior teeth. Most of the arrested lesions were smooth surface lesions. In posterior teeth, a higher number of ECL progressed to cavitations and fillings compared with anterior teeth.

Discussion

In the past few years, there has been a growing interest in diagnosing non-cavitated lesions in epidemiological studies involving children.[14,15,16] This has been the case because a diagnosis limited to cavitations is no longer in accordance with the current understanding of the carious process.

The increase in the caries index with age (Table 2) is in agreement with previous studies.[3,17] This trend is observed because early childhood caries is a

rapid and progressive disease, especially considering primary teeth characteristics regarding teeth size as well as enamel and dentin compositions. In this context, a study by Weinstein et al.,[18] verified that even with fluoride varnish applications during semiannual visits, children experienced an average of 8.6 new surfaces of primary tooth decay (even using WHO criteria) in a 3-year period. In the present study, the majority of children who developed carious lesions (being them cavitated or not) after one year were already caries-active (had ECL) at baseline, as only 6 children who were caries-free in the baseline developed caries. This is in accordance with the results found in Table 3, where caries-active children at baseline had a higher risk of developing caries (OR=17.3, OR=24.5), corroborating studies by Sclavos et al.,[19] Grindefjord et al.*,* [20] and Peretz et al.[17]

With regard to ECL changes, we observed that the majority of ECL remained active/unchanged after one year or were arrested, whereas a minority became cavitated, were filled or were missing due to the carious process (Figure 1). This is an interesting finding in terms of non-cavitated lesions management, since it supports that the pedodontist does not need to perform restorative treatment (which have a finite longevity) immediately, and can attempt management of the ECL lesion. This is in line with AAPD guideline[21] that states that modern management of dental caries should be more conservative, including identification of an individual's risk for caries progression, understanding of the disease process for that individual, and active surveillance of enamel lesions. The active surveillance is based on the concept that treatment of disease may only be necessary if there is disease progression. Additionally, in the present study, the

majority of the arrested ECL were found on smooth surfaces in both anterior and posterior teeth (Figures 3 and 4). A possible reason for this result is that smooth surfaces are more accessible to the fluoride effect, as well as to oral hygiene and saliva.[22] It is plausible that this also explains why caries in occlusal and approximal surfaces progressed to cavitations or fillings more frequently in the present study (Figure 3), which is in line with previous results.[20,22]

It is worth mentioning that on the occlusal surfaces (Figure 3), new ECL were the major type of carious lesion that were developed. Children with ECL had a 17.3 higher chance of continued development of ECL or sustained ECL activity (Table 3). Moreover, children presenting ECL at baseline showed a 24.5 times higher likelihood of having cavities or fillings at follow up. Also, the increment of active cavities (cavitation+ECL and fillings+ECL+active cavity) and fillings without cavity was predominant in occlusal and approximal surfaces (Figure 2). With regard to fillings on oclusal surfaces, one has to remember that dentists are more likely to restore non-cavitated pits and fissure lesions than smooth surface lesions. As such, if a particular dentist merely filled the questionable lesion, the lesion will be considered to have progressed in a longitudinal evaluation, which could overestimate the rate of caries progression[22].

Considering approximal surfaces, most ECL continued to be active/unchanged or were newly developed lesions in both the anterior and posterior teeth (Figures 3 and 4). In this respect, the study by Ekstrand et al.,[23] involving proximal caries in primary enamel or in outer primary dentin revealed that after one year, 31% of the lesions sealed and subsequently treated by vanish

application and 67% of the lesions treated with varnish only had progressed according to ICDAS (International Caries Detection and Assessment System) scores. Although radiographs were not used to assess proximal caries, the present study identified ECL in proximal surfaces because most children showed spaced arches, which enables a direct view of the anterior teeth. In the posterior teeth, ECL were identified in the proximal surfaces when the adjacent surface was lost due to caries and when the extension of ECL could be seen in the vestibular or palatine surfaces. This is a limitation of the current study. However, it should be emphasized that unless there is enamel cavitation, radiographs do not show ECL.

Taking into account the few studies that have evaluated the ability of ECL to progress to cavitated lesions in children with early childhood caries (0-71 months), the study by Warren et al.,[22] revealed that of the 144 non-cavitated pit and fissure lesions at the primary dentition examinations, 29% had progressed to either filled or frank decay status in the mixed dentition after 4 years. Our results showed a lower progression rate of ECL on the occlusal surfaces (Figure 3). This may have happened because in the one year follow up in the current study, lesions may have been less likely to progress than in 4 years. However, the study by Grindefjord et al.,[20] with younger children (2.5 years at baseline and 3.5 years at follow up), showed that of the lesions diagnosed at baseline as initial caries, 64% progressed to manifest lesions over one year. This is not in line with our results (Figures 3 and 4) probably because they worked with suburban children with very high risks and a large proportion of immigrant backgrounds, where dietary habits, oral hygiene and fluoride exposure are not as good as in non-immigrant populations. Moreover, that

study was performed more than 10 years ago before the recent decrease in early childhood caries prevalence in Brazil that occurred between 1996 and 2006.[16]

As was previously mentioned, caries diagnosis criteria used in the present investigation was based on field clinical examinations. As such, radiographs and compressed air drying were not used unlike in dental clinics, where they are commonly applied. Thus, one must be careful when extrapolating these results to other populations.

As caries profiles continue to change with time, it is important to develop studies to evaluate caries progressions in young children. Obtaining current data may provide additional insights into the implementation of effective measures to prevent and control early childhood caries.

Conclusion

1. Our findings support the conservative management of early caries lesions since after one year, the majority of ECL were kept active/unchanged or were arrested on smooth surfaces of anterior and posterior primary teeth.

Acknowledgments

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Table 1. Codes used in the World Health Organization criteria with an additional measurement criterion for early caries lesions (ECL)

WHO + ECL Codes	
A	Sound, excluding early caries lesions
ECL	Early caries lesion (white chalky spot lesion)
B	Cavitated, with no ECL
BECL	Cavitated+ECL
C	Filled+chronic cavity
CECL	Filled+cavity +ECL
D	Filled, no cavity
DECL	Filled+ECL
4	Missing, as a result of caries
5	Missing due to any other reason

Adapted from Nyvad et al.,[13] Assaf et al.[12] and Parisotto et al.[11]

Table 2. Mean numbers and standard deviations of decayed, missing and filled surfaces and teeth according to the WHO+ECL criteria

Caries index	Baseline (n=179)	Follow up (n=179)	One year increment (n=179)
dmfs+ECL	5.60±8.22	7.20±9.85	1.60±1.64
dmft+ECL	3.66±4.22	4.27±4.59	0.61±0.37

WHO: World Health Organization; ECL: early caries lesions.

Table 3. Risk for the development of carious lesions

	Follow up Condition			Estimated risks					
	Caries- free	**ECL**	**Cavitations/Fillings**	ECL development/ECL maintenance			Cavitations/fillings development		
	n=50	n=43	n=17	OR	95%CI	p_Wald	OR	95%CI	p_Wald
ECL at baseline									
Present	8	33	14	17.3	6.9 – 42.9	<0.001	24.5	8.6 – 69.6	<0.001
Absent	42	10	3	1			1		

Model-fitting information: -2 Log Likelihood (334.5), Chi-square (46.2), degrees of freedom (2), significance (< 0.001). ECL: early caries lesion; OR: odds ratio; CI: confidence interval.

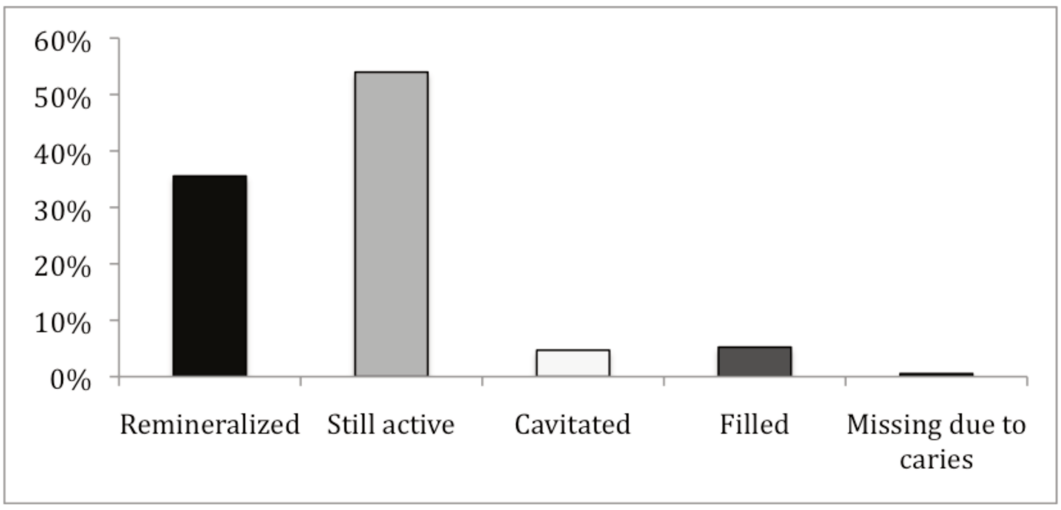

Figure 1. Changes in ECL after the one-year follow-up period

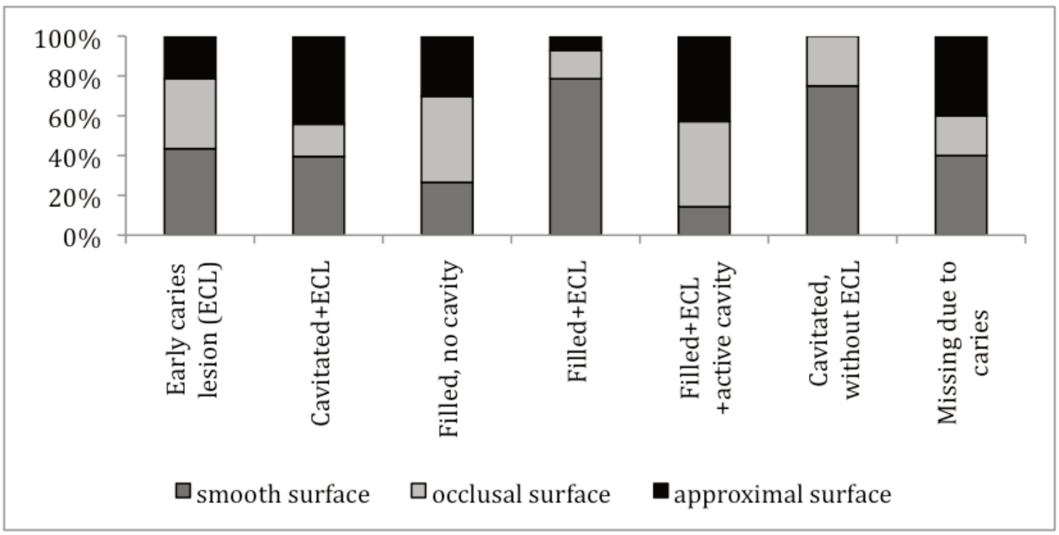

Figure 2. Distribution of dmfs increments by surface type in the children after the one-year follow-up period

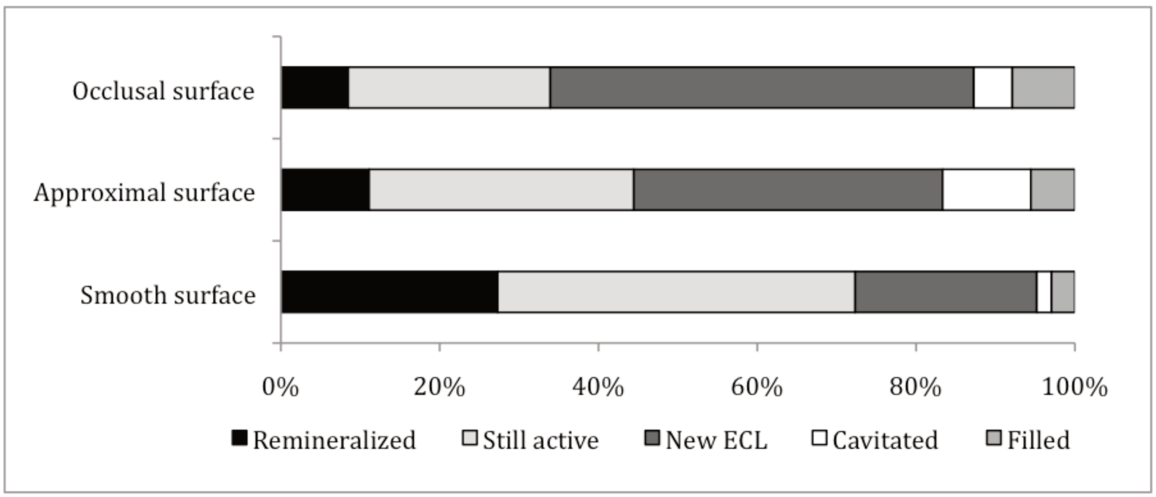

Figure 3. Changes in ECL according to surface type in posterior teeth after the one-year follow-up period

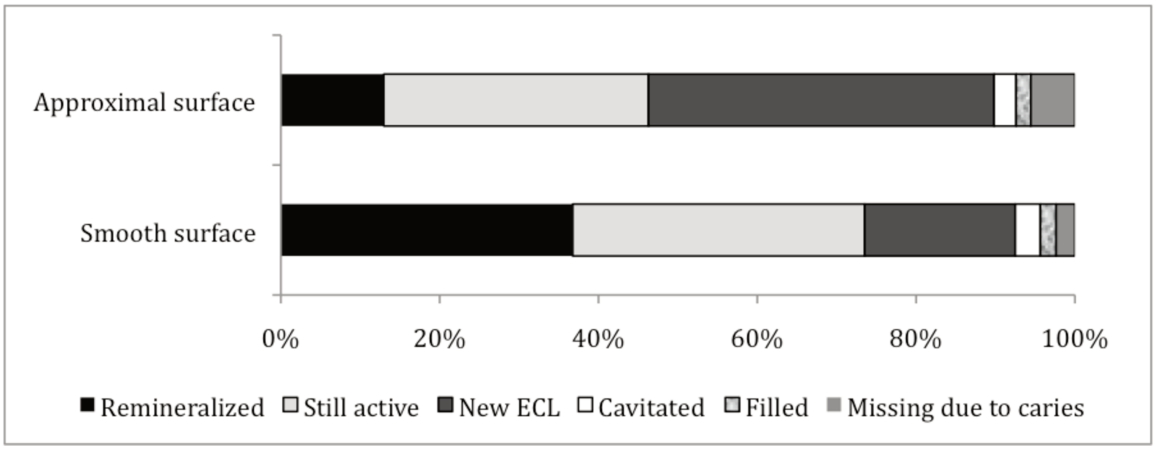

Figure 4. Changes in ECL according to surface type in anterior teeth after the one-year follow-up period

