Remberto Marcelo Argandoña Valdez

Papel de cepas de *Streptococcus mutans* e *Bifidobacterium* spp. na etiologia ou proteção contra doenças bucais

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Tese apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, como requisito 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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Epígrafe

Quando você quer alguma coisa, todo o Universo conspira para que você realize o seu desejo.

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Resumo geral

Valdez, RMA. Papel de cepas de *Streptococcus mutans* e *Bifidobacterium* spp. na etiologia ou proteção contra doenças bucais. 2016. 110f. Tese (Doutorado em Ciência Odontológica) Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2016.

A boca é um ecossistema complexo composto por centenas de espécies microbianas que apresentam características genotípicas e fenotípicas distintas que permitem sua adaptação e sobrevivência às adversidades desse ambiente. *Streptococcus mutans* é a espécie bacteriana mais relacionada à etiologia da cárie dentária, devido principalmente ao seu potencial acidogênico, acidúrico e na aderência e formação de biofilme dental. Outras espécies bacterianas, como as bifidobactérias, foram detectadas em diversos sítios bucais incluindo lesões cariosas iniciais ou cavitadas e estão sendo relacionadas à etiologia da cárie precoce da infância (CPI). Contrariamente, efeito benéfico na prevenção da doença periodontal tem sido observado para as bifidobactérias pelo fato dessas espécies competirem e interferirem na aderência e formação do biofilme de patógenos periodontais. Assim, ambiguidade em relação ao papel das bifidobactérias na etiologia ou prevenção de doenças bucais está sendo observada na literatura. Este trabalho foi dividido em três capítulos. Os objetivos foram: 1) avaliar a diversidade genotípica e as características fenotípicas de cepas de *S. mutans*, isoladas do biofilme dental de crianças CPI e CPI-severa (CPI–S) em comparação com crianças livres de cárie (LC); 2) avaliar a capacidade de produzir e tolerar ácidos, de formar biofilme e de induzir lesões iniciais de cárie *in vitro* por espécies de bifidobactérias comparadas à de espécies bacterianas já reconhecidas no contexto da cárie dentária e 3) investigar o efeito antagonista *in vitro* de algumas espécies de bifidobactérias sobre biofilmes de *Fusobacterium nucleatum, Porphyromonas gingivalis* e *Streptococcus oralis.* No capítulo 1, cepas de *S. mutans* de amostras de biofilme de crianças com CPI, CPI-S e LC foram isoladas em meio Agar Mitis Salivarius com bacitracina e avaliadas geneticamente pelo método de AP-PCR (Reação em Cadeia da Polimerase usando Iniciadores Arbitrários) e fenotipicamente pelos métodos de acidogenicidade, verificando o pH final das culturas após exposição a alta concentração de glicose; de aciduricidade, medida pelo crescimento bacteriano após exposição à pHs ácidos e de formação de biofilme *in vitro* com a quantificação da biomassa do biofilme em leitor de ELISA. No Capítulo 2, as seguintes espécies bacterianas foram incluídas: *Bifidobacterium lactis, B. longum, B. animalis, B. dentium, S. mutans, S. sobrinus*,

Lactobacillus acidophilus, L. casei e *Actinomyces israelli*. Para avaliação do potencial cariogênico dessas espécies bacterianas foram realizados os testes de acidogenicidade, aciduricidade, formação de biofilme *in vitro* e ensaios de indução de lesão de cárie inicial em dentes bovinos verificando a dureza superficial do esmalte em microdurômetro. Os ensaios de biofilme e de indução de lesão de cárie foram realizados com todas as espécies isoladas ou combinadas com *S. mutans* ou *S. mutans/S.sobrinus.* No capítulo 3, os periodontopatógenos *F. nucleatum, P. gingivalis* e uma espécie da microbiota bucal indígena, *S. oralis*, foram cultivados em microplacas para formar biofilmes na presença de *B. longum, B. lactis, B. infantis,* isoladamente ou em combinação. A capacidade de competição dessas espécies foi avaliada por meio das contagens das bactérias após 24, 72 e 168 h de crescimento empregando a técnica de *checkerboard DNA-DNA hybridization*. Os resultados do capítulo 1 mostraram que não houve diferença na diversidade genotípica nem na capacidade acidogênica de cepas de *S. mutans* obtidas de crianças CPI e CPI-S em comparação com aquelas isoladas de crianças LC. Os genótipos de *S. mutans* de CPI-S formaram mais biofilmes e foram mais tolerantes a ácidos que os isolados LC. Com relação ao capítulo 2, *B. animalis* e *B. longum* foram as espécies mais acidogênicas e acidúricas, comparáveis à *S. mutans* e *L. casei*. Todas as espécies tiveram um aumento significativo na biomassa do biofilme quando combinados com *S. mutans* ou com *S. mutans*/*S. sobrinus*. A maior perda de esmalte superficial foi produzida quando *B. longum* ou *B. animalis* foram inoculados com *S. mutans* ou *S. mutans*/*S. sobrinus.* No capítulo 3, os resultados mostraram que *B. infantis* e *B. lactis* tiveram um melhor efeito antagonista sobre crescimento de *F. nucleatum* (24h e 72h) e *P. gingivalis* (168 horas) e não apresentaram influência sobre o crescimento de *S. oralis* (168 horas). Conclui-se que a tolerância ácida parece ser o fator de virulência de *S. mutans* mais importante para a patogênese da cárie precoce da infância. Assim como as bactérias cariogênicas, *B. longum* e *B. lactis* apresentam potencial acidogênico e acidúrico e também capacidade de formar biofilmes e induzir a desmineralização de esmalte em associação com *S. mutans*. Enquanto, *B. lactis* e *B. infantis* exerceram efeito inibitório sobre o crescimento de patógenos periodontais.

General Abstract

Valdez, RMA. The role of *Streptococcus mutans* strains and *Bifidobacterium* spp. in the etiology or protection against oral diseases. 2016. 110f. Tese (Doutorado em Ciência Odontológica) Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2016.

The mouth is a complex ecosystem composed of hundreds of microbial species with different genotypic and phenotypic characteristics that allow their adaptation and survival in the adversity of that environment. *Streptococcus mutans* is the bacterial species more closely related to the etiology of dental caries, mainly due to its acidogenic and aciduric potential and adherence and formation of dental biofilm. Other bacterial species, such as bifidobacteria, have been detected in oral sites including initial and cavitated carious lesions and have been related to the etiology of early childhood caries (ECC). In contrast, beneficial effect of bifidobacteria in the prevention of periodontal disease has been observed because they compete and interfere with the adherence and biofilm formation by periodontal pathogens. Thus, ambiguity regarding the role of bifidobacteria in the etiology or prevention of oral diseases has been observed in the literature. This work was divided into three chapters. The objectives were: 1) to evaluate the genotypic diversity and phenotypic characteristics of *S. mutans* strains isolated from dental biofilm of ECC and severe-ECC (S-ECC) children compared to caries-free children (CF); 2) to evaluate the ability to produce and tolerate acids, to form biofilm and inducing in vitro initial carious lesions by species of bifidobacteria compared to the bacterial species already recognized in the context of dental caries and 3) to investigate the in vitro antagonistic effect of some bifidobacteria species on biofilms of *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Streptococcus oralis*. In Chapter 1, *S. mutans* strains from biofilm samples of children with ECC, S-ECC and CF were isolated in Agar Mitis Salivarius with bacitracin and genetically evaluated by AP-PCR method (Arbitrary Primers - Polymerase Chain Reaction) and phenotypically by acidogenicity methods, verifying the final pH of the cultures after exposure to high glucose concentration; aciduricity, measured by bacterial growth after exposure to acid pH and in vitro biofilm formation by means the quantification of the biofilm biomass in ELISA reader. In Chapter 2, the following bacterial species were included: *Bifidobacterium lactis, B. longum, B. animalis, B. dentium, S. mutans, S. sobrinus, Lactobacillus acidophilus, L. casei* and *Actinomyces*

israelli. Acidogenicity, aciduricity and biofilm formation assays were performed for evaluating the cariogenic potential of these bacterial species and initial caries lesion induction tests on bovine teeth for checking the surface enamel hardness in microdurometer. Biofilm formation and carious lesion induction assays were performed with all species either alone or in combination with *S. mutans* or *S. mutans* / *S.sobrinus*. In Chapter 3, the periodontal pathogens *F. nucleatum, P. gingivalis* and a species of indigenous oral microbiota, *S. oralis*, were cultured in microtiter plates to form biofilms in the presence of *B.* longum, B. lactis, B. infantis, either alone or in combination. The competition ability of these species was assessed by bacterial counts after 24, 72 and 168 h of growth using the DNA-DNA hybridization checkerboard technique. Results from Chapter 1 showed no difference in the genotypic diversity or acidogenic ability of *S. mutans* strains collected from ECC and S-ECC children compared to those from CF children. The genotypes of *S. mutans* formed higher biofilm biomasses and were more tolerant to acids than those isolated from CF. With respect to chapter 2, *B. animalis* and *B. longum* were the most acidogenic and aciduric species, comparable to *S. mutans* and *L. casei*. All species had a significant increase in biomass of biofilm when combined with *S. mutans* or *S. mutans* / *S. sobrinus*. The greatest loss of surface enamel was produced when *B. longum* or *B. animalis* were inoculated with *S. mutans* or *S. mutans / S. sobrinus*. In Chapter 3, the results showed that *B. infantis* and *B. lactis* had a better antagonistic effect on *F. nucleatum* (24h and 72h) and *P. gingivalis* (168 hours) growth and showed no influence on the growth of *S. oralis* (168 hours). It is concluded that acid tolerance seems to be the most important virulence factor of *S. mutans* for the pathogenesis of early childhood caries. As well as the cariogenic bacteria, *B. longum* and *B. lactis* have acidogenic and aciduric potential and also ability to form biofilms and induce enamel demineralization in association with *S. mutans*. While *B. infantis* and *B. lactis* presented inhibitory effect on the growth of periodontal pathogens.

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‡Statistical difference between 24 and 168h, considering each group separately, according to ANOVA and Tukey tests.

¥ Statistical difference between 72 and 168h, considering each group separately, according to ANOVA and Tukey tests.

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Lista de Abreviaturas

AAPD: The American Academy of Pediatric Dentistry; Academia Americana de Odontopediatria.

ANOVA: Analysis Of Variance; Análise de variância

AP-PCR: Arbitrary Primer Polymerase Chain Reaction; Reação em cadeia da polimerase com utilização de iniciadores aleatórios.

AUC: Area Under the Curve; área sob a curva

ATCC: American Type Culture Collection: Coleção de microorganismos norte-americana

ATP: Adenosine triphosphate; Trifosfato de adenosina.

