Natália Helena Colombo

FATORES MICROBIOLÓGICOS E IMUNOLÓGICOS ENVOLVIDOS NO DESENVOLVIMENTO DA CÁRIE PRECOCE DA INFÂNCIA

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FATORES MICROBIOLÓGICOS E IMUNOLÓGICOS ENVOLVIDOS NO DESENVOLVIMENTO DA CÁRIE PRECOCE DA INFÂNCIA

Tese apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, para obtenção do título de Doutor em Ciência Odontológica – Área de Concentração: Saúde Bucal da Criança.

Orientadora: Profa. Dra. Cristiane Duque

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Dedicatória

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“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas graças a Deus, não sou o que era antes”.

Marthin Luther King

Resumo geral

Colombo, NH. Fatores microbiológicos e imunológicos envolvidos no desenvolvimento da cárie precoce da infância. 2015. 101f. Tese (Doutorado em Ciência Odontológica) Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2015.

A cárie precoce da infância (CPI) é ainda um grave problema de saúde pública no mundo, principalmente em países em desenvolvimento. Estudos têm sugerido a associação da ingestão frequente de carboidratos fermentáveis como a sacarose, altas contagens de microrganismos cariogênicos e maior vulnerabilidade imunológica da criança na etiologia da CPI. O objetivo deste estudo foi avaliar os aspectos microbiológicos e imunológicos associados ao desenvolvimento da cárie precoce da infância. Crianças com idade entre 36 e 60 meses foram selecionadas e divididas em três grupos: LC - livres de cárie, CPI e CPI-S (CPI-severa). Questionário sobre os aspectos socioeconômico-culturais, hábitos de higiene bucal e diários de dieta foram respondidos pelos responsáveis. Foram coletadas amostras de saliva e biofilme dental das crianças e processadas para subsequentes avaliações laboratoriais. Em seguida, os níveis de IgA salivar total e contra GbpB de *S. mutans* foram determinados por ELISA e Western blot, respectivamente, as concentrações salivares dos peptídeos catiônicos antimicrobianos (PCAM): defensinas hBD-2 e hBD-3, catelicidina LL-37 e histatina 5 (HTN-5) por ELISA e a presença e os níveis salivares de *Streptococcus mutans, Streptococcus sobrinus*, *Lactobacillus* spp., *Bifidobacterium* spp. e *Scardovia wiggsiae* por qRT-PCR, sendo que estes dados foram correlacionados com os níveis salivares e no biofilme dental de estreptococos mutans (SM) e *Lactobacillus* spp. por meio de cultivo em meios específicos. Os resultados mostraram que as crianças com CPI-S apresentaram menor renda familiar quando comparadas às crianças LC ou CPI. Contudo, a ingestão de açúcar não diferiu entre os grupos. O grupo CPI-S apresentou maior contagem de SM na saliva/biofilme em relação aos grupos LC e CPI. Houve uma correlação positiva entre a resposta de IgA contra GbpB e os níveis de SM, quando a população geral foi avaliada. Quando apenas crianças com altos níveis de SM foram comparadas, o grupo CPI-S mostrou redução significante na resposta do IgA

contra GbpB em relação ao grupo LC. Esta alteração não foi observada para o grupo CPI. O presente estudo mostrou correlação positiva de hBD-2 e HTN-5 com os níveis salivares de SM. Além disso, houve uma correlação positiva entre hBD-2, LL-37 e HTN-5, sugerindo uma ação combinada desses peptídeos na proteção do organismo. Contudo, as concentrações salivares de cada PCAM não diferiram entre os grupos. Os resultados do qRT-PCR mostraram que a frequência de detecção de *S. mutans, Bifidobacterium* spp. e *S. wiggsiae* aumentou com a severidade da cárie. Os níveis de *S. mutans*, *S. sobrinus, S. wiggsiae* e *Bifidobacterium* spp. foram maiores no grupo CPI-S em relação aos grupos LC e CPI. Não houve diferença nos níveis de *Lactobacillus* spp. e em sua frequência de detecção entre os grupos. Este estudo sugere que crianças com CPI-S apresentam resposta imunológica reduzida representada pelos níveis de IgA contra GbpB de *S. mutans*, mas não relacionadas com as concentrações salivares dos PCAM. Em relação aos aspectos microbiológicos, conclui-se que, além de *S. mutans*, outras espécies bacterianas como *S. sobrinus, S. wiggsiae* e *Bifidobacterium* spp., estão associadas com a severidade da cárie na infância.

Palavras-chave: Cárie dentária. Sistema imunológico. Peptídeos catiônicos antimicrobianos. Bactérias. Scardovia wiggsiae.

General Abstract

Colombo, NH. Microbiological and immunological factors for the development of early childhood caries. 2015. 101f. Tese (Doutorado em Ciência Odontológica) Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2015.

Early childhood caries (ECC) is still a serious public health problem worldwide, especially in developing countries. Studies have been suggested the association among frequent intake of fermentable carbohydrates such as sucrose, high cariogenic microorganism's counts and child's immune vulnerability in the etiology of ECC. The objective of this study was to evaluate the microbiological and immunological factors for the development of early childhood caries. 36 to 60 month-old children were selected and distributed into three groups: caries free (CF), ECC and S-ECC (severe-ECC). Questionnaires about socio-economic-cultural data, oral hygiene habits and food-frequency diary were completed by the parents. Saliva and dental biofilm were collected from children and processed for subsequent laboratorial tests. The following analyses were determined: total IgA and IgA response against *S. mutans* GbpB by ELISA and Western blot, respectively; salivary concentrations of antimicrobial peptides (AMPs): defensins hBD-2 and hBD-3, cathelicidin LL-37 and histatin 5 (HTN-5) by ELISA; salivary detection and quantification of *Streptococcus mutans, Streptococcus sobrinus*, *Lactobacillus* spp., *Bifidobacterium* spp. and *Scardovia wiggsiae* by qRT-PCR, and these data were correlated with mutans streptococci (MS) and *Lactobacillus* spp. levels by culture in specific medium. Results showed that S-ECC children had reduced family income compared to ECC and CF. However, sugar intake did not differ among the groups. S-ECC group had higher MS count than CF/ECC groups. Positive correlations between salivary IgA response against GbpB and MS counts were found when the entire population was evaluated. When children with high mutans streptococci counts were compared, S-ECC group showed a significant decrease in IgA antibody levels against GbpB compared to CF group. This finding was not observed for ECC group. The present study showed positive correlations between salivary hBD-2 and HTN-5 with salivary mutans streptococci levels. In addition, results showed a positive correlation among hBD-2, LL-37 and HTN-5, suggesting a combined action of these peptides in the host

protection. However, salivary concentration of AMPs did not differ among the groups. The results of qRT-PCR showed that the frequency of *S. mutans, Bifidobacterium* spp. and *S. wiggsiae* detection increased with caries severity. The levels of *S. mutans*, *S. sobrinus, Bifidobacterium spp*. and *S. wiggsiae* were significantly higher in S-ECC children compared to CF and ECC children. There was no statistical difference among the groups considering the levels or frequency of *Lactobacillus* spp. This study suggested that children with S-ECC have reduced immunological response, represented by the salivary IgA levels against *S. mutans* GbpB, but not with salivary concentrations of AMPs. In relation to microbiological aspects, it was concluded that, in addition to *S. mutans*, other bacterial species such as *S. sobrinus*, *S. wiggsiae* and *Bifidobacterium* spp., are associated with the severity of early childhood caries.

Keywords: Dental caries. Immune system. Antimicrobial cationic peptides. Bacteria. Scardovia wiggsiae.

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Lista de Abreviaturas

AMPs: Antimicrobial peptides; Peptídeos catiônicos antimicrobianos

Ags: Antigens; Antígenos

BHI: Brain Heart Infusion; Infusão de Cérebro e Coração

ATCC: de American Type Culture Collection; Coleção Americana de tipos de cultura

CF: Caries Free; Livres de cárie

CFU: Colony-forming units; Unidades formadoras de colônia

dmfs: Decayed Missing Filled Surfaces; Superficies cariadas, perdidas ou restauradas

DNA: Deoxyribonucleic acid; Ácido desoxirribonucleico

ECC: Early childhood caries; Cárie precoce da infância

hBD1: Human β-defensin 1; β-defensina 1 humana

hBD-2: Human β-defensin 2; β-defensina 2 humana

hBD-3: Human β-defensin 3; β-defensina 3 humana

HMS – high mutans streptococci count; alta contagem de *estreptococos mutans*

hNP1-3: Human neutrophil defensins (α-defensin); α-defensina

HTN-5: Human-Histatin 5; Histatina 5 humana

GtfB: Glucosyltransferase B; Glicosiltransferase B

GtfC: Glucosyltransferase B; Glicosiltransferase C

GtfD: Glucosyltransferase B; Glicosiltransferase D

GbpA: Glucan-binding proteins A; Proteína ligante de glicano A

GbpB: Glucan-binding proteins B; Proteína ligante de glicano B

GbpC: Glucan-binding proteins C; Proteína ligante de glicano C

HMS: High *Mutans streptococci* count; Alta contagem de estreptococos mutans

IgA: Immunoglobulin A; Imunoglobulina A

KDa: Kilo Daltons; Quilo Daltons (unidade de massa atômica).

LL-37: Human cathelicidin LL-37; Catelicidina humana LL-37

LMS: Low *mutans streptococci* count; Baixa contagem de estreptococos mutans

MS: *mutans streptococci*

MSA: Mitis Salivarius Agar;

PCR: Polymerase Chain Reaction; Reação em cadeia da polimerase

qRT-PCR: Quantitative Real-Time PCR; PCR quantitativo em tempo real

S-ECC: Severe early childhood caries; Cárie severa da infância

Sumário

Introdução geral

A cárie dentária é uma doença infecciosa, causada por ácidos provenientes da fermentação microbiana dos carboidratos da dieta que, com o tempo, causam a desmineralização dos tecidos duros do dente (1). Quando ocorre em crianças com menos de 6 anos esta doença é chamada de cárie precoce da infância (CPI ou ECC do inglês *early childhood caries*). A Academia Americana de Odontopediatria (2008) (2) determina a severidade da cárie precoce da infância de acordo com idade da criança. Em crianças menores de 3 anos de idade, qualquer sinal de cárie em superfície dental lisa é indicativo de cárie severa da infância (CPI-S ou S-ECC). Entre os 3 e 5 anos de idade, uma ou mais superfícies lisas cavitadas, perdidas (devido à cárie) ou restauradas em dentes decíduos anteriores constitui CPI-S. Crianças apresentando ≥4 (aos 3 anos de idade), ≥5 (aos 4 anos de idade), ou ≥6 (aos 5 anos de idade) superfícies cariadas, perdidas ou restauradas também são classificadas com CPI-S.

A cárie precoce da infância continua sendo um grave problema de saúde pública no mundo, principalmente em países em desenvolvimento (3). No Brasil, a CPI apresenta prevalência entre 14,8% e 43,4% (4-6). Devido à rápida destruição dentária em curto período de tempo, tem sido sugerida a associação entre os seguintes fatores na etiologia da CPI: ingestão frequente de carboidratos fermentáveis como a sacarose, altas contagens de microrganismos cariogênicos e maior vulnerabilidade imunológica da criança (6-10).

O grupo bacteriano considerado mais cariogênico é o dos estreptococos mutans (SM), especialmente *Streptococcus mutans* (9, 10). Embora a associação entre *S. mutans* e CPI pareça convincente, grande percentual das crianças colonizadas por essa espécie bacteriana não manifesta a doença (6, 8). Assim, outras espécies acidogênicas e acidúricas, incluindo estreptococos não mutans e *Actinomyces,* estão envolvidas com o início das lesões de cárie (11, 12). Van Ruyven *et al.*(13) detectaram também outras espécies bacterianas, entre elas, *Lactobacillus* e *Bifidobacterium,* em biofilmes dentários cobrindo lesões de mancha branca. Tanner et al. (14) avaliaram por PCR as espécies mais frequentes na saliva de crianças com CPI e notaram alta prevalência de bactérias da família

Bifidobacteriaceae, entre elas, *Scardovia wiggsiae* ou ainda grande associação entre *Streptococcus mutans* e bifidobactérias.

Para se aderir à superfície dental, *Streptococcus mutans* sintetizam três tipos de proteínas: adesina Atg I/II; glucosiltransferases (Gtfs), e proteínas ligantes de glucano (Gbps A,B, C e D, referente à "glucan-binding proteins") (15). Diversos estudos têm demonstrado que a indução de anticorpos específicos contra esses antígenos proteicos pode prevenir o desenvolvimento de cárie em modelos animais (16, 17) e em humanos (18). Na cavidade bucal, as imunoglobulinas salivares, principalmente IgA secretória, apresentam grande importância na resistência da mucosa às infecções. Nogueira et al. (19) mostraram que a resposta de IgA contra GbpB esteve relacionada ao atraso na infecção bucal por *S. mutans*. A intensidade dos padrões de IgA aos antígenos de *S. mutans* foi estudada por Parisotto et al.(7) que verificaram baixos níveis de anticorpos IgA contra GbpB associados com alto risco de cárie. Entretanto, para crianças com padrões típicos de cárie severa da infância, os níveis de IgA contra esses antígenos ainda não foram avaliados .

O sistema imunológico apresenta diversas formas de defesa contra a microbiota. As mucosas, além de apresentarem a função de barreira física contra a entrada de organismos estranhos, são fonte de potentes peptídeos catiônicos antimicrobianos (PCAM). Os PCAM fazem parte da resposta imune inata, participando da primeira linha de defesa em vários locais do corpo (20). Na cavidade oral, PCAM estão presentes na saliva, epitélio gengival e fluido gengival (21) Alguns PCAM têm se destacado na literatura por sua atividade bactericida ou bacteriostática contra patógenos orais, entre eles estão as α- e β-defensinas, catelicidina humana LL-37 e histatinas (22).

As defensinas são peptídeos pequenos, de 15 a 45 aminoácidos, que dependendo do padrão de pareamento de seus resíduos de cisteína, são subdivididas em duas principais subfamílias: α e β-defensinas. Foram identificadas seis α-defensinas em humanos, sendo que quatro são produzidas pelos neutrófilos e denominadas de peptídeo neutrofílico humano (HNP-1 a 4) e as outras duas são produzidas por células de Paneth nas criptas intestinais. As β-defensinas (hBDs) são produzidas por células epiteliais de diversos órgãos como olhos, pele, pulmão, rim, pâncreas, mucosa nasal e oral e embora tenham sido encontradas quase 40 regiões gênicas potenciais para hBDs, as mais bem caracterizadas são HBD- 1 a -4. As α e β-defensinas apresentam função imunomoduladora, modificando a migração e

maturação celular, induzindo citocinas e a liberação de histamina e prostaglandina A2 de mastócitos (23-25).

O peptídeo catiônico humano (hCAP-18) é a única catelicidina identificada em seres humanos isolada primeiramente em grânulos de neutrófilos. hCAP-18 é produzida também por células epiteliais do pulmão, intestino, cavidade bucal e trato urogenital, sendo encontrada no plasma seminal e plasma sanguíneo. Após a secreção, ocorre a quebra de hCAP-18 pela ação de proteases em pequenos fragmentos de peptídeos RK-31 e KS-30 e em um peptídeo de cadeia longa LL-37, todos com ação antimicrobiana. Esse último fragmento do peptídeo hCAP-18, o LL-37, é um modulador multifuncional da imunidade inata, envolvendo a função antibacteriana, estímulo de angiogênese, cicatrização cutânea e quimiotaxia de células inflamatórias e do sistema imune. Esse fragmento de peptídeo causa a formação de poros na membrana das bactérias e a lise celular, entretanto, em altas concentrações (>13µM) pode se tóxica para as células eucarióticas (23, 24).

As histatinas são proteínas catiônicas ricas em histidina, produzidas pelas glândulas salivares, que apresentam ação bactericida e fungicida. Existem, pelo menos, 12 histatinas na saliva, como resultado de ligações ou proteólise das histatinas 1 e 3. O fragmento de 24 aminoácidos da porção N-terminal da histatina 3 é denominado de histatina 5. Esta é a histatina salivar com maior ação fungicida. As histatinas também podem inibir proteinases e prevenir a co-agreagação bacteriana (24).

Alguns estudos relacionaram a presença de PCAM e cárie na infância. Tao *et al* (26) observaram que baixos níveis de HNP1-3 podem representar um fator biológico para a susceptibilidade à cárie, pois foram detectadas maiores concentrações de HNP1-3 em crianças livres de cárie. Davidopoulou *et al.* (27) avaliaram os níveis de LL-37 em crianças com dentição decídua, mista e permanente com ou sem lesões de cáries e gengivite e verificaram que crianças com dentição decídua tiveram concentrações significantemente menores do peptídeo que aquelas na dentição mista e permanente. O mesmo foi verificado para crianças com alta atividade de cárie quando comparadas às crianças com baixa ou moderada atividade de cárie.