CAPÍTULO 2

Immunological and Microbiologic Changes during Caries Development in Young Children

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Key words: children, caries increment, microbiology, immunology, longitudinal study

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Abstract

We explored the association between caries development, colonization with caries-associated microflora, and immunity as children begin the transition to mixed dentition. Forty children received dental examinations at 3-4 years of age, repeated a year later. Children were grouped into caries-free (n=23;CF) and caries-active (n=17; CA≥3 new lesions on follow-up). Salivary IgA and IgA antibody to *Streptococcus mutans* virulence epitopes were measured by Luminex assay. Mutans streptococci (MS), lactobacilli and total microorganisms were enumerated on selective media from plaque samples. There was no significant difference in baseline levels of MS or lactobacilli between CF and CA groups. However, both MS and lactobacilli levels were higher at follow-up in the CA group. Furthermore, children with detectable lactobacilli at baseline had significantly higher caries risk. Salivary IgA concentrations increased significantly in both groups during the study. Both CF and CA groups also displayed significant increases in salivary IgA antibody levels to glucosyltransferase, glucan-binding protein(Gbp) and Antigen I/II SBR. CF antibody levels to 7 peptides associated with domains of biological importance increased at follow-up, in contrast to increases to only three peptides in CA salivas. Multivariate modeling showed that a lower baseline level of salivary IgA anti-GbpB was associated with higher caries-risk. These data indicate that MS and lactobacilli are associated with caries in this population, that the secretory immune system is undergoing significant maturation during this period, and that the breadth of mucosal IgA response to epitopes of *S.mutans* virulence components may influence the degree to which these cariogenic microorganisms can cause disease.

Introduction

Early childhood caries (ECC) is an infectious disease affecting children aged 71 months or younger [Drury et al., 1999; American Academy of Pediatric Dentistry, 2005-2006]. In the carious process, the dental plaque/biofilm supports a micro-ecosystem of bacteria that exhibit a variety of physiological characteristics which are related to their adherence characteristics, aciduric nature and resistance to low pH levels [Berkowitz, 1996; Gudiño et al., 2007]. Since mutans streptococci (MS) are frequently isolated from cavitated caries lesions, induce caries formation in animals when fed with sucrose rich diet and are highly acidogenic and aciduric, these microorganisms are considered major pathogens in the initiation and progression of dental caries [Loesche, 1986; Mattos-Graner et al., 1998; Mattos-Graner et al., 2001; Nobre dos Santos et al., 2002; Vachirarojpisan et al., 2004; Takahashi and Nyvad, 2008]. Additionally, mutans streptococci are able to produce water-insoluble-glucans, which are central for bacterial adherence to the tooth structure as well to other bacteria [Hamada and Slade, 1980; Loesche 1986]. However, the presence of other non-MS acidogenic bacteria, such as lactobacilli (LB), could also moderate caries outcomes in children [van Houte et al., 1994; Kanasi et al., 2010].

The MS ability to adhere and accumulate on host surfaces is the most significant virulence factor of MS in colonization [Könönen et al.,1994; Law et al., 2007]. In light of this, the role played by three antigen groups associated with the cellular surface of these microorganisms have been studied: antigen I/II, glucosyltranferases (GtfB, GtfC, GtfD) and glucan-binding-proteins (GbpA, GbpB, GbpC) [Smith and Mattos-Graner, 2008]. The adhesins interact with salivary proteins of the acquired pellicle on the tooth surface to promote adherence, glucosyltransferases catalyze glucan synthesis; glucan-binding-proteins increase the binding of MS to glucans deposited on tooth surfaces contributing to the sucrose dependent adherence to teeth [Lamont et al., 1991; Jenkinson et al, 1997].

Studies have shown that specific antibody induction to the above antigens [Katz et al., 1993; Taubman and Smith, 1977; Smith and Taubman, 1996], as well

as to intrinsic peptides related to these antigens (such as QGQ, VAR, SYI, SIG, GGY, Pep 7, Pep 16, LVK, GLU) might prevent dental caries development in animal models [Smith et al., 1994a, Smith et al., 1994b; Taubman et al., 1995; Smith et al., 1997; Smith et al., 1999, Taubman et al., 2001; Smith et al., 2003; Smith et al., 2005; Culshaw et al., 2007b]. Furthermore, some clinical trials [Smith and Taubman, 1990; Childers et al., 1999] suggest that adhesins I/II and Gtfs could be important in development of vaccines [Smith 2002; Smith and Mattos-Graner 2008].

In the oral cavity, the importance of the salivary immunoglobulins (secretory IgA) in mucosal resistance to infection has been recognized [Abbas and Lichtman, 2009]. Secretory IgA is responsible for the first line of adaptative immunity against MS antigens. Additionally, IgA can enhance lactoferrin, peroxidases and lysozyme activities, can reduce the hydrophobicity, block microbial adhesins, neutralize viruses and toxins, inactivate enzymes, and exclude antigen in saliva, activities which may affect MS colonization [Law et al., 2007]. The study of Nogueira et al. [2005], indicated that an immunologically dominant salivary IgA response to GbpB may occur in the first year of life. They also showed that this response was often associated with a delay in infection with MS.

Despite the fact that immunological factors are likely to play an important role in infections in general, much remains to be understood regarding the influence of these factors on MS colonization and dental caries [Koga-Ito et al., 2004]. While some studies show that high anti-MS antibody activity is associated with caries presence in children [de Farias and Bezerra, 2003; Koga-Ito et al., 2004], others show the opposite [Bolton and Hlava, 1982; Kirtaniya et al., 2009]. Moreover, the identification of factors involved in mutans streptococcal colonization could be a key point in the pathogenesis of ECC that can be targeted for caries prevention in early childhood. Thus, the purpose of the present study was to explore the association between caries development, colonization with caries-associated microflora, and salivary immunity as children begin the transition to mixed dentition.

Material and methods

Ethical considerations

The present longitudinal study was approved by the Ethical Committee in Research of Piracicaba Dental School/UNICAMP (Protocol numbers 015/2006 and 017/2008) and was ethically conducted in accordance with the Declaration of Helsinki (World Medical Association). Children's parents as well as the preschools involved granted written permission for the study.

Subjects

As part of a larger study, 188 children (3-4 years old at baseline) were clinically evaluated (baseline and one-year follow-up) for dental caries and 40 children were selected for the present study. These children were selected based in the following two criteria:

1. caries active group (n=17): the child must have developed 3 or more caries lesions (dmfs>=3) in the one year follow up period;
2. caries free group (n=23): the child must never have developed any caries lesion, including white spot lesions.

All children attended public preschools in the fluoridated (0.7ppm), urban area of a city with approximately 91,000 inhabitants (Itatiba, Brazil), which is located 80 kilometers from the state capital (São Paulo).

Clinical examination

Dental examinations of each preschooler were performed at baseline and after one year from the start of the study. One calibrated dentist (baseline Kappa value: 0.75, follow up Kappa value: 0.78) carried out all the examinations at the preschools, sitting behind the child, who was lying on a table. Mouth mirror, gauze, ball-ended dental probe and focusable flashlight were used. Cross-infection control measures were followed rigorously.

The criteria used for ECC diagnosis was the World Health Organization`s with an additional measurement of active white spot lesions [Nyvad et al., 1999; Assaf

et al., 2006; Kassawara et al., 2007]. The units of evaluation used in the clinical exams were decayed, missing, or filled surfaces (dmfs).

Dental plaque collection and microbiological assays

Pooled supragingival plaque samples were collected at baseline and after one year from all buccal and lingual smooth surfaces, except from the interior of the cavities, at least one hour after food intake in the afternoon. In order to standardize plaque amount, a sterile plastic disposable inoculating loop (Greiner, Frickenhausen, Germany) with a circular opening of about 1μL capacity was used for the collection. Collection was stopped when the opening was filled. Plaque samples were placed immediately into a 1 mL centrifuge tube containing prereduced transport fluid, then transferred on ice (4ºC) to a microbiological lab and processed within 6 h. The samples were vortexed for 1 minute and, after serial 10-fold dilution (10^{-1}-10^{-7}) with saline solution 0.9%, the bacteria were plated in triplicate as follows: mutans streptococci on Mitis Salivarius agar (Difco, Sparks, MD) containing 0.2 units/mL bacitracin (Sigma, Poole, UK), lactobacilli on Rogosa agar (Difco, Sparks, MD) supplemented with 0.13% glacial acetic acid, and total microorganisms on Brain Heart Infusion agar (Difco, Sparks, MD) containing 5% defibrinated sheep blood. The plates were incubated for 24h at 37°C in a sealed jar with a 5-10% CO_2, except for the Rogosa agar plates, which were incubated for 48h under the same conditions. The enumeration of microorganisms was performed using a stereomicroscope, and the results were expressed as colony forming units (CFU)/mL. Additionally, MS and LB counts were divided by total microorganism counts to normalize the data among samples.

Saliva collection and immunological assays

A minimum amount of 250 μL of nonstimulated whole saliva was collected in disposable plastic cups at baseline and follow up. Afterwards, saliva was transferred to microcentrifuge tubes. These tubes were sealed and transported in refrigerated boxes (4ºC) to the Pediatric Dentistry Laboratory at the Piracicaba

Dental School, where they were frozen at -70ºC. Saliva sampling was always performed between 1:30 and 3:30pm and at least one hour after food intake.

After all saliva samples had been obtained, they were thawed and 250 mM EDTA solution was added (to minimize salivary IgA aggregation) in the proportion of 5µL for each 250 µL of saliva. Saliva samples were then clarified by centrifugation at 11,000 x g at 4ºC for 10 minutes and the supernatants were collected and frozen at -70ºC until laboratory analysis.

Protein antigens for immunological analysis included *S. mutans* SJ32 GbpB and *S. sobrinus* strain 6715 Gtf, both prepared as previously described [Taubman et al., 1988; Smith et al., 1994a]. The *S. mutans* GbpB was homogeneous in Western blot assays. The *S. sobrinus* Gtf preparation contained a mixture of Gtf isotypes, approximately 30% of which was Gtf-I, which shares >80% homology with *S. mutans* GtfB and GtfC. *S. sobrinus* Gtf-S isotype comprised the balance of the Gtf preparation. Also included for immunological analysis was the salivary binding region (SBR) of the *S. mutans* adhesin antigen I/II. This was a gift from the laboratory of Dr. Noel Childers (University of Alabama-Birmingham, USA).

Peptide antigen constructs for immunological analysis were prepared byAnaSpec, San Jose, CA. The peptides were synthesized as multiple antigenic peptides using the stepwise solid-phase method of Merrifield with a core matrix of lysines, which yielded macromolecules with four identical peptides, as previously described [Smith et al., 1993]. Based on predicted human MHC class II epitopes [Germain and Margulies, 1993] that were in the GbpB sequence (using the ProPred algorithm – [Singh and Raghava, 2001], four monoepitopic peptide construct sequences (QGQ, VAR, SYI and SIG) were selected for the study. With regard to predicted MHC class II epitopes that were in the Gtf sequence using the ProPred algorithm, 2 monoepitopic peptide construct sequences (Pep 7 and Pep 16) were used. Also used were two monoepitopic peptide construct sequences (GGY and LVK) which were based on Gtf sequences associated with putative catalytic interactions [Mooser et al., 1991; Tsumori et al., 1997] and 1 monoepitopic peptide construct sequences (GLU) was based on Gtf sequence associated with

putative glucan binding domains in the C-terminal third of the Gtf protein [Wong et al., 1990]. Table 1 shows the Gtf/GbpB sequences of the peptides and putative importance. Each of the peptides used in this study has previously been shown to induce an immune response, which in some cases can result in caries protection in the rat model [Taubman et al., 1995; Smith et al., 1997; Taubman et al., 2001; Smith et al., 2003; Smith et al., 2005; Culshaw et al., 2007a; Culshaw et al., 2007b]

For IgA antibody measurement, saliva samples were diluted 1:10 in PBS-BN-Pr (phosphate-buffered saline [Sigma Chemical], 1% bovine serum albumin-BSA [Sigma Chemical], 0.05% sodium azide [pH 7.4], 2.0% Prionex Stock [Centra Chem, Inc.]). A cellulose Wek-Cel surgical sponge (Weck-USA) treatment was performed in order to reduce the mucin content and consequently the assay background [Haneberg et al., 1994]. Then, the samples were filtered with 0.22 μm centrifuge tube filters (Spin-X; CoStar). The reactions of salivary IgA antibody to Gtf, GbpB, peptides derived from these proteins, or SBR were tested using a particle-based multiplex fluorescent immunoassay - Luminex Corporation [Nogueira et al., 2008]. Fluorescently tagged microspheres with different fluorescent signatures were coated with Gtf, GbpB, 1 of 9 protein-derived peptides, or SBR. Protein and peptide antigens were covalently attached to these beads via their amines using the protein coupling protocol (www.luminexcorp.com/support/protocols/protein.html). Optimal coating of 2.5 $x10^6$ beads required 5μg protein, or 5μg (QGQ, VAR, SYI, GGY, GLU and Pep 16 peptide), 25μg (LVK, Pep 7 peptide) or 2.5μg (SIG) of peptide. Each saliva assay well contained 2500 beads of each antigen. The mixtures used for assay contained a combination of all GbpB peptide-coated microspheres, all Gtf peptide-coated microspheres, or native Gtf protein, SBR and GbpB protein-coated microspheres. Uncoated microspheres were added to each mixture as controls. The assays with proteins and peptides were performed in different plates. Bead mixtures (50 μl) were added to wells in 1.2 μm-pore-size filter plates (Millipore Corp., Bedford, MA) and drained under vacuum. Then, microspheres were washed twice with PBS-BN under a vacuum and re-suspended in 50 μl of PBS-BN-Pr. Fifty microliters of each

diluted saliva sample (diluted 1:10 in PBS-BN-Pr) were then added and incubated at room temperature with shaking for 2 hours. After the buffer was drained under a vacuum, microspheres were incubated with a 1:500 solution of goat anti-human IgA (Jackson ImmunoResearch) in PBS-BN for 30 min. After washing and draining under vacuum again, microspheres were incubated with a 1:125 solution of R-phycoerythrin conjugate donkey anti-sheep IgG (Jackson ImmunoResearch Laboratories) for 30 min (Jackson Lab's anti-sheep IgG reagent has similar reactivity as their anti-goat reagent – personal communication with Jackson Lab). All incubations were performed in the dark. After washing, the samples were resuspended in PBS-BN and read with a Luminex 100 analyzer to obtain a median fluorescence intensity (MFI). The appropriate value was subtracted to account for the background in the wells without saliva for each antigen. The MFI values were transformed into nanogram of antibody per milliliter of saliva. For that, secretory IgA standards in different concentrations (0.1 to 2μg/mL) were used to conjugate beads. These beads were tested in the same way as the proteins and related peptides (except for the saliva sample addition, which was replaced by PBS-BN-Pr) and a standard curve was obtained, from which the approximate nanograms of antibodies per milliliter were calculated [Cappelli et al., 2009].