ATR: Acid tolerance response; resposta de tolerância ácida

BHI: Brain Heart Infusion Agar; Agar Infusão de Cérebro e Coração

CD: Conducted by One Examiner; Conduzido por um examinador

CF: Caries Free; Livre 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

dNTPs: desoxirribonucleotídeo trifosfatado

ECC: Early childhood caries; Cárie precoce da infância

EDTA: Ethylenediamine tetraacetic acid; ácido etilenodiamino tetra-acético

EPS: extracellular polysaccharides; polissacarídeos extracelulares

FAO/WHO: Food and Agriculture Organization of the United Nations/ World Health Organization; Organização para Alimentação e Agricultura das Nações Unidas/ Organização Mundial de Saúde

GtfB: Glucosyltransferase B; Glicosiltransferase B

MRS: Man, Rogosa and Sharpe

MS: *mutans streptococci*

MSB: Mitis Salivarius Agar with bacitracin/Agar Mitis Salivarius com bacitracina

OD: Optical density; Densidade óptica

PCR: Polymerase Chain Reaction; Reação em cadeia da polimerase

pH: potencial Hidrogeniônico

RNA: Ribonucleic acid; ácido ribonucléico

SDS: sodium dodecyl sulfate; Dodecil sulfato de sódio

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

SSM: Simple Matching Coefficient; Coeficiente de simililaridade

SH: Surface Hardness; dureza de superfície

TE buffer: Tris-EDTA buffer; tampão Tris-EDTA

TSA: Trypticase Soy Agar; Ágar de soja Trypticase

TBE: Tris-borato-EDTA; Base Tris, ácido Bórico e EDTA.

WHO: World Health Organization; Organização Mundial de Saúde

Sumário

Introdução geral

A boca é um ecossistema complexo composto por mais de 700 espécies bacterianas (Paster et al., 2006). Essas espécies interagem entre si e com outros microrganismos formando comunidades microbianas embebidas em uma matriz de polímeros de origem bacteriana ou do próprio hospedeiro denominadas de biofilme dental (Socransky e Haffajee, 2002). Alterações em fatores que regulam a homeostase no ambiente bucal, como a integridade do sistema imune e a composição da dieta, por exemplo, podem modificar a proporção dos microrganismos permitindo a proliferação de espécies patogênicas que levam a formação de doenças bucais (Marsh, 2009). Entre essas doenças, as mais prevalentes são a cárie dentária e a doença periodontal (WHO, 2012).

A cárie dentária é conceituada como uma doença biofilme-dependente associada à ingestão frequente de carboidratos da dieta. A fermentação desses carboidratos pelos microrganismos do biofilme leva à produção de ácidos o que desequilibra a homeostase do biofilme e pode causar a dissolução dos tecidos minerais do dente (Marsh, 2009). Quando atinge a dentição decídua em crianças muito jovens é denominada de cárie precoce da infância – CPI (Drury et al.,1999; AAPD, 2002). No Brasil, a CPI apresenta-se como grave problema de saúde pública, com prevalência nacional de 26,85% nas crianças aos cinco anos de idade (SBBrasil, 2010). A CPI é caracterizada pela presença de uma ou mais superfícies dentárias cariadas, cavitadas ou não, perdidas ou restauradas, em menores de seis anos (Drury et al., 1999; AAPD, 2002). Os fatores etiológicos mais associados a CPI são: ingestão frequente de carboidratos fermentáveis, altas contagens de estreptococos mutans e maior vulnerabilidade imunológica da criança (Mattos-Graner et al., 1998; 2001a; Ramos-Gomez et al., 2002; Mohebbi et al., 2008).

O grupo bacteriano considerado mais cariogênico é o dos estreptococos mutans (SM), especialmente *Streptococcus mutans* e secundariamente *Streptococcus sobrinus* (van Houte et al., 1991; Mattos-Graner et al., 2001a). Embora a associação entre *S. mutans* e CPI pareça convincente, grande porcentagem das crianças colonizadas por essa espécie bacteriana não manifestam a doença (Mattos-Graner et al., 1998; Ramos-Gomez et al., 2002), sugerindo que, entre outras possibilidades, *S. mutans* variam em sua habilidade de iniciar a cárie dentária. Estudos utilizando técnicas de biologia molecular têm demonstrado que cepas de *S. mutans* associadas com CPI diferem em sua

composição genômica comparadas aos grupos controle livres de cárie (Saxena et al., 2005;2008; Duque et al., 2009). Além disso, características fenotípicas específicas podem determinar o estabelecimento da espécie na complexa comunidade microbiana do biofilme dental. Os principais fatores de virulência de *S. mutans* que determinam sua cariogenicidade são: sua habilidade em formar biofilme na superfície dentária (por meio da aderência e produção de polissacarídeos extra e intracelulares), acidogenicidade (produção de ácidos a partir de uma variedade de açúcares fermentáveis) e aciduricidade (tolerância ao baixo pH do ambiente) (Smith, 2002; Lemos et al., 2005). Entretanto, outras espécies acidogênicas e acidúricas, incluindo estreptococos não mutans, *Actinomyces* e *Lactobacillus* também foram relacionadas ao desenvolvimento das lesões de cárie (Sansone et al., 1993; van Houte et al., 1996). Adicionalmente, espécies de *Bifidobacterium* foram identificadas na composição do biofilme dental em lesões de mancha branca (Van Ruyven et al., 2000) e observada forte associação entre *S. mutans* e bifidobactérias na saliva de crianças com CPI (Tanner et al., 2011).

A família Bifidobacteriaceae é composta por sete gêneros: *Bifidobacterium, Aeriscardovia, Falcivibrio, Gardnerella, Parascardovia, Scardovia* e *Alloscardovia*, isoladas de animais ou humanos. São microrganismos anaeróbios Gram-positivos, não filamentosos, em forma de bastonete, que habitam o trato gastrointestinal. O gênero *Bifidobacterium* ou das bifidobactérias tem recebido muita atenção, principalmente do mercado alimentício, por seu papel benéfico para a saúde humana, incluindo o aumento da resposta imune adaptativa, tratamento ou prevenção de infecções do trato respiratório e urogenital, além da prevenção de alergias e doenças atópicas na infância (Saxelin et al., 2005). Diversos estudos sugerem que o consumo de produtos contendo probióticos, ou seja, microrganismos vivos, entre os quais, as bifidobactérias, poderia ter efeito sobre a redução de *S. mutans* na saliva (Caglar et al., 2005, 2008; Cildir et al., 2009). Entretanto, outros estudos demonstram não haver diferença na contagem de estreptococos na saliva ou redução da incidência de lesões de cárie após o consumo de probióticos (Nozari et al., 2015; Taipale et al., 2013).

As bifidobactérias estão entre os primeiros anaeróbios a colonizarem a cavidade bucal, sendo encontradas precocemente no leite materno (Abrahamsson et al., 2009) ou adicionadas a alimentos, como os iogurtes (Caglar et al., 2005, 2008). Essas espécies foram isoladas do biofilme dental, saliva e lesões de cárie (Modesto et al., 2006, Beighton et al., 2008, Mantzourani et al., 2009), sendo que as mais abundantes foram:

B. dentium, B. longum, B. breve, B. subtile, B. adolescentis e B. urinalis e a maioria delas não é exclusiva da cavidade bucal. Mantzourani et al. (2009) avaliaram por PCR a presença de bifidobactérias em amostras de lesões de cárie oclusal em comparação com superfícies oclusais livres de cárie em crianças e verificaram que as espécies mais prevalentes nas lesões de cárie foram: *B. dentium, B. longum e B. breve*, sendo que não foi detectada a presença dessas espécies em superfícies livres de cárie, sustentando a sugestão de que essas bactérias poderiam ter um papel na progressão das lesões de cárie oclusal. Esses achados indicam que esse grupo pode estar relacionado ao processo de cárie possivelmente por sua habilidade acidogênica e acidúrica em sobreviver e proliferar em ambientes ácidos de lesões de cárie, como ocorre no trato gastrointestinal (van Houte et al., 1996; Nakajo et al., 2010).

Estudo mostrou que, de modo similar à *Streptococcus mutans*, uma espécie de bifidobactéria encontrada em animais e utilizada em laticínios, *Bifidobacterium animalis*, subespécie *lactis*, apresentou mecanismos de manutenção do pH intracelular, como a bomba translocadora de íons que excreta H+ sob condições acídicas (Gibson e Wang, 1994). Nakajo et al. (2010) avaliaram os efeitos da acidificação sobre *B. dentium* e *B. longum* e compararam com *S. mutans, S. sanguinis* e *L. paracasei.* Os autores verificaram alto padrão de sobrevivência e manutenção do pH das bifidobactérias em comparação com *S. mutans* em ambientes ácidos, demonstrando porque essas espécies vivem estáveis junto com estreptococos mutans em lesões de cárie. Além desse fator de virulência, a maioria das espécies de bifidobactérias consegue degradar diversos tipos de carboidratos, dentre eles, glicose, frutose, manose, n-acetilglicosamina, sorbitol, entre outros. Muitas delas apresentam glicosidases, transportadores e enzimas metabólicas para fermentar até açúcares que não são absorvidos por humanos ou animais (Fushinobu, 2010), o que favorece sua sobrevivência e proliferação em ambientes hostis. Recentemente, Torlakovic et al. (2012) estudaram *in vivo* longitudinalmente a sucessão microbiana em lesões de esmalte dental e notaram que várias espécies, além de *S. mutans* podem estar envolvidas com o início da lesão de cárie, entre elas, as bifidobactérias. Desta forma, seria importante verificar o potencial cariogênico desses microrganismos, representado pela acidogenicidade, aciduricidade, capacidade de formar biofilme e até induzir a lesões iniciais de cárie na superfície dentária.

De modo contrário ao que ocorre com a cárie dentária, estudos foram unânimes em relacionar o impacto positivo *in vitro* e *vivo* de cepas probióticas na redução de periodontopatógenos e dos sinais clínicos das doenças periodontais (Twetman et al., 2009; van Essche et al., 2013, Teughels et al 2013; Baca-Castañon et al., 2015). No entanto, poucos estudos têm-se focado na relação entre espécies de *Bifidobacterium* e patógenos periodontais, mas, foi demonstrada a capacidade de coadesão e coagregação e efeito antagonista de algumas espécies de bifidobactérias quando cultivadas com periodontopatógenos (Haukioja et al., 2006; Hojo et al., 2007; Nagaoka et al., 2008; Zhu et al., 2010). Zhu et al. (2010) observaram que um bioiogurte contendo probióticos e algumas espécies de bactérias probióticas isoladas, incluindo as bifidobactérias, foram capazes de inibir o crescimento de patógenos periodontais.