Capítulo 1

Relationship between the Immunoglobulin A Antibody Response to *Streptococcus mutans* Glucan-Binding Protein B and the Severity of Early Childhood Caries

Natália Helena Colombo[1], Jesse Augusto Pereira[1], Márjully Eduardo Rodrigues da Silva[1], Laís Fernanda Fonseca Ribas[1], Thaís Manzano Parisotto[2], Renata de Oliveira Mattos-Graner[3], Daniel J Smith[4], Cristiane Duque[1*]

[1] Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Univ Estadual Paulista (UNESP), Araçatuba, SP, Brazil

[2] Laboratory of Microbiology and Molecular Biology, Sao Francisco University Dental School, Bragança Paulista, SP, Brazil

[3] Department of Oral Diagnosis, Piracicaba Dental School, University of Campinas (UNICAMP), Piracicaba, SP, Brazil.

[4] Department of Immunology and Infectious Disease, Forsyth Institute, Cambridge, MA, USA.

Running head: IgA antibody response to *S. mutans* GbpB and caries

Keywords: IgA, Dental decay, Glucan-binding proteins, GbpB, Immunity

*Correspondence: Dr Cristiane Duque, Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Univ Estadual Paulista (UNESP)

Address: R. José Bonifácio, 1193, CEP: 16015-050, Araçatuba-SP, Brazil

Tel: (+55) 1836363315

E-mail: cristianeduque@yahoo.com.br

* According to guidelines for authors of Molecular Oral Microbioly (Anexo D).

SUMMARY

Lower levels of salivary IgA antibody against *S. mutans* GbpB have been associated with higher risk to develop dental caries. The objective of this study was to explore the associations between the severity of dental caries in childhood and putative etiological factors: socio-economic-cultural aspects, dietary habits, mutans streptococci (MS)/lactobacilli colonization and IgA antibody response against *S. mutans* GbpB. 36 to 60 month-old children were grouped into Caries-Free (n=19, CF), Early Childhood Caries (n=17, ECC) and Severe Early Childhood Caries (n=21, S-ECC). Questionnaires were completed by the parents, which assessed socio-economic-cultural data, oral hygiene habits and dietary patterns. Saliva was collected from children for microbiological analysis (MS and lactobacilli levels) by culture and for detection of salivary IgA antibody reactive with *S. mutans* GbpB in western blot. Results showed that S-ECC children had reduced family income compared to ECC and CF. There was a difference between CF/ECC and S-ECC children in MS counts. Positive correlations between salivary IgA antibody response against GbpB and MS counts were found when the entire population was evaluated. When children with high mutans streptococci counts were compared, the S-ECC group showed a significant decrease in IgA antibody levels to GbpB compared to the CF group. This finding was not observed with the ECC group. This study suggests that children with S-ECC have reduced salivary IgA immune responses to *S. mutans* GbpB, potentially compromising their ability to modify MS infection and its cariogenic potential. Furthermore, a reduced family income and high levels of SM was also associated with S-ECC.

INTRODUCTION

Dental caries is an infectious disease that results from the dissolution of tooth mineral by acids derived from bacterial fermentation of sucrose and other dietary carbohydrates (Loesche, 1996). When occurring in young children less than 71 months of age, this disease is called early childhood caries (ECC). ECC remains a serious worldwide public health problem, particularly in developing countries. According to the American Academy of Pediatric Dentistry (2008), from ages three through five, one or more cavitated, missing (due to caries) or filled smooth surfaces in primary maxillary anterior teeth or a decayed, missing or filled score of greater than or equal to four (age 3), five (age 4) or six (age 5) surfaces constitutes severe ECC (S-ECC). The disease can progress and lead to destruction of the primary dentition affecting negatively children's physical and mental health, as well as increasing the risk of new caries lesions in the permanent dentition (Ng & Chase, 2013, Isaksson *et al.*, 2013).

The mutans streptococci (MS) group, mainly *Streptococcus mutans*, is most strongly associated with the pathogenic process of ECC because of its high presence in the biofilms and saliva of the affected children (Parisotto *et al.*, 2010b, Ge *et al.*, 2008). Two important cell-associated antigens (Ags) are correlated directly with the ability of *S. mutans* to adhere and accumulate in the tooth surfaces forming dental biofilm: glucosyltransferases (GtfB, GtfC, GtfD) and glucan-binding proteins (GbpA, GbpB, GbpC) (Smith, 2002). Gtf catalyzes glucan synthesis and Gbps increase the binding of *S. mutans* to each other and to glucans deposited on tooth surfaces, contributing to the sucrose-dependent adherence to teeth (Smith, 2002). Several studies have demonstrated that induction of specific antibodies against these antigens can prevent the development of dental caries in animal models (Jespersgaard *et al.*, 1999, Koga *et al.*, 2002, Smith & Taubman, 1996) and modify infection in humans (Smith & Taubman, 1987).

Salivary immunoglobulins, particularly secretory IgA have major importance in the resistance of the mucosa to oral infections. The secretory IgA immune response represents the first line of adaptive immune defense against mutans streptococci, blocking microbial adhesins and potentially reducing oral colonization with this cariogenic microflora. Salivary IgA has also been shown to enhance the activity of several enzymes such as lactoferrins and lysozymes (Law *et al.*, 2007). A clinical

study showed that the IgA antibody response against *S. mutans* GbpB was predominant in the first year of life and frequently correlated with the delay in the oral infection with *S. mutans* (Nogueira *et al.*, 2005). (Parisotto *et al.*, 2011) studied the intensity of IgA patterns against antigens of *S. mutans* and found that lower levels of salivary IgA against GbpB were associated with higher caries risk. However, the relationship between IgA antibody levels against this antigen and different levels of caries has not yet been evaluated. The objective of this study was to explore the associations among the severity of ECC and caries-related etiological factors: socio-economic-cultural aspects, dietary habits, mutans streptococci/lactobacilli colonization and IgA response against *S. mutans* GbpB.

METHODS

Subjects

The study population included 36- to 60-month-old children who attended the four public nursery schools in the city of Araçatuba, São Paulo, Brazil (21° 12′ 32″ S, 50° 25′ 58″ W). The city´s population has access to public water supplied with fluoride to a level of 0.7 ppm. Children's parents as well as the preschools involved granted written permission for the study which had been previously approved by the Research Ethics Committee of Univ. Estadual Paulista (UNESP), Brazil (Certificate of Presentation for Ethical Consideration (CAAE) # 13079213.4.0000.5420). Seventy-five 36 to 60 month-old children were selected to participate in this study. Questionnaires were supplied to the parents to assess socio-economic-cultural data and oral hygiene habits. The socio-economic-cultural data analyzed were family income and mother's education. Dietary data were obtained from a food-frequency diary filled out by parents for three days during the week. Clinical examination was carried out at the school by a single calibrated examiner using mouth mirror and probe under natural light. Children were separated into three groups according to their caries status: Caries-Free group (CF), Early Childhood Caries group (ECC) and Severe Early Childhood Caries group (S-ECC). ECC was defined for this study as the presence of 1-3 decayed tooth surfaces (cavitated lesions) and S-ECC was defined as the presence of decayed surfaces score of ≥ 4 (age 3) and ≥ 5 (age 4) (American Academy of Pediatric Dentistry, 2008). Children suffering from systemic diseases, using long-term medication or antibiotics less than one month before examination, or

children with mucosal breaks were excluded from the study. Children with restored or absent teeth when saliva was collected for microbiological and immunological analysis were also excluded. Thus, the final number of children who participated in this study was fifty-seven, distributed in the following groups: 19 (CF), 17 (ECC) and 21 (S-ECC). Children were encouraged and instructed on dental hygiene and received all other necessary oral care.

Saliva samples

Unstimulated whole saliva was collected from each subject into a 50 mL sterile falcon conical tube for 5-10 min. Collections were performed at least 1 h after feeding to avoid contamination with non salivary components. Tubes were transported on ice to the laboratory and processed within 1 h. After agitation, one hundred microliters of saliva were separated for microbiological procedures. The remaining saliva was clarified by centrifugation at 10000 rpm at 4ºC for 10 min. The supernatants were collected and 250 mM EDTA was added to minimize salivary IgA aggregation. Aliquots of 50µl of each saliva samples were frozen at −70°C until immunological analysis.

Microbiological procedures

Aliquots of saliva were homogenized by vortexing for 1min and the suspensions were serially diluted (10^{-1} to 10^{-7}) in 0.9% saline solution. Each dilution was cultivated in triplicate on the surface of two selective media: Mitis Salivaris Agar (Difco Laboratories, Detroit, MI, USA) with sucrose and bacitracin for isolation of mutans streptococci and Rogosa agar (Oxoid, Basingstoke, Hampshire, England) for lactobacilli. All plates were incubated at 37°C for 48 h in 5% CO_2 atmosphere. After 48h of incubation, the total number of colony-forming units (CFU) was counted from a representative area of each agar plate yielding 30–300 colonies using a stereoscopic microscope. Results were expressed as CFU/ml.

Total salivary IgA level measurement

The concentration of total IgA in saliva samples were determined by an enzyme-linked immunosorbent assay (ELISA) kit using a commercially available analysis kit (Mabtech Inc, Cincinnati OH, USA) and following the manufacturer's instructions.

Western blot analysis of salivary antibody to *S. mutans* GbpB

In order to analyze the influence of patterns of specificity of IgA response to *S. mutans* GbpB, levels of infection, and caries status, Western blot assays were performed using saliva samples from children and tested against Ags extracted from a standard *S. mutans* strain (ATCC 25175). For Ags preparation, colonies of *S. mutans* from fresh Brain Heart Infusion Agar (BHI, Difco) were inoculated in 5ml BHI broth and incubated for 18h. Bacterial cells were then harvested from 1 ml of cultures previously adjusted to an absorbance of 1.0 (A550nm). Cells were ressuspended in TE containing 100-µm-diameter zirconia/silica beads and mechanically disrupted using a Mini-BeadBeater (BioSpec) at maximum speed (2,800 rpm) for 1-min pulses, three times, with a 30-s rest on ice between pulses. Cell pellets were then boiled in Laemmli buffer for 5 min, and protein extracts were separated by centrifugation at 4°C (10000 rpm for 4 min). Protein concentrations were determined by the method of Bradford and a total of 16 µg of protein extract was used for Western blot analysis (Nogueira *et al.*, 2005). Ags extracts were loaded per lane, separated by sodium dodecyl sulfate–6% polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. After transference, membranes were washed and blocked overnight at 4°C (in Tris-buffered saline–Tween, pH 7.5, 5% nonfat milk). Incubations with saliva samples diluted 1:100 were performed at room temperature for 2 h. As negative controls, membranes were incubated only with blocking buffer, and as positive controls, membranes were incubated with a standard saliva sample obtained from an adult subject whose pattern of reaction with *S. mutans* antigen extracts had been previously measured. The secondary antibody was HRP-Goat Anti-Human IgA (1:4000 dilution) (Invitrogen, Life Technologies, USA). Immunoreactive bands were detected by autoradiography using ECL chemiluminescent substrate reagent kit (Invitrogen, Life Technologies, USA) according to the manufacturer's instructions. X-ray films were scanned in a transluminator using a White Light Converter Plate (UVP, LLC, Upland, CA, USA) and the patterns of antigen recognition, including the number and intensity of reactive bands were analyzed with UVP Image software. The molecular weight of *S. mutans* GbpB was about 60 kDa. Migration position of GbpB were determined in parallel western blot assays performed with specific polyclonal rat antiserum to GbpB (Smith & Taubman, 1996).

Statistical analysis

The statistical analysis was performed using the three groups of children with dental caries (CF, ECC and S-ECC) as the dependent variables. The comparisons among the groups were performed according to data distribution. ANOVA and Tukey tests were applied for caries levels (dmfs), age and sugar intake. Kruskal-Wallis and Mann-Whitney tests were applied for gender comparison, family income, mother´s education level, mother's helping with tooth brushing, artificial (bottle) feeding, mutans streptococci/lactobacilli counts and total IgA levels. Medians and ranges of bacterial counts were expressed as log (CFU +1) and the constant 1 was added to CFU counts, when the sample showed zero CFUs. Pearson correlation tests were conducted to compare IgA levels against *S. mutans* GbpB and bacterial counts for the entire population. The reactivities of salivary IgA antibody with *S. mutans* GbpB were compared with respect to caries severity (CF, ECC and S-ECC) and mutans streptococci levels (low mutans streptococci - LMS and high mutans streptococci - HMS) using Mann-Whitney tests.

RESULTS

With respect to caries status, there were no statistical differences among the groups in relation to age, gender, mother´s education level, mother´s help with tooth brushing and diet habits (artificial feeding and sugar intake) and total IgA levels (Table 1). Families of S-ECC children had reduced income compared to families of ECC and CF children showing a relationship between high levels of caries and economic condition of family. The colonization with caries-associated microbiota in saliva of children demonstrated statistical difference between CF or ECC and S-ECC children only for mutans streptococci (MS) counts. S-ECC children were heavily colonized by MS. There was a gradual increase in the MS count with respect to the severity of the disease (Table 1). Considering the total population, positive correlations between salivary IgA levels to GbpB and MS counts were found (Figure 1). For these reasons, children were paired according to levels of MS within each group. Considering the mean of MS counts of the population, the groups of children (CF, ECC and S-ECC) were subdivided as follows: LMS - low mutans streptococci count (with MS counts ≤ log 4 CFU/ml) and HMS - high mutans streptococci count (with MS counts ≥ log 5 CFU/ml). Comparing LMS and HMS, there was no significant

difference among the groups in relation to total IgA levels (Table 2). The levels of IgA antibody reactive with GbpB were lower in S-ECC compared to ECC and CF children, only for HMS (Figure 2). IgA antibody levels to GbpB increased for all groups, when compared with the same groups of children (CF, ECC and S-ECC) with LMS and HMS counts (Figure 2). However, S-ECC children with high mutans streptococci counts showed a significant decrease in IgA antibody levels to GbpB compared to the caries free group with HMS. No statistically significant differences were observed comparing ECC with the other groups with HMS (Figure 2).

DISCUSSION

Early childhood caries has a complex etiology with biological, behavioral, and socioeconomic influences (Arora *et al.*, 2011, Ng & Chase, 2013). In this study, various factors determining oral health were analyzed. S-ECC children had a reduced family income compared to CF and ECC groups. This result is in accordance with the study of Oliveira *et al.* (2008) that demonstrated greater prevalence of dental caries in children with adverse socio-economic conditions and in children whose mothers had less than 8 years of education. However, our results showed no difference among groups in relation to mother's education level. These findings corroborated with the study of Parisotto *et al.* (2010a) that found no difference in mother's education level between CF and caries group. This lack of difference probably occurred because children were selected from the schools with similar social characteristics.

A strong association has been reported between high frequency of sugar exposure and occurrence of dental caries (Parisotto et al., 2010a, Kalsbeek & Verrips, 1994, Milgrom *et al.*, 2000). However, the present study did not find a significant difference in sugar exposure among the groups. Our results are according to Ohlund *et al.* (2007) who found that caries experience was not correlated with intake frequency or total intake of sugary foods. One possible explanation for this finding in the present study is that the responses from questionnaires may have reflected present, not historical experience, which would have better revealed habits during the period when caries had started.

Several previous studies found mutans streptococcus is a significant factor for the presence of ECC (Ohlund et al., 2007, Ge et al., 2008, Milgrom et al., 2000). Our

results showed that S-ECC children were highly colonized by mutans streptococci compared with CF and ECC children. Children with high level of *S. mutans* counts have 5 times more risk to develop dental caries than children with a lower level of these microorganisms (Milgrom et al., 2000). A recent systematic review and meta-analysis showed that there is scientific evidence of *S. mutans* transmission from mother to child especially when the mother is the primary caregiver (da Silva Bastos *et al.*, 2015). Kozai *et al.* (1999) showed both mother and father can be sources of *S. mutans* transmission. However, 18% of bacterial strains were from an unknown source. The American Academy of Pediatric Dentistry recommended the reduction of mutans streptococci levels of mothers, primary caregivers and sibling(s), educating them on avoiding saliva-sharing behaviors (e.g., sharing spoons and other utensils, sharing cups, cleaning a dropped pacifier or toy with their mouth), to decrease the child's risk of ECC (American Academy of Pediatric Dentistry, 2008).