The total salivary IgA in each saliva sample was measured in a separate plate. Salivas were diluted 1:500 and incubated with beads coated with monoclonal mouse anti-human IgA (Invitrogen). The reactions were developed with goat anti-human IgA and then with R-phycoerythrin conjugate donkey anti-sheep IgG, as described above. The results of the total salivary IgA test were obtained in MFI after reading in a Luminex 100. The MFI values were transformed into micrograms of total sIgA per milliliter of saliva. For that, secretory IgA standards (obtained from human colostrum - Sigma Chemical Co - St. Louis, MO, USA) in different concentrations (0.1-1μg/mL) were assayed with beads conjugated with monoclonal mouse anti-human IgA, also as described above. A standard curve was obtained, from which the micrograms of sIgA per milliliter were calculated.

Antibody levels to protein and peptide antigens were expressed as ng/mL divided by total IgA concentration and then transformed into parts per million (ppm) of specific antibody.

Statistics

The means and standard errors were obtained for the microbiological data (MS and LB per total microorganisms) as well as for immunological data (GbpB, Gtf, SBR and peptides divided by total salivary IgA). The comparison between baseline and follow up inside each group were performed using Wilcoxon`s test, and the comparison between the groups in the baseline and follow up were done using Mann-Whitney`s test. In addition, a chi-square analysis was performed after the dichotomization of the independent variables (amount of antibodies to GbpB, GtF, SBR and 9 peptides as well as MS and LB counts) based on their median values. The variables that reached a p-value lower than 0.2 were selected for the multivariate logistic regression analysis. The multivariate model, expressed by odds ratios (OR), was used to identify which variables were significant factors for ECC development. The Hosmer & Lemeshow test was used to check the model`s goodness of fit. The analyses were performed using SPSS 17.0 (Statistical Package for Social Science Inc., Chicago, IL, USA) using a level of significance of 5% and a confidence interval of 95%.

Results

Salivary IgA concentrations increased significantly (p=0.012) in the total population of 40 children. The baseline-follow-up comparisons of salivary IgA concentrations in caries-free and caries-active group also revealed increases in each, although only the caries-free groups IgA concentration changed significantly (p=0.0118)–(Table 2).

Caries free and CA groups did not differ significantly from one another in salivary IgA antibody levels to the three MS protein antigens at baseline. After the one year follow up period, both caries free (CF) and caries active (CA) groups

displayed significant increases in salivary IgA antibody levels (adjusted for IgA concentration) to GbpB (CA:1.44; CF:1.46 fold-increase), SBR (CA:2.34; CF:2.45 fold-increase) and Gtf (CA:3.22; CF:1.81 fold-increase) (Table 3). Antibody levels at the follow-up did not differ significantly between the groups.

Salivary IgA antibody activity levels to seven of the nine peptides associated with domains of biological importance in GbpB (VAR, SYI, SIG) and Gtf (GGY, LVK, GLU, peptide16) increased significantly at follow-up in the CF group (Table 4), This contrasted with increases in antibody activity to only three peptides in salivas of the CA group. Interestingly, both CA and CF groups shared significant increases to VAR, SIG and GLU peptides (Table 4).

Oral bacteria were also enumerated at baseline and at the one-year follow-up (Table 5). Baseline levels of respective mutans streptococci (MS) or lactobacilli (LB) counts (adjusted for total microorganism count) showed no statistical difference between CF and CA groups ($p>0.05$). In contrast, at follow up, LB levels were significantly higher in the CA group, compared with the CF group ($p=0.009$). In addition, both MS and LB levels were significantly higher than baseline MS and LB levels at follow-up only in the CA group.

The multivariate modeling indicated that children with detectable lactobacilli at baseline had significantly higher caries risk, with odds ratio of 16.2. Moreover, it was shown that a lower baseline level of salivary IgA anti-GbpB was associated with higher caries-risk during the period of study (OR=7.5) (Table 6).

Discussion

Mutans streptococcal infection is an important etiologic factor of early childhood caries. However, it is not sufficient for initiating ECC, as caries is a multifactorial disease [Selwitz et al., 2007]. In light of this, host genetic differences and their effect on saliva characteristics may be part of the reason why some children develop ECC while others do not [Bagherian et al., 2008] when exposed to similar cariogenic challenges.

In the present longitudinal study, total salivary IgA concentration increased in the population studied (Table 2). This may be partly explained by the normal growth process, because previous studies have already shown that secretory IgA levels increase with age [Everhart et al., 1982; Gleeson et al., 1991; Weemaes et al., 2003; Childers et al., 2003]. At follow up, by 5 years, children were beginning the mixed dentition transition, also characterizing the growth process, which could be linked to maturation of salivary glands as part of general development of systems of the body. Whereas some studies showed that by age of 6-9 years the levels of salivary IgA reach their peak [Burgio et al., 1980; Thaweboon et al., 2008] others showed that IgA reaches adult levels at adolescence [Berdicevsky et al., 1984; Ben Aryeh et al., 1990]. A study with Thai children aging 5 to 10 years showed salivary IgA levels means of 114.96 and 86.47μg/mL [Thaweboon et al., 2008] for children with and without caries, respectively, which is similar to our findings. Moreover, it could be observed in our research that MS increased in the one year follow up (Table 5). Thus, this increased antigenic load may have contributed to the expansion of total salivary IgA levels. Specific salivary antibodies to GbpB, Antigen I/II, SBR and Gtf were observed to increase in both CF and CA groups after one year (Table 3). Furthermore, it was found that preschoolers with a lower baseline level of salivary IgA antibody reactive with GbpB had a 7.5 higher chances to develop caries during the period of study (Table 6). This finding emphasizes that specific antibodies could play a role in oral/bacterial homeostasis. In support of this concept, Nogueira et al. [2007], found that children infected with *S. mutans* at an early stage showed a delay in the immune response to the *S. mutans* GbpB antigen. The glucan-binding-protein B is of great importance to bacteria accumulation and aggregation in the dental biofilm, as this protein antigen participates in cell-surface interaction with glucan, favoring primary or secondary colonization events [Stipp et al., 2008]. It may also be involved with cell wall synthesis and cell growth. Furthermore, animal models have already demonstrated that immunization with GbpB confers protection against dental caries [Smith and Taubman, 1996].

It is interesting to note that in the follow up, antibody response in the CF group increased significantly to a larger number of peptides in comparison with the caries group (Table 4). This suggests that caries free children were able to mount a broader mucosal immune response during the one year period studied. Our results agree with Kirtaniya et al. [2009] and Bolton and Hlava [1982], who also found a better response in caries free children or children presenting low caries index. However, different data were found by Koga-Ito et al. [2004]. The discrepancy in the results could be due to differences in the methodology. It is important to note that the use of purified antigens derived from cariogenic microorganisms is likely to provide a more reliable method for evaluating the contribution of salivary IgA to caries resistance [Bolton and Hlava 1982] compared to analysis using non-purified antigens and may explain the difference between Koga-Ito et al's [2004] data and ours.

With regard to glucosyltransferase-related peptides, GLU was the only one to which salivary IgA antibody levels were shown to increase significantly at follow up (Table 4) in both groups. This result could be explained by the fact that GLU is a peptide located in the glucan binding domain, and that this motif is repeated several times within the C-terminal sequence, which could lead to increased frequency of binding of the IgA antibody with Gtf [Nogueira et al., 2008]. Gtf is essential for *S. mutans* accumulation as it catalyzes the synthesis of glucan from sucrose. The good response to the GLU peptide seen in our study, suggests that epitope may have promise for inclusion in immunological approaches to dental caries prevention in young children.

With regard to microbiological data, an increase in the mutans streptococci and lactobacilli levels, from baseline to follow up, was verified in both groups. However, only in the CA group did this increase reach statistical significance (Table 5). This suggests that the development of new carious lesions may serve as retentive sites for additional bacterial colonization [Ge et al., 2008]. Furthermore, at follow up, children were starting the mixed dentition transition, so that they presented loose teeth, leading to difficulties in tooth brushing and favoring biofilm

accumulation. Also, about 10% of our sample population had first permanent molars in the beginning of the eruptive process, providing more area for microbial retention. Still, the multivariate modeling showed that children with detectable lactobacilli at baseline had 16.2 more chance to develop new caries lesions than those who did not. Others that used regression models also showed that lactobacilli and MS are related to caries [Ramos-Gomez et al., 2002; Parisotto et al., 2010].

Although the main etiologic factors related to early childhood caries are well known and well established in the scientific literature, this disease is still a public health problem in developing countries. It is very difficult to establish healthy habits in families, particularly among the economically deprived ones. Thus ECC preventive measures that do not rely only on the patient, family or community compliance should be encouraged. Therefore, the development of vaccines could be a good strategy to interfere in the virulence factors of the bacteria, thus its cariogenicity.

In conclusion, this longitudinal study supports that mutans streptococci and lactobacilli are associated with caries in this population, that the secretory immune system is undergoing significant maturation during this period, and that the breadth of mucosal IgA response to epitopes of *S.mutans* virulence components may influence the degree to which these cariogenic microorganisms can cause disease.

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Table 1. Peptides used for detection of salivary IgA antibody

Source	Peptide	Sequence	Putative importance	Reference(s)
S. mutans **GbpB**	QGQ	52-KHKLITIQGQVSALQTQQAG-71	MHC class II binding peptide	Smith et al., 2003
	VAR	92-TLSSKIVARNESLKQQARSAQ-111	MHC class II binding peptide	Smith et al., 2003
	SYI	113-KSNAATSYINAIINSKSVSD-132	MHC class II binding peptide	Smith et al., 2003
	SIG	412-SIGNYRGWFNPGSVSYIYPN-431	MHC class II binding peptide	Smith et al., 2003
S. mutans **Gtf**[a]	GGY	402-GGYEFLLANDVDNSNPV**V**Q-418	Catalytically implicated residues	Funane et al., 1993
	Pep 7	505-**L**M**N**MDNK**F**RLS**M**L**W**SLAKP**T**-525	MHC class II binding peptide	Culshaw et al., 2007b
	LVK	911-LVKAIKALH**S**KG**I**KVMADW-937	Catalytically implicated residues	Monchois e tal., 2000
	GLU	1297-TGAQTI**K**GQKLYFK**A**NGQQVKG-1318	Glucan-binding domain	Abo et al., 1991, Lis et al 1995, Smith et al., 1993, Wong et al., 1990
	Pep 16[b]	1364-SG**ALRFY**NLKGQLV**T**GSGWY-1383	MHC class II binding peptide	Abo et al., 1991, Lis et al 1995, Smith et al., 1993, Wong et al., 1990

[a] Gtf peptide sequence numbers are shown for *S. mutans* GtfB. Bold type: residues which are different in *S. sobrinus* GTF-I

[b] Peptide found only in *S. sobrinus* GTF-I. Underline type: residues which are complementary to *S. mutans* GtfB residues. Bold type: residues which are different in GtfB and Gtf-I

Based on Nogueira et al., 2008

Table 2. Means, medians and standard errors of total salivary IgA immunoglobulin

	Total salivary IgA (μg/mL)	
	Baseline	*One year follow up*
Both groups together (n=40)	116.46(84.06)±18.91	153.36(115.53)±18.36*
Caries free group (n=23)	91.44(64.75)±13.09	132.22(95.55)±19.03*
Caries active group (n=17)	150.30(93.49)±40.06	181.97(116.93)±34.18

Values indicated by asterisks are statistically different by Wilcoxon`s test (α=0.05) (comparison baseline x follow up inside each group).

Table 3. Means, medians and standard errors of salivary IgA antibodies against MS protein antigens

Salivary antibodies (ppm)	Caries free group (n=23)		Caries active group (n=17)	
	Baseline	One year follow up	Baseline	One year follow up
Anti-GbpB	96.57(54.41)±33.64	140.81(57.67)±43.08*	92.86(19.87)±51.50	134.26(41.75)±61.32*
Anti-SBR	5.31(1.14)±2.35	13.00(8.41)±3.29*	5.85(2.43)±3.62	13.72(3.91)±7.03*
Anti-Gtf	6.94(2.21)±2.43	12.54(5.28)±3.19*	6.89(1.61)±4.23	22.18(3.95)±9.76*

Values indicated by asterisks are statistically different by Wilcoxon`s test (α=0.05) (comparison baseline x follow up inside each group). All salivary specific antibodies were adjusted by dividing them by total salivary IgA, thus are indicated as ppm:parts per million. GbpB: glucan-binding-protein B; SBR: salivary binding region; Gtf: glucosyltransferase.