Este trabalho foi dividido em três capítulos. Os objetivos foram: 1) avaliar a diversidade genotípica e as características fenotípicas de cepas de *S. mutans*, isoladas do biofilme dental de crianças CPI e CPI-S (CPI severa) em comparação com crianças livres de cárie (LC); 2) Avaliar a capacidade de produzir e tolerar ácidos, de formar biofilme e de induzir lesões iniciais de cárie *in vitro* de espécies de bifidobactérias comparada à de espécies bacterianas já reconhecidas no contexto da cárie dentária; 3) investigar o efeito antagonista *in vitro* de algumas espécies de bifidobactérias sobre biofilmes de *Fusobacterium nucleatum, Porphyromonas gingivalis* e *Streptococcus oralis.*

Capítulo 1

Capítulo 1

Genotypic diversity and phenotypic traits of *Streptococcus mutans* isolates and their relation to severity of early childhood caries

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Running title: *S. mutans* virulence traits and children caries status

Abbreviations: ECC, Early childhood caries; CF, Caries free; S-ECC, Severe Early childhood caries.

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Abstract

Aim: This study aimed to evaluate genotypic diversity and phenotypic traits of *S. mutans* isolated from dental biofilms of children with different caries status in comparison with caries free (CF) children.

Design: *Streptococcus mutans* strains were isolated from supragingival biofilm samples of CF, ECC and severe-ECC (S-ECC) children and genotyped by AP-PCR. *S. mutans* genotypes were tested for their ability to reduce the suspension pH through glycolysis, to tolerate extreme acid challenge and by their ability to form biofilm. Response variables were analyzed by ANOVA/Tukey or Kruskal-Wallis/Mann-Whitney tests at a 5% of significance.

Results: There was an increase in the prevalence of *Streptococcus mutans* in biofilms with the severity of dental caries. No differences in genotypic diversity and in acidogenicity of genotypes were found among CF, ECC and S-ECC children. Genotypes isolated from S-ECC were highly acid tolerant. Genotypes from ECC and S-ECC formed more biofilms than those isolated from CF children.

Conclusion: Although *S. mutans* genotypic diversity was similar among the groups of children, phenotypic traits of *S. mutans* could explain caries lesion development at individual level.

Keywords: early childhood caries, *Streptococcus mutans*, dental biofilm, genotypic diversity, acidogenicity, aciduricity.

Introduction

Dental caries is a biofilm-associated disease caused by frequent intake of dietary sugars. Fermentation of these sugars by biofilm microbiota leads to acid production, which disrupt microbial homeostasis and cause dissolution of tooth minerals[1]. Mutans streptococci, mainly *Streptococcus mutans*, have been considered as the most important bacteria implicated in dental caries etiology and progression. These microorganisms are frequently isolated from cavitated caries lesions and induce caries formation in sucrose-fed animals[2]. *S. mutans* have important features which contribute to their virulence, such as their ability to metabolize a wide variety of carbohydrates and produce large amounts of acid (acidogenicity), to tolerate extreme acidic environments (aciduricity) and ability to synthesize extracellular polysaccharides (EPS), mainly from sucrose, which improve their adherence to other microorganisms and to tooth surface[3].

Early childhood caries (ECC) is an aggressive condition of dental caries that affects young children. ECC is classified by The American Academy of Pediatric Dentistry (AAPD) as the presence of one or more decayed, missing (due to caries) or filled tooth surfaces in any primary tooth in a child 71 months of age or younger, while severe ECC (S-ECC) is represented by one or more cavitated, missing (due to caries) or filled smooth surface in primary maxillary anterior teeth or decayed, missing or filled surfaces greater than or equal to four (age of 3), five (age of 4) or six (age of 5)[4]. ECC disease has rampant, acute and progressive characteristics and lead to destruction of the primary dentition affecting negatively children's physical and mental health in addition to increase the risk of new caries lesions in the permanent dentition[5].

It is well known that saliva and dental biofilm harbors different genotypes of *S. mutans* and caries-active individuals seems to have more genotypes than caries-free ones[6-8]. Only few studies evaluated genetic diversity of *S. mutans* from ECC children[8-11]. It has been discussed that the simultaneous action of several strains with possibly different cariogenic potential could increases the risk of caries[8]. Phenotypic traits of different *S. mutans* genotypes would be associated with their ability to colonize tooth surface or express factors that could induce the formation of caries lesions[10]. In this way, Lembo et al.[11] demonstrated that caries-active children harbor more *S. mutans* isolates able to survive under acidic environment than those from caries-free group. However, it is still unknown if genotypic diversity and phenotypic traits of *S. mutans* are related to different caries status or caries severity in children. Therefore, this study aimed to evaluate genotypic diversity, acidogenicity, aciduricity and biofilm formation of *S. mutans* isolated from dental biofilms of caries free (CF), of ECC and of S-ECC children in an effort to understand caries lesion development at individual level. Tested alternative hypotheses were: 1) ECC and S-ECC children harbor more genotypes than CF children; 2) genotypes from ECC and S-ECC children are more acidogenic, acid-tolerant and present higher ability to form biofilm than those isolated from CF children. 3) Genotypes from S-ECC children are more

acidogenic, acid-tolerant and present higher ability to form biofilm than those isolated from ECC children.

Material and methods

Study design

This study was approved by the Research Ethics Committee of Araçatuba Dental School, State University of Sao Paulo, Brazil (CAAE 0041.0.258.000-10 ANEXO A). Signed informed consent was obtained from the parents/ legal tutors of children previously to the beginning of the study. Twenty and seven children enrolled in public preschools in peripherical area of the city of Araçatuba (São Paulo, Brazil) participated in the study. Children were included if they were in good general health, without syndromes or chronic systemic diseases. Children whose parents/legal tutors refused to sign the informed consent, or who did not cooperate with the clinical exams, were excluded from the study. Dental examinations were conducted by one examiner (CD) using a mouth mirror and a ball-ended dental probe under a focusable flashlight, after biofilm removal and drying with gauze. World Health Organization (WHO) criteria was used to classify children caries status, considering the total number of decayed, missing or filled teeth surfaces (dmfs). Children were divided into three groups according to their caries status (CF, ECC, and S-ECC) according to AAPD guidelines [4]. All pateents received dental treatment, when it was necessary.

Samples collection and microbiological procedures

Supragingival biofilm from CF, ECC and S-ECC children were collected from all buccal and lingual smooth surfaces at least 1 h after food intake. Biofilm samples were pooled in order to have a representative sample of each individual. No biofilm was collected from caries cavities. In order to standardize the amount of biofilm, a sterilized plastic disposable handle (Greiner, Frickenhausen, Germany) with a circular opening of about 1 µL of volume capacity was used for biofilm collection immediately after sample pooling. Biofilm samples were placed in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) that was kept on ice for no longer than 2 hours. Biofilm suspensions were vortexed for 1 min, an aliquot of each sample was serially diluted in 0.9% NaCl sterile solution and plated on Mitis Salivarius Agar with 0.2 U Bacitracin (Sigma Aldrich) for the isolation of *mutans* streptococci (MS)[12]. All agar plates were incubated at 37 ^{o}C for 48 h in an atmosphere of 5% CO_2. The number of colony-forming units (CFU) was determined from a representative area of each agar plate yielding 30–300 colonies using a stereoscopic microscope and the results were expressed as CFU/ml. Up to six representative colonies of MS were then selected from agar plates and individually transferred to tubes containing 5 mL of Brain Heart Infusion broth (BHI) which were incubated

for 24 h under the same conditions described above. The purity of the cultures was checked by Gram-staining. Aliquots of MS isolated strains were frozen at -20 ^{o}C in BHI containing 20% glycerol for further use in molecular analysis[13].

Genotypic analysis

DNA extraction and Polymerase Chain Reaction (PCR)

Frozen aliquots of each MS colony isolated from biofilms were grown on MSB (Mitis Salivarius Agar with bacitracin, as described above) and incubated 37 ^{o}C for 24-48 h in an atmosphere of 5% CO_2. The colonies that grew on BHI agar were incubated into BHI broth (Difco) and incubated at 37 ^{o}C for 18h at the same conditions. Cells from these cultures were then harvested and genomic DNA of MS isolates was extracted using a protocol modified by Nascimento et al.[14]. Briefly, samples were submitted to a lysing solution (extraction buffer and proteinase K) and then purified using chloroform:isoamil-alcohol, followed by DNA precipitation with isopropanol and 70% ethanol. The DNA was ressuspended in TE buffer (10 mM Tris–Hcl, 0.1 mM EDTA, pH 7.5, with 10 mg/mL RNAse). DNA was quantified in a spectrophotometer at 260 nm for obtaining a standard concentration of 100 ng of DNA/mL from each isolate. DNA samples were stored at -70^{o}C for subsequent PCR reactions.

In order to confirm *Streptococcus mutans* molecular identity, DNA of MS isolates were submitted to PCR method, using specific primers for portions of the glucosyltransferase B gene (gtfB) following the bases sequences: 5' – ACT ACA CTT TCG GGT GGC TTG G – 3' e 5' – CAG TAT AAG CGC CAG TTT CAT C – 3', to amplify a 517 bp DNA fragment[15]. Each PCR mixture contained 5µl of the DNA template, 5µl of X 10 PCR amplification buffer (100mM Tris-HCl, 500 mM KCl, pH 8.3), 0.2 mM of dNTPs (DNA Polymerization Mix), 3.0 mM $MgCl_2$, 1 µM of each primer and 2.5 U de Taq *DNA* Polymerase and sterile distilled water to obtain a final volume of 25 µl. Positive and negative controls of PCR reactions were purified genomic DNA of *S. mutans* (ATCC 25175) and sterile water, respectively. The amplification of DNA was performed in a thermocycler (GeneAmp PCR System 2400, Perkin Elmer, Applied Biosystems, USA) with an initial denaturation at 95^{o} C for 5 minutes, followed by 30 cycles of denaturation at 95^{o} C for 30 seconds, annealing at 59^{o} C for 30 seconds and extension at 72^{o} C for 1 minute, besides the final extension at 72^{o} C for 7 minutes (18). The PCR amplification products were separated by electrophoresis in 2% agarose gels in Tris-borato-EDTA (TBE) running buffer (pH 8.0) at 100 V for 45 min. Gels were stained with SYBR Green 1.6% and visualized under ultraviolet light illumination (UltraLum – Labtrade do Brasil). A 100 bp DNA ladder was included as a molecular-size marker in each gel. All PCR reagents were obtained from Invitrogen, Life Technologies, São Paulo, Brazil [13, 14].