The literature has shown that a better immune response to oral microorganisms, mainly *Streptococcus mutans,* may be a protective factor against the development of dental caries (Nogueira et al., 2005, Tao *et al.*, 2005, Davidopoulou *et al.*, 2012). This observation may be valid for both innate and adaptive immune responses. Reduced levels of some antimicrobial peptides, components of innate immune response, are associated with caries in childhood (Tao et al., 2005, Davidopoulou et al., 2012). The action of salivary IgA against specific surface proteins of cariogenic bacteria such as *S. mutans* has been the subject of many studies (Nogueira et al., 2005, Nogueira *et al.*, 2007, Parisotto et al., 2011), with focus on GbpB and Gtf expression. These proteins may be targets in the development of vaccines against dental caries (Smith & Taubman, 1996, Kim *et al.*, 2011). Smith & Taubman (1996) showed that the immunization of rats with GbpB induces an immune response that interferes with the accumulation of *S. mutans* and reduce the levels of dental caries. The caries protection resulting from immunization of rats with Gtf was lower than observed after immunization with *S. mutans* GbpB. The same study showed that saliva of sham-immunized/*S. mutans* infected rats contained antibody to GbpB in saliva at the end of the experiment, indicating that infection with *S. mutans* alone can induce an immune response to this antigen.

Furthermore, Parisotto et al. (2011) showed that preschoolers with a lower baseline level of salivary IgA antibody reactive with GbpB had 7.5 higher risk to develop caries, but the study did not find differences between groups CF and ECC in

relation to salivary IgA against *S. mutans* antigens (GbpB, Gtf). Our results are in accordance with this study, we did not find a difference in IgA antibody levels against GbpB between CF and ECC children, regardless of their mutans streptococci levels. We only found a difference in anti-GbpB antibodies between CF and S-ECC children (with HMS), suggesting that reduced levels of IgA against GbpB may be related to severity of dental caries. Although IgA levels against GbpB were lower in the S-ECC (HMS) group, total salivary IgA levels did not differ between groups. Thus, heavily-infected S-ECC children have a reduced immune response, and this immunological failure could have contributed to the severity of caries status. Bolton & Hlava (1982) demonstrated that salivary IgA levels to *S. mutans* antigens were higher in a caries-free group than in caries group, a difference that persisted in children from 3 to 11 years old. A recent study comparing specific IgA levels in three-year old children revealed increased concentrations of anti-*S.mutans* IgA and anti-*S. sanguinis* IgA in children who were culture positive for *S. mutans* compared with those who were culture negative (Malcolm *et al.*, 2014).

Nogueira et al., (2005) showed that salivary IgA response to GbpB was often associated with a delay in infection with *S. mutans*, and this response may occur during the first year of life. The same group found that children infected with *S. mutans* showed a delay in the immune response to the *S. mutans* GbpB antigen (Nogueira et al., 2007). These studies paired children according to mutans streptococci infection and they were divided in two groups: infected or non-infected children. In the present study, children were paired according to the mutans streptococci (MS) counts (CFU/ml) because a positive correlation was observed for this variable and IgA response to *S. mutans* GbpB, for the total population. It is expected that with the increasing of bacterial infection, higher host immune response to pathogen will occur (Nogueira et al., 2007).

The importance of GbpB for *S. mutans* viability has been studied by several investigators (Fujita *et al.*, 2007, Matsumoto-Nakano *et al.*, 2007, Duque *et al.*, 2011). Using a GbpB-deficient mutant strain, authors suggested that GbpB may have an important role in cell-wall construction, as well as in the cell separation and cell-wall maintenance in *S. mutans*, similar to murein hydrolases (Fujita *et al*., 2007). Furthermore, a GbpB-deficient mutant was more sensitive to acid pH in the acid killing assays (Matsumoto-Nakano et al., 2007), had decreased autolysis, increased cell hydrophobicity, and increased sensitivity to antibiotics and osmotic and oxidative

stresses (Duque *et al*., 2011). These functions, associated with binding of *S. mutans* to glucans deposited on tooth surfaces, highlight the important role of GbpB in the biofilm formation and survival of *S. mutans* in the oral cavity (Matsumoto-Nakano *et al*., 2007). Thus a good strategy to control dental caries could be the interference in the virulence factors of *S. mutans*, such as GbpB, by means the development of vaccines to reduce its cariogenicity (Smith & Mattos-Graner, 2008).

In conclusion, this study suggests that children with severe early childhood caries and high levels of mutans streptococci have reduced salivary IgA response to *S. mutans* GbpB showing that this parameter may influence the severity of caries status. Furthermore, a reduced family income and high levels of SM was associated with S-ECC.

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Table 1 Comparative analysis between the severity of early childhood caries and related etiological factors.

		CF	ECC	S-ECC	*p* value
Dmfs (mean±SD)		0[a]	2 ± 1.06[b]	23.43 ± 17.17[c]	**0.00**
Age (months) Mean±SD		46.26 ± 5.05[a]	45.94 ± 9.83[a]	48.10 ± 8.59[a]	0.664
Gender (%)	Female	47.37[A]	35.29[A]	52.38[A]	0.571
	Male	52.63	64.71	47.62	
Family income per month	≤ R$ 1448.00*	41.1[A]	25[B]	64.71[C]	**0.05**
Mother's education (%)	Up to 8 years	37.50[A]	35.71[A]	47.62[A]	0.737
Mother's help with tooth brushing (%)		82.35[A]	80.0[A]	65.0[A]	0.961
Bottle feeding (%)		82.36[A]	66.67[A]	85[A]	0.692
Sugar intake (mean±SD)	liquid	4.21 ± 1.37[a]	4.02 ± 1.18[a]	4.56 ± 1.38[a]	0.465
	solid	2.70 ± 0.90[a]	3.17 ± 1.43[a]	2.97 ± 1.40[a]	0.565
	total	6.88 ± 1.62[a]	7.19 ± 1.66[a]	7.53 ± 2.32[a]	0.579
mutans streptococci median (range)	(log UFC+1)	3.35 (0 - 7.29)[A]	3.74 (0 - 6.66)[A]	5.63 (3.15 - 8.3)[B]	**0.012**
total lactobacilli median(range)	(log UFC+1)	0 (0 - 6.79)[A]	3.30 (0 - 6.47)[A]	1.97 (0 - 6.72)[A]	0.447
Total IgA Median (range)		99.15(24.52-114.24)[A]	83.24 (32.27-114.56)[A]	88.04 (18.17-109.31)[A]	0.125

[a] Different lower case letters show statistical difference among the groups, according to ANOVA and Tukey tests.

[A] Different upper case letters show statistical difference among the groups, according to Kruskal-Wallis and Mann-Whitney tests.

CF – caries free, ECC – early childhood caries, S-ECC – severe early childhood caries

* R$ - Brazilian real. 1 US$ ~ R$ 2.70 (2015, January).

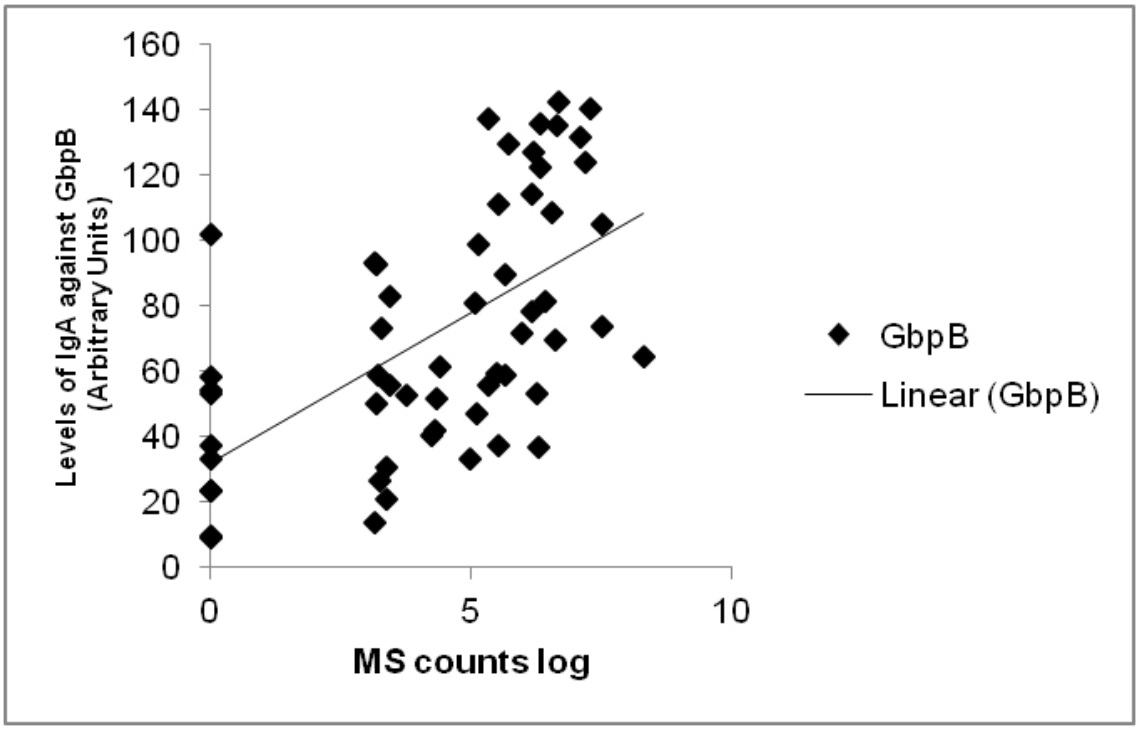

Fig. 1 Scatter plot showing correlation between levels of IgA antibody reactive with GbpB and mutans streptococci (MS). The analysis was carried out for the total population, regardless the caries status. Positive Pearson correlation (R=0.583, p=0.00) was observed for MS counts.

Table 2. Medians (range) of total salivary IgA, distributed according to mutans streptococci levels.

	MS levels*#		
	LMS	**HMS**	***p* value**
CF	101.9 (24.52-114.24)	91.05 (60.04-107.43)	0.375
ECC	81.93 (32.27-107.43)	84.55 (48.02-114.56)	0.341
S-ECC	98.92 (18.17-104.14)	84.10 (26.01-109.31)	0.357
***p* value**	0.380	0.243	

* There was no statistical difference among groups of children (columns), considering each MS levels separately (LMS or HMS), using Kruskal-Wallis tests.

There was no statistical difference inside each group of children (rows), comparing MS levels (LMS x HMS), using Mann-Whitney tests.

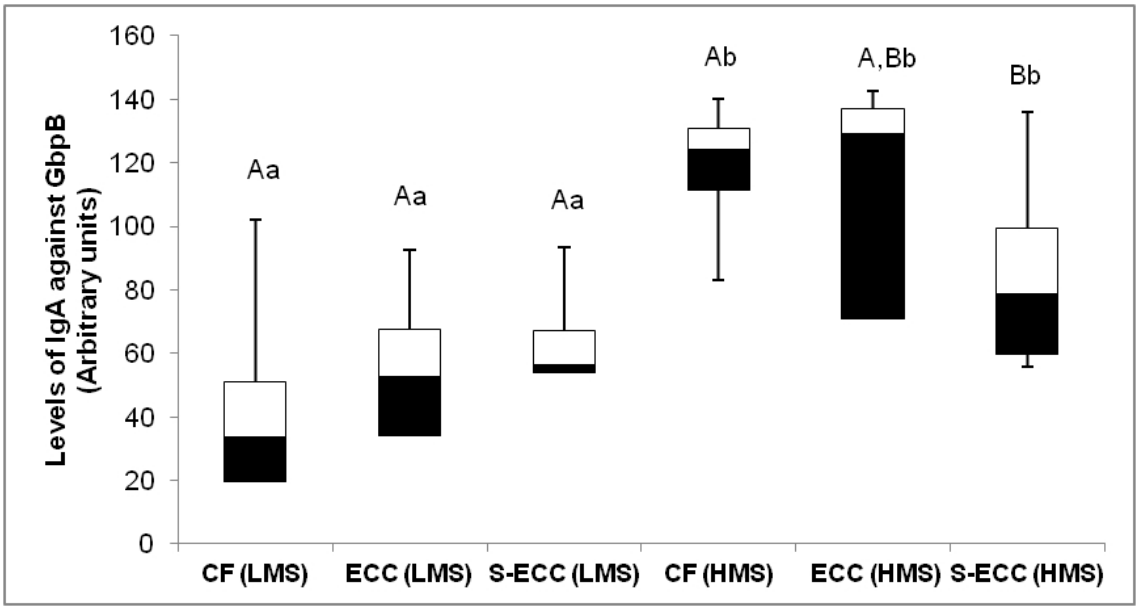

Fig. 2 Box plots of the western blot reactivity of salivary IgA antibody with *S. mutans* GbpB among the groups of children, distributed according to caries status (CF, ECC and S-ECC) and mutans streptococci levels (LMS and HMS). *Bars indicate minimum and maximum values. Black and white boxes indicate lower and upper quartiles, respectively. Line in the middle of boxes is median.*

[A] - Different upper case letters show statistical difference among groups of children considering each MS level separately (LMS or HMS), according to Mann-Whitney tests. For example: GbpB CF (LMS) x GbpB ECC (LMS)

[a] - Different lower case letters show statistical difference inside each group of children, comparing MS level (LMS x HMS), according to Mann-Whitney tests. For example: GbpB CF (LMS) x GbpB CF (HMS).

CF – caries free, ECC – early childhood caries, S-ECC – severe early childhood caries.

LMS- Low mutans streptococcus levels

HMS – High mutans streptococcus levels

Capítulo 2

Salivary levels of the antimicrobial peptides in children with severe early childhood caries

Natália H. Colombo[1],
Laís F. F. Ribas[1]
Jesse A. Pereira[1]
Paula F. Kreling[1]
Christine A. Kressirer[2,3]
Anne C. R.Tanner[2,3]
Cristiane Duque[1]*

[1] Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, University Estadual Paulista (UNESP), Araçatuba, SP, Brazil
[2] Department of Microbiology, The Forsyth Institute, Cambridge, MA, USA
[3] Harvard School of Dental Medicine, Harvard University, Boston, MA, USA

Running head: Antimicrobial peptides and severe early childhood caries

Correspondence: Dr. Cristiane Duque, Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Univ Estadual Paulista (UNESP)
Address: R. José Bonifácio, 1193, CEP: 16015-050, Araçatuba-SP, Brazil
Tel: (+55) 1836363315
E-mail: cristianeduque@yahoo.com.br, cduque@foa.unesp.br

* According to guidelines for authors of Archives of Oral Biology (Anexo E).

Abstract

Objective: Controversies are still exist regard the relationship between the concentrations of some antimicrobial peptides (AMPs) and presence of dental caries in children. Thus, the aim of this study was to examine AMPs levels in saliva of caries-free (CF), early childhood caries (ECC) and severe early childhood caries (S-ECC) children, in order to determine if the levels of these salivary peptides isolated or combined could be related to caries severity and mutans streptococci levels.

Design: 36 to 60 month-old children were selected to participate in this study. Children were grouped into CF group (n=29), ECC group (n=25) and S-ECC group (n=29). Saliva was collected from children for microbiological analysis by culture. Salivary concentration of cathelicidin LL-37, Human β-defensin 2 (hBD-2), Human β-defensin 3 (hBD-3) and Human-Histatin 5 (HTN-5) were determined by ELISA.

Results: Salivary concentration of AMPs did not differ among CF, ECC and S-ECC groups. The present study showed positive correlations between mutans streptococci levels and salivary hBD-2 or HTN-5. Positive correlation also was found among hBD-2, hBD-3, LL-37 and HTN-5. Combinations among AMPs, mainly with LL-37, were positively correlated with caries levels.

Conclusions: Salivary concentrations of AMPs separately are not associated with the severity of early childhood caries. The stimulus of caries can trigger a biological response with a combined action of these peptides.

Keywords: human cathelicidin LL-37, defensins, histatin-5, children, innate immunity, dental caries

Introduction

Antimicrobial peptides (AMPs) constitute an important class of molecules belonging to the innate immune system, which participates in the first line defence reactions at various sites of the human body (1). AMPs are produced by epithelial tissues, phagocytic cells (2), parotid gland, gingival and lateral tongue tissue (3) and exhibit a broad spectrum of activity against bacteria, fungi, and viruses as well as chemotactic activities and induction of cytokine release (1). In the oral cavity, AMPs are present in saliva, gingival epithelia and gingival crevicular fluid (4). Among AMPs, cationic peptides, especially α- and β-defensins, human cathelicidin LL-37 and histatins, have been highlighted in the literature by their typical bactericidal and /or bacteriostatic activities against oral pathogens (5).

Defensins (α- and β) are peptides with three disulfide bonds which differ from each other in the spacing and the pairing of the cysteine residues. Alpha-defensins are expressed in neutrophils and have been commonly identified in the gingival crevicular fluid. Beta-defensins (hBD1, hBD-2 and hBD-3) are expressed in epithelial cells of the oral cavity and are found in the gingival crevicular fluid and in the saliva. LL-37 is a long cationic alpha-helical peptide from human cathelicidin CAP18, expressed in neutrophils and epithelial cells and consequently present in saliva and gingival crevicular fluid. Histatins are expressed in salivary glands and found in saliva. Histatin 5 is derived from histatin 3 (1, 4, 5). Histatin 5 showed potent inhibition against *C. albicans* growth and bacterial co-aggregation (6). Ouhara et al.(7) evaluated antimicrobial activity of defensins and LL-37 and found that six Gram-positive bacteria, oral streptococci and *L. casei*, showed similar susceptibility to these peptides. Except for hBD1, all peptides demonstrated nearly 100% bactericidal activity with concentrations less than 10 mg/l.