Table 4. Means, medians and standard errors of salivary antibodies against MS GbpB-related and Gtf-related peptides

Salivary antibody (ppm)	Caries free group (n=23)		Caries active group (n=17)	
	Baseline	*One year follow up*	*Baseline*	*One year follow up*
Anti-QGQ	0.69(0.15)±0.26	1.09(0.26)±0.31	0.81(0.16)±0.23	0.96(1.18)±0.26
Anti-VAR	1.52(0.67)±0.40	4.10(2.30)±1.48*	1.52(0.27)±0.55	7.29(1.43)±3.51*
Anti-SYI	1.12(0.46)±0.36	2.04(1.73)±0.38*	1.02(0.58)±0.28	1.88(1.20)±0.63
Anti-SIG	2.51(1.29)±0.55	5.24(4.12)±0.076*	2.46(2.26)±0.44	5.11(3.06)±1.20*
Anti-GGY	1.63(0.00)±1.55	4.93(0.41)±3.94*	0.24(0.00)±0.14	0.62(0.52)±0.27
Anti-Peptide 7	0.58(0.48)±0.15	1.74(0.93)±0.66	0.66(0.47)±0.21	1.27(1.10)±0.36
Anti-LVK	4.26(2.25)±1.02	9.39(7.50)±1.72*	3.84(2.51)±1.03	6.19(4.84)±1.27
Anti-GLU	1.52(0.42)±0.47	3.00(2.41)±0.44*	1.27(0.89)±0.26	3.56(2.11)±1.50*
Anti-Peptide 16	8.89(5.63)±2.11	14.13(13.38)±2.00*	6.81(5.88)±0.95	10.35(7.86)±1.90

Values indicated by asterisks are statistically different by Wilcoxon`s test (α=0.05) (comparison baseline x follow up inside each group). All salivary specific antibodies were adjusted by dividing them by total salivary IgA. ppm:parts per million.

Table 5. Means, medians and standard errors of microbiological data

Microorganisms (CFU/mL)	Caries free group (n=23)		Caries active group (n=17)	
	Baseline	One year follow up	Baseline	One year follow up
mutans streptococci	0.10(0.01)±0.04	0.39(0.02)±0.15	0.02(0.01)±0.01	0.59(0.07)±0.46*
lactobacilli	$0.001x10^{6}$ $(0.0)\pm0.001x10^{-6}$	$0.15x10^{6}$ $(0.0)\pm0.15x10^{-6}$	$0.13x10^{-6}$ $(0.0)\pm 0.06x10^{-6}$	$78.22x10^{-6}$ $(0.23x1^{6})\pm71.39x10^{-6}$*

Values indicated by asterisks are statistically different by Wilcoxon`s test (α=0.05) (comparison baseline x follow up inside each group). The microorganism levels were adjusted by dividing them by total microorganism levels. CFU: colony forming units.

Table 6. Multivariate modeling of caries lesions development in the early childhood

Variables	Caries lesions development				
	No (%)	Yes (%)	OR_{crude} (95%CI)	$OR_{adjusted}$ (95%CI)	Model p-value*
Salivary antibody anti-GbpB (ppm)					
< 29.41	9(43)	12(57)	3.7(0.98-14.28)	7.5(1.26-50.00)	
≥ 29.41	14(74)	5(26)	1	1	0.005
Lactobacilli					
Present	1(67)	6(86)	12(1.28-114.42)	16.2(1.12-233.36)	
Absent	22(14)	11(33)	1	1	

OR: odds ratio; CI: confidence interval. *Likelihood test χ^2 with 4 freedom-degrees = 14.97; p-value of the Hosmer & Lemeshow test = 0.58. Model adjusted by salivary antibody anti-SIG and anti-SBR. As the median of lactobacilli/total microorganism was 0, this variable was dichotomized into presence or absence.

CAPÍTULO 3

Is plaque fluoride a good caries predictor in early childhood?

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Short title: plaque fluoride and caries prediction

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Abstract

This longitudinal study aimed to assess the predictive power of dental plaque fluoride (F) in early childhood caries development as related to sugar exposure conditions. Thirty-one preschool children (3–4 years) were followed for one year and divided into two groups: caries (CA n=16) and no caries development (NC n=15). Before the clinical examinations (World Health Organization + early caries lesions criteria), the presence of clinically visible dental plaque was recorded. Plaque F concentration, expressed as μg F/mg of dry weight, was determined by measurement with an ion-specific electrode. A diet chart was used to estimate the mean daily sugar exposure. The results were statistically analyzed by the Mann-Whitney and Wilcoxon tests, the Spearman correlation and logistic regression analyses (α=0.05). The results revealed statistical differences between groups with respect to sugar/sucrose exposure in the solid+liquid form and sucrose exposure in the liquid form only at follow-up ($p<0.05$). Positive correlations were identified between caries increment and liquid sucrose and solid+liquid sucrose/sugar ($p<0.05$). When plaque F concentrations were compared, no difference was identified between groups ($p>0.05$). However, children with plaque F concentrations ≤ 0.1 μg/mg at baseline were 10 times more likely to develop caries during the period of study. Solid sugar/sucrose and solid+liquid sugar exposures at baseline showed significant positive correlations with the presence of plaque at follow-up ($p<0.05$).

In conclusion, this study demonstrates for the first time that in the presence of high sugar exposure, dental plaque fluoride concentration is a strong predictor of caries development in early childhood.

Introduction

In the past decades, dental caries has declined and is restricted to some segments of the population [Narvai et al., 2006]. However, early childhood caries (ECC), which affects children under 6 years of age, continues to be highly prevalent, especially in developing countries [Cariño et al., 2003; Sutthavong et al., 2010; Begzati et al., 2010; Parisotto et al., 2011].

The development of carious lesions reflects a prolonged imbalance between episodes of de- and remineralization with progressive net tooth mineral loss, which could lead to tooth-surface cavitation [Lingström et al., 2000; Selwitz, 2007; Rodrigues et al., 2010]. This dynamic bacteria-mediated de- and remineralization relationship reflects the consumption frequency of cariogenic substrates, such as sugars [Touger-Decker and van Loveren, 2003]. If consumption increases, it could cause an unfavorable shift toward an increased demineralization time and a decreased remineralization time [Lingström et al., 2000].

The role played by fluoride (F) in the caries process should be highlighted with regard to the de- and remineralization of tooth surfaces [Buzalaf et al., 2011]. It is well known that, in the presence of F, there is a decrease in the dissolution of dental tissues. This is the case because even when the plaque/plaque fluid pH is under the critical value for hydroxyapatite, but not lower than pH 4.5, a certain amount of calcium and phosphate is recovered by enamel/dentin as fluorapatite. Additionally, remineralization is favored when the pH rises, enhancing the redeposition of these ions on demineralized tissues [Cury and Tenuta, 2009]. Thus, the key point in the availability and maintenance of F in the oral cavity is to reduce the mineral potential of demineralization during acid attacks and to promote mineral transfer into the tooth following acid attacks.

Many *in vitro* and *in situ* studies [Santos et al., 2009; Castellan et al., 2007; Thaveesangpanich et al., 2005; Issa et al., 2003; Cury et al., 2010; de Mazer Papa et al., 2010] have been conducted focusing on F and dental caries in primary teeth. However, not as many *in vivo* studies have been conducted [Nobre dos Santos et al., 2002; Bayrak et al., 2011], especially as longitudinal studies. Longitudinal

investigations are important because they consider the response of the child to certain factors during the disease process and, unlike cross-sectional investigations, they do not assume that certain factors preceded caries development. Moreover, F, calcium and phosphate positively influence dental plaque composition, whereas sugar exposure negatively influences plaque composition. The understanding of changes occurring in dental biofilms, which are closely related to caries development, and the identification of children who are at risk are important tools for caries prevention. Thus, this study aimed to assess the predictive power of dental plaque fluoride in early childhood caries development as related to sugar exposure conditions.

Materials and methods

Ethical considerations

This longitudinal study was approved by the Ethical Committee in Research of Piracicaba Dental School/UNICAMP (Protocols #015/2006 and #017/2008) and was conducted in accordance with the Declaration of Helsinki. The parents of the children granted written consent for the study. The preschools and nurseries also granted permission.

Sample

Thirty-one children in good general health, 3–4 years old at baseline, were selected to take part in the study. The children were from both genders, all of low socioeconomic backgrounds, and were attending public preschools in the urban area of Itatiba, in the state of São Paulo, Brazil. The urban area of Itatiba has been fluoridated since 1980. Heterocontrol of the fluoridation process revealed that the concentration of F in the tap water was 0.5–0.8 ppm during the study. The 31 children were followed for one year and were divided into two groups according to caries development at follow-up: 1. the caries group (CA, n=16, 8 girls and 8 boys), in which the children had caries at baseline and had developed at least one cavitation or one filling with or without active white spot lesions in the one-year

follow-up period (decayed, missing, or filled surfaces- dmfs≥1); and 2. the no caries development group (NC, n=15, 10 girls and 5 boys), in which the children never developed any caries lesions (dmfs=0) or had active white spot lesions at baseline that were arrested/remineralized at follow-up. The children stayed a minimum of 4 hours per day at the preschools, and they had their teeth brushed at least once a day with fluoridated dentifrice.

Clinically visible dental plaque recording

Before the clinical examinations and teeth cleaning, the presence or absence of clinically visible plaque on the maxillary incisors was recorded. This procedure was performed under artificial light with the child lying on a table.

Clinical examination

Dental examinations of each child were performed at baseline and after one year. After receiving instructions regarding the caries diagnosis criteria, one pedodontist was calibrated by re-examination of 12 children with a one-week interval period. The baseline Kappa value at the surface level was of 0.78, and the follow-up Kappa value at the surface level was of 0.82. The pedodontist conducted all the examinations following rigorous cross-infection control measures. The examinations were performed at the preschools with the child lying on a table and the examiner sitting behind the child. A focusable flashlight, a ball-ended dental probe, a mouth mirror and gauze for cleaning and drying the teeth were used. The criteria from the World Health Organization (WHO) were used for ECC diagnosis [Parisotto et al., 2011], with an additional measurement of active white spot lesions/early caries lesions (ECL). The units of evaluation were decayed, missing, or filled surfaces.

Dental plaque collection and fluoride assay

Pooled supragingival plaque samples were collected at baseline and after one year from smooth surfaces (buccal and palatine), except from the interiors of

the cavities, at least one hour after food intake and tooth brushing in the afternoon period. A wooden stick was employed for the plaque collection, which was placed into a microcentrifuge tube. The tubes were then transported in refrigerated boxes (4°C) to the Pediatric Dentistry Laboratory at Piracicaba Dental School, where they were frozen (–20 °C) until the analysis. Dental plaque was dried for 24 h in vacuum over P_2O_5 [Pearce, 1984], and the dry weight was obtained using an analytical balance (BelEngineering, Via Venezia Giulia, Monza). Then, 0.5 M HCl was added to the tube in the proportion of 0.1 mL/mg plaque dry weight. After extraction for 3 h at room temperature under constant agitation, the same volume of TISAB II (Total Ionic Strength Adjustor Buffer), pH 5.0 (containing 20 g NaOH/L), was added to the tube as a buffer [Nobre dos Santos et al., 2002]. The samples were centrifuged (12,000 g) for 3 min, and the supernatant was retained for determination of the acid-soluble F concentration. Fluoride determination was performed using an Orion 96-09 ion-selective electrode (Orion Research Inc., Boston, MA, USA) and an Orion EA-940 digital ion analyzer that were previously calibrated with various F standard solutions (0.025 to 2.00 µg F/mL). The readings were expressed in millivolts (mV) and were then transformed to µg F/mL through linear regression of the calibration curve. The results were expressed as µg F/mg of plaque dry weight.

Dietary sugar exposure evaluation

Mothers and health agents of the preschoolers that took part in this study were asked to fill out a diet chart for three consecutive days during the work week [Parisotto et al., 2010] at baseline and at follow-up. This chart included the times of day that the children ate and drank anything and the contents of all meals/snacks. The daily frequencies of total (solid + liquid) sugar and total (solid + liquid) sucrose exposure, solid sucrose, solid sugar exposure and liquid sucrose exposure were calculated using this chart. Whether the child slept with a baby bottle containing sweetened liquids was also recorded.

Statistics

The statistical analyses were performed using the Statistical Package for Social Science 13.0 (SPSS Inc., Chicago, IL, USA). The comparison between the two groups (CA x NC) at baseline and at follow-up was performed using the Mann-Whitney test. When the same groups were compared at different time points (baseline x follow-up), the Wilcoxon matched pairs signed ranks test was used. To assess the correlation between caries increment and sugar/sucrose consumption increment, the Spearman correlation was used. This test was also used to evaluate the correlation between the sugar/sucrose exposure, dental plaque presence and dental plaque F concentration at baseline with these same variables at follow-up. Logistic regression was also employed to individually identify risk factors for early childhood caries development. All analyses were conducted using a level of significance of 5% and a confidence interval of 95%.

Results

Children in the caries group exhibited a mean dmfs score of 8.0±4.9 at baseline and 10.4±4.9 at follow-up (increment=2.4); ECL lesions were the most prevalent lesions. The means, medians and standard errors of dental plaque F concentration and sugar/sucrose consumption frequency in the solid, liquid and total forms are displayed in Table 1. This table also shows the statistical differences ($p<0.05$) between groups regarding total sucrose, total sugar and liquid sucrose consumption at follow-up. Within the two groups, significant increases at follow-up were revealed for the following variables: NC – solid sugar and solid sucrose consumption; CA – solid sugar, solid sucrose, total sugar and total sucrose consumption. A significant decrease in dental plaque F concentration and liquid sucrose consumption was identified only in the NC group. No statistically significant differences were identified between the CA and NC groups regarding sugar/sucrose consumption increments and plaque fluoride increments (Table 1).

Table 2 indicates a positive significant correlation between caries increment and liquid sucrose, total sugar and total sucrose consumption increments. The

correlations among variables evaluated at baseline versus variables evaluated at follow-up are shown in Table 3. This table also reveals that the solid sugar, solid sucrose and total sugar exposure at baseline had significant positive correlations with the presence of dental plaque at follow-up ($p<0.05$).

Finally, Table 4 shows that children with plaque F concentrations ≤ 0.1 μg/mg at baseline were more likely to develop caries in one year. This table also shows that sugar/sucrose exposure and the practice of sleeping with a baby bottle containing sugary liquids at baseline could not predict caries development.