Arbitrary-Primed Polymerase Chain Reaction (AP-PCR)

Isolates molecularly identified as *Streptococcus mutans* were genotyped by AP-PCR technique. Amplification was performed with primer OPA-02 (5'- TGCCGAGCTG – 3')[6]. All reactions were processed in a volume of 50µl, containing 1x PCR buffer, 5U/µl of Taq DNA polymerase, 10mM DNTp, 20µM primer, 50mM MgCl2 and 2µl of template DNA [6]. The amplification was performed in the same thermocycler with an initial denaturation at 95 ^{o}C for 2 minutes and 45 cycles consisting of 94 ^{o}C for 30 seconds, 36 ^{o}C for 30 seconds and 72 ^{o}C for 1 minute, concluding with a final extension of 72 ^{o}C for 5 minutes[6]. Amplicons generated by AP-PCR were analyzed eletrophoretically in 1.5% agarose gel in TBE running buffer and stained with SYBR Gren 1.6%. A 1Kb DNA ladder was used as molecular-size marker. The gels were photographed and their images captured with a digital imaging system (Kodak Digital Science 1D). The molecular weights for each band or amplicon were computed and analyzed by the Sigma Gel software program[13, 14].

Phenotypic analysis

Acidogenicity assays (glycolytic curves)

Frozen stocks of *S. mutans* genotypes (n=14 (CF); n=12 (ECC); n=8 (S-ECC)) identified in the dental biofilms were grown on BHI agar plates and incubated at 37 ^{o}C for 48 h in an atmosphere of 5% CO_2. CFU were collected and inoculated into BHI broth which was incubated at 37^{o}C for 18h. These assays were conducted according protocol described by Arthur et al.[15], to evaluate the ability of *S. mutans* genotypes to lower the suspension pH through glycolysis (pH drop). Aliquots of cultures (approximately 10^8 CFU/ml) grown in BHI broth were centrifuged and resuspended in 50 mM KCl supplemented with 1 mM $MgCl_2$ (Fluka, Steinheim, Germany). The pH of the solution was adjusted to 7.2, and glucose was added to a final concentration of 55.6 mM. The decrease in pH was then assessed during 300 min using a glass electrode previously calibrated with pH standards (pH 4.0 and 7.0). The area under the curve (AUC) for the drop in pH after 300 min considering pH 3.0 as a cut-off point was also determined. The acidogenicity was expressed as AUC (total pH drop) or means/standard deviation of pH for each period of time (0 to 300 min). Analyses were performed in triplicate.

Aciduricity assays (acid killing assays)

The ability of *S. mutans* genotypes (n=14 (CF); n=12 (ECC); n=8 (S-ECC)) to tolerate acid challenge was evaluated using the acid killing assay[11,15-16]. Briefly, aliquots (approximately 10^8 CFU/mL) grown for 18 h in BHI broth were transferred into fresh BHI broth and grown to mid-exponential phase (OD550 = 0.5). The suspensions were then centrifuged, and the pellets

were washed once with 0.1 M glycine buffer (pH 7.0) (Fluka). In addition, the washed pellets were resuspended in 0.1 M glycine buffer pH 2.8, 5.0 and 7.0 (control). Immediately after resuspension (T0) and after 60 min (T60) of incubation at 37 ^{o}C, aliquots were serially diluted in phosphate buffer (pH 7.2), plated on BHI agar and incubated at 37 ^{o}C for 48 h in an atmosphere of 5% CO_2. Cell viability at each time point was expressed as the percentage of bacterial counts reduction at pH 2.8 and 5.0 in relation to pH 7.0. Analyses were performed in triplicate.

Biofilm assays

Biofilm assays were conducted for *S. mutans* genotypes (n=14 (CF); n=12 (ECC); n=8 (S-ECC)) according to Mattos-Graner et al.[17] with slight modifications. An aliquot from a BHI broth culture of each MS isolate (prepared as described above) was diluted 1:100 in fresh BHI, and 200 µl of this dilution was transferred to sterile polystyrene U-bottom microtiter plates. Plates were incubated for 18 h at 5% CO_2, and biofilm growth was revealed and quantified by staining with crystal violet. Crystal violet absorbance was determined with a plate reader at 575 nm (Eon Microplate Spectrophotometer, BioTek Instruments, USA). The absorbance (A550) of planktonic cultures grown under the same conditions was measured to monitor growth. Biofilm formation for all strains was measured in triplicate plates.

Statistical analysis

The studied groups (CF, ECC and S-ECC) were considered as dependent variables. The constant "1" was added to CFU because no growth was detected in some samples. A counts were then transformed to log10 (CFU+1). Age, dmfs, mutans streptococci counts, acidogenicity and biofilm assays were compared among the groups by ANOVA followed by Tukey tests. Aciduricity, number of isolates and genotypes of *S. mutans* were compared among the groups by Kruskal-Wallis and Mann-Whitney tests. Electrophoretic bands previously scored in the AP-PCR gels were converted into binary data and submitted to NTSYS-pc software (Applied Biostatistics, Inc.), using coefficient SSM (Simple Matching Coefficient) and UPGMA cluster analysis (Unweighted Pair-Group method with Mathematic Average) to generate dendrogram showing genetic similarity among the bacterial strains isolated from CF, ECC and S-ECC children. Statistical analysis was performed using the program SPSS version 17.1. ($P<0.05$).

Results

Twenty-seven children, 12 girls (44.5%) and 15 boys (55.5%) between 36 and 65 months of age (mean 45.21 ± 12.04) were included in this study, corresponding to 10 (37.7%) CF, 9 (33.4%) ECC and 8 (29.6%) S-ECC children. There was no statistical difference among

CF, ECC and S-ECC children regarding gender and age. Dmfs values differed statistically among the tested groups with S-ECC children presenting the highest values. Counts of MS in biofilms of ECC and S-ECC children did not differ from each other but they were higher than those found in CF children **(Table 1)**.

One hundred and nine MS strains (38 for CF, 35 for ECC and 36 for S-ECC) were isolated from biofilms. Ninety-eight isolates were molecularly identified as *S. mutans*. S-ECC children presented the highest percentage of *S. mutans* isolates compared with CF and ECC children, which were not different between them **(Table 2)**. Fifty-nine out of 98 *S. mutans* isolates (21 CF, 20 ECC and 18 S-ECC) were re-isolated and genotyped by AP-PCR. A total of 22 clusters (identical or highly related) were detected in biofilm samples after analysis of dendrogram **(Figure 1).** Although S-ECC children seems to harbor lesser genotypes compared to other groups, the number of genotypes found in biofilms was not statistically different among CF, ECC and S-ECC children **(Table 2)**. Most of children (53.6%) exhibited two different genotypes. One genotype was found in 35.7% of children, while three or more genotypes were present in 10.7% of children.

Regarding phenotypic analysis, no statistical difference was observed among the tested groups for acidogenicity of *S. mutans* genotypes considering both pH values at each time point (0 to 300 minutes) and the values of area under the curve – AUC (**Figure 2**; $p>0.05$). Final pH achieved by genotypes (mean±SD) of CF children (3.84±0.48) ranged from 4.67 to 3.48; from 4.0 to 3.47 for ECC children (3.69±0.19) and from 3.75 to 3.51 for S-ECC children (3.63±0.12), but no difference was found among the groups evaluated. Considering aciduricity, counts of viable cells of *S. mutans* genotypes were not statistically different among CF, ECC and S-ECC children at pH 5.0 and 7.0 for both time points (**Figure 3**). However, genotypes of S-ECC children presented higher counts of viable cells than CF and ECC genotypes at pH 2.8 at time zero and time 60. Under exposure to pH 2.8 for 60 minutes, counts of viable cells of *S. mutans* were statistically lower than those found at pH 5.0 and 7.0 for CF, ECC and S-ECC genotypes. Genotypes isolated from ECC and S-ECC children presented higher biofilm formation than those isolated from CF children (**Figure 4**).

Discussion

In the present study, differences were not found in genotypic diversity among CF, ECC and S-ECC children. Therefore, the tested hypothesis 1 was rejected. Available evidences are inconclusive regarding genotypic diversity and caries. Some studies reported a decrease in genotypic diversity in caries-active subjects or absence of difference between caries-free and caries-active children[11] while multiple genotypes were associated with caries activity in infants[7, 9,18]. In the present study, no difference on *S. mutans* genotypic diversity was found in dental

biofilm of caries-active and caries-free children, agreeing with aforementioned investigation[11]. Previous studies have suggested that constant sugar stress provided by dietary sugars, a condition found in caries-active individuals, may select some salivary genotypes make them more prone to colonize dental biofilms[15,19]. These divergences in genotypic diversities among studies could be attributed to differences in the studied population mainly in respect to their age and caries status. Additionally, altogether, these data reinforce that phenotypic traits may be more important than the presence/absence of specific genotypes in biofilm[18].

In respect to the genotyping of MS isolates, Argimón et al.[20] have suggested that *S. mutans* strains derived from caries-active or caries-free individuals cannot be differentiated only based on the presence or absence of specific genetic elements. In order to circumvent this problem, we chose AP-PCR analysis with primer OPA-02 that showed a considerable number of amplicons and an efficient differentiation of genotypes. Previous studies have proved superior efficiency of OPA-02 in the identification of distinct genotypes when compared with other arbitrary primers[6, 7, 11, 17]. Genetic polymorphism among closely related species is determined by changes in base pairing, by deletion or insertion of new genetic sequences and the cloning transmission from external sources[21]. Some strains of *S. mutans* can acquire various cariogenic properties and antibiotic resistance by transformation[22]. Cvitkovitch[22] suggested that bacterial transformations may occur in environments, which suffer changes and extreme fluctuations in population dynamics, such as the oral cavity. Bacteria in these environments are often exposed to different stress conditions, such as excess or lack of nutrients, low pH, high osmolarity and the use of antimicrobial agents by the host[23]. Therefore, the natural genetic transformation could be considered an important mechanism of cell adaptation to environmental changes, providing microbial resistance, genetic variation and rapid evolution of virulence factors [22, 23]. All these microbial traits could favor survival and proliferation and contribute in the development and/or progression of caries lesions.