Some authors have suggested that the reduced concentration of some peptides could be associated with the presence of caries in children (8-10). The level of α-defensin (HNP1-3) was shown to be lower in saliva from caries-active children compared to caries-free children (8). The same was observed for the salivary levels of LL-37 in another study (9). Although the antimicrobial properties of β-defensins are proven, the literature does not show changes in the salivary concentrations of these peptides in caries-active compared to caries-free children (10). No studies were found revealing the salivary concentrations of histatin-5 in children yet.

Dental caries is an infectious disease that cause the dissolution of tooth mineral by acids derived from bacterial fermentation of dietary carbohydrates (11). When tooth decay affects children less than 71 months this disease is called early childhood caries (ECC) (12). If the disease progression is not interrupted, it may cause the destruction of several deciduous teeth, denominated as severe ECC (S-ECC), resulting in local, systemic, psychological and social consequences (13).

Limited information can be found about the salivary antimicrobial peptides concentration in children and most of them evaluated children with mixed or permanent dentition. Since the salivary level of AMPs may contribute to caries susceptibility, these peptides could be a new and useful measure of the risk for caries in children. Thus, we aimed to examine the levels of hBD-2, hBD-3, LL-37 and HTN-5 in saliva of caries-free, ECC and S-ECC children, in order to determine if the levels of these salivary peptides could be related to caries severity. Furthermore, this study aimed to explore the associations among the severity of ECC and caries-related etiological factors: socio-economic-cultural aspects, dietary habits, mutans streptococci/lactobacilli colonization.

Material and methods

Subjects

The study population included 36- to 60-month-old children who attended the four public nursery schools in the city of Araçatuba, São Paulo, Brazil. The city´s population has access to public water supply with fluoride level of 0.7 ppm. Children's parents as well as the preschools involved granted written permission for the study which was previously approved by the Research Ethics Committee of Univ. Estadual Paulista (UNESP), Brazil (Certificate of Presentation for Ethical Consideration (CAAE) # 13079213.4.0000.5420). Questionnaires were applied to the parents to assess socio-economic-cultural data and oral hygiene habits. The socio-economic-cultural data analyzed were family income and mother's education. Dietary data were obtained from a food-frequency diary filled out for three consecutive days during the workweek. The diet chart was filled during the workweek because in the weekend the diet can be modified. In addition to the diet data reported by parents, the preschools informed infant feeding in the school period, and many of them were in school full time (14). Clinical examination was carried out at the school by a single calibrated

examiner using mouth mirror and probe under natural light. Children suffering from systemic diseases, or using long-term medication or antibiotics less than one month before the examination and children with mucosal breaks were excluded from the study. Children with only presented filling or absent teeth were also excluded. Initially ninety children from both genders, 36-60 month-age were selected to participate to this study. Seven of the selected children did not attend for saliva collection. So, eighty-three children were divided into three groups according to oral health: caries-free group (CF) (n=29), early childhood caries group (ECC) (n=25) and severe early childhood caries group (S-ECC) (n=29). ECC was defined for this study as the presence of 1 through 3 decayed tooth surface (cavitated lesions), S-ECC was defined as the presence of decayed surfaces score of ≥ 4 (age 3 years), ≥ 5 (age 4 years), must also have at least one smooth-surface caries surface (15). Children were encouraged and instructed on dental hygiene and received all other necessary oral care.

Saliva samples

Unstimulated whole saliva was collected from each subject into a 50-ml sterile falcon conical tube for 5-10 min. Collections were performed at least 1 h after feeding to avoid contamination with no salivary components. Tubes were transported on ice to laboratory and processed within 1 h. After agitation, one hundred microliter of saliva was separated for microbiological procedures. The remaining saliva was clarified by centrifugation at 10000 rpm at 4^{o}C for 10 min. The supernatants were collected and 250 mM EDTA was added to minimize salivary protein aggregation. Aliquots of 100µl of each saliva samples were frozen at −70°C until immunological use. The aliquots was frozen at −70°C until use for antimicrobial peptides level measurement by ELISA

Microbiological procedures

Aliquots of saliva were homogenized by vortexing for 1min and the suspensions were serially diluted (10^{-1} to 10^{-7}) in 0.9% saline solution. Each dilution was cultivated in triplicate on the surface of two selective media: Mitis Salivaris Agar (Difco Laboratories, Detroit, MI, USA) with sucrose and bacitracin for isolation of mutans streptococci and Rogosa agar (Oxoid, Basingstoke, Hampshire, England) for lactobacilli. All plates were incubated at 37°C for 48 h in 5% CO_2 atmosphere. After 48h of incubation, the total number of colony-forming units (CFU) was counted from a

representative area of each agar plate yielding 30–300 colonies using a stereoscopic microscope and the results were expressed as CFU/ml.

Antimicrobial Peptides Level Measurement

The concentration of antimicrobial peptides was determined by enzyme-linked immunosorbent assay (ELISA) using specific kits and according the manufacturer's instructions. LL-37, Human β-defensin 2 (hBD-2) and Human histatin-5 (HTN-5) were purchased from MyBioSource Inc. (San Diego, CA, USA) and Human β-defensin 3 (hBD-3) from Assay Biotechnology Company Inc. (Sunnyvale, CA, USA).

Statistical analysis

The statistical analysis was performed considering three groups of children according to dental caries status (CF, ECC and S-ECC) as the dependent variables. The comparisons among the groups were performed according to data distribution. ANOVA/Tukey tests were applied for caries levels (dmfs), age, sugar intake. Kruskal-Wallis/Mann-Whitey tests were applied for gender, mother´s education level, adult's help with tooth brushing, family income, breastfeeding, bottle feeding, mutans streptococci/lactobacilli counts and salivary levels of AMPs. Data were tested using Pearson or Spearman correlation tests.

RESULTS

The results did not show gender difference among the groups of children (Table 1) and neither gender difference in relation to the concentrations of salivary AMPs (Kruskal-Wallis test, $p = 0.393$). S-ECC presented a reduced family income compared to CF children. However, in this present study no significant difference in maternal education was found between groups. Sugar intake did not differ among the groups, but ECC and S-ECC children were breastfed for longer than CF children (Table 1).

LL-37, hBD-2, hBD-3 and HTN-5 were detected in all saliva samples. Salivary concentration of AMPs does not differ among CF, ECC and S-ECC groups (Figure 1). The present study showed positive correlations between salivary hBD-2 and HTN-5 with salivary MS levels and there was no relationship between MS levels and other AMPs tested (Figure 2). Lactobacilli counts could not be correlated with AMP levels

because of high number of children with counting zero. Other positive correlation was found between dmfs and LL-37 and dmfs and hBD-2 (Table 2). A combination among hBD-2, hBD-3, LL-37 and HTN-5 was correlated with dmfs too (Table 2). Positive correlation also was found among hBD-2, hBD-3 and LL-37. HTN-5 was only correlated with LL-37 and hBD-2 (Figure 3).

DISCUSSION

The present study shows that there was positive correlation between hBD-2 and HTN-5 with salivary mutans streptococci levels (Figure 2). Other positive correlation found was between hBD-2 and LL-37 levels and dmfs. This correlation was not found for hBD-3 and HTN-5 (Table 2). These results confirmed that innate immune system of children, represented by antimicrobial peptides, reacted to *S. mutans* aggression and their production is increased in the presence of dental caries. In addition, results showed a positive correlation between among hBD-2, hBD-3, LL-37 and HTN-5 (except for the combination of HTN-5 and hBD-3), suggesting a combined action of these peptides in host protection. Malcolm et al. (16) showed that salivary concentrations of AMPs (hNP1–3 and LL-37) in 3-year-old children were positively correlated with detection of *S. mutans* by qPCR. Although the present study has shown that there was a positive correlation between some AMPs e dmfs, the results demonstrated that salivary concentration of any AMP studied differ among CF group, ECC group and S-ECC group. Our results are in agreement with the study of Tao et al.(8) and Phattarataratip et al. (10). Tao et al. (8) studied children between 11 and 15 years of age and showed no significant differences in LL-37 and hBD-3 levels in saliva of caries-free compared to caries-active subjects. Phattarataratip et al.(10) studied children with 13 years old and did not find statistically significant differences between salivary levels of LL-37, hBD-2, hBD-3 and hNP1-3 between caries-free and caries-active subjects. In contrast, there are studies showing that salivary concentration of hNP1-3 (8) and LL-37 (9) are lower in saliva from caries-active compared to caries-free children.

Few studies have associated salivary peptides and caries experience and the majority of the available information in the literature refers to older children with mixed and permanent dentition (8-10). A recent study compared AMPs and bacteria-specific IgA levels in younger children (1-3 years old) and revealed higher

concentrations of hNPs 1–3, LL-37, anti- *S. mutans* IgA and anti-*S. sanguinis* IgA in children who had positive culture for mutans streptococci compared with those who had negative culture (16). In this present study, few children had negative culture for mutans streptococci making impossible the statistical analysis of the concentration of AMPs over this variable.

Ribeiro et al. (17) evaluated protein composition of saliva from caries free children and children with caries experience (10-71 months age). Identification of molecular masses (chromatograms) suggested the presence of nine peptides, but only three of them were related to caries experience. The suggestive presence of α-defensin-3 and β-defensin-3 reduced the chances of caries experience, and the presence of proline-rich peptides IB-4 expressed a positive association with dental caries. The methodology (chromatograms) used in aforementioned study does not provide the concentrations of salivary peptides, only reveals its presence or absence.

This present study provides the first investigation of salivary concentration of histatin 5 in children. The results showed that the salivary concentration of AMP in children is lower compared to that found in adults in the literature (18, 19). This difference was expected since the immune system is immature in young children. Johnson and co-workers (18) analyzed adults and elderly people and showed significant age-associated decrease for salivary histatin concentration. The authors suggested that the salivary concentration of histatin is compromised with the increasing of the age. Histatins are best known for their antifungal activity and the most of studies have concentrated on this aspect. Histatin 5 showed potent antifungal properties including inhibition of growth of *C. albicans* (6). *Candida albicans* is not only involved in fungal infections such as candidiasis. Several studies have demonstrated that this yeast presented a cariogenic potential (20-22). There is a significant association between the presence of *C. albicans* and early childhood caries. Carvalho et al. (23) showed that *C. albicans* was significantly more prevalent in children with early childhood caries than in caries-free group. Considering this information and anti-*Candida* activity presented by Histatin-5 we expected that the concentration of this peptide could be reduced in children with early childhood caries. However, no difference among the groups was identified. These data corroborate with the study of Dodds et al. (24) that evaluated a population of caries-active and caries-free young adults and no difference between the groups was found considering the histatin concentration from parotid saliva. In contrast, a recent study

showed increased levels of salivary histatin 5 in young adults (18 years old) with high caries activity compared to subjects with low caries activity (25)

The literature has showed that caries activity may not be associated with salivary AMPs concentration, but associated with virulence characteristics of *S. mutans* strains identified in caries active children and to be more resistant to AMPs. *S. mutans* strains isolated from caries-active subjects showed greater resistance to salivary hNP-1-2, hBD-2, hBD-3 and LL-37 than those from caries-free subjects (10). Furthermore, *S. mutans* strains isolated from children with S-ECC have higher sucrose-dependent adhesion, water-insoluble glucan synthesis and gene expression levels of *gtfB* and *gtfC* than *S. mutans* strains isolated from caries-free children (26). These results showed that *S. mutans* strains from children with S-ECC can be more virulent than those found in caries-free children.

Early childhood caries is multifactorial disease caused by oral bacteria and influenced by various social and behavioral factors (27-29). In the present study S-ECC group presented a reduced family income compared to CF group. This result is in accordance with study of Oliveira *et al* (30) and Corrêa-Faria *et al* (29) that demonstrated higher prevalence of dental caries in children with low-income family. Oliveira *et al* (30) also reported that children whose mothers had less than 8 years of education presented the highest prevalence of dental caries. However, in this present study no significant difference in maternal education was found between groups.

High frequency of sugar exposure is a dietary habit strongly associated with dental caries (14, 31, 32). However, the present study did not found significant difference in sugar intake among the groups. Our results corroborate with the results obtained by Köhler *et al* (33) who demonstrated that caries experience was not correlated with sugar exposure. In the present study, the lack of difference among the groups in relation to sugar intake may be related to the time that the questionnaire was applied to the parents. The answers of parents about sugar intake reflected the present time, not historical experience, which would have better revealed habits during the period when caries had started. Moreover, information obtained from parents may be biased. Parents of children with ECC, who have some knowledge about the risk of sugary foods consumption in the development of dental caries, may not be ready to admit that they practiced habits that can increase the caries risk.

Despite eating habits are not different among the groups at the present, the question about breastfeeding habits revealed that ECC and S-ECC children were breastfed for longer than CF children. No statistical difference among the groups was observed for the bottle feeding time. This result provides evidence of the association of prolonged breastfeeding and early childhood caries. Our results are in accordance with study of Azevedo *et al*. (34) that demonstrated that breastfeeding after 1 year of age was associated with ECC. Kato *et al* (35), conducted a longitudinal study with more than 43,000 babies and found association between breastfeeding for at least 6 or 7 months and risk of dental caries at age 30 months. Prolonged breastfeeding may be associated with ECC because many mothers know the benefits of breast milk, such as the presence of antibodies (secretory IgA) for baby protection against a variety of diseases, but they often unaware that it also may cause tooth decay, and thus neglect oral hygiene of their babies after breastfeeding.

Our results showed that S-ECC children had higher mutans streptococci levels in saliva and biofilm in relation CF and ECC children. High levels of mutans streptococci (MS) in the saliva of children, especially *S. mutans,* are considered a significant risk factor for the presence of ECC (32, 33, 36). Children with a high level of MS have 5 times more risk to develop dental caries than children with a lower level (32). The prediction of caries risk has been of long-standing interest and is very important for development of new strategies for caries prevention. This is especially significant for young children. In this present study, among caries-related etiological factors analyzed, a reduced family income and prolonged period of breastfeeding were associated with S-ECC. However, our results do not support the measurement of salivary levels of LL-37, hBD-2, hBD-3 and Histatin 5 as a reliable tool to predict caries risk. Thus, salivary concentrations of these AMPs are not associated with severity of early childhood caries.

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Table 1. Comparative analysis between the severity of early childhood caries and related etiological factors.

		CF	ECC	S-ECC	*p* value
Dmfs (mean±SD)		0[a]	2.36 ± 0.95[b]	19.34 ± 14.97[c]	**0,000**
Dmfs + white spot (mean±SD)		0[a]	2.76 ± 1.23[b]	24.41 ± 17.94[c]	**0,000**
Age in months (mean±SD)		48.31 ± 8.59	49.6 ± 7.78	50.21 ± 9.93	0,664
Gender (%)	Female	55.2	44	51.7	0,593
	Male	44.8	56	48.3	0,593
Family income per month (%)	< R$ 1448.00*	59.3[A]	82.6[A]	92.6[B]	**0.016**
Mother's education (%)	Up to 8 year	20.7	24	31	0,516
Adult's help with tooth brushing (%)		72.4	68	69	0,851
Sugar exposure (mean±SD)	Liquid	4.4 ± 1.62	5.12 ± 3.04	4.96 ± 1.56	0,729
	Solid	3.71 ± 1.34	3.64 ± 1.33	3.27 ± 1.80	0,227
	Total	8.12 ± 2.20	8.76 ± 3.01	8.23 ± 2.30	0,534
Breast feeding (%)		79.3	88	96.4	0,169
Bottle feeding (%)		96.6	96	92.9	0,524
Breastfeeding duration in months (mean±SD)		5.62 ± 6.68[A]	13.68 ± 15.77[B]	17.21 ± 12.80[B]	**0,000**
Bottle feeding duration in months (mean±SD)		37.9 ± 15.96	31.67 ± 16.72	31.46 ± 16.77	0,178
Bottle early age (mean±SD)		5.69 ± 5.52[A]	10.33 ± 9.83[B]	13.00 ± 10.03[B]	**0,015**
Mutans streptococci count (log CFU+1) median (range)	saliva	4.08 (0 - 6.62)[A]	4.05 (0 - 6.41)[A]	5.08 (0 - 8.30)[B]	**0,034**
	biofilm	4.08 (0 - 6.23)[A]	4.08 (0 - 6.10)[A]	4.71 (0 - 6.72)[B]	**0,026**
Lactobaccili count (log CFU+1) median (range)	saliva	0 (0 - 7.08)	3 (0 - 6.47)	3.7 (0 - 6.88)	0,335
	biofilm	0 (0 - 5.29)	0 (0 - 7.05)	0 (0 - 6.33)	0,887

[a] Different lower case letters show statistical difference (p<0,05) among the groups, according to ANOVA and Tukey tests.
[A] Different upper case letters show statistical difference (p<0,05) among the groups, according to Kruskal-Wallis and Mann-Whitney tests.
CF – caries free, ECC – early childhood caries, S-ECC – severe early childhood caries
* R$ - Brazilian real. 1 US$ ~ R$ 2.70 (2015, January).