Discussion

Fluoride is a critical ion involved in the dynamics of de- and remineralization of dental tissues, enhancing remineralization and reducing demineralization when the oral environment is oversaturated with respect to fluorapatite [Buzalaf et al., 2011]. To our knowledge, for the first time, this study demonstrated longitudinally and *in vivo* that preschool children with plaque F concentrations lower than 0.1 μg/mg at baseline were 10 times more likely to develop caries lesions in primary teeth in a one-year follow-up period than those who had higher F concentrations (Table 4). However, regarding dental plaque F concentrations at baseline or at follow-up, no statistically significant difference could be observed between the group that developed caries and the group that did not. These findings are in line with studies by Bayrak et al. [2011] assessing primary and permanent dentitions and Pearce et al. [2002] assessing permanent dentition. However, they do not corroborate the results obtained by Gaugler and Bruton [1982] and Nobre dos Santos et al. [2002]. Compared to the Nobre dos Santos et al. study [2002], the smaller sample size and consequently higher data variability of our investigation could be a plausible explanation for the lack of statistical significance between groups. In addition, the caries diagnosis criteria used in their study did not include ECL. Therefore, the caries index of their caries group might have been much higher than it appeared, which might have favored achieving a significant difference. Regarding the Gaugler and Bruton study [1982], the difference in the

target population of the study could be a possible reason for the difference compared to this study, as their study involved adults.

In the one-year follow-up period, although the F concentrations in dental plaques decreased within groups, this study revealed that the solid sugar/sucrose exposures increased significantly in both groups (Table 1). The group that did not develop caries exhibited a higher plaque F concentration at baseline and a significant decrease of plaque F concentration at follow-up. The decrease in F concentration at follow-up may be related to the depletion of the inorganic pools by organic acids as a result of the pH decrease during sugar fermentation. It may also be related to possible changes in the structure of dental plaque. These changes might include several factors. First, the uptake of fluoride from dental plaque fluid into enamel might be involved because, during falls in pH, the biofilm fluid would be undersaturated relative to hydroxyapatite but would be still oversaturated relative to fluorapatite, which precipitates on the enamel. Second, the release of fluoride bound to bacterial cells, which were depleted during the falls in pH, might be a factor. Third, low bacterial density might contribute to a low proportion of ion-binding sites because when the frequency of sucrose exposure is increased, a higher concentration of extracellular polysaccharides occupies a larger volume of dental plaque, thereby reducing the number of bacteria. Finally, a low concentration of specific proteins might be involved because there are differences in the patterns of the matrix protein compositions of the dental plaque formed under distinct conditions (i.e., the absence of sugar, the presence of glucose and fructose, and the presence of sucrose), and changes in the protein profiles could affect the binding sites [Paes Leme et al., 2006].

The comparison between CA and NC groups regarding sugar/sucrose consumption frequency revealed statistically significant differences with respect to total sugar exposure and to sucrose exposure in the total and liquid forms at follow-up ($p<0.05$, Table 1). This is in line with the positive and significant correlations between dmfs increment and liquid sucrose, total sucrose and the total sugar increments, which are shown in Table 2. This is also in accordance with studies by

Grindefjord et al. [1996] and Law and Seow [2006], who also identified an association between dental caries development in young children and sugar/sucrose consumption. Interestingly, the baseline conditions of sugar/sucrose exposure and the habit of sleeping with a baby bottle containing sweetened liquids could not predict caries development in our study (Table 4). These results were unexpected but emphasize the importance of evaluating a determined variable and its behavior for a certain period of time, as significant correlations of dmfs increments and some sugar/sucrose exposure increments (such as those described above) were identified. The fact that sleeping with a baby bottle could not predict caries might be because the parent or responsible party could not have included this item in the diet chart according to a prevailing social norm rather than reporting the factual situation, as they might want to adhere to what would be desirable for good oral health [Sjostrom and Holst, 2002].

The oral hygiene conditions were also evaluated in this study by recording the presence or absence of dental plaque on the maxillary incisors. However, no association between dental caries development and the presence of dental plaque at baseline was identified in this study (Table 4). This finding is not in agreement with the cohort study by Leroy et al. [2011], which used a different criterion for recording the visible dental plaque. These authors investigated plaque accumulation on teeth 52, 55, 72 and 75 in large numbers of preschoolers while, in our study, we recorded the presence or absence of clinically visible plaque on the maxillary incisors. Recognizing the role of dental plaque and sugar exposure in the caries process, especially in its initiation, a significant positive correlation was identified between solid sugar and sucrose exposure at baseline and the presence of dental plaque at follow-up ($p<0.05$, Table 3). The higher the sucrose consumption, the higher the concentration of insoluble extracellular polysaccharide in dental plaque [Nobre dos Santos et al., 2002], which favors bacterial adherence to the tooth surface [Rölla, 1989; Schilling and Bowen, 1992], which in turn contributes to the structural integrity of dental biofilm [Paes Leme et al., 2006].

In summary, it should be highlighted that evaluating factors involved in caries development, especially in early childhood, can provide a better understanding of caries dynamics, which may favor the implementation of preventive measures. Moreover, the identification of children who are at risk is very relevant for caries prevention. The preventive measures for early childhood caries are important because this disease englobe serious consequences, such as high levels of pain and infection, and can impact the quality of life.

In conclusion, this *in vivo* longitudinal study demonstrated that in the presence of high sugar exposure, dental plaque fluoride concentration is a strong predictor for caries development in early childhood.

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Table 1. Means, medians and standard errors of fluoride concentration in dental plaque and sugar/sucrose consumption frequencies at baseline and at follow-up in the group of children that developed caries and in the group that did not.

Variables	No caries development group (n=15)			Caries development group (n=16)		
	Baseline	One year follow up	Increment	Baseline	One year follow up	Increment
Fluoride (µg/mg of plaque)	0.11(0.03)±0.04a	0.01(0.00)±0.0b	-0.10(-0.03)±0.04	0.04(0.02)±0.01a	0.02(0.01)±0.00a	-0.02(-0.01)±0.01
Solid sugar	2.08(1.99)±0.17a	4.49(4.67)±0.50b	2.41(2.34)±0.53	2.32(2.33)±0.28a	6.04(6.00)±0.61b	3.72(3.68)±0.69
Solid sucrose	1.15(1.00)±0.14a	3.58(3.67)±0.50b	2.43(2.67)±0.56	1.22(1.00)±0.18a	4.92(5.17)±0.59b	3.69(3.67)±0.65
Liquid sucrose	3.99(3.33)±0.36a	2.93(3.33)±0.35b*	-1.05(-1.00)±0.37	3.99(4.16)±0.38a	4.15(4.17)±0.43a*	0.15(0.00)±0.58
Total sugar	6.07(6.65)±0.36a	7.42(8.00)±0.82a*	1.35(0.75)±0.76	6.31(6.65)±0.48a	10.19(10.33)±1.00b*	3.88(3.85)±1.2
Total sucrose	5.14(5.65)±0.39a	6.51(7.00)±0.82a*	1.37(1.08)±0.82	5.22(5.16)±0.45a	9.06(9.33)±0.99b*	3.85(3.68)±1.2

Different letters in lines indicate statistical difference (the Wilcoxon test, comparison inside groups: baseline x follow-up). *Asterisks indicate a statistical difference (the Mann Whitney test, comparison between groups: baseline x baseline, follow-up x follow-up)

Table 2. Correlations between caries increment (dmfs) and sugar/sucrose consumption frequency increment.

Variables	Liquid sucrose		Total sugar		Total sucrose		Solid sugar		Solid sucrose	
	r(p)									
dmfs	0.463	(0.009*)	0.429	(0.016*)	0.414	(0.021*)	0.353	(0.052)	0.354	(0.051)

* Asterisks indicate statistical significance; dmfs: decayed, missing or filled surfaces

Table 3. Correlations between fluoride concentrations in dental plaque, sugar/sucrose consumption frequency and presence of dental plaque at baseline versus these same variables at follow-up.

Variables	Baseline						
Follow up	**Total sugar**	**Total sucrose**	**Solid sugar**	**Solid sucrose**	**Liquid sucrose**	**Fluoride (µg/mg of plaque)**	**Presence of dental plaque**
				r(p)			
Total sugar	0.143(0.444)	0.093(0.617)	-0.012(0.949)	-0.154(0.407)	0.177(0.340)	0.101(0.588)	0.041(0.825)
Total sucrose	0.104(0.579)	0.103(0.582)	-0.086(0.645)	-0.179(0.336)	0.203(0.274)	0.110(0.555)	0.021(0.912)
Solid sugar	0.171(0.359)	0.061(0.745)	0.074(0.692)	-0.066(0.725)	0.098(0.598)	0.105(0.573)	-0.037(0.842)
Solid sucrose	0.077(0.679)	0.070(0.708)	-0.095(0.610)	-0.174(0.349)	0.175(0.345)	0.097(0.603)	-0.087(0.642)
Liquid sucrose	0.173(0.351)	0.178(0.339)	-0.039(0.836)	-0.185(0.319)	0.274(0.136)	0.137(0.463)	0.187(0.315)
Fluoride (µg/mg of plaque)	0.051(0.784)	0.107(0.565)	-0345(0.057)	-0.324(0.076)	0.252(0.172)	0.040(0.831)	0.085(0.651)
Presence of dental plaque	0.422(0.018*)	0.314(0.085)	0.525(0.002*)	0.365(0.044*)	0.203(0.274)	-0.142(0.446)	0.158(0.397)

* Asterisks indicate statistical significance.

Table 4. Risks for caries development, considering fluoride concentration, sugar/sucrose consumption frequency, and presence of dental plaque.

Variables	Caries development n(%)		Odds Ratio (95%CI)	p - value
	No	Yes		
Fluoride (µg/mg of plaque)				0.047*
>0.1	6(86)	1(14)	1	
≤0.1	9(38)	15(63)	10.0(1.03 -100.00)	
Dental plaque				0.095
absent	5(63)	3(38)	1	
present	10(43)	13(57)	4.67(0.77-28.41)	
Solid sugar				0.054
>2	5(31)	11(69)	4.40(0.98-19.85)	
≤2	10(67)	5(33)	1	
Solid sucrose				0.886
>1	6(50)	6(50)	0.90(0.21-3.82)	
≤1	9(47)	10(53)	1	
Liquid sucrose				0.853
>4	7(47)	8(53)	1.14(0.28-4.68)	
≤4	8(50)	8(50)	1	
Total sugar				0.606
>6	8(44)	10(56)	1.46(0.35-6.11)	
≤6	7(54)	6(46)	1	
Total sucrose				0.853
>5	8(50)	8(50)	0.87(0.21-3.57)	
≤5	7(47)	8(53)	1	
Sleep with a baby bottle containing sweetened liquids				0.577
No	9(53)	8(47)	1	
Yes	6(43)	8(57)	1.50(0.36-6.23)	

CI: confidence interval

CAPÍTULO 4

Relationships between early childhood caries and insoluble polysaccharide concentration in dental plaque, sugar exposure and cariogenic microorganisms

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Abstract

This study aimed to assess the relationships between early childhood caries and extracellular insoluble polysaccharides (IP) in dental plaque, sugar exposure and cariogenic microorganisms (ECC). The study also performed a screening of the abilities of *S. mutans* strains to synthesize glucan *in vitro*. Clinically visible plaque on maxillary incisors was recorded, followed by caries diagnosis in sixty-five preschoolers (3-4 years of age) at baseline and after one year. Plaque was collected for mutans streptococci (MS), total microorganism (TM) and lactobacilli (LB) enumerations in selective media, as well as for IP analysis, which was later assessed by colorimetry. *In vitro* glucan synthesis and sugar/sucrose exposure were assessed by a zymographic assay and diet chart, respectively. Positive correlations were found among the prevalence of caries and MS, TM, LB, solid sucrose and dental plaque. Additionally, children with IP concentrations in dental plaque higher than 2.36 μg/mg, with visible plaque on maxillary incisors, harboring LB and exposed to solid sugar more than twice/day showed higher risk of developing caries than children without these conditions ($p<0.05$). No direct correlation could be demonstrated between IP concentrations in dental plaque and the ability of *S. mutans* strains to synthesize glucan. However, the strains with higher glucan production were found in children who developed caries. In conclusion, IP, solid sugar/sucrose, visible dental plaque and cariogenic microorganisms could predict ECC, while the ability of *S. mutans* strains to synthesize glucan *in vitro* could not reflect the IP concentration in dental plaque in this population.

Introduction

Early childhood caries (ECC) is a multifactorial disease characterized by mineral transfer from the tooth to the surrounding environment and vice versa in children aged 0 to 71 months old [Drury et al., 1999; American Academy of Pediatric Dentistry, 2011–2012]. This dynamic process is intimately related to dental plaque, which covers tooth surfaces as a tightly adherent layer consisting of bacterial, inorganic and organic components [Paes Leme et al., 2006].

With respect to the organic components/matrix, the role played by glucans should be highlighted. Glucans are extracellular polysaccharides resulting from glucose molecule polymerization after sucrose is hydrolyzed by the *Streptococcus mutans* (*S. mutans*) glucosyltransferases [Loesche, 1986; Koo et al., 2010]. Depending on the linkage type alpha 1-3 or alpha 1-6, the glucan or extracellular polysaccharide could be water-insoluble or soluble, respectively. A sticky and porous matrix is formed mostly by the insoluble glucans, which are very important to *S. mutans* adherence and accumulation [Wiater et al., 1999; Paes Leme et al., 2006]. The ability of *S. mutans* to adhere and accumulate on dental surfaces via glucan production is a very significant virulence factor, which could lead to an increased number of infected tooth sites [Napimoga et al., 2004].

Mutans streptococci are the main pathogens behind dental caries, as demonstrated by several studies involving these bacteria [Loesche, 1986; Mattos-Graner et al., 1998; Nobre dos Santos et al., 2002; Takahashi and Nyvad, 2004; Parisotto et al., 2010_a]. Young children with ECC are often colonized by mutans streptococci and usually have inappropriate feeding practices, such as frequent consumption of carbohydrates and sweetened fluids [Hallett and O'Rourke, 2006; Mattos-Graner et al., 1998; Selwitz, 2007]. Inappropriate feeding habits with a high frequency of sugar consumption provide sucrose, the specific substrate for glucan production. As studies involving extracellular insoluble polysaccharide (IP) and ECC *in vivo* are lacking in the scientific literature, the purpose of this study was to assess the relationships between ECC and dental plaque IP, sugar exposure and

cariogenic microorganisms. The study also performed a screening of the abilities of *S. mutans* strains to synthesize glucan *in vitro*.