An important characteristic of virulence of *S. mutans* is its ability to metabolize a variety of dietary carbohydrates such as sucrose and produce a large amount of organic acids by fermentation, which acidify the dental biofilm. In this study, no statistical difference in relation to acidogenicity (pH drop) was found among the *S. mutans* genotypes isolated from dental biofilm of CF, ECC and S-ECC children. Previous studies reported no correlation between caries activity and *in vitro* acid production of *S. mutans* isolates monitored by records of final pH reached after sugar fermentation[7, 10]. Regarding aciduricity, our data showed that genotypes isolated from S-ECC children are more acid-tolerant under extreme acidic pH challenge (such as the tested pH 2.8 condition) than those isolated from CF and ECC children. This data is in consonance with Lembo et al.[11] that demonstrated that genotypes from caries-active children presented low-susceptibility to acid challenge. However, it is important to mention that no

criterion for the assessment of caries lesions severity was adopted in that study[11]. Therefore, we were able to show indeed that acid-tolerance of genotypes is increased with increased caries severity, since no difference was found in cell viability between CF and ECC children under distinct acidic environments. The frequent acidification of oral cavity as a result of sugar fermentation by biofilm microbiota is followed by expression of numerous proteins responsible for maintenance of cell viability by a process called acid-tolerance response – ATR[24]. This mechanism is dependent on a number of virulence traits including the ability to carry out glycolysis at a lower pH[16]. The adaptation process to acidic environments in *S. mutans* is progressive, indicating not a genetic selection but a gradual physiologic process, changing membrane lipid composition, incorporating unsaturated fatty acids into the plasma membrane[25] and affecting activities of membrane ATPases[26]. *S. mutans* controls proton intracellular influx increasing proton extrusion via end-product efflux and F_1−F_0-ATPase activity. The *S. mutans* F_1−F_0-ATPase can operate at low pH much more efficiently than the ATPase of several other competing oral bacterial species such as *Streptococcus salivarius* and *Streptococcus sanguinis*[27]. Besides, *S. mutans* are able to encode several enzymes to protect DNA or repair DNA damage from the harmful effects of intracellular acidification[24]. As a response to all these physiological changes, the frequent pH fall found in oral cavity of S-ECC children allowed a more efficient acid adaptation of their *S. mutans* genotypes, which contributed to the enhanced aciduricity. Considering acidogenicity and aciduricity, it seems based on our results that the latter one plays a more important role on the development of cariogenic biofilms compared with the ability of strains in acid production from dietary sugars.

The present study also evaluated the ability of *S. mutans* genotypes to form *in vitro* biofilm in a sucrose-rich medium. ECC and S-ECC genotypes formed higher amount of biofilm biomass than CF genotypes. This finding corroborated with those obtained by other researches using animal model[28] and *in vitro* study[10]. This last study showed that *S. mutans* isolates exhibited variability in their ability to form biofilm, but five of the six high biofilm-forming isolates were obtained from caries-active children[11]. The potential to form biofilm can be also indirectly determined by the production of insoluble extracellular polysaccharides (EPS)[10]. Individuals with high caries activity are often infected by *S. mutans* strains that produce significantly higher amounts of EPS compared to strains infecting caries-free children[2] and adults[7]. EPS improve bacterial adherence to dental surfaces[10]. Additionally, EPS modify the matrix of biofilms making them more porous facilitating the diffusion of acids and carbohydrates throughout the biofilm allowing pH fall in tooth/biofilm interface[29]. It may be possible though that ECC and S-ECC genotypes produce more EPS than CF genotypes. This question will be addressed in further studies. Therefore, hypotheses 2 and 3 could be partially accepted.

In the current study, in agreement with Kouidhi et al.[30], we showed that the detection of *S. mutans* in biofilms increased depending on severity of dental caries, since all MS isolates from S-ECC children were positively identified as *S. mutans*. As discussed above, *S. mutans* are highly acidogenic, aciduric and able to form thick biofilms in the presence of sucrose. Then it is important to discuss that up to 80% of MS isolates were identified as *S. mutans* in biofilm samples of CF children which means that phenotypic traits of *S. mutans* are more important for caries development than their relative numbers in biofilms. This is supported by our data in a way that biofilm forming ability and acid tolerance were higher in genotypes isolated from ECC and S-ECC children.

Conclusions

In conclusion, no difference in genotypic diversity and acidogenicity was found in biofilm samples of CF, ECC and S-ECC children. However, genotypes isolated from S-ECC were highly acid tolerant. *S. mutans* genotypes isolated from ECC and S-ECC formed more biofilms than those isolated from CF children.

Why this paper is important to paediatric dentists

- *S. mutans* genotypic diversity is similar in children, regardless of caries status.
- *S. mutans* genotypes from caries-active children are more acid-tolerant and present higher ability to form biofilm than those isolated from caries-free children.
- Acid tolerance seems to be the most important *S. mutans* trait related to the pathogenesis of severe early childhood caries.

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Conflict of interests

The authors declare no conflict of interest.

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Table 1. Description of the study population

	CF	**ECC**	**S-ECC**	***p* value**[¥]
Age (months; mean±SD)	44.4±8.19[A]	45.88±9.42[A]	46.37±6.32[A]	0.67
Dmfs* (mean±SD)	0[A]	3.11±1.83[B]	22.6±23.21[C]	**0.00**
Mutans streptococci counts (log CFU+1;mean±SD)	4.12±1.35[A]	5.58±1.12[B]	5.13±1.17[B]	**0.04**

*tooth surfaces with caries, indicated for extraction and filled

[A] Different uppercase letters shows statistical difference among CF, ECC and S-ECC groups, according to ANOVA/Tukey tests ($p<0.05$).

Table 2. Number of isolates and genotypes of *S. mutans* from CF, ECC and S-ECC groups.

	CF	**ECC**	**S-ECC**	***p* value**
S. mutans isolates n (%)**	30 (78.9)	32 (91.42)	36 (100)*	**0.00**
Total *S. mutans* genotypes (identical genotypes)	21(14)	20(12)	18(8)	0.62

* Statistical difference between CF x S-ECC and ECC x S-ECC groups, according to Kruskal-Wallis and Mann-Whitney tests ($p<0.05$).

** Percentage calculated in relation to total number of MS isolated in each tested condition.

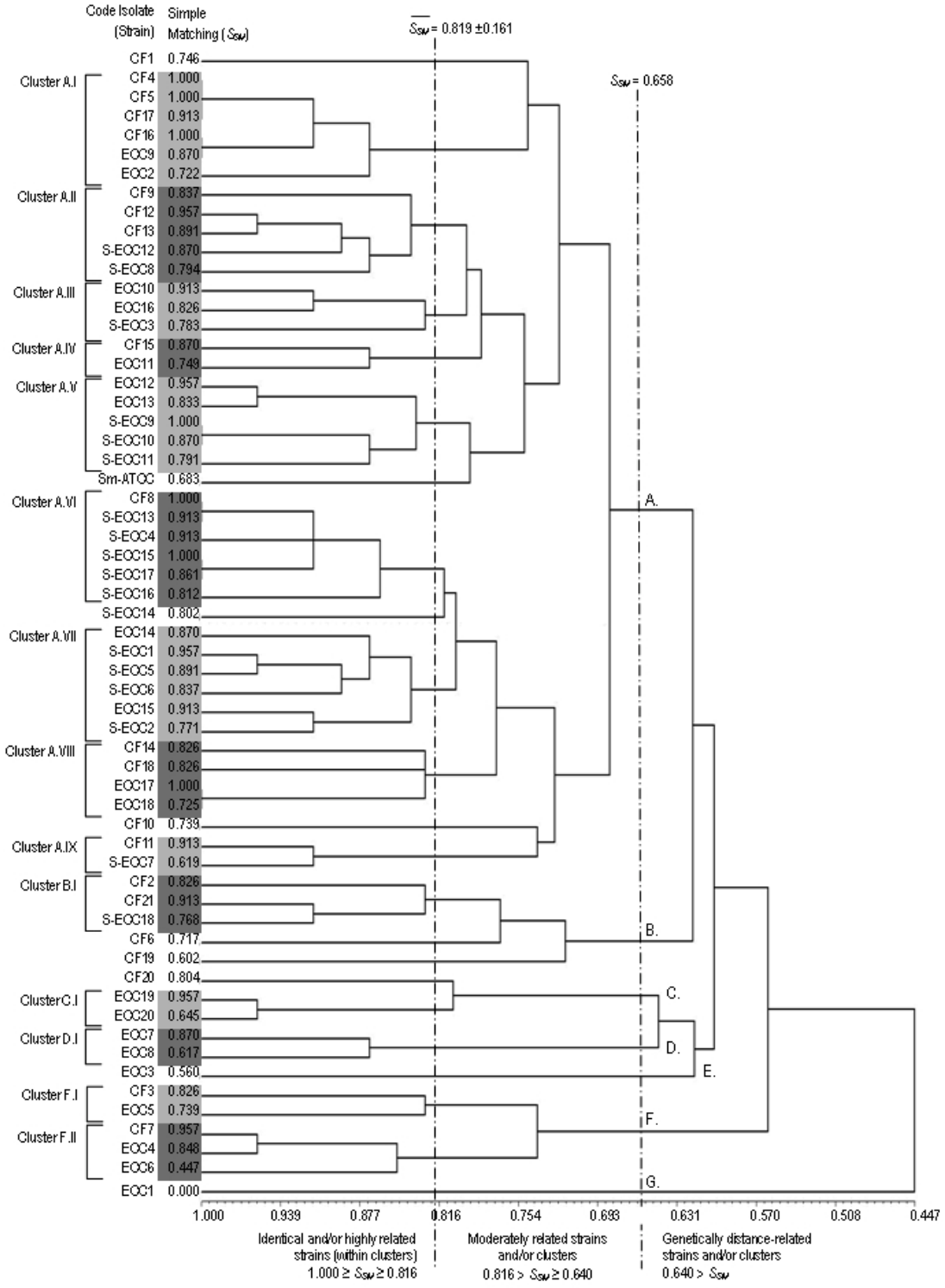

Figure 1. Dendrogram with genetic similarity indices (AP-PCR method, primer OPA-02) verified among *S. mutans* strains sampled from biofilm of CF, ECC and S-ECC groups. Individual bands were analyzed by matrices generated by UPGMA analysis using coefficient SSM (simple matching). Tonalities of gray in the dendrogram illustrate identical or highly related isolates (SSM ≥ 0.819±0.161)