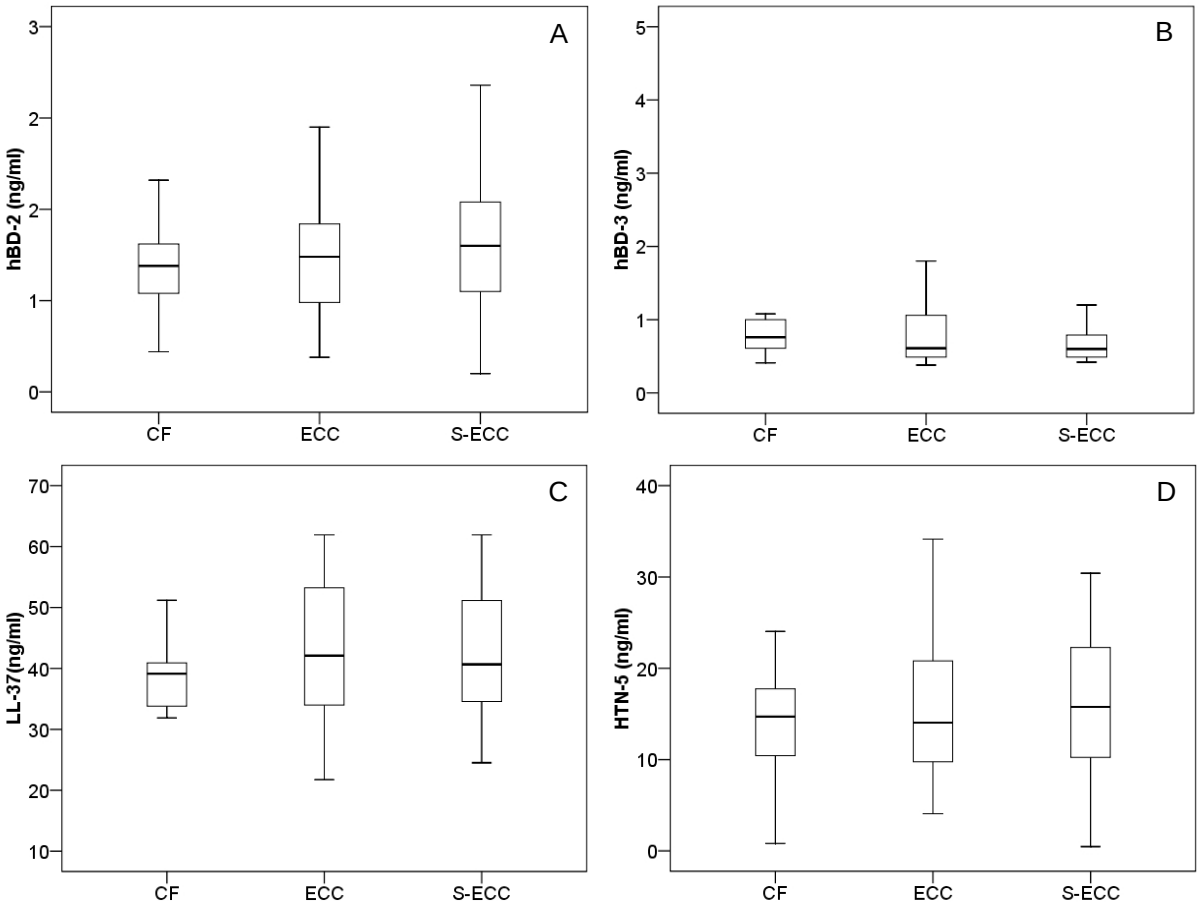

Figure 1. Levels of antimicrobial peptides (hBD-2, hBD-3, LL-37 and HTN-5) detected in saliva samples of children.

*There was no statistical difference ($p>0.05$) among the groups, considering each AMP separately, according to Kruskal-Wallis tests.

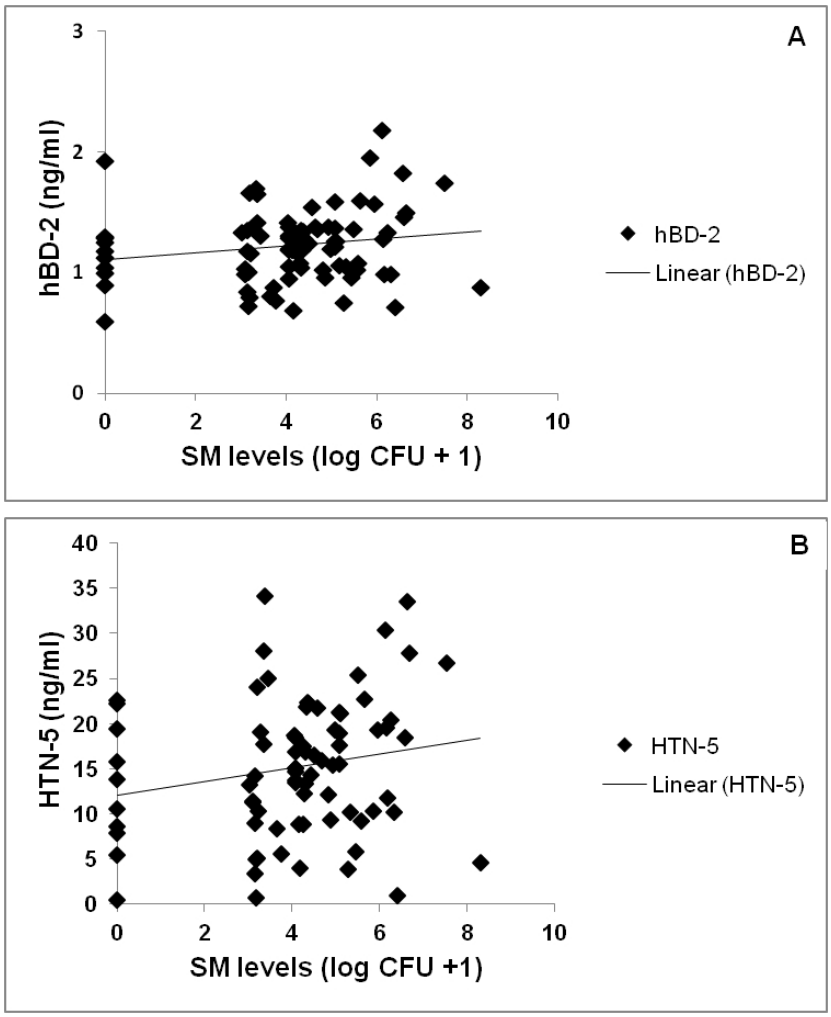

Figure 2. Only significant correlations are shown. A. Relationship between hBD-2 and salivary mutans streptococci levels (Spearman correlation, r= 0.228, p=0.043). B. Relationship between HTN-5 and salivary mutans streptococci levels (Spearman correlation, r= 0.235, p=0.039).

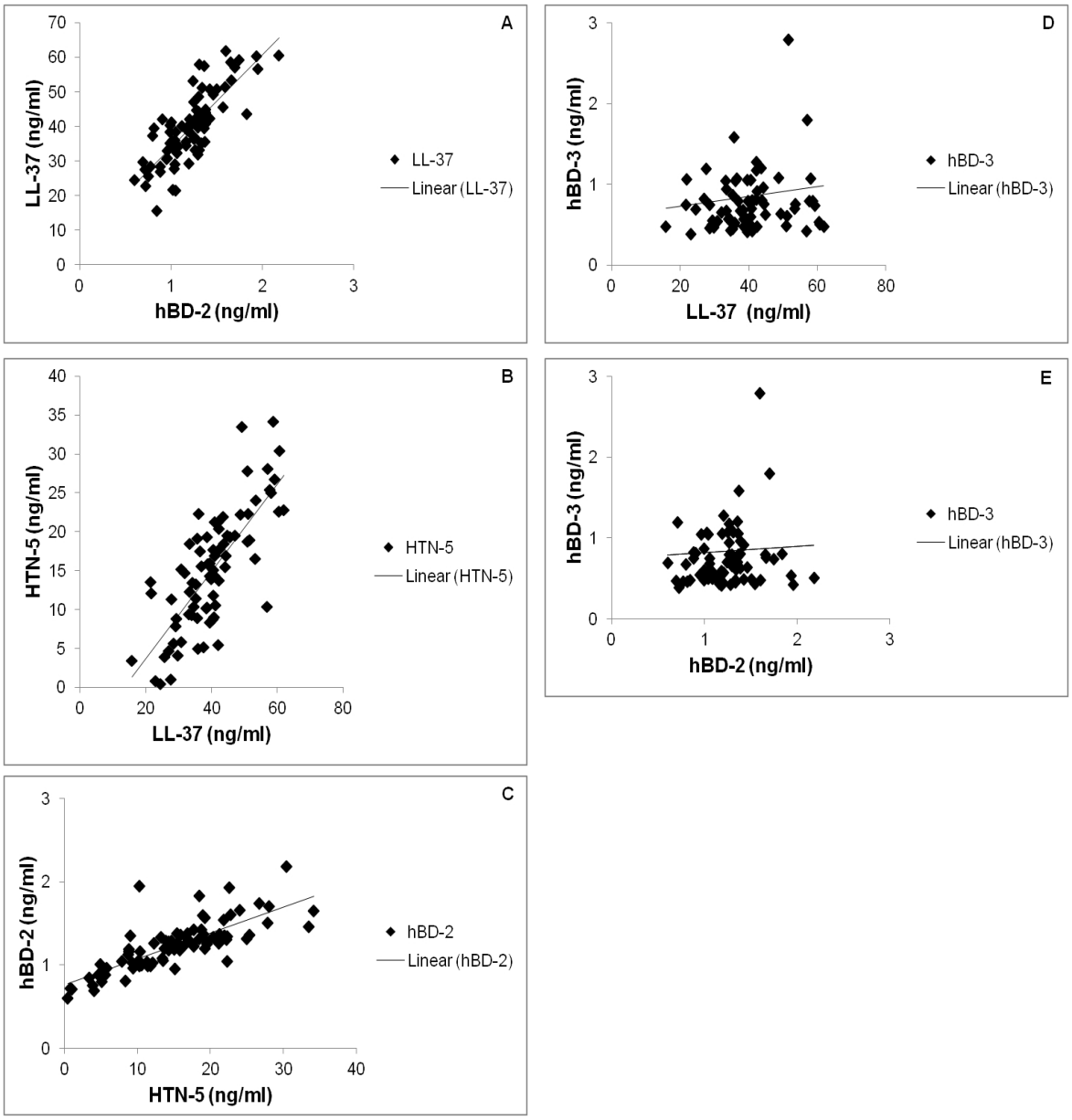

Figure 3. Only significant correlations are shown. A. Relationship between LL-37 and hBD-2 (Spearman correlation, r= 0.831, p=0.000). B. Relationship between HTN-5 and LL-37 (Spearman correlation, r= 0.765, p=0.000). C. Relationship between hBD-2 and HTN-5 (Spearman correlation, r= 0.796, p=0.000). D. Relationship between hBD-3 and LL-37 (Spearman correlation, r= 0.188, p=0.053). E. Relationship between hBD-3 and hBD-2 (Spearman correlation, r= 0.193, p=0.048).

Table 2. Relationship between antimicrobial peptides (hBD-2, hBD-3, LL-37 and HTN-5) and your combinations and dmfs.

	dmfs	
	Pearson Correlation	**p**
LL-37	0,297	0,007
hBD-2	0,268	0,015
hBD-2 e LL-37	0,307	0,005
hBD-3 e LL-37	0,293	0,008
LL-37 e HTN-5	0,238	0,031
hBD-2, hBD-3 e LL-37	0,295	0,007
hBD-2, LL-37 e HTN-5	0,24	0,030
hBD-3, LL-37 e HTN-5	0,231	0,037
AMPs	0,232	0,036

Only significant correlations are shown.

Molecular bacterial detection and severity of early childhood caries

Natália H. Colombo[1]
Paula F. Kreling[1]
Laís F. F. Ribas[1]
Jesse A. Pereira[1]
Christine A. Kressirer[2,3]
Anne C. R.Tanner[2,3]
Cristiane Duque[1]*

[1] Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, University Estadual Paulista (UNESP), Araçatuba, SP, Brazil
[2] Department of Microbiology, The Forsyth Institute, Cambridge, MA, USA
[3] Harvard School of Dental Medicine, Harvard University, Boston, MA, USA

Keywords: Dental caries, microbial ecology, *Streptococcus mutans*, *Scardovia wiggsiae*, *Bifidobacterium.*

*Correspondence: Dr. Cristiane Duque, Department of Pediatric Dentistry and Public Health, Araçatuba Dental School, Univ Estadual Paulista (UNESP)
Address: R. José Bonifácio, 1193, CEP: 16015-050, Araçatuba-SP, Brazil
Tel: (+55) 1836363315
E-mail: cristianeduque@yahoo.com.br

* According to author´s instructions of Journal of Clinical Microbiology (Anexo F).

Abstract

Streptococcus mutans is the bacterial species most frequently associated with dental caries. However, its presence alone is not sufficient to establish carious lesions in children. Many other oral bacteria are also acidogenic and aciduric and are caries-associated. This study aimed to identify and quantify differences in oral microbiota among caries-free children, early childhood caries and severe-early childhood caries. One hundred and thirty-six children, 36 to 60 months old were divided into three groups according to caries status: early childhood caries (ECC) (n=40), severe-early childhood caries (S-ECC) (n=49), caries-free (CF) (n=47). Questionnaires were completed by the parents, which assessed socio-economic-cultural data, oral hygiene habits and dietary patterns. Saliva was collected for detection and quantification of *S. mutans*, *Streptococcus sobrinus*, *Lactobacillus* spp. *Bifidobacterium* spp. and *Scardovia wiggsiae* by q-PCR. The results showed that the frequency of *S. mutans, Bifidobacterium spp.* and *S. wiggsiae* detection increased with caries severity. The levels of *S. mutans*, *S. sobrinus, Bifidobacterium* spp. and *S. wiggsiae* were significantly higher in S-ECC children compared to CF children. There was no difference in *Lactobacillus* spp. levels or frequency of detection among the groups. We conclude that in addition to *S. mutans*, other bacterial species, such as *Bifidobacterium spp, Scardovia wiggsiae* and *S. sobrinus* are important in severe-early childhood caries.

Introduction

Despite the decline in dental caries prevalence in the world, this disease is still the most common chronic infectious disease of childhood and remains a problem in both developed and developing countries (1, 2). In the USA caries prevalence in 2-5 years old children, however, increased from 24% in 1988-1994 to 28% in 1999-2004 (3). When tooth decay affects primary teeth of children under 6 years old, this disease is called early childhood caries (ECC). Severe forms of ECC significantly contribute to the burden of pain and are associated with a marked decrease in the quality of life and general health (4, 5). Untreated carious primary teeth in ECC can affect children physically and psychologically, influencing speech, nutrition, sleeping, as well as socialization, ability to learn and concentrate and reduction of self-esteem (2). ECC can be associated with lower height and weight due to insufficient food consumption from eating discomfort with decayed teeth resulting in insufficient physical development (6).

The etiology of ECC is considered multifactorial because microbial, genetic, immunological, behavioral, environmental and socioeconomic factors contribute to risk of the occurrence and severity of clinical disease (7). Although ECC is frequently associated with a poor diet and bad oral health habits (8, 9), microorganisms are responsible for the biochemical alterations particularly acid production on the tooth surface and consequently are essential for the development of this oral disease (10).

Streptococcus mutans is the bacterial species most frequently associated with dental caries. However, its presence is not enough to establish carious lesions in children (11). Many other oral bacteria including *Lactobacillus spp.,* non-mutans streptococci, *Actinomyces* spp. and *Bifidobacterium* spp. are also acidogenic and aciduric and are associated with caries (12). Recent research on the microbiota of severe-early childhood caries have shown that besides *S. mutans* (13, 14), other bacteria such as *Streptococcus sobrinus* (15), *Bifidobacteriaceae* (14, 15), *Slackia exigua* (14) and a new species, *Scardovia wiggsiae* (14, 16) are associated with severe-ECC.

To our knowledge, no studies have compared the bacteria newly associated with severe early childhood caries in children with lower degrees of caries severity. This study aimed to identify and quantify differences in oral microbiota among caries-free children, early childhood caries and severe-early childhood caries.