Methods

Ethical considerations

This study was approved by the Ethical Committee in Research of Piracicaba Dental School/UNICAMP (Protocols #015/2006 and #017/2008). The preschools granted permission for the study and the children's parents signed a written positive consent form.

Sample

Sixty-five children, from both genders and aged 3-4 year olds, took part in this study. These children were from low socioeconomic backgrounds and attended public preschools in the urban and fluoridated (0.5-0.8 ppm) area of Itatiba, state of São Paulo, Brazil. Children were excluded from the study if they refused to cooperate with the clinical examinations or if they had systemic diseases. In the preschools, the children ate the same meals, stayed a minimum of 4 hours/day and had their teeth brushed at least once a day with dentifrice containing fluoride.

The children were submitted to clinical examinations for caries diagnosis; plaque collection for IP and microbiological analyses; dental plaque assessments of the maxillary incisors; and sugar exposure investigation. In order to determine the changes in the prevalence of caries of the studied population, the clinical examinations for caries diagnosis were repeated after one year. Thus, according to the changes (follow up scores – baseline scores) in the prevalence of caries, the children were assigned into 3 groups:

1. Caries arrestment (AR): children who had early carious lesions that arrested (decayed, missing, or filled surfaces-dmfs change<0, mean number of dmfs: -2.3) (n=11);

2. Caries free (CF): children who were always free of caries and never showed early carious lesions (ECL), cavitations or fillings (dmfs=0) (n=19).
3. Caries active (CA): children who continued to develop carious lesions (dmfs changes>0, mean number of dmfs: 4.9) (n=35).

Clinically visible dental plaque recording

The presence or absence of clinically visible plaque on the maxillary incisors was recorded under artificial light, with the child lying on a table. This was performed before teeth cleaning for the clinical examinations.

Clinical examination

ECC diagnosis was made according to the World Health Organization's criteria [Parisotto et al., 2010_a], with an additional measurement of active early carious lesions.

Before the clinical examinations, a pedodontist was calibrated by replicate examinations on a random sample of 12 children from the population studied, with different clinical situations, including ECL. Clinical photographic slides combined with theoretical discussions were performed to provide visual examples for the examiner of the examination criteria. The calculated Kappa values, according to the surface levels, were 0.78 and 0.82 for the first and second calibrations, respectively.

Dental examinations were performed at the preschools, following the cross-infection control measures and using a focusable flashlight, a ball-ended dental probe, a mouth mirror and gauze to clean and dry the teeth. During the examinations, the child lied on a table while the examiner sat behind the child.

The units of evaluation were decayed, missing, or filled surfaces.

Insoluble extracellular polysaccharide assay

Pooled supragingival plaque samples were collected from smooth surfaces with a wooden stick in the afternoon period, at least one hour after food intake and tooth brushing. The dental plaque was placed into microcentrifuge tubes and transported in refrigerated boxes (4°C) to the Pediatric Dentistry Laboratory at Piracicaba Dental School, where they were frozen (-20°C) until analysis. The dental plaque was dried for 24 h in vacuum over P_2O_5 [Pearce, 1984] and its dry weight was obtained using an analytical balance (BelEngineering, Via Venezia Giulia, Monza). Then, 0.1 mL of 0.5 M HCl per 1 mg of plaque was added to the tube and after 3 h of constant agitation at room temperature, an equal volume of TISAB II, pH 5.0 (containing 20 g NaOH/L), was added to the tube [Cury et al., 1997; Nobre dos Santos et al., 2002]. The samples were centrifuged (12,000 g) for 3 min and 1 N NaOH (0.1 mL/mg plaque dry weight) was added to the precipitate. The samples were homogenized for 1 min and agitated for 3 h at room temperature. To the supernatant, 3 volumes of 75% ethanol (0.3 mL/mg plaque dry weight) were added, [Ccahuana-Vásquez et al., 2007] and the samples were incubated at -20°C overnight and subsequently centrifuged (12,000 g) for 3 min. The precipitate was then resuspended in 1 N NaOH (0.1 mL/mg plaque dry weight) and the concentration of the insoluble extracellular polysaccharide was determined by colorimetry [Dubois et al., 1956]. The readings expressed in absorbance units were transformed to µg IP/mL through linear regression of the calibration curve (3.12 to 21.25 µg of glucose). The results are expressed as $\log_{10}$ of micrograms of IP per milligram of plaque dry weight.

Cariogenic microorganism enumeration

Pooled supragingival plaque was collected with a sterilized plastic disposable handle (Greiner, Frickenhausen, Germany) from all smooth surfaces, except for the interior of the cavities. The collection was performed in the afternoon period, at least 1 h after food intake and tooth brushing. A disposable handle was used to standardize the amount of plaque collected, as the collection stopped

when the handle opening was full. Dental plaque samples were immediately placed in reduced transport fluid [Syed and Loesche, 1972] and transported in refrigerated boxes (4°C) to the Pediatric Dentistry Laboratory at Piracicaba Dental School. Within 6 h, microbiological analysis was performed using serial dilutions (10^1–10^7) with 0.9% saline solution. Each dilution was placed in triplicate in three different media: (1) Mitis salivarius agar (Difco, Sparks, MD) with 0.22 units/mL of bacitracin (Sigma, Poole, UK) for mutans streptococci (MS); (2) Rogosa agar (Difco, Sparks, MD) supplemented with 0.13% glacial acetic acid for lactobacilli (LB); and (3) Brain Heart Infusion agar (Difco, Sparks, MD) with 5% defibrinated sheep blood to assess total microorganisms (TM). The plates were incubated for 24 h at 37°C in a candle-extinguishing jar with 5-10% CO_2 atmosphere, except for the Rogosa agar plates, which were incubated for 48 h [Ersin et al., 2006]. The colony-forming units (CFU) were enumerated using a stereomicroscope and the results are expressed as $\log_{10}$ of CFU/mL.

Streptococcus mutans identification and glucan production in vitro

Streptococcus mutans growth and identification

Mutans streptococci were recovered in Mitis salivarius agar (Difco, Sparks, MD) with 0.2 units/mL of bacitracin (Sigma, Poole, UK) [Gold et al., 1973]. Eight isolated strains were maintained as frozen stocks in 10% skim milk (Difco, MD, USA) at -20°C and routinely grown in Brain Heart Infusion (BHI) (Difco) at 37°C/10% CO_2. These colonies were selected from four children: two who developed carious lesions (CA group) in one year and the other two who had ECL (AR group) that arrested in one year.

The chromosomal DNA was purified by phenol-chloroform extraction and the integrity of the DNA samples was examined by GelRed (Biotium)-stained 1% agarose gels. Identification of *S. mutans* was carried out by PCR (polymerase chain reaction) of the *ddl* (D-alanine: D-alanine ligase) gene according to Garnier et al. [1997].

In vitro glucan synthesis

Strains identified as *S. mutans* were grown in BHI (5 mL) for 15 h. The densities of the cultures were measured spectrophotometrically (A_{550nm}) and the same quantity of bacterial cells was inoculated into 25 mL of BHI. Cells were collected at A_{550nm}=0.500 (±0.050, approximately 3.5 h of growth), centrifuged and stored at -20°C. For protein extraction, mechanical disruption of the cells was carried out with 350 µL of H_2O and 0.16 g of zirconium beads (0.1 mm diameter, Biospec) on a Mini-Beadbeater (Biospec) at maximum power at 3 cycles of 60 sec with 1 min of rest on ice. The extracts were briefly centrifuged (30 sec/7000 g) and the supernatants containing the whole cell protein fraction were stored at -80^{o}C. Protein concentrations were measured by Bradford assay (Sigma) according to the manufacturer's protocol.

Equal volumes of extracted protein (2.5 µg) were separated in 6% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) with a Mini Protean III apparatus (Bio-Rad) and developed as previously described [Mattos-Graner et al., 2004]. Briefly, gels were washed twice for 15 min with a renaturing buffer containing 2.5% of Triton X-100. The gels were incubated for 18 h at 37°C with 0.2 M sodium phosphate buffer (pH 6.5) containing 0.2% dextran T70 (Sigma) and 5% sucrose. The enzymatic reaction was stopped by washing the gels with water. The resulting white opaque glucan bands, synthesized by glucosyltransferases (Gtf) within the gels, were photographed against a dark background to enhance visibility. The band intensities were measured using the Image G software and expressed as arbitrary densitometric units (AU). The assays were performed in duplicate, with at least three independent growths.

Sugar exposure evaluation

During the workweek, a diet chart was filled for 3 consecutive days (Parisotto et al., 2010_a). On this chart, mothers and health professionals included the content of all meals and snacks, as well as the period of the day when the children ate and drank. The daily frequency means for the total (solid+liquid) sugar

and total (solid+liquid) sucrose exposures, as well as solid sucrose, solid sugar and liquid sucrose exposures, were calculated using this chart.

Statistical analysis

The statistical analyses were performed using the Statistical Analysis System-SAS version 9.1.3 with an alpha level of 0.05 and a confidence interval of 95%. The correlation between the prevalence of caries (dmfs) and each of the tested variables, including concentration of IP (log_{10}) in dental plaque, MS counts (log_{10}), TM counts (log_{10}), presence of LB in dental plaque, clinically visible dental plaque on the maxillary incisors and the mean frequencies of solid sugar, solid sucrose, liquid sucrose, total sugar and total sucrose consumption, was assessed using Spearman's correlation. Additionally, chi-square tests, followed by multiple logistic regressions, were performed in order to identify the variables that could explain the development of caries after one year. The chi-square test was used to select the variables with p values ≤0.2, which would enter the model. Also, descriptive statistics and Spearman's correlations were used to evaluate the relationship between the IP concentration in dental plaque and the ability of the *S. mutans* strains to synthesize glucan *in vitro*.

Results

Caries prevalence in the studied population was 67.7%. The correlations between the prevalence of caries and sugar/sucrose consumption frequency, cariogenic microorganisms and plaque IP concentrations are shown in Table 1. This table reveals positive significant correlations between caries prevalence and MS, TM, LB, solid sucrose and dental plaque presence on the maxillary incisors.

Table 2 displays the variables that reached p values ≤0.2, comparing CF versus the CA group as well as CA versus the AR group. These variables were selected for the models shown in Table 3. According to Model 1 (AR x CA group), children with plaque IP concentrations higher than 2.36 µg IP/mg in dry weight and with the presence of LB showed a higher risk of developing caries in one year than

children with lower concentrations of IP and an absence of detectable levels of LB ($p<0.05$). According to Model 2 (CA x CF group), children who had clinically visible dental plaque on the maxillary incisors and who were exposed to solid sugar more than twice a day were more likely to develop caries in one year compared with children who did not show these conditions.

After performing a screening with eight *S. mutans* strains in four children (two from the CA group and two from the AR group) to evaluate a possible relationship between the strains' ability to synthesize glucan *in vitro* and the concentration of the IP found in dental plaque, no direct correlation could be demonstrated ($r=-0.2$, $p=0.73$).

Discussion

This study of preschool children with a high prevalence of caries and low socioeconomic backgrounds showed significant relationships between early childhood caries and extracellular insoluble polysaccharide, solid sucrose/sugar exposure, cariogenic microorganisms and the presence of dental plaque.

The presence of dental plaque on the maxillary incisors displayed a positive correlation with caries prevalence (Table 1). Furthermore, children who had visible dental plaque on the maxillary incisors were 4.3 times more likely to develop caries (Table 3) than children who did not. The accumulation of clinically visible dental plaque is not only related to oral hygiene status [Parisotto et al., 2010_a] but also reflects the consumption of sucrose and colonization by cariogenic microorganisms, both of which are important factors behind early childhood caries [Cury et al., 1997; Nobre dos Santos et al., 2002; de Mazer Papa et al., 2010].

With respect to dental plaque composition, our results showed that children with dental plaque IP concentrations higher than 2.36 μg/mg were more likely (odds ratio-OR=6.8) to develop caries than children with lower concentrations (Table 3). This is in line with Nobre-dos Santos et al., [2002], who demonstrated that plaque from preschoolers with caries had significantly higher IP and higher mutans streptococci. Similarly, Bayrak et al., [2011] also found that IP

concentration was significantly higher in children with caries. It is important to highlight that dental plaque rich in IP has an increased porosity, facilitating the transport of bacterial substrates, such as sugar, acids and ions [Van Houte, 1994; Paes Leme et al., 2006].

The present study also found a positive correlation between solid sucrose and caries prevalence (Table 1). This is in line with previous studies that found an association between sugar and dental caries [Tsai et al., 2006; Johansson et al., 2010; Parisotto et al., $2010_{a;}$ Parisotto et al., 2010_{b}]. Moreover, children exposed to solid sugar more than twice a day were 5 times more likely to develop carious lesions (Table 3) than those with lower sugar exposure. Because the solid physical form of sucrose/sugar needs to be triturated by the teeth, it can be retained on the tooth surfaces for a prolonged period of time [Gustafsson, 1954; Touger-Decker and van Loveren, 2003] in comparison with the liquid form, for example. A higher exposure of the cariogenic plaque bacteria to sucrose leads to decreased plaque pH. More importantly, sucrose is a specific substrate for IP production; the higher the IP plaque content, the larger the volume of the matrix diffusion pathways, which increases the extent of acidification within the biofilm [Van Houte, 1994; Paes Leme et al., 2006; Koo et al., 2010]. It should be emphasized that the ability of MS to adhere and accumulate on tooth surfaces is influenced by IP production, which depends on the action of Gtf enzymes on sucrose [Smith et al., 1997]. These enzymes are very important for the virulence of *S. mutans* [Yamashita et al., 1993].

In addition, caries is a multifactorial disease. As such, the roles played by the acidogenic and aciduric bacteria are also relevant. In this respect, the present study found a positive significant correlation among caries prevalence, MS counts and the presence of LB (Table 1). Furthermore, children harboring LB were 13 times more likely to develop caries than children harboring undetectable levels of these bacteria. This is in accordance with previous studies [Ramos-Gomez et al., 2002; Parisotto et al., 2011]. It is worth noting that the levels of not only MS and LB but also TM (Table 1) reached statistical significance in the correlation tests. This may suggest that bacteria other than MS and LB, such as *Actinomyces* [Milnes and

Bowden, 1985; Marchant et al., 2001], could also influence the carious process in young children. In addition, *Actinomyces* species and non-mutans streptococci microorganisms could be bound by Gtf [Vacca-Smith and Bowen, 1998]. Glycosyltransferases promote the binding of *S. mutans* to other organisms and concomitantly provide additional structural support for microcolony development [Koo et al., 2010].