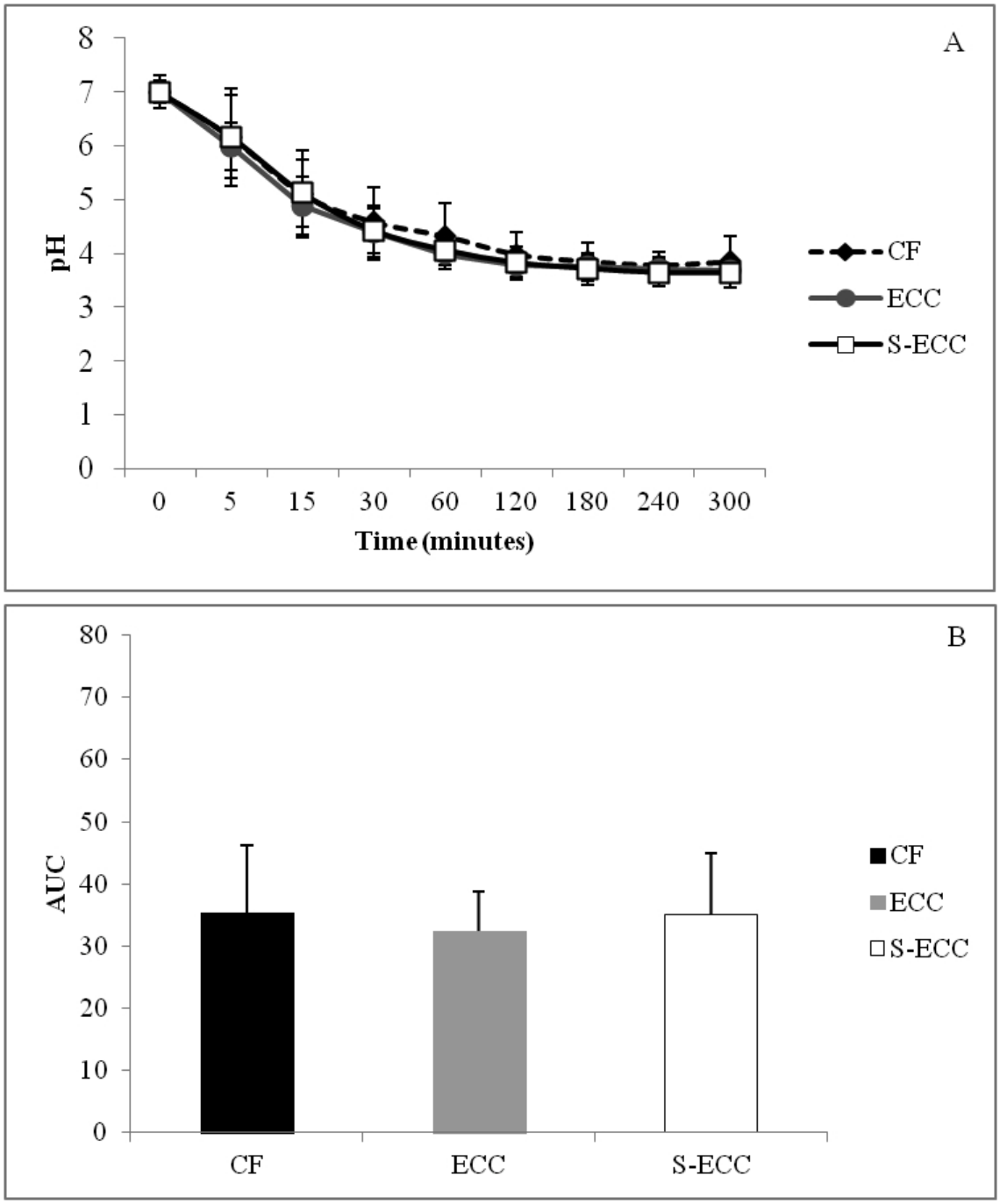

Figure 2. Acidogenicity (acid production). A. Glycolytic curves. Means of pH values through glycolysis. B. Means of the area under the curve (AUC) of glycolytic pH fall. Bars denote standard deviation for both Figures A and B.

*There was no difference in means of pH values in any time points (A) or means of AUC (B) among CF, ECC and S-ECC groups, according to ANOVA and Tukey tests, $p>0.05$.

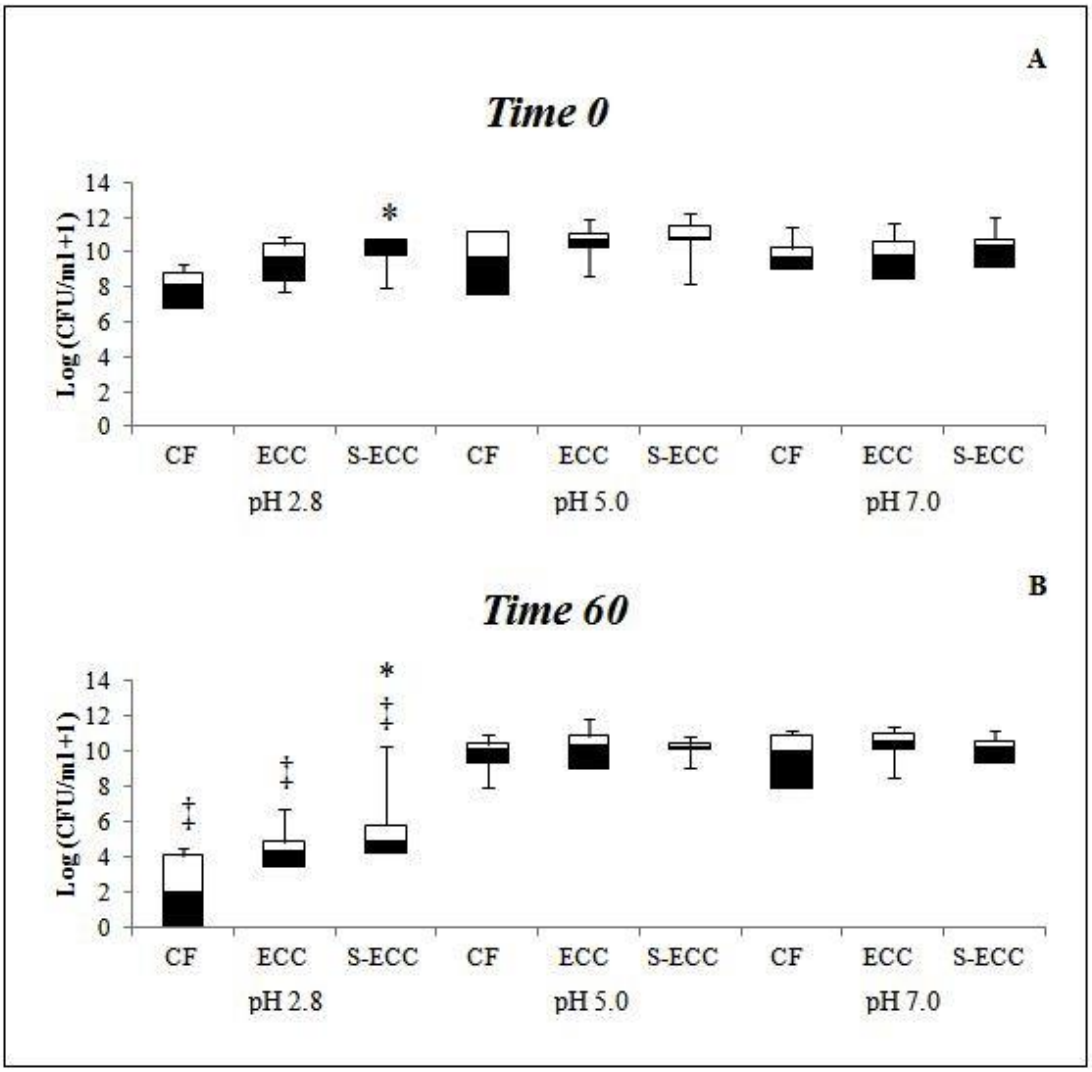

Figure 3. Aciduricity (acid tolerance). Box plots of *S. mutans* counts (converted in log CFU +1) obtained after baseline (Figure A - Time 0) and 60 min (Figure B - Time 60) of exposition to glycine buffer in different pHs.

* Statistical difference among CF, ECC and S-ECC groups, considering each pH separately, according to Kruskal-Wallis and Mann-Whitney tests ($p<0.05$).

‡ Statistical difference among pHs, considering each group (CF, ECC or S-ECC) separately, according to Kruskal-Wallis and Mann-Whitney tests ($p<0.05$).

Bars indicate minimum and maximum values. Black and white boxes indicate lower and upper quartiles, respectively. Line in the middle of boxes is median.

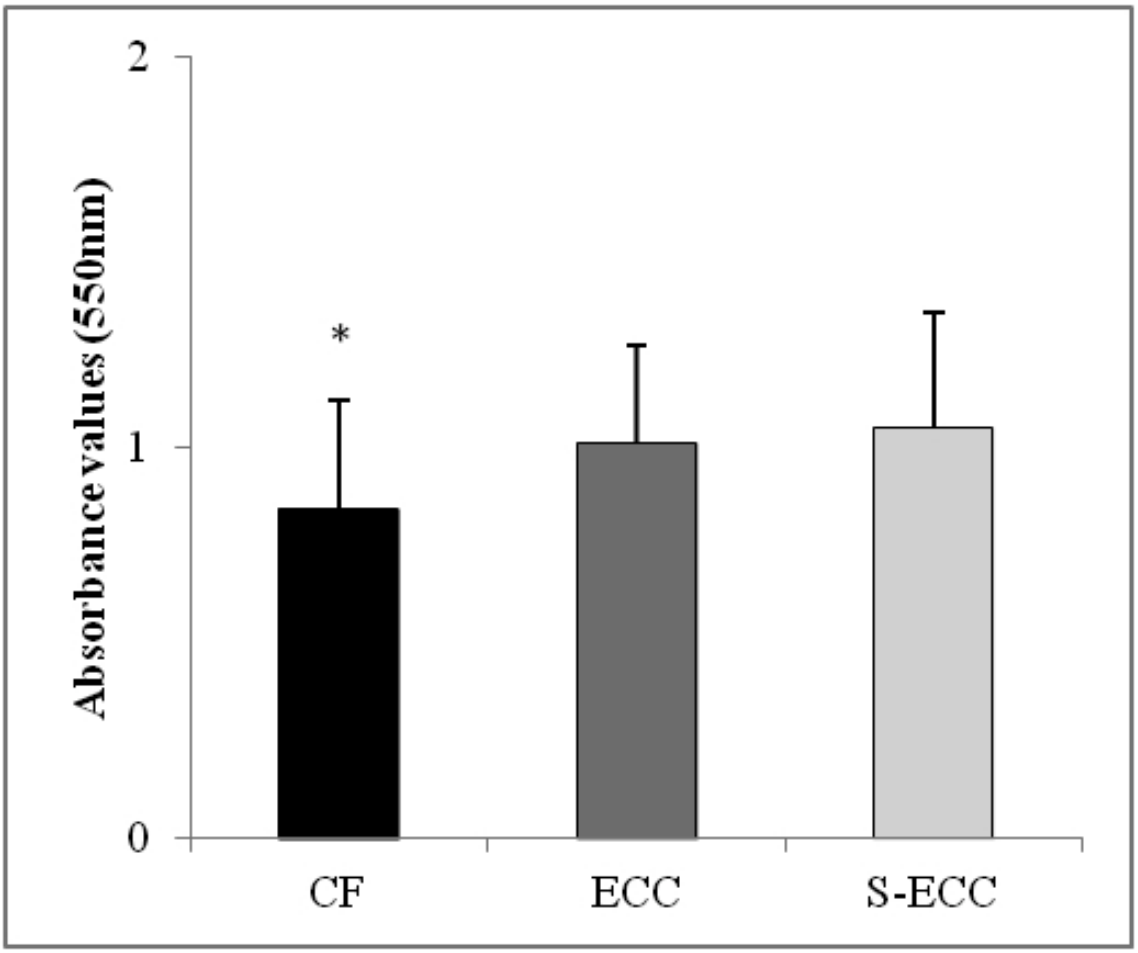

Figure 4. Biofilm formation. Absorbance values (550nm) obtained for 48h biofilm of *S. mutans* genotypes from CF, ECC and S-ECC children.