Material and methods

Subjects

The study population included 36- to 60-month-old children who attended the four public nursery schools in the city of Araçatuba, São Paulo, Brazil. The city´s population has access to a public water supply which contains fluoride levels of 0.7 ppm. The children's parents as well as the preschoolers involved granted written permission for the study, which had been previously approved by the Research Ethics Committee of Univ. Estadual Paulista (UNESP), Brazil (Certificate of Presentation for Ethical Consideration (CAAE) #13079213.4.0000.5420). Questionnaires were supplied to the parents to assess socio-, economic, cultural and oral hygiene habits. Dietary data was obtained from a food-frequency diary filled out for three days. Clinical examinations were carried out at the nursery schools by a single calibrated examiner using mouth mirrors and probes under natural light.

One hundred and forty children from both genders, 36- 60 months of age were selected to participate in this study, four of which did not give saliva samples. One hundred and thirty-six children were divided into three groups according to oral health status: early childhood caries (ECC) (n=40), severe-early childhood caries (S-ECC) (n=49), and caries-free (CF) (n=47). ECC was defined for this study as the presence of 1 to 3 decayed tooth surface (cavitated lesions), S-ECC was defined as the presence of decayed surfaces score of ≥4 (age 3), ≥5 (age 4) (17) and CF was defined as no lesions. Children suffering from systemic diseases, using long-term medications or had taken antibiotics less than one month before the examination, and children with mucosal lesions were excluded from the study. Children were encouraged and instructed on dental hygiene and received all other necessary oral care.

Saliva samples

Unstimulated whole saliva was collected from each subject by direct expectoration into a 50 mL sterile falcon conical tube for 5-10 min. Collections were performed at least 1 h after food intake to avoid contamination with non-salivary components. Tubes were transported on ice to the laboratory and processed within 1 h. After agitation, 100 µL of saliva was reserved for microbiological assay. The remaining saliva was clarified by centrifugation at 10000 rpm at 4ºC for 10 min. The

supernatant was stored at -70ºC for future analysis and the resulting pellet was used for DNA extraction.

Microbiological procedures

Aliquots of saliva were homogenized in a tube agitator (Vortex, Phoenix AT 56, Munising, MI, USA) for 1 min and the suspensions were serially diluted (10^{-1} to 10^{-7}) in 0.9% saline solution. Each dilution was cultivated in triplicate on the surface of two selective media: Mitis Salivaris Agar (Difco Laboratories, Detroit, MI, USA) with sucrose and bacitracin for isolation of mutans streptococci and Rogosa agar (Oxoid, Basingstoke, Hampshire, England) for lactobacilli. All plates were incubated at 37°C for 48 h in 5% CO_2 atmosphere. After 48 h of incubation, the total number of colony forming units (CFU) was counted from a representative area of each agar plate yielding 30–300 colonies using a stereoscopic microscope. Results were expressed as CFU/ml.

DNA extraction

DNA was extracted using a protocol described by Sardi *et al* (18). Briefly, samples were lysed with extraction buffer and proteinase K (Sigma-Aldrich, St. Louis, MO, USA) and then purified using chloroform and isoamyl alcohol, followed by DNA precipitation with isopropanol and washing with 70% ethanol. The DNA was re-suspended in TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.5, with 10 µg/mL RNAse). DNA was quantified in a BioTek Eon Microplate spectrophotometer at 260 nm (BioTek, Winooski, VT, USA), in order to obtain a standard concentration of 100 ng/mL and stored at −20 C for subsequent qPCR reactions (18).

Quantitative-PCR analysis

DNA concentration and quality were analyzed in a Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at 260 nm. For samples less than 12.5 ng/ul, DNA was amplified using Ready-to-go GenomiPhi V3 DNA Amplification Kit (GE Healthcare, Piscataway, NJ, USA). Samples were analyzed for detection and quantification of: *S. mutans* (19), *S. sobrinus* (19), *Lactobacillus* spp. (20), *Bifidobacterium* spp. (21) and *Scardovia wiggsiae* (14) using a Roche Lightcycler 480 (Roche, USA) for quantitative PCR (qPCR). The reaction

mixture (20 µL) was composed of 10 µL of SYBR Green qPCR Master (Roche), 2 µL of each specific primers (20 µM), 4 µL of diluted template DNA and 4 µL of deionized water. The amplification program was consistent with the corresponding published primer sets (Table 1).

Statistical analysis

The statistical analysis was performed using the three groups of children according to caries status (CF, ECC and S-ECC) as the dependent variables. The comparisons among the groups were performed according to data distribution. ANOVA and Tukey tests were applied for caries levels (dmfs), age and sugar intake. The Kruskal-Wallis and Mann-Whitey U tests were applied for gender comparison, mother´s education level, tooth brushing assisted by an adult, family income, bottle feeding, mutans streptococci counts and detection and quantification of microorganisms by q-PCR. Medians and ranges of bacterial counts were expressed as log (CFU +1) and the constant 1 was added to the CFU count, when the sample showed zero CFUs.

Results

The S-ECC group had a lower family income compared to families with CF children. Mothers of children with S-ECC had a lower education level than ECC and CF, but the difference was not statistically significant. Sugar intake did not differ among the groups (Table 2). The results showed that the frequency of *S. mutans, Bifidobacterium* spp*.* and *S. wiggsiae* detection increased with caries severity (Figure 1). The levels of *S. mutans*, *S. sobrinus, Bifidobacterium spp*. and S. *wiggsiae* were significantly higher in S-ECC children compared to CF children (Table 3). There was no difference in *Lactobacillus* spp. levels or frequency of detection among the groups.

Discussion

Previous studies have reported differences in the microbiota of caries-free compared to S-ECC children (14, 16, 22). Our results corroborated with findings of those studies. It was found that the frequency of *S. mutans*, *Bifidobacterium spp.* and

S. wiggsiae detection was increased with the severity of dental caries. Among the bacterial species studied, the major difference between ECC and S-ECC groups was found for the levels of *Bifidobacterium* spp. Species of *Bifidobacterium have been associated with the progression of dental caries* (16, 23)*. Becker et al. (23) reported that Bifidobacterium* spp. were the most frequently bacterial species identified in carious dentin, more than *S. mutans.* In the literature study bifidobacteria were not detected in plaque from intact surface or white spot lesions of ECC. These authors highlighted the high prevalence of *Bifidobacterium* spp. in deep caries lesions in deciduous teeth. The same study *(23)* reported that the levels of *Lactobacillus fermentum* was increased in carious dentin when compared to plaque from intact surface. However, these levels were lower than *Bifidobacterium* species. The authors suggested that the major secondary pathogens in S-ECC are not *Lactobacillus* spp. but rather *Bifidobacterium* spp. *S. wiggsiae* was observed to be the major species detected in plaque biofilm of ECC children in the absence of *S. mutans* (16).

Studies using culture (16) and PCR methods (14) have reported the association of *S. wiggsiae* with S-ECC. These studies, however, did not report bacterial levels. In the present study, the qPCR allowed bacterial quantitation. Our results found higher levels of *S. wiggsiae* in S-ECC group compared to CF group in addition to higher frequencies with increasing caries severity. *S. wiggsiae* in dental plaque detected by PCR showed a stronger association with S-ECC than *S. mutans* (14). Further, *Scardovia* was one of the eight genera which increased in cavitated dentin lesions, in addition to *Streptococcus* and *Lactobacillus* (24).

Xu *et al* (25) studied the microbiota from ECC and CF children and reported that some genera could only be detected in either the caries or the caries-free group. *Lactobacillus* spp. were detected only in ECC group. Kanasi *et al* (26) reported that *Lactobacillus gasseri*, *Lactobacillus fermentum* and *Lactobacillus vaginalis* were associated with childhood caries, whereas other probiotic *Lactobacillus* species showed negative associations with dental caries. In the current study, salivary levels of lactobacilli did not differ between CF, ECC and S-ECC children.

Although other bacteria have been highlighted in ECC etiology, the importance of *S. mutans* in this disease cannot be underestimated. Zhou *et al* (22) showed that children presenting with *S. mutans* and *S. sobrinus* had higher caries scores (dmft) than children who presented only with *S. mutans,* and further the prevalence and

levels of *S. mutans* and *S. sobrinus* were significantly higher in S-ECC children compared with CF children. Our results are consistent with this studies finding, showing that the frequency of *S. mutans* and *S. sobrinus* were increased in children with higher severities of dental caries.

Li *et al* (2007) reported that microbial diversity in dental biofilm was significantly less in S-ECC children than in CF children. The authors suggested that microbiota of S-ECC children becomes less diverse compared with the microbiota of CF children; maybe because only some groups of bacteria dominate the dental biofilm in the decay progress. Investigations about composition of the oral microorganisms are important for an understanding of the etiology of early childhood caries. Zhou *et al* (22) reported that CF children presented less genotypes of *S. mutans* and *S. sobrinus* than S-ECC children. However, three children with high score of caries lesions were not colonized with *S. mutans* and *S. sobrinus* (22). *In contrast,* the study of Becker *et al* (23) did not found an association of *S. sobrinus* with dental caries. *S. sobrinus* was found only in 30% of children with dental caries, and it was detected at low levels compared to others caries-associated species.

Early childhood caries is multifactorial disease. Besides cariogenic oral microorganisms, socioeconomic-cultural factors may contribute to caries risk (7). The literature shows that children from low-income families have a higher prevalence of dental caries (27, 28). Furthermore, when mothers had less than 8 years of education, their children presented with a higher prevalence of dental caries (27). In the current study, the severity of dental caries was associated with lower family income. However, no significant difference in maternal education was found between groups.

A strong risk factor for ECC is the high consumption of sweet snacks (29, 30). However, the present study did not found significant differences in sugar intake among the groups. Our results corroborated with those found in other studies which demonstrated that caries experience was not correlated with sugar exposure (31, 32). It is important to recognize however that some parents may minimize the consumption of sweets of their children when reporting to questionnaires. Besides, dietary data reflects the present time, thus, the questionnaire may not reflect previous eating habits, corresponding to a period when dental caries may have started. The detrimental effect of bottle feeding was not found in our study. Our

results corroborate with study of Parisotto *et al* (29) that did not find difference in bottle feeding usage between caries-free children and caries children.

We conclude that in addition to *S. mutans*, other bacterial species, such as *Bifidobacterium spp., Scardovia wiggsiae* and *S. sobrinus*, are associated with the severity of early childhood caries.

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Table 1 – Primers used in q-PCR analysis

Species	Primer	Primer sequence (5'-3')	Amplicon size (bp)	Reference
S. mutans	Forward	CTACACTTTCGGGTGGCTTG	261	(19)
	Reverse	GAAGCTTTTCACCATTAGAAGCTG		
S. sobrinus	Forward	AAAACATTGGGTTACGATTGCG	156	(19)
	Reverse	CGTCATTGGTAGTAGCCTGA		
Lactobacillus spp.	Forward	TGGAAACAGRTGCTAATACCG	231–233	(20)
	Reverse	GTCCATTGTGGAAGATTCCC		
Bifidobacterium spp.	g-Bifid-F	CTCCTGGAAACGGGTGG	549-563	(21)
	g-Bifid-R	GGTGTTCTTCCCGATATCTACA		
Scardovia wiggsiae	Scar448F	GTGGACTTTATGAATAAGC	200	(14)
	Scar619R	CTACCGTTAAGCAGTAAG		

Table 2. Comparative analysis between the severity of early childhood caries and related etiological factors.

	CF	ECC	S-ECC
dmfs (mean±SD)	0[a]	2.28 ± 1.36[b]	18.02 ± 15.62[c]
dmfs + white spot (mean±SD)	0[a]	3.38 ± 3.11[b]	21.55 ± 18.16[c]
Age in months (mean±SD)	48.16 ± 7.99[a]	48.43 ± 9.10[a]	49.53 ± 9.75[a]
Gender (%) Female	55.6[a]	47.5[a]	63.3[a]
Male	44.4[a]	52.5[a]	36.7[a]
Family income per month (%) < R$ 1448.00*	35.6[a]	45[ab]	63.3[b]
Mother's education (%) (Up to 8 year)	31.1[a]	30[a]	44.9[a]
Adult's help with tooth brushing (%)	77.8[a]	65[a]	63.3[a]
Total sugar exposure (mean±SD)	7.61 ± 2.09[a]	8.34 ± 3.58[a]	7.49 ± 2.17[a]
Bottle feeding (%)	88.9[a]	75[a]	81.6[a]
Mutans streptococci count (log CFU+1) median (range) (from selective media)	4.17 (0 – 7.29)[a]	4.37 (0 – 8.26)[ab]	5.12 (0 – 7.52)[b]

[a] Different lower case letters show statistical difference ($p<0.05$) among the groups, according to ANOVA and Tukey tests.

[A] Different upper case letters show statistical difference ($p<0.05$) among the groups, according to Kruskal-Wallis and Mann-Whitney U tests.

CF – caries-free, ECC – early childhood caries, S-ECC – severe-early childhood caries

* R$ - Brazilian reais. 1 US$ ~ R$ 2.70 (2015, January).

Table 3. Microorganism levels [Means (medians) standard errors] obtained by qPCR (ng/µL)

	CF	**ECC**	**S-ECC**
S. mutans	0.058 (0,000) ± 0.035[a]	0.085 (0.007) ± 0.030[b]	79.036 (7.450) ± 27.367[c]
Lactobacillus spp.	0.978 (0.111) ± 0.305[a]	1.188 (0.199) ± 0.493[a]	88.742 (63.370) ± 14.174[a]
S. sobrinus	0.000 (0.000) ± 0.000[a]	0.000 (0.000) ± 0.000[a]	0.489 (0.000) ± 0.328[b]
Bifidobacterium spp.	0.022 (0.000) ± 0.018[a]	0.008 (0.000) ± 0.004[a]	2.316 (0.621) ± 0.503[b]
Scardovia wiggsiae	0.072 (0.000) ± 0.058[a]	0.052 (0.000) ± 0.026[b]	1.952 (0.175) ± 0.514[b]

[a] Different lower case letters show statistical difference ($p<0.05$) among the groups, according to Kruskal-Wallis and Mann-Whitney U tests.

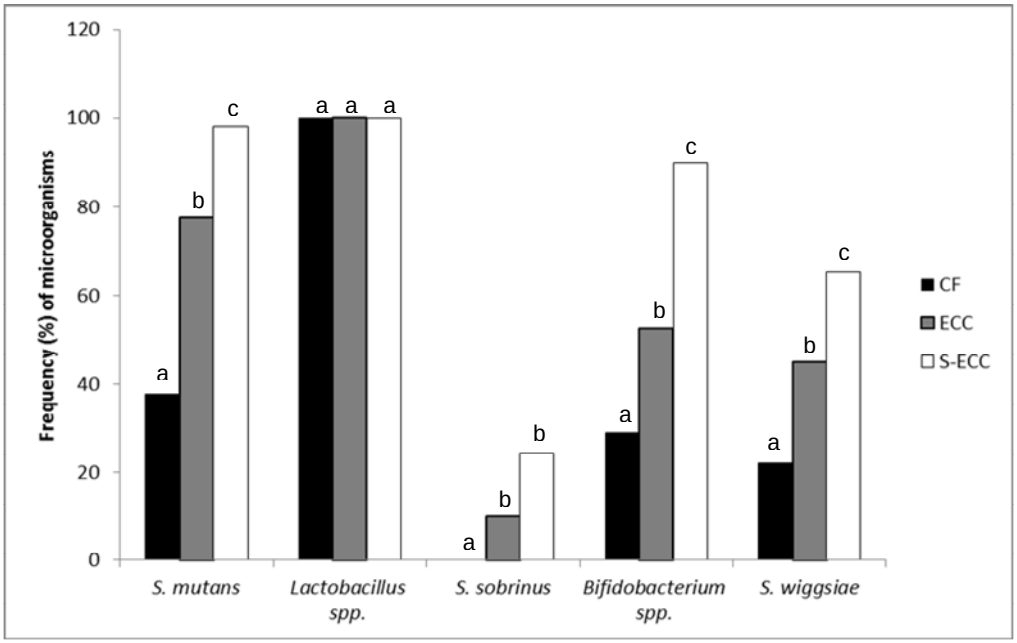

Figure 1. Frequency (%) of microorganisms detected by qPCR.

[a] Different lower case letters show statistical difference ($p<0.05$) among the groups, according to Chi-square test.

ANEXO A

Parecer de aprovação do comitê de ética

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

Comitê de Ética Em Pesquisa (CEP)
Committee for Ethical Research (CEP)

CERTIFICADO

Certificamos que o projeto "Estudo dos fatores de risco para o desenvolvimento da cárie da primeira infância e efeito clínico/microbiológico do tratamento restaurador atraumático em crianças pré-escolares" sob responsabilidade da pesquisadora **CRISTIANE DUQUE** e colaboração de Natália Helena Colombo (FOA-UNESP), Laís Fernanda Fonseca Ribas (FOA-UNESP), Marjully Eduardo Rodrigues da Silva (FOA-UNESP), Dinah Fressato Silva (FOA-UNESP), Anne C. R. Tanner (Forsyth Institute, EUA) e Christine A. Kressirer (Forsyth Institute, EUA) está de acordo com os princípios éticos em pesquisa e foi aprovado pelo CEP, de acordo com CAAE 13079213.4.0000.5420.