No correlation could be demonstrated between the ability of the *S. mutans* strains to synthesize glucan *in vitro* and the IP concentration found in the dental plaque of children colonized by these bacteria. The lack of a direct correlation may be a result of the large variability (3.99-22.83 AU; 4.5 fold increase) in the individual glucan production displayed by the strains. Therefore, future studies involving a large number of strains are strongly encouraged, and young children could be a suitable target population.

Interestingly, the strains with higher glucan production (22.83 and 12.07 AU) were in the group that developed carious lesions in one year (CA group). This result agrees with a previous study that showed insoluble glucan synthesis to be higher in isolates from caries-active children [Mattos-Graner et al., 2000]. This interesting finding suggests that glucans (especially the insoluble type) could be an important factor in the field of caries prevention given its influence on the ecological and structural changes of biofilms [Paes Leme et al., 2006], which could increase *S. mutans* adherence and accumulation on dental tissues.

In conclusion, insoluble extracellular polysaccharide, solid sugar/sucrose and cariogenic microorganisms are associated with caries development in young children, thus partially explaining early childhood caries. Moreover, the ability of the *S. mutans* strains to synthesize glucan *in vitro* could not reflect the IP concentration in dental plaque in this population.

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Table 1. Spearman correlation coefficients (r) and probabilities of statistical significance (p) between caries and the variables analyzed - baseline conditions

Variables	**dmf-s**	
	r	p
polysaccharide	-0.013	0.918
mutans streptococci	0.304	0.014*
total microorganisms	0.339	0.006*
lactobacilli	0.435	<0.001*
liquid sucrose	-0.113	0.372
solid sucrose	0.254	0.044*
solid sugar	0.174	0.166
total sucrose	-0.014	0.912
total sugar	-0.003	0.979
presence of dental plaque	0.258	0.038*

dmfs: decayed, missing or filled surfaces. The mean and standard error of dmfs was 6.6±1.26.

Table 2. Bivariate analysis of the relationships between caries development and the related variables at baseline

Variables	AR x CA		CF x CA	
	n(%)		n(%)	
Polysaccharide[1] (µg/mg of plaque)	p = 0.082		p = 0.933	
> 2.36	2(10)	18(90)	10(36)	18(64)
≤ 2.36	9(35)	17(65)	9(35)	17(65)
Mutans streptococci[1]	p = 0.749		p = 0.315	
> 6.70	5(26)	14(74)	5(26)	14(74)
≤ 6.70	6(22)	21(78)	14(40)	21(60)
Total microorganisms[1]	p= 0.460		p = 0.346	
> 9.11	9(28)	23(72)	10(30)	23(70)
≤ 9.11	2(14)	12(86)	9(43)	12(57)
Lactobacilli	p = 0.032*		NA	
absent	10(36)	18(64)	19(51)	18(49)
present	1(6)	17(94)	NA	17(100)
Solid sugar consumption frequency	p = 0.307		p = 0.073	
> 2	4(17)	20(83)	6(23)	20(77)
≤ 2	7(32)	15(68)	13(46)	15(54)
Solid sucrose consumption frequency	p = 1.000		p = 0.229	
> 1	4(21)	15(79)	5(25)	15(75)
≤ 1	7(26)	20(74)	14(41)	20(59)
Liquid sucrose consumption frequency	p = 0.396		p = 0.208	
> 4	6(30)	14(70)	11(44)	14(56)
≤ 4	5(19)	21(81)	8(28)	21(72)
Total sugar consumption frequency	P = 0.857		p = 0.776	
> 6	5(23)	17(77)	10(18)	17(82)
≤ 6	6(25)	18(75)	9(33)	18(67)
Total sucrose consumption frequency	p = 0.988		p = 0.907	
> 5	5(18)	16(82)	9(18)	16(82)
≤ 5	6(24)	19(76)	10(34)	19(66)
Dental biofilm	p = 1.000		p = 0.048*	
Absent	2(18)	9(82)	10(53)	9(47)
Present	9(26)	26(74)	9(26)	26(74)

*Significant results were evaluated using the chi-square test or Fisher's exact test (α=0.05). Fisher's exact test was applied when the frequencies were smaller than 5. CF: caries-free group; AR: caries arrestment group; CA: caries development group. [1]values expressed by $\log_{10}$. NA: not available.

Table 3. Risk factors for early childhood caries development

	Variables	Caries development		OR (95%CI)	p-value
		No (%)	Yes(%)		
Model 1: CA x AR[1]	**Lactobacilli**				
	absent	10(36)	18(64)	1.00	
	present	1(6)	17(94)	13.0 (1.39 – 121.99)	
	Polysaccharide (µg/mg of plaque)				0.003
	> 2.36	2(10)	18(90)	6.8 (1.15 – 40.30)	
	≤ 2.36	9(35)	17(65)	1.00	
Model 2: CA x CF[2]	**Dental plaque**				
	Present	9(26)	26(74)	4.3 (1.12 – 16.37)	
	Absent	10(53)	9(47)	1.00	
	Solid sugar consumption frequency				0.007
	> 2	6(23)	20(77)	5.0 (1.19 – 21.02)	
	≤ 2	13(46)	15(54)	1.00	

NA: not available; OR: odds ratio; CI: confidence interval.

[1] Likelihood Ratio Test = 11.81 (2 degrees of freedom); Hosmer & Lemeshow: p=0.95.

[2] Adjusted by liquid sugar exposure frequency. Likelihood Ratio Test = 12.05 (3 degrees of freedom). Hosmer & Lemeshow: p=0.72.

III – CONCLUSÃO GERAL

1. Após um ano de acompanhamento, a maioria das lesões iniciais de cárie (manchas brancas) mantiveram-se ativas ou paralisaram em superfícies lisas livres de dentes decíduos anteriores e posteriores.

2. O sistema imunológico sofreu maturação significativa dos 3-4 aos 4-5 anos e a amplitude de resposta da imunoglobulina A secretora da saliva contra os epítopos de *Streptococcus mutans* pode influenciar o grau com que esses microrganismos causam doença.

3. Na presença de uma alta exposição ao açúcar, a concentração de flúor na placa dentária é um preditor de risco significativo para o desenvolvimento da cárie precoce da infância.

4. A concentração de polissacarídeos extracelulares insolúveis no biofilme, os microrganismos cariogênicos e a exposição aos açúcares/sacarose na forma sólida foram capazes de predizer o desenvolvimento da cárie precoce da infância. Além disso, a capacidade das cepas de *Streptococcus mutans* produzirem glucano *in vitro* não pode refletir a concentração de PECIs no biofilme na população estudada.

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· *De acordo com a norma da UNICAMP/FOP, baseadas na norma do International Committee of Medical Journal Editors – Grupo deVancouver. Abreviatura dos periódicos em conformidade com o Medline.

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V – ANEXOS

ANEXO 1 - Resolução CCPG/002/06 da UNICAMP que aprova o formato alternativo das teses de doutorado (Parte I)

INFORMAÇÃO CCPG/OO2/06[6]

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG n° 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "impar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2° desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

[6] Disponível em: http://www.prpg.unicamp.br/ccpg_inf002_06.pdf

ANEXO 1 - Resolução CCPG/002/06 da UNICAMP que aprova o formato alternativo das teses de doutorado (Parte II)

§ único - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

Artigo 3º - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em formato alternativo, desde que observados os incisos I, II, III IV, V e VII do artigo 1º.

Artigo 4º - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Um original da dissertação ou tese, em versão definitiva, impresso em folha tamanho carta, em uma só face, deve ser encaminhado à gráfica da Unicamp acompanhado do formulário "Requisição de Serviços Gráficos", onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão encaminhados à Unidade em, no máximo, cinco dias úteis.

§ 4º - No formulário "Requisição de Serviços Gráficos" deverão estar indicadas as páginas cuja reprodução deva ser feita no padrão "cores" ou "foto", ficando entendido que as demais páginas devam ser reproduzidas no padrão preto/branco comum.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

§ 6º - As páginas fornecidas para inserção deverão ser impressas em sua forma definitiva, ou seja, apenas frente ou frente/verso.

§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 13 de setembro de 2006

Profa. Dra. Teresa Dib Zambon Atvars
Presidente
Comissão Central de Pós-Graduação

ANEXO 2 - Certificado do comitê de ética em pesquisa - *baseline*

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Associação entre cárie precoce na infância e variáveis clínicas, bioquímicas, microbiológicas e comportamentais em pré-escolares de 24 a 60 meses**", protocolo nº **015/2006**, dos pesquisadores **THAÍS MANZANO PARISOTTO e MARINÊS NOBRE DOS SANTOS UCHÔA**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 31/03/2006.

The Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas, certify that project "**Association between early childhood caries and clinical, biochemical, microbiological and behavioral variables in preschool children aging 24 to 60 months**", register number **015/2006**, of **THAÍS MANZANO PARISOTTO and MARINÊS NOBRE DOS SANTOS UCHÔA**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for researching in human subjects and was approved by this committee at 31/03/2006.

Profa. Cecília Gatti Guirado
Secretária
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 3 - Certificado do comitê de ética em pesquisa – *follow up*

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**A relação entre a composição bioquímica e microbiológica do biofilme dentário, fatores imunológicos e ambientais e a incidência de cárie em pré-escolares com cárie precoce da infância**", protocolo nº **017/2008**, dos pesquisadores **THAÍS MANZANO PARISOTTO, JULIANA PAIOLA e MARINÊS NOBRE DOS SANTOS UCHÔA**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 19/09/2008.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**The relationship among biochemical and microbiological composition of dental biofilm, immunological and ambiental factors and caries incidence in preschool children with early childhood caries**", register number **017/2008**, of **THAÍS MANZANO PARISOTTO, JULIANA PAIOLA and MARINÊS NOBRE DOS SANTOS UCHÔA**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 19/09/2008.

Prof. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 4 - Ficha clínica utilizada na avaliação do índice de cárie

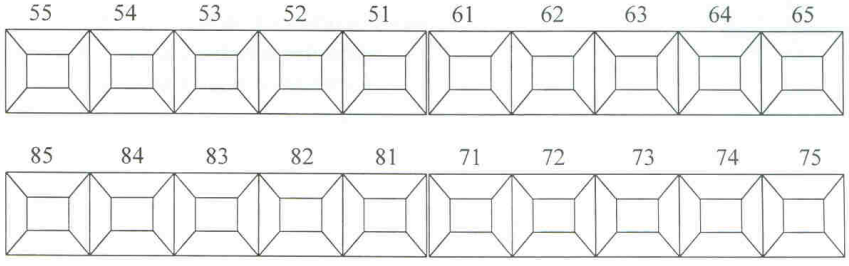
FICHA CLÍNICA

Nome:_____Nº da Ficha:_____
Creche:_____Data exame:_____

Data nasc.:_____Idade (meses):_____Sexo: (F) (M): Cor: (B) (N) (P)

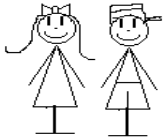
A: hígido
ECL: mancha branca ativa
B: cavitado com lesão crônica
BECL: cavitado com lesão ativa
C: restaurado com cavidade crônica de cárie
CW: restaurado com cavidade ativa de cárie
D: restaurado sem lesão de cárie

DW: restaurado com mancha branca
4: perdido devido à cárie
5: perdido por outra razão



ANEXO 5 - Diário de Dieta

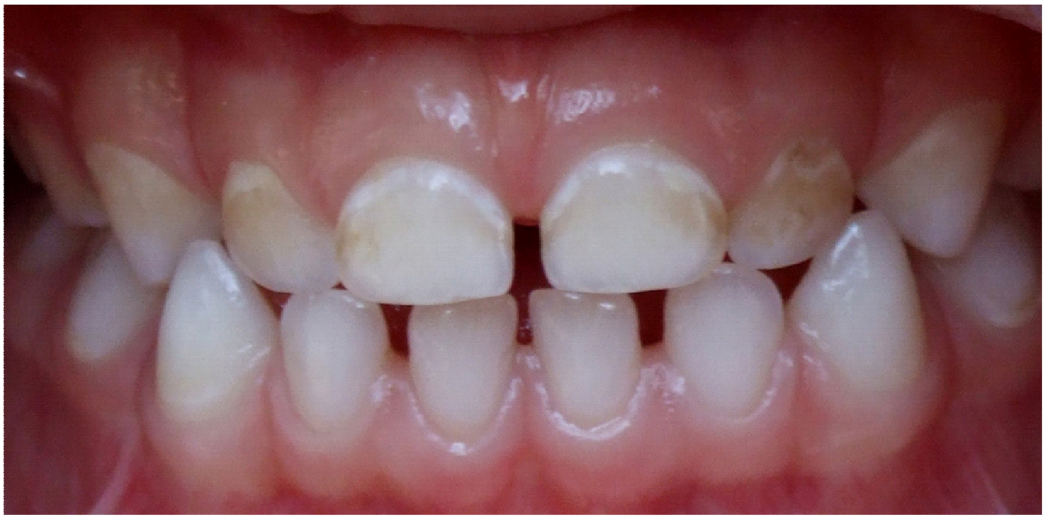
ANOTAR TUDO O QUE A CRIANÇA **COMER E BEBER** POR 3 DIAS SEGUIDOS!
NÃO PREENCHER DE SÁBADO E DOMINGO!