* Statistical difference between CF x ECC and CF x S-ECC, according to ANOVA/Tukey tests ($p<0.05$).

Capítulo 2

In vitro investigation of the cariogenic potential of bifidobacteria compared with caries associated bacteria

Running Title: Cariogenic potential of bifidobacteria

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Abstract

Objective: This study aimed to assess the *in vitro* cariogenic potential of some *Bifidobacterium* species in comparison with caries-associated bacteria.

Design: *Bifidobacterium lactis*, *Bifidobacterium longum*, *Bifidobacterium animalis*, *Bifidobacterium dentium, Lactobacillus acidophilus*, *Lactobacillus casei*, *Actinomyces israelii*, *Streptococcus sobrinus* and *Streptococcus mutans* were tested for acidogenicity and aciduricity by measuring the pH of the cultures after growth in glucose and bacterial growth after exposure to acid solutions. Biofilm biomass was determined for each species either alone or associated with *S. mutans* or *S. mutans*/*S. sobrinus*. Bovine enamel superficial hardness was analyzed before and after 7-days biofilm formation using bacterial combinations.

Results: *B. animalis* and *B. longum* were the most acidogenic and aciduric strains, comparable to caries-associated bacteria, such as *S. mutans* and *L. casei*. All species had a significantly increased biofilm when combined either with *S. mutans* or with *S. mutans*/*S. sobrinus*. The greatest enamel surface loss was produced when *B. longum* or *B. animalis* were inoculated with *S. mutans*, similar to *L. casei* and *S. sobrinus.* All strains induced similar enamel demineralization when combined with *S. mutans*/*S. sobrinus*, except by *B. lactis*.

Conclusion: *B. animalis* and *B. longum* had acidogenic and aciduric potential, ability to form biofilms and to induce enamel demineralization when associated with mutans streptococci, comparable to other caries-associated bacteria.

Keywords: Biofilms, *Bifidobacterium, Lactobacillus, Streptococcus, Actinomyces*, Dental Caries.

Introduction

Dental caries is a biofilm-associated disease highly associated with frequent intake of dietary sugars. Fermentation of these sugars by biofilm microorganisms leads to acid production, which disrupts biofilm microbial homeostasis and can causes dissolution of tooth minerals (Marsh, 2003). The bacteria considered the most cariogenic are the mutans streptococci (MS), especially *Streptococcus mutans* (van Houte, Sansone, Joshipura, & Kent, 1991; Mattos-Graner, Correa, Latorre, Peres, & Mayer, 2001a). Other acidogenic and aciduric bacterial species, including *Actinomyces* spp. and *Lactobacillus* spp., have been observed in dental caries lesion development (van Houte, Lopman, & Kent, 1996; Dige, Gronnkjaer & Nyvad 2014). Additionally, *Lactobacillus* and *Bifidobacterium* species have been identified as part of the dental biofilm on white spot lesions (Van Ruyven, Lingstrom, van Houte, & Kent, 2000).

The *Bifidobacteriaceae* family consists of seven genera: *Bifidobacterium*, *Aeriscardovia*, *Falcivibrio*, *Gardnerella*, *Parascardovia*, *Scardovia* and *Alloscardovia*, which have been isolated from both animals and humans. These bacteria are anaerobic, Gram-positive, rod-shaped, and principally colonize the gastrointestinal tract. Species in the genus *Bifidobacterium* have recently received significant attention in the consumer food industry due to their beneficial roles in human health. They have been shown to have a role in increasing the adaptive immune response, in treating or preventing respiratory and urogenital infections, and in the prevention of allergies and atopic diseases during childhood (Saxelin, Tynkkynen, Mattila-Sandholm, & de Vos, 2005). Several studies suggest that the consumption of products containing probiotics, such as bifidobacteria, could suppress *S. mutans* levels in saliva (Caglar *et al*., 2005, 2008; Cildir *et al*., 2009). However, recent clinical randomized studies have shown that the administration of probiotics did not affect the MS levels or the occurrence of dental caries in children (Taipale, Pienihäkkinen, Salminen, Jokela, & Söderling, 2012; Taipale, Pienihäkkinen, Alanen, Jokela, & Söderling, 2013; Nozari, Motamedifar, Seifi, Hatamizargaran, & Ranjbar, 2015). A confounding issue is that levels of caries-related microorganisms are not always related to the severity of dental caries (Mattos-Graner, Zelante, Line, & Mayer, 1998), making the beneficial effects of probiotics on the control of caries difficult to elucidate.

Bifidobacterium species have been isolated from dental plaque, saliva and dentinal caries (Modesto, Biavati, & Mattarelli, 2006; Beighton *et al*., 2008;

Mantzourani *et al*., 2009). *B. dentium*, *B. longum* and *B. breve* were only detected in the oral cavities of children with occlusal caries and not in those of caries-free children (Mantzourani *et al*., 2009). Those authors suggested that these bacteria might have a role in the progression of occlusal caries lesions. These findings suggest that this group of bacteria may be related to caries development, possibly due to their acidogenicity and acid-tolerance (van Houte, Lopman, & Kent, 1996; Nakajo, Takahashi, & Beighton, 2010). Tanner *et al.,* (2011) reported a high prevalence of the *Bifidobacterium* spp. in addition to *Streptococcus* in children with advanced dental caries and severe early childhood caries.

Studies have shown that, unlike *Streptococcus mutans*, *B. animalis* subspecies *lactis*, a common species of bifidobacteria found in animals and used in dairy products, have mechanisms of intracellular pH maintenance. One of these mechanisms is a F1F0-ATPase proton *pump* that can facilitate the expulsion of protons (H^+) under acidic conditions (Gibson & Wang, 1994; Nakajo, Takahashi, & Beighton, 2010) which is directly related to their acid-tolerance. In addition to this virulence factor, most species of bifidobacteria can utilize various carbohydrates including glucose, fructose, mannose, N-acetylglucosamine, and sorbitol (Fushinobu, 2010), contributing to their proliferation in disease sites. Thus, this study aimed to determine the cariogenic potential of *Bifidobacterium* species in comparison with currently recognized caries-associated bacteria. The null hypotheses formulated was that the cariogenic potential of *Bifidobacterium* species, represented by acidogenicity, aciduricity, and the ability to form biofilms and to induce enamel demineralization, is not different compared with cariogenic potential of caries-associated bacteria.

Materials and Methods

Bacterial strains and growth conditions

Assays were performed using the following *Bifidobacterium* species: *B. animalis* (from ACTIVIA®), *B. longum* (ATCC 15707), *B. lactis* (LMG 18905) and *B. dentium* (ATCC 27678); *Lactobacillus* species: *L. acidophilus* (ATCC 4356), *L. casei* (ATCC 393); *Streptococcus* species: *S. mutans* (ATCC 25175 and 3VF2), *Streptococcus sobrinus* (ATCC 27607); *Actinomyces* species: *A. israelli* (ATCC 12102). All ATCC strains were obtained from Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, RJ, Brazil) or André Tosello Foundation (Campinas, SP, Brazil). *B. animalis* was isolated

from the yogurt ACTIVIA® in Transgalactosylated Olygosaccharides-Propionate agar with supplement lithium-muporicina (50mg/L) (TOS-MUP agar; Merck Millipore, Darmstadt, Germany). *S. mutans* 3VF2 is highly acidogenic clinical strain kindly provided by Dr. Renata de Oliveira Mattos-Graner (FOP-UNICAMP) (Mattos-Graner, Napimoga, Fukushima, Duncan, & Smith, 2004). Reactivation of strains on selective media was done as follow: *S. mutans* and *S. sobrinus* on Mitis Salivarius agar with bacitracin (0.2U/mL) (Difco), *L. casei* and *L. acidophilus* on Rogosa agar (Difco)*; B. animalis*, *B. longum*, *B. lactis* and *B. dentium* on TOS-MUP agar (Merck) and *A. israelii* on *Actinomyces* Garrod medium (HiMedia Laboratories, Mumbai, India). Plates were incubated anaerobically with Anaerocult system (Merck).

Acidogenicity tests

Up to 5 colonies of each species were individually transferred from agar plates to Brain Heart Infusion broth (BHI, Difco) and incubated at 37°C for 24 h. Bacterial cultures were diluted 1:20 in BHI and the bacterial growth was monitored by turbidimetry. When the optical density reached 0.5 (approximately 10^8-10^9 CFU/mL) at 550 nm, tubes were centrifuged and the pellet was resuspended in a solution of 50 mM KCl and 1 mM $MgCl_2$. The suspension was centrifuged and the pellet was resuspended in 20 mM phosphate buffer and incubated at 37^{o}C for 60 min for starvation. After centrifugation, the pellet was resuspended in a solution of 50 mM KCl and 1 mM $MgCl_2$, the pH of the solution was adjusted to 7.0 and glucose was added to a final concentration of 55.5 mM. The pH of the suspension was evaluated immediately and 5, 15, 30, 60, 120, 180 and 240 minutes after the addition of glucose using a glass electrode (Orion 720 A+; Orion Research Inc.) previously calibrated with pH standards of pH 4.0 and 7.0. The area under the curve (AUC) was calculated of pH drop, considering pH 2.8 as a cut-off point, using UTHSCSA ImageTool software, version 3.0. The acidogenicity was expressed as the AUC (cm^2). Each analysis was performed in three distinct experiments (Belli & Marquis, 1991; Arthur *et al*., 2011).

Aciduricity tests

Similar to acidogenicity tests, 24h broth cultures were diluted and grew until optical density at 550 nm of 0.5. 3 mL of each culture were individually distributed into tubes, which were centrifuged and resuspended in 0.1 M glycine buffer with a pH of 7.0, pH 5.0 or 2.8. Immediately after resuspension (Time 0) and after 60 minutes (Time 60), 100 µl of each sample was serially diluted and plated on BHI agar plates which were incubated for 24 hours at 37°C. Colony Forming Units/mL (CFU/mL) were then counted (Duarte *et al*., 2008; Arthur *et al*., 2011).

In vitro biofilm formation

After reaching an optical density at 550 nm of 0.5, aliquots of 200 µl of each strain were individually transferred to the wells of 96-well plates (Dynatech Lab). Additionally, aliquots of each strain were also combined in equal proportion (at the same optical density) with *S. mutans* (3VF2) or *S. mutans* (3VF2)/*S. sobrinus*. Triplicate of samples were incubated at 37°C for 48 h. Wells containing only the culture medium were used as negative controls. After incubation, the plates were washed by immersion in distilled water three times to remove the non-adherant cells. After a brief drying, 150 µl of aqueous 1% crystal violet was added to each well, and the plates were incubated at room temperature for 30 min. The crystal violet solution was then removed and the plates washed again 3 times. The plates were inverted on paper towels and remained for 2 h at room temperature to dry. The crystal violet dye that stained the biofilm was then solubilized by incubating with 200 µl of ethanol per well for 30 min. Then, 100 µl of the dye in ethanol was transferred to wells of a new plate, and read at absorbance of 575 nm in a microplate spectrophotometer (BioTek Instruments. USA) to quantify the biomass of the biofilm (Mattos-Graner *et al*., 2001b).