CERTIFICATE

We certify that the research "Evaluation of the risk factors and clinic/microbiologic effectiveness of the minimum restorative treatment in children with early childhood caries", CAAE number 13079213.4.0000.5420, under responsibility of **CRISTIANE DUQUE** and with collaboration of Natália Helena Colombo (FOA-UNESP), Laís Fernanda Fonseca Ribas (FOA-UNESP), Marjully Eduardo Rodrigues da Silva (FOA-UNESP), Dinah Fressato Silva (FOA-UNESP), Anne C. R. Tanner (Forsyth Institute, USA) and Christine A. Kressirer (Forsyth Institute, USA) agree with Ethical Principles in Research and was approved by CEP.

Profª Drª ANA CLAUDIA DE MELO STEVANATO NAKAMUNE
CEP Coordinator

Faculdade de Odontologia - Seção Técnica Acadêmica
Rua José Bonifácio, 1193 CEP 16015-050 Araçatuba – SP
Tel (18) 3636-3234 E-mail: cep@foa.unesp.br

ANEXO B

Questionário aplicado aos responsáveis

Questionário

Escola_____ Série_____

Nome:_____Data de nascimento:_____Idade: _____

Sexo: (F) (M) Endereço: _____

_____ Telefone: _____

DADOS DO RESPONSÁVEL:

Nome: _____

Idade: _____ Profissão: _____ Números de filhos: _____

Nível de escolaridade do responsável: ( ) não estudou ( ) 1º grau incompleto ( ) 1º grau completo (terminou a 8ª série) ( ) 2º grau incompleto ( ) 2º grau completo (terminou o 3º colegial) ( ) superior incompleto ( ) superior completo

Renda familiar: ( ) menos de 1 salário mín (0) ( ) 1 a 2 salários mín (1) ( ) 2 a 3 salários mín(2) ( ) 3 salários mín (3) ( ) 4 salários mín (4) ( ) mais de 5 sal mínimos (5)

nº de pessoas que vivem dessa renda: _____

DADOS DA CRIANÇA:

Nasceu como: ( ) Parto normal ( ) cesárea ( ) normal com forceps

MAMOU NO PEITO ? : ( ) sim ( ) não ( ) ainda mama no peito

Mamou até quantos meses? _____

Quantas vezes por dia? _____ ( ) manhã ( ) tarde ( ) noite ( ) de madrugada

AMAMENTAÇÃO ARTIFICIAL (uso de mamadeira) : ( ) sim ( ) não ( ) ainda mama

Idade que iniciou a amamentação artificial: _____ Até quantos meses? _____

Quantas vezes por dia? _____ ( ) manhã ( ) tarde ( ) noite ( ) madrugada

O que você coloca na mamadeira? _____

HIGIENE BUCAL

Escova os dentes? ( ) sim ( ) não Quantas vezes/dia? _____ Quantas vezes em casa: _____

Quem escova? ( ) criança ( ) mãe ou pai ( ) outro

Usa pasta de dente? ( ) sim ( ) não Qual? _____

Usa fio dental? ( ) sim ( ) não

Com que idade começou a escovar os dentes do seu filho? ( ) Assim que os primeiros dentes nasceram ( ) durante o 1º ano de idade ( ) durante o 2º ano de idade ( ) durante o 3º ano de idade

Biofilme visível: _____

Data da entrevista: ____/_____/_____

Data da 1ª coleta: ____/_____/_____ Local da coleta: _____

ANEXO C

Diário de dieta entregue ao responsável

DIÁRIO DE DIETA		Frutas, legumes e verduras 		Sucos e refrigerantes 		Arroz, feijão batatas e cereais 		Pães e massas 		Leite, iogurte, ovos, queijos e carnes 		Bolos e Doces 		Mamadeiras 	
		Quais alimentos?	Quantas porções?	Quais alimentos e quantos copos?	Com açúcar?	Quais alimentos?	Quantas porções?	Quais alimentos?	Quantas porções?	Qual alimento e quantos copos?	Qual alimento e quantas porções?	Qual doce?	Quantas porções?	Quantas?	Com açúcar ou chocolate?
Dia 1	Manhã														
	Tarde														
	Noite														
Dia 2	Manhã														
	Tarde														
	Noite														
Dia 3	Manhã														
	Tarde														
	Noite														

ANEXO D

Molecular Oral Microbiology
Instructions to authors

5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Format

Language: The language of publication is English. It is preferred that the manuscript is professionally edited before submission. We recommend that you have your paper professionally edited for English language by a service such as Wiley's at http://wileyeditingservices.com. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.

Abbreviations, Symbols and Nomenclature: This journal follows the recommendations of:
1) Council of Biology Editors Style Manual, 5th ed., Council of Biology Inc., Bethesda, MD, 1983: and
2) Instructions to Authors *molecular microbiology* (January issue of each year)
Scientific Names: Scientific names of bacteria should be binomials, only the generic name with an initial capital, and should be italicized (or underlined) in the typescript. A name should be given in full upon first mention in a paper; the generic name may be abbreviated thereafter, but the abbreviation must be unambiguous. With regard to drugs, generic names should be used instead of proprietary names. If proprietary names are used, they should be attached when the term is first used, and should be followed by a superscript ®.

5.2. Structure

All manuscripts submitted to Molecular Oral Microbiology should include Title Page, Summary, Main Text, Acknowledgements, References, Tables, Figure Legends and Figures as appropriate. You are encouraged to view a recent paper published to resolve any formatting issues or use the Submission Template.

Title Page: should contain the following information in the order given: 1) the article title; 2) authors' full names (without degrees or titles); 3) authors' institutional affiliations including city and country; 4) a running title, not exceeding 40 letters and spaces; 5) 4-6 keywords; 6) name, address, telephone, fax and e-mail address of the author responsible for correspondence.

Summary: A separate summary (not abstract) should not exceed 250 words.

The Main Text of Original Research Article should include Introduction, Methods, Results, Discussion and References.

Introduction: Clearly state the purpose of the article. Summarize the rationale for the study or observation. Give only strictly pertinent references, and do not review the subject extensively.

Methods: The objective of writing the Methods is that there is sufficient information presented for a reader to be able to repeat the work. As the author, you will be very familiar with what has been done, but the challenge is to present information clearly for others.

Results: Present your results in logical sequence in the text, tables, and illustrations. Do not repeat in the text all the data in the tables, illustrations, or both: emphasize or summarize only important observations.

Discussion*:* Emphasize the new and important aspects of the study and conclusions that follow from them. Do not repeat in detail data given in the Results section. Include in the Discussion the implications of the findings and their limitations and relate the observations to other relevant studies.

The Main Text of Review Articles should be structured with appropriate headings. The summary should not exceed 250 words. Please do not go over the same ground as previous reviews on the topic. Your review should be fair and balanced and avoid too much emphasis on your own work. Inform the reader what the main questions are and why they are important, and where new technologies are likely to have a major impact in the future. Careful attention should be paid to both factual content and presentation. The article should be no longer than 10-15 pages double spaced text, have a maximum of 3 Figures or Tables (or combination of), and carry no more than 50 references. For longer articles, please contact the Reviews Editor for approval.

Acknowledgements: Under Acknowledgements please specify contributors to the article other than the authors accredited. Acknowledge only persons who have made

substantive contributions to the study. Please also include specifications of the source of funding for the study and any potential conflict of interests if appropriate. Suppliers of materials should be named in the body of the text along with their location (town, state/county, country). The information must be included in the first citation.

5.3. References

References should be kept to the pertinent minimum, and arranged in alphabetical order by first author. For papers with up to six authors, the names of all authors should be listed. For papers with seven or more authors, the first three names should be listed followed by "et al.". Identify references in text, tables and legends by: Jones (2000), Jones et al. (2000), Jones & Michel (2000), or (Jones et al., 2000) etc. Do not use abstracts as references. Include manuscripts accepted but not published; designate the abbreviated title of the journal followed by (in press). Information from manuscripts not yet accepted should be cited in the text as (unpublished). The references must be verified by the author(s) against the original documents. Titles should be abbreviated in accordance with the style used in Index Medicus.

Journals

Savitt, E.D., and Socransky, S.S. (1984) Distribution of certain subgingival microbial species in selected periodontal conditions. *J Periodont Res* 19: 111-123.
He, J., Miyakazi, H., Anaya, C., Yu, F., Yeudall, W.A. and Lewis, J.P. (2006) Role of *Porphyromonas gingivalis* Feob2 in metal uptake and oxidative stress protection. *Infect Immun* 74: 4214-4223.
Colombo, A.P., Haffajee, A.D., Dewhirst, F.E. et al. (1998) Clinical and microbiological features of refractory periodontitis subjects. *J. Clin Periodontol* 25: 169-180.

Chapter in a book

Taubman, M.A., Ebersole, J.L., and Smith, D.J. (1982) Association between systemic and local antibody and periodontal diseases In *Host-Parasite Interactions in Periodontal Diseases*. Genco, R.J, and Mergenhagen, S.E. (eds). Washington, DC: American Society for Microbiology, pp. 283-298.

Personal author(s)

Touchstone, J.C., and Dobbins, M.F. (1983) *Practice of Thin Layer Chromatography*, 2nd edn. New York: Wiley.

We recommend the use of a tool such as Reference Manager for reference management and formatting. Reference Manager reference styles can be searched for here:www.refman.com/support/rmstyles.asp.

5.4. Tables, Figures and Figure Legends

Tables: Tables should be numbered consecutively with Arabic numerals. **Figures**: All figures (abbreviated to Fig(s).) should clarify the text and their number be kept to a minimum. Details must be large enough to retain their clarity after reduction in size. Illustrations should preferably fill single column width (54 mm) after reduction, although in some cases 113 mm (double column) and 171 mm (full page) widths will be accepted. Micrographs should be designed to be reproduced without reduction, and a linear size scale incorporated. Line drawings should be professionally drawn; half-tones should exhibit high contrast.

Figure Legends: should be numbered and listed after the Tables.

Preparation of Electronic Figures for Publication: Although low quality images are adequate for review purposes, print publication requires high quality images to prevent the final product being blurred or fuzzy. Submit EPS (lineart) or TIFF (halftone/photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Do not use pixel-oriented programmes. Scans (TIFF only) should have a resolution of 300 dpi (halftone) or 600 to 1200 dpi (line drawings) in relation to the reproduction size (see below). EPS files should be saved with fonts embedded (and with a TIFF preview if possible). For scanned images, the scanning resolution (at final image size) should be as follows to ensure good reproduction: lineart: >600 dpi; half-tones (including gel photographs): >300 dpi; figures containing both halftone and line images: >600 dpi. Only minimal processing of computer-generated images is permissible and should not alter the interpretation of the data. Any in silico manipulation must be applied to all parts of the image, including the controls. Descriptions of image adjustments and the software used must be included in the text. Composite images representing parts of more than one original image (or a rearrangement of different parts of the same image) must be clearly labeled as such. Original data must be retained and made available to the editors on request.

Further information can be obtained at Wiley Blackwell's guidelines for figures:http://authorservices.wiley.com/bauthor/illustration.asp.

Check your electronic artwork before submitting

it:http://authorservices.wiley.com/bauthor/eachecklist.asp

Permissions: If all or parts of previously published illustrations are used, permission must be obtained from the copyright holder concerned. It is the author's responsibility to obtain these in writing and provide copies to the Publishers.

ANEXO E

Archives of Oral Biology
Instructions to authors

Article structure

Manuscript Structure

Follow this order when typing manuscripts: Title, Authors, Affiliations, Abstract, Keywords, Main text (Introduction, Materials & Methods, Results, Discussion for an original paper), Acknowledgments, Appendix, References, Figure Captions and then Tables. Do not import the Figures or Tables into your text. The corresponding author should be identified with an asterisk and footnote. All other footnotes (except for table footnotes) should be identified with superscript Arabic numbers.

Introduction

This should be a succinct statement of the problem investigated within the context of a brief review of the relevant literature. Literature directly relevant to any inferences or argument presented in the Discussion should in general be reserved for that section. The introduction may conclude with the reason for doing the work but should not state what was done nor the findings.

Materials and Methods

Enough detail must be given here so that another worker can repeat the procedures exactly. Where the materials and methods were exactly as in a previous paper, it is not necessary to repeat all the details but sufficient information must be given for the reader to comprehend what was done without having to consult the earlier work.

Authors are requested to make plain that the conditions of animal and human experimentation are as outlined in the "Ethics" and "Studies on Animals" sections above

Results or Findings

These should be given clearly and concisely. Care should be taken to avoid drawing inferences that belong to the Discussion. Data may be presented in various forms such as histograms or tables but, in view of pressure on space, presentation of the same data in more than one form is unacceptable.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

• **Title.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• **Author names and affiliations.** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• **Corresponding author.** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**

• **Present/permanent address.** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

As titles frequently stand alone in indexes, bibliographic journals etc., and indexing of papers is, to an increasing extent, becoming computerized from key words in the titles, it is important that titles should be as concise and informative as possible. Thus the animal species to which the observations refer should always be given and it is desirable to indicate the type of method on which the observations are based, e.g. chemical, bacteriological, electron-microscopic, histochemical, etc. A "running title" of

not more than 40 letters and spaces must also be supplied. A keyword index must be supplied for each paper.

Structured abstract

The paper should be prefaced by an abstract aimed at giving the entire paper in miniature. Abstracts should be no longer than 250 words and should be structured as per the guidelines published in the Journal of the American Medical Association (JAMA 1995; 273: 27-34). In brief, the abstract should be divided into the following sections: (1) Objective; (2) Design - if clinical, to include setting, selection of patients, details on the intervention, outcome measures, etc.; if laboratory research, to include details on methods; (3) Results; (4) Conclusions.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

As Archives of Oral Biology is a journal with a multidisciplinary readership, abbreviations, except those universally understood such as mm, g, min. u.v., w/v and those listed below, should be avoided if possible. Examples of abbreviations which may be used without definition: ADP, AMP, ATP, DEAE-cellulose, DNA, RNA, EDTA, EMG, tris.

Other abbreviations used to improve legibility should be listed as a footnote on the title page. Chemical symbols may be used for elements, groups and simple compounds, but excessive use should be avoided. Abbreviations other than the above should not be used in titles.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the

references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Bacterial nomenclature

Organisms should be referred to by their scientific names according to the binomial system. When first mentioned the name should be spelt in full and in italics. Afterwards the genus should be abbreviated to its initial letter, e.g. 'S. aureus' not 'Staph. aureus'. If abbreviation is likely to cause confusion or render the intended meaning unclear, the names of microbes should be spelt in full. Only those names which were included in the Approved List of Bacterial Names, Int J Syst Bacteriol 1980; 30: 225?420 and those which have been validly published in the Int J Syst Bacteriol since 1 January 1980 have standing in nomenclature. If there is good reason to use a name that does not have standing in nomenclature, the names should be enclosed in quotation marks and an appropriate statement concerning the nomenclatural status of the name should be made in the text (for an example see Int J Syst Bacteriol 1980; 30: 547?556). When the genus alone is used as a noun or adjective, use lower case Roman not italic, e.g.'organisms were staphylococci' and 'streptococcal infection'. If the genus is specifically referred to use italics e.g. 'organisms of the genus Staphylococcus'. For genus in plural, use lower case roman e.g. 'salmonellae'; plurals may be anglicized e.g.'salmonellas'. For trivial names, use lower case Roman e.g. 'meningococcus'

Artwork

Image manipulation

Whilst it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

Electronic artwork

General *points*

• Make sure you use uniform lettering and sizing of your original artwork.

• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Size the illustrations close to the desired dimensions of the published version.
• Submit each illustration as a separate file.
A detailed guide on electronic artwork is available on our website: http://www.elsevier.com/artworkinstructions.

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.
Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings, embed all used fonts.
TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Illustration services

Elsevier's WebShop (http://webshop.elsevier.com/illustrationservices) offers Illustration Services to authors preparing to submit a manuscript but concerned about

the quality of the images accompanying their article. Elsevier's expert illustrators can produce scientific, technical and medical-style images, as well as a full range of charts, tables and graphs. Image 'polishing' is also available, where our illustrators take your image(s) and improve them to a professional standard. Please visit the website to find out more.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

References

All manuscripts should use the 'Vancouver' style for references, which should be numbered consecutively in the order in which they are first cited in the text and listed at the end of the paper.

For journal references, all authors should be included when there are six or fewer (first six followed by 'et al.' when seven or more), followed by the title of article, name of journal abbreviated according to Index Medicus, or left in full, year, volume with part number in brackets, and first and last pages. For example:

1. Walsh NP, Montague JC,Callow N and Rowlands AV. Saliva flow rate, total protein concentrationand osmolality as potential markers of whole body hydration statusduring progressive acute dehydration in humans. Arch Oral Biol2004;49(2):149-154.