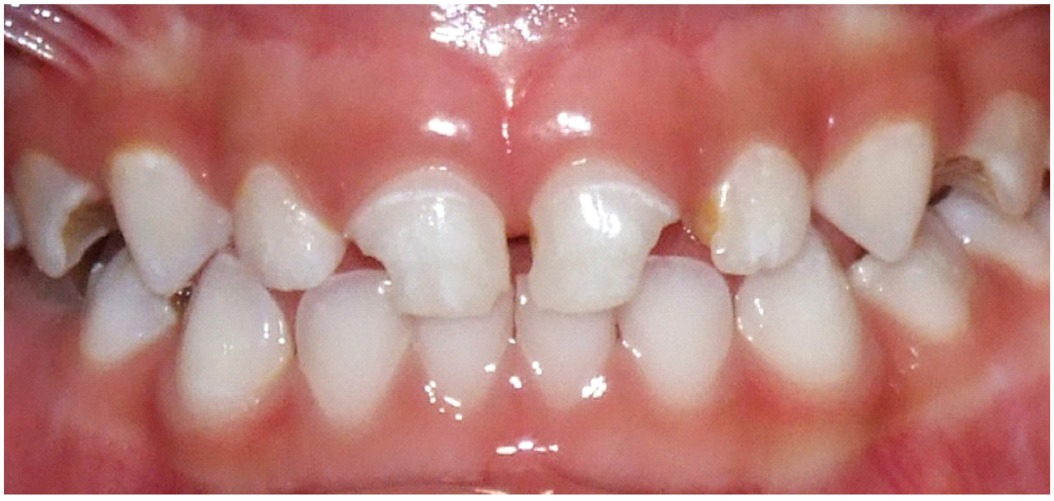
Nome da criança:_____Telefone:_____ Escola:_____

	_____/_____ _____- feira	_____/_____ _____- feira	_____/_____ _____- feira
Café da manhã			
Lanche da manhã			
Almoço			
Lanche da tarde			
Antes do Jantar			
Jantar			
Antes de dormir			
Madrugada			

Figura 1. Exame clínico para determinação do índice de cárie em pré-escolares de escolas públicas do município de Itatiba – SP (Capítulos 1, 2, 3 e 4)



A



B

Figura 2. Manifestação clínica inicial (A) e lesão já cavitada (B) da cárie precoce da infância. As manchas brancas ativas foram incluídas no critério de diagnóstico utilizado nessa tese (Capítulos 1, 2, 3 e 4

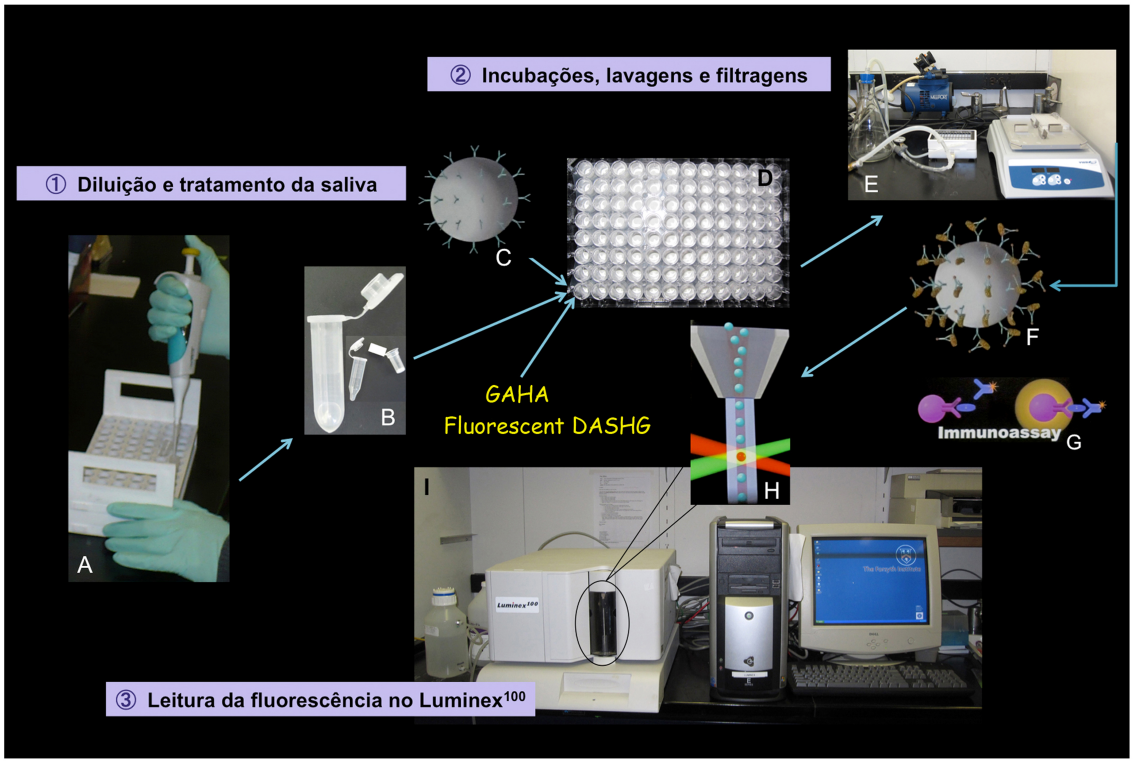


Figura 3. Metodologia empregada na análise imunológica da saliva (Capítulo 2)

A: Diluição da saliva com tampão fosfato salino contendo 1% de albumina sérica bovina, 0,05% de azida sódica e 2% de Prionex

B: Saliva diluída pronta a análise após remoção de mucinas. A remoção foi obtida por meio do uso de esponja de celulose acoplada a sistema de filtragem

C: Microesfera fluorescente conjugada com antígeno de *S. mutans*

D: Placas com 96 poços contendo filtro em sua base (Millipore). Nessa placa foram realizadas as incubações, as lavagem e as filtragens à vácuo

E: Sistema de filtragem a vácuo e *shaker* utilizado nas incubações

F: Anticorpos salivares (elipses marrons) ligados à microesfera fluorescente conjugada com antígeno de *S. mutans*

G: Representação das ligações: anticorpo salivar - microesfera fluorescente conjugada com antígeno de S. mutans - GAHA - DASHG

GAHA: Anticorpos de cabra anti-IgA humana; DASHG: Anticorpo de jumento anti-IgG de ovelha conjugado com o composto fluorescente R- Ficoeritrina

H: Laser vermelho e laser verde do sistema Luminex [100]. O laser vermelho identifica a microesfera e o laser verde mede a fluorescência decorrente das ligações antígeno-anticorpo

I: Sistema Luminex[100]

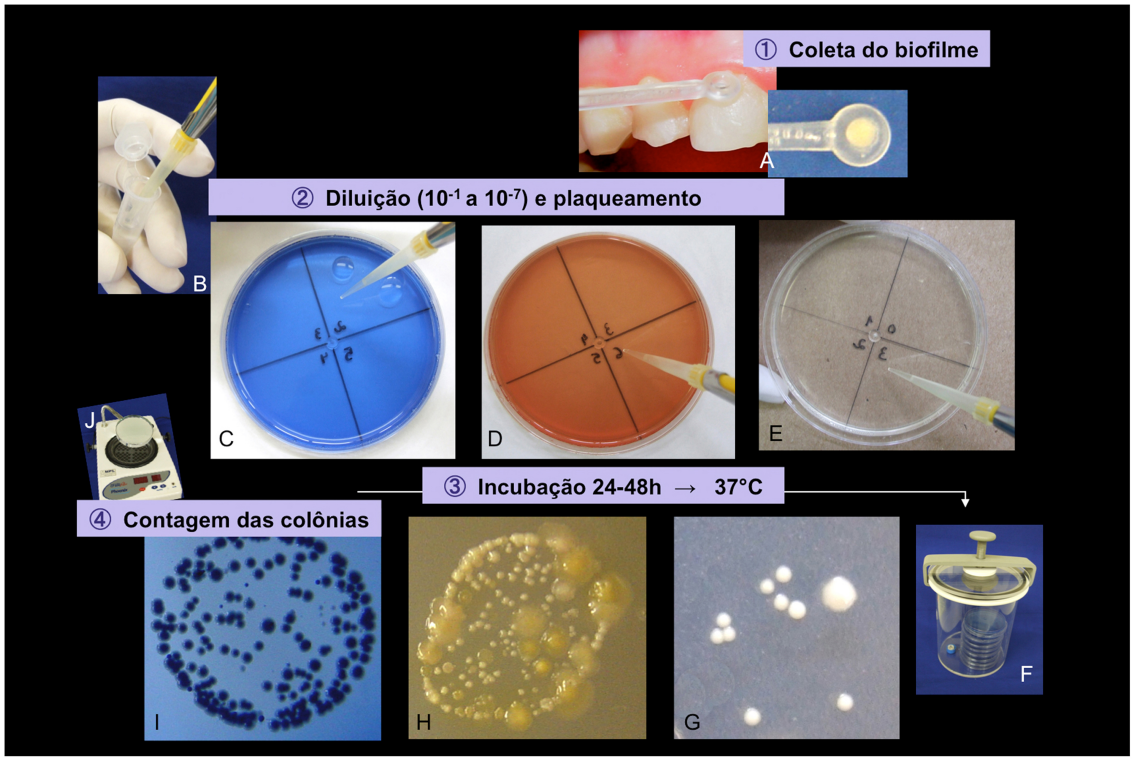


Figura 4. Metodologia empregada na análise microbiológica do biofilme (Capítulos 2 e 4)

A: Alça esterilizada utilizada para padronizar a quantidade de biofilme coletada

B: Diluição do biofilme dentário com solução salina 0,9%

C: Meio de cultura Mitis Salivarius + Bacitracina

D: Meio de cultura Ágar Sangue

E: Meio de cultura Rogosa

F: Jarra de anaerobiose

G: Unidades formadoras de colônias (UFC) de lactobacilos

H: UFC de microrganismos totais

I: UFC de estreptococos do grupo Mutans

J: Lupa esteroscópica utilizada para contagem de UFC

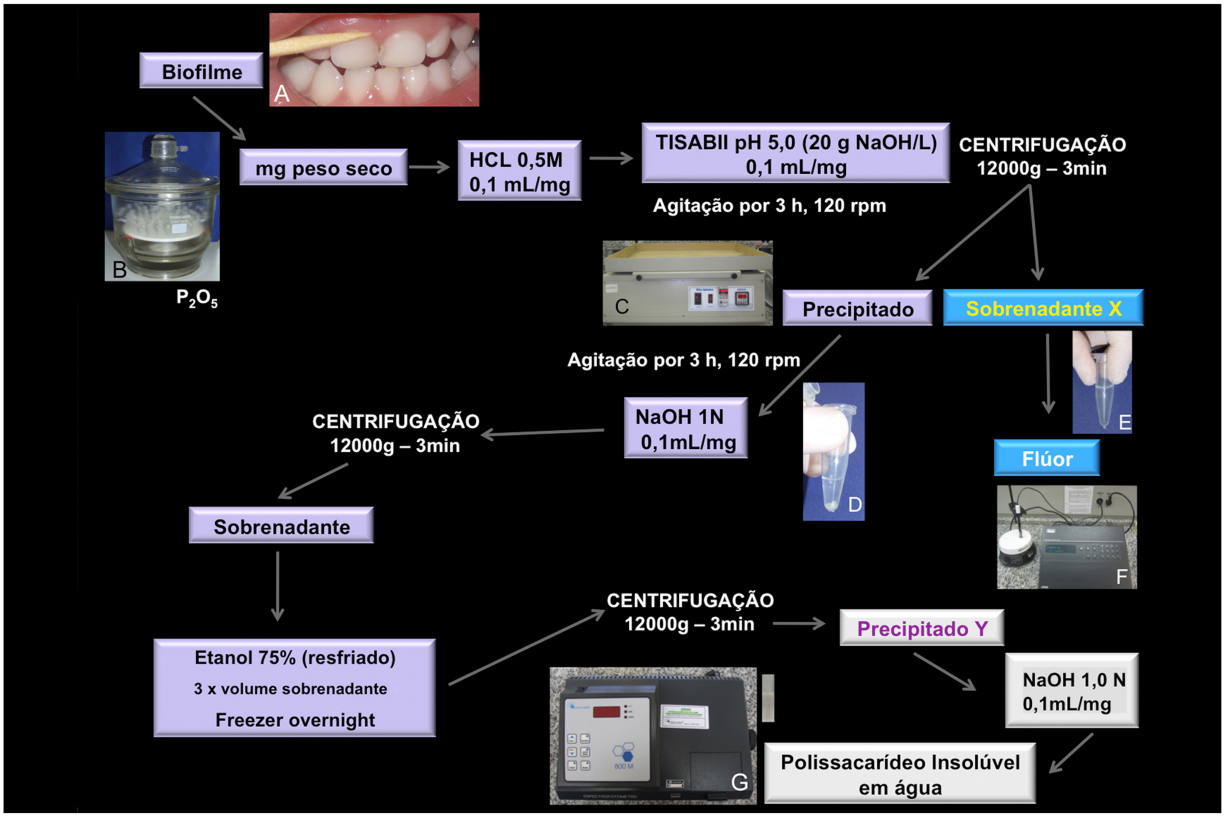


Figura 5. Metodologia empregada na análise bioquímica do biofilme (Capítulos 3 e 4)

A: Coleta do biofilme dentário com palitos de madeira

B: Dessacadora contendo P_2O_5 (pentóxido de fósforo) utilizada para secar o biofilme

C: Mesa agitadora

D: Precipitado em que foi adicionado NaOH 1N

E/Sobrenadante X: Sobrenadante utilizado para a dosagem de fluoreto

F: Eletrodo íon específico acoplado ao potenciômetro utilizados para dosagem de fluoreto

G: Espectrofotômetro e cubeta utilizados para a dosagem de polissacarídeos extracelulares insolúveis em água (PEIs) por colorimetria

Precipitado Y: precipitado ressuspenso em NAOH 1N para dosagem PECIs

Figura 6. Metodologia empregada na análise de produção de glucano pelas cepas de *Streptococcus mutans* do biofilme (Capítulo 4)

A: Retirada de uma alçada de colônias conservadas em Skin Milk

B: Estriamento na placa de Mitis Salivarius + Bacitracina

C: Jarra de anerobiose

D: Estufa

E: Termociclador

F: Cuba horizontal de eletroforese e fonte

G: Imagem do gel de agarose 2% após eletroforese: 1ª coluna marcador, 2ª controle negativo, 3ª controle positivo, 4ª amostra (colunas da esquerda para a direita)

H: cuba de eletroforese vertical e fonte

I: Imagem do gel de acrilamida 6% contendo bandas de glucano de diferentes intensidades. O glucano foi sintetizado pelas enzimas glucosiltransferases contidas no gel utilizando os açúcares contidos no tampão de incubação (fosfato de sódio 0.2M, pH 6.5, com dextrano 0.2% e sacarose 5%).