In vitro initial caries lesion induction

This part of the study was approved by the Ethics Committee of Araçatuba Dental School - UNESP (protocol 197/2013 ANEXOS B/C). Bovine permanent central incisors were maintained one month in 2% formaldehyde solution, pH 7.0, for disinfection. Enamel blocks were prepared from the most flat portion of the labial surface of the crown using a precision saw (IsoMet 1000, Buehler, Lake Bluff, IL, USA) with two diamond disks (series 15HC 11–4243—Diamond. Buehler) separated by

a spacer disk (thickness, 4 mm) under refrigeration with distilled water. Dentin was adjusted to obtain parallel surfaces between enamel and dentin (± 2 mm thick), using sandpaper (CARBIMET, Buehler) under low speed and cooling. Then, the blocks were sequentially polished using 600-, 800- and 1200-grade water-cooled silicon carbide paper disks (Buehler) with a final polish using a felt disk (Buehler Polishing Cloth 40–7618) moistened with a 1-μm diamond polishing suspension (Extec Corp., Enfield, CT, USA) (Vieira *et al*., 2005). Blocks with a initial surface hardness (SHi) between 320 and 380 KHN were selected and randomized using Excel program (Microsoft Inc.) into study groups. Enamel blocks were used per group, ten of them or the combination of the bacterial species with *Streptococcus mutans* (3FV2) and another ten specimens in combination with *S. mutans* and *S. sobrinus*. Control groups of *S. mutans* and *S. mutans*/*S. sobrinus* were also analyzed. The modified protocol of an previous study (Lima, Motisuki, Spolidorio, & Santos-Pinto, 2005) was used to induce artificial caries lesions. The bovine enamel blocks were completely isolated with a thin layer of nail varnish, except for the external surface (area = 16 mm^2) and individually placed in modified artificial caries solution (BHI supplemented with 1% yeast extract, 0.5% glucose, 1% sucrose and 2% of the bacterial culture (10^8 cells/mL) for 7 days at 37°C. exchanging the culture medium every 48 h. The blocks were measured for microhardness of the enamel surface before (SHi) and after demineralization (SHf) by means of five impressions alongside the initial five impressions, at a distance of 100 microns using a Shimadzu HMV-2000 microhardness tester (Shimadzu Corp., Kyoto. Japan), with a load of 25 g for 10 s. The percentage of surface hardness loss (%SHL=[[SHif-SHi]/SHi]x100) was then calculated.

Statistical analysis

Means (standard deviations) of area under the curve (AUC) of pH values obtained for bacterial strains during pH drop (acidogenicity) were calculated and the comparison among bacterial species were tested using ANOVA and Tukey tests. For aciduricity assays, CFU/mL counts were transformed to $\log_{10}$ (CFU). The means of counts obtained at pH 7.0 T0 and T60 were calculated and considered as 100% of cell growth. Counts of viable cells at pH 5.0 and pH 2.8 were also performed and the percentage of viable cells was calculated based on counts at pH 7.0 and data was analyzed using ANOVA and Tukey tests. Absorbance values (550 nm) obtained after 48 h of bacterial

biofilm formation were compared among the groups of bacteria and between each species combined (dual or multi-species) or single species according to ANOVA and Tukey tests. Percentage of surface hardness loss (SHL) induced by dual-species and multi-species biofilm exposure after 7 days was analyzed using ANOVA and Tukey tests. Virulence factors comparisons among the groups of bacteria *(Streptococcus, Lactobacillus* and *Bifidobacterium*) were performed using ANOVA and Tukey (acidogenicity/aciduricity/mono-species biofilm) or unpaired *t*-Student tests (dual/multi-species biofilm). Statistical analysis was performed using the program SPSS version 17.1, considering $p<0.05$ as significant.

Results

Acidogenicity tests

Figure 1 shows the results of the AUC for the bacteria tested in this study. Statistically lower values of AUC (high acidogenicity) were found for *S. sobrinus, S. mutans* (3VF2 and ATCC), *L. casei, B. animalis, B. longum* and *A. israelii*, which were not statistically different from each other. *B. lactis*, *B. dentium* and *L. acidophilus* did not differ from each other but they had the highest values of AUC (low acidogenicity).

Aciduricity tests

Figure 2 shows means/standard deviations of the percentage of bacterial growth at pH 5.0 and pH 2.8 relative to growth at pH 7.0 (considered 100%). Analyzing each bacterial strain separately (upper case letters), no difference on percentage of bacterial growth at pH 5.0 between T0 and T60 was found for all the tested strains. The percentage of viable cells decreased at pH 2.8 for all bacteria, except for *B. animalis*, *B. dentium, L. casei* and *L. acidophilus*, comparing T0 with T60. The percentage of viable cells at pH 2.8 was statistically lower when compared to pH 5.0 at T0 for all tested bacteria, except for *B. longum*, *L. casei* and *S. mutans* ATCC. Additionally, the percentage of viable cells was statistically lower at pH 2.8 after T60 in comparison with pH 5.0 T60 for all strains tested, except for *L. casei*.

Considering each pH and time separately (lower case letters) at pH 5.0 and T0 the percentage of viable cells of *S. mutans* (ATCC) was not different compared with *B. animalis*, but it was statistically lower compared with the other strains. At pH 2.8 and T0 the highest percentage of viable cells was observed for *B. longum*, *L. casei* and *S.*

sobrinus, but without statistical difference from *S. mutans* (ATCC and 3VF2). At pH 5.0 and T60, no difference was found in the percentage of viable cells among all tested strains. At pH 2.8 and T60, *L. casei* presented the highest aciduricity, followed by *B. longum, B. animalis* and *S. mutans* 3VF2, followed by *B. dentium, L. acidophilus, A. israelli* and *S. sobrinus*, while no growth was observed for *B. lactis* and *S. mutans* (ATCC).

In vitro biofilm formation

Table 1 shows the optical density values (Abs 550 nm) for the bacterial strains evaluated. For mono-species biofilms, the ability of *S. sobrinus* to form biofilms was similar to that of *S. mutans* 3VF2, which was higher than biofilms for the other strains. In the dual-species biofilms (associated with *S. mutans* 3VF2), *B. animalis* and *L. casei* had the lowest rates of biofilm formation. In the multi-species biofilm, the association of *S. mutans* 3VF2 + *S. sobrinus* + *B. dentium* presented lower biofilm ability than the combination of *S. mutans* 3VF2 + *S. sobrinus* and *A. israelli, L. acidophilus or B. longum.* but it was not different compared with the other strains combinations. For all the tested strains, the biofilm formation ability was statistically lower as monospecies compared with dual- and multi-species biofilms except for *S. sobrinus* mono-species compared with *S. sobrinus* dual-species. Multi-species growth of *B. longum, B. animalis, L. casei* and *L. acidophilus* presented higher biofilm formation ability compared with their growth as dual-species while no difference was found in biofilm formation ability for *B. lactis, B. dentium* and *A. israelli* between dual- or multi-species growth.

In vitro initial caries lesion induction

Figure 3 shows surface hardness loss (%SHL) after exposure of enamel specimens to dual and multi-species biofilms. Under dual-species biofilms, higher demineralization was found in the presence of *B. longum. B. animalis, L. casei* and *S. sobrinus* while under multi-species biofilms. *B. lactis* associated with *S. mutans* 3VF2 and *S. sobrinus* presented the lowest cariogenic potential compared with the other tested conditions.

Comparison of the virulence factors among the groups of microorganisms

Table 2 summarizes the comparisons among the groups of bacteria in virulence factors evaluated. *Lactobacillus* and *Streptococcus* species were more aciduric than *Bifidobacterium* species. No difference was found among the groups of microorganisms in acidogenicity. *Lactobacillus* and *Bifidobacterium* species presented similar biofilm formation and induced similar enamel demineralization either in dual-species or in multi-species biofilms.

Discussion

Studies have assessed the microbial succession of tooth enamel lesions and noted that various species including *Bifidobacterium* species, in addition to *S. mutans,* may be involved in the development of early caries lesions. (Modesto, Biavati, & Mattarelli, 2006; Beighton *et al*., 2008; Mantzourani *et al*., 2009; Torlakovic *et al*., 2012). Specific phenotypic characteristics can influence establishment of species in complex microbial biofilms. The main virulence factors of *S. mutans* that determine their cariogenicity include acidogenicity (production of acids from a variety of fermentable sugars), aciduricity (tolerance to a low pH) and ability to form biofilms on the tooth surface, through adhesion and production of extra and intracellular polysaccharides (Lemos, Abranches, & Burne, 2005).

Data from acidogenicity tests in this current study indicated that, in addition to *S. mutans* (clinical strain). *L. casei*, *B. animalis* and *B. longum* were more acidogenic than the other bacterial strains analyzed. *S. sobrinus* and *A. israelii* were among the most acidogenic strains, but had intermediate acid tolerance. The pH drops caused by probiotics, including *B. longum, B. lactis, L. casei* and *L. acidophilus*, from the degradation of sucrose and other sugars were evaluated by Haukioja, Loimaranta, & Tenovuo (2008). The authors found that all of the strains produced acids from glucose with a reduction of pH comparable to that of *S. mutans,* similar to the present results. Those authors also found that all lactobacilli and bifidobacteria tested, except for *L. rhamnosus* GG and *B. lactis* Bb12, caused a significant decrease in pH in the presence of lactose.

The current study showed that the growth of all strains, including species of bifidobacteria, was not affected by pH 5.0 after 60 minutes of acid exposure. The same was not observed at pH 2.8. Among bifidobacteria, *B. longum* and *B. animalis* were the

most acid-resistant strains and *B. dentium* was the least acid-resistant strain, at pH 2.8. These results are in agreement with those obtained by Nakajo, Takahashi, & Beighton (2010) who evaluated the effects of acidification on the survival of *B. dentium* and *B. longum* in comparison with *S. mutans*, *S. sanguinis* and *L. paracasei*. These authors showed that high levels of survival in acidic environments were observed for bifidobacteria, which were comparable to *S. mutans*, demonstrating how these species could co-exist with *Streptococcus mutans* in acidic carious lesions. *B. longum* was the most resistant bacteria at pH 5.0 when compared to the others strains, while *L. paracasei* was the most resistant at pH 4.0. In contrast, *B. dentium* was less resistant to acids among all tested bacteria.

Bacterial aciduricity is maintained by a mechanism in the cell membrane called proton-translocating ATPase (