For book references, the author(s) should be followed by the chapter title (if appropriate), editor(s) (if applicable), book title, place of publication, publisher, year and page numbers. For example:

Nanci A. Ten Cate's Oral Histology: Development, Structure and Function. 6th ed. St. Louis: Mosby; 2003.

Papers in the course of publication should only be entered in the references if the paper has been accepted by a journal, and then given in the standard manner in the text and list of references but with the words "In press" following the name of the journal.

Reference management software

Most Elsevier journals have a standard template available in key reference management packages. This covers packages using the Citation Style Language, such as Mendeley (http://www.mendeley.com/features/reference-manager) and also others like EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to word processing packages which are available from the above sites, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style as described in this Guide. The process of including templates in these packages is constantly ongoing. If the journal you are looking for does not have a template available yet, please see the list of sample references and citations provided in this Guide to help you format these according to the journal style.

If you manage your research with Mendeley Desktop, you can easily install the reference style for this journal by clicking the link below:
http://open.mendeley.com/use-citation-style/archives-of-oral-biology
When preparing your manuscript, you will then be able to select this style using the Mendeley plug-ins for Microsoft Word or LibreOffice. For more information about the Citation Style Language, visit http://citationstyles.org.

ANEXO F

Journal of Clinical Microbiology

Instructions to authors

ORGANIZATION AND FORMAT

Editorial Style

The editorial style of ASM journals conforms to the *ASM Style Manual for Journals* (American Society for Microbiology, 2015, in-house document) and *How To Write and Publish a Scientific Paper*, 7th ed. (Greenwood, Santa Barbara, CA, 2011), as interpreted and modified by the editors and the ASM Journals Department.

The editors and the Journals Department reserve the privilege of editing manuscripts to conform with the stylistic conventions set forth in the aforesaid publications and in these Instructions.

On receipt at ASM, an accepted manuscript undergoes an automated preediting, cleanup, and tagging process specific to the particular article type. To optimize this process, manuscripts must be supplied in the correct format and with the appropriate sections and headings.

Type every portion of the manuscript double-spaced (a minimum of 6 mm between lines), including figure legends, table footnotes, and References, and number all pages in sequence, including the abstract, figure legends, and tables. Place the last two items after the References section. Manuscript pages must have continuous line numbers and page numbers. Manuscripts without line and page numbers will be returned to authors for provision of this information prior to processing. The font size should be no smaller than 12 points. It is recommended that the following sets of characters be easily distinguishable in the manuscript: the numeral zero (0) and the letter "oh" (O); the numeral one (1), the letter "el" (l), and the letter "eye" (I); and a multiplication sign (×) and the letter “ex.” (x). Do not create symbols as graphics or use special fonts that are external to your word processing program; use the "insert symbol" function. Set the page size to 8.5 by 11 inches (ca. 21.6 by 28 cm). Italicize any words that should appear in italics, and indicate paragraph lead-ins in boldface type.

Manuscripts may be editorially rejected, without review, on the basis of poor English or lack of conformity to the standards set forth in these Instructions.

Authors who are unsure of proper English usage should have their manuscripts checked by someone proficient in the English language or engage a professional language editing service for help.

Manuscript Submission Checklist

- Double-space all text, including references and figure legends.
- Number pages.

- Number lines continuously.
- Present statistical treatment of data where appropriate.
- Format references in ASM style.
- Provide accession numbers for all newly published sequences in a dedicated paragraph, and if a sequence or sequence alignment important for evaluation of the manuscript is not yet available, provide the information as supplemental material not for publication or make the material available on a website for access by the editor and reviewers.
- Confirm that genetic and chemical nomenclature conforms to instructions.
- Include as supplemental material not for publication in-press and submitted manuscripts that are important for judgment of the present manuscript.

Full-Length Papers

Full-length papers include the elements described in this section.

Title, running title, byline, affiliation line, and corresponding author.. Each manuscript should present the results of an independent, cohesive study; thus, numbered series titles are not permitted. Exercise care in composing a title. Avoid the main title/subtitle arrangement, complete sentences, and unnecessary articles. On the title page include the title, the running title (not to exceed 54 characters and spaces), the name of each author, all authors' affiliations at the time the work was performed, the name(s) and e-mail address(es) of the corresponding author(s), and a footnote indicating the present address(es) of any author(s) no longer at the institution where the work was performed. Place a number sign (#) in the byline after the name of the author to whom inquiries regarding the paper should be directed (see**"Correspondent footnote"** below). **Please review this sample title page for guidance.**

Study group in byline. A study group, surveillance team, working group, consortium, or the like (e.g., the Active Bacterial Core Surveillance Team) may be listed as a coauthor in the byline if its contributing members satisfy the requirements for authorship and accountability as described in these Instructions. The names (and institutional affiliations if desired) of the contributing members may be given as a separate paragraph in Acknowledgments.

If the contributing members of the group associated with the work do not fulfill the criteria of substantial contribution to and responsibility for the paper, the group may not be listed in the author byline. Instead, it and the names of its contributing members may be listed in the Acknowledgments section.

Correspondent footnote. The e-mail address for the corresponding author should be included on the title page of the manuscript. This information will be published in the article as a footnote to facilitate communication and will be used to notify the corresponding author of the availability of proofs and, later, of the PDF file of the published article. No more than two authors may be designated corresponding authors.

Abstract. Limit the abstract to 250 words or fewer and concisely summarize the basic content of the paper without presenting extensive experimental details. Avoid abbreviations and references, and do not include diagrams. When it is essential to include a reference, use the format shown under “References” below (see

the **"Citations in abstracts"**section). Conclude the abstract with a summary statement. Because the abstract will be published separately by abstracting services, it must be complete and understandable without reference to the text.

Introduction. The introduction should supply sufficient background information to allow the reader to understand and evaluate the results of the present study without referring to previous publications on the topic. The introduction should also provide the hypothesis that was addressed or the rationale for the present study. Choose references carefully to provide the most salient background rather than an exhaustive review of the topic.

Materials and Methods. The Materials and Methods section must include sufficient technical information to allow the experiments to be repeated. The sources of all media (i.e., name and location of manufacturer) or components of a new formulation must be provided. When centrifugation conditions are critical, give enough information to enable another investigator to repeat the procedure: make of centrifuge, model of rotor, temperature, time at maximum speed, and centrifugal force (x *g* rather than revolutions per minute). For commonly used materials and methods (e.g., media and protein concentration determinations), a simple reference or specifically recommended product or procedure is sufficient. If several alternative methods are commonly used, it is helpful to identify the method briefly as well as to cite the reference. For example, it is preferable to state "cells were broken by ultrasonic treatment as previously described (9)" rather than to state "cells were broken as previously described (9)." This allows the reader to assess the method without constant reference to previous publications. Describe new methods completely, and give sources of unusual chemicals, reagents, equipment, or microbial strains. When large numbers of microbial strains or mutants are used in a study, include tables identifying the immediate sources (i.e., sources from whom the strains were obtained) and properties of the strains, mutants, bacteriophages, and plasmids, etc.

A method or strain, etc., used in only one of several experiments reported in the paper may be described in the Results section or very briefly (one or two sentences) in a table footnote or figure legend. It is expected that the sources from whom the strains were obtained will be identified.

Results. In the Results section, include the rationale or design of the experiments as well as the results; reserve extensive interpretation of the results for the Discussion section. Present the results as concisely as possible in one of the following: text, table(s), or figure(s). Avoid extensive use of graphs to present data which might be more concisely presented in the text or tables. For example, except in unusual cases, double-reciprocal plots used to determine apparentK_m values should not be presented as graphs; instead, the values should be stated in the text. Similarly, graphs illustrating other methods commonly used to derive kinetic or physical constants (e.g., reduced-viscosity plots and plots used to determine sedimentation velocity) need not be shown except in unusual circumstances. All tabular data must be accompanied by either standard deviation values or standard errors of the means. The number of replicate determinations (or animals) used for making such calculations must also be included. All statements concerning the significance of the differences observed should be accompanied by probability values given in parentheses. The statistical procedure used should be stated in Materials and Methods. Limit illustrations (particularly photomicrographs and electron micrographs) to those that are absolutely necessary to show the experimental findings. Number

figures and tables in the order in which they are cited in the text, and be sure to cite all figures and tables.

Discussion. The Discussion section should provide an interpretation of the results in relation to previously published work and to the experimental system at hand. It must not contain extensive repetition of the Results section or reiteration of the introduction. In short papers, the Results and Discussion sections may be combined.

Acknowledgments. The source of any financial support received for the work being published must be indicated in the Acknowledgments section. (It will be assumed that the absence of such an acknowledgment is a statement by the authors that no support was received.) The usual format is as follows: "This work was supported by Public Health Service grant CA-01234 from the National Cancer Institute."

Recognition of personal assistance should be given as a separate paragraph, as should any conflict of interest statements and statements disclaiming endorsement or approval of the views reflected in the paper or of a product mentioned therein.

Appendixes. Appendixes that contain additional material to aid the reader are permitted. Titles, authors, and reference sections that are distinct from those of the primary article are not allowed. If it is not feasible to list the author(s) of the appendix in the byline or the Acknowledgments section of the primary article, rewrite the appendix so that it can be considered for publication as an independent article, either full-length paper or Short-Form style. Equations, tables, and figures should be labeled with the letter “A” preceding the numeral to distinguish them from those cited in the main body of the text.

References

In the reference list, references are numbered in the order in which they are cited in the article (citation-sequence reference system); ASM no longer uses the citation-name system with an alphabetized reference list. In the text, references are cited parenthetically by number in sequential order. Data that are not published or not peer reviewed are simply cited parenthetically in the text (see section ii below).

(i) References listed in the References section. The following types of references must be listed in the References section:

- Journal articles (both print and online)
- Books (both print and online)
- Book chapters (book title is required)
- Patents
- Theses and dissertations
- Published conference proceedings
- Meeting abstracts (from published abstract books or journal supplements)
- Letters (to the editor)
- Company publications
- In-press journal articles, books, and book chapters (publication title is required)

Provide the names of all the authors and/or editors for each reference; names should not be abbreviated with “et al.” Since title and byline information that is downloaded from PubMed does not always show accents, italics, or special characters, authors should refer to the PDF files or hard-copy versions of the articles and incorporate the necessary corrections in the submitted manuscript. Abbreviate

journal names according to the **PubMed Journals Database**(National Library of Medicine, National Institutes of Health), the primary source for ASM style (do not use periods with abbreviated words). The EndNote output style for ASM Journals' current reference style can be found **here**; click "Open" and then "Download and Install" to save it to your EndNote Styles folder (it should replace any earlier output styles for ASM journals [all ASM journals use the same reference style]).

Follow the styles shown in the examples below for print references.

1. **Caserta E, Haemig HAH, Manias DA, Tomsic J, Grundy FJ, Henkin TM, Dunny GM.** 2012. *In vivo* and *in vitro*analyses of regulation of the pheromone-responsive *prgQ* promoter by the PrgX pheromone receptor protein. J Bacteriol**194**:3386-3394.

2. **Falagas ME, Kasiakou SK.** 2006. Use of international units when dosing colistin will help decrease confusion related to various formulations of the drug around the world. Antimicrob Agents Chemother **50:**2274-2275. (Letter.) {"Letter" or "Letter to the editor" is allowed but not required at the end of such an entry.}

3. **Cox CS, Brown BR, Smith JC.** J Gen Genet, in press.* {Article title is optional; journal title is mandatory.}

4. **da Costa MS, Nobre MF, Rainey FA.** 2001. Genus I. Thermus Brock and Freeze 1969, 295,[AL] emend. Nobre, Trüper and da Costa 1996b, 605, p. 404-414. *In* Boone DR, Castenholz RW, Garrity GM (ed), Bergey's manual of systematic bacteriology, 2nd ed, vol 1. Springer, New York, NY.

5. **Stratagene.** 2006. Yeast DNA isolation system: instruction manual. Stratagene, La Jolla, CA. {Use the company name as the author if none is provided for a company publication.}
6. **Forman MS, Valsamakis A.** 2011. Specimen collection, transport, and processing: virology, p 1276-1288. *In* Versalovic J, Carroll KC, Jorgensen JH, Funke G, Landry ML, Warnock DW (ed), Manual of clinical microbiology, 10th ed, vol 2. ASM Press, Washington, DC.

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9. **Carlson E.** 2013. Selective penicillin-binding protein imaging probes reveal substructure in bacterial cell division, p 59. Final Program 113th Gen Meet Am Soc Microbiol. American Society for Microbiology,

Washington, DC. {Abstract title is optional.}

10. **Rotimi VO, Salako NO, Mohaddas EM, Philip LP.** 2005. Abstr 45th Intersci Conf Antimicrob Agents Chemother, abstr D-1658. {Abstract title is optional.}

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13. **Odell JC.** April 1970. Process for batch culturing. US patent 484,363,770. {Include the name of the patented item/process if possible; the patent number is mandatory.}

14. **Elder BL, Sharp SE.** 2003. Cumitech 39, Competency assessment in the clinical laboratory. Coordinating ed, Sharp SE. ASM Press, Washington, DC.

*A reference to an in-press ASM publication should state the control number (e.g., JCM00123-15) if it is a journal article or the name of the publication if it is a book.
Online-only references must provide essentially the same information that print references do. For online journal articles, posting or revision dates may replace the year of publication; a DOI (preferred) or URL is required for articles with nontraditional page numbers or electronic article identifiers.

1. **Bina XR, Taylor DL, Vikram A, Ante VM, Bina JE.** 2013. *Vibrio cholerae* ToxR downregulates virulence factor production in response to cyclo(Phe-Pro). mBio **4**(5)**:**e00366-13. doi:10.1128/mBio.00366-13.
2. **Winnick S, Lucas DO, Hartman AL, Toll D.** 2005. How do you improve compliance? Pediatrics **115:**e718-e724. doi:10.1542/peds.2004-1133.
3. **Dionne MS, Schneider DS.** 2002. Screening the fruitfly immune system. Genome Biol **3:**reviews1010-reviews1010.2. doi:10.1186/gb-2002-3-4-reviews1010.
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Note: a posting or accession date is required for any online reference that is periodically updated or changed.
Citations of ASM Accepts manuscripts should look like the following example.

Wang GG, Pasillas MP, Kamps MP. 15 May 2006. Persistent transactivation by Meis1 replaces Hox function in myeloid leukemogenesis models: evidence for co-occupancy of Meis1-Pbx and Hox-Pbx complexes on promoters of leukemia-associated genes. Mol Cell Biol doi:10.1128/MCB.00586-06.

Other journals may use different styles for their publish-ahead-of-print manuscripts, but citation entries must include the following information: author name(s), posting

date, title, journal title, and volume and page numbers and/or DOI. The following is an example:

Zhou FX, Merianos HJ, Brunger AT, Engelman DM. 13 February 2001. Polar residues drive association of polyleucine transmembrane helices. Proc Natl Acad Sci U S A doi:10.1073/pnas.041593698.

(ii) References cited in the text. References that should be cited in the text include

- Unpublished data
- Manuscripts submitted for publication
- Unpublished conference presentations (e.g., a report or poster that has not appeared in published conference proceedings)
- Personal communications
- Patent applications and patents pending
- Computer software, databases, and websites

These references should be made parenthetically in the text as follows:

... similar results (R. B. Layton and C. C. Weathers, unpublished data).

... system was used (J. L. McInerney, A. F. Holden, and P. N. Brighton, submitted for publication).

... as described previously (M. G. Gordon and F. L. Rattner, presented at the Fourth Symposium on Food Microbiology, Overton, IL, 13 to 15 June 1989). {For nonpublished abstracts and posters, etc.}

... this new process (V. R. Smoll, 20 June 1999, Australian Patent Office). {For non-U.S. patent applications, give the date of publication of the application.}

... available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/index.html).

... using ABC software (version 2.2; Department of Microbiology, State University [http://www.state.micro.edu]).

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(P. S. Satheshkumar, A. S. Weisberg, and B. Moss, J Virol 87:10700–10709, 2013, doi:10.1128/JVI.01258-13)

(J. H. Coggin, Jr., p. 93–114, *in* D. O. Fleming and D. L. Hunt, ed., *Biological Safety. Principles and Practices*, 4th ed., 2006)

". . . in a recent report by D. A. Hopwood [mBio 4(5):e00612-13, 2013, doi:10.1128/mBio00612-13] . . . ."

This style should also be used for Addenda in Proof.

(iv) References related to supplemental material. If references must be cited in the supplemental material, list them in a **separate** References section within the supplemental material and cite them by those numbers; do not simply include citations of numbers from the reference list of the associated article. If the same reference(s) is to be cited in both the article itself and the supplemental material, then that reference would be listed in both References sections.

ANEXO G

Referências

Introdução Geral

1. Loesche WJ. Microbiology of Dental Decay and Periodontal Disease. In: Baron S e, editor. Medical Microbiology. 4th edition ed. Galveston (TX): University of Texas Medical Branch at Galveston1996.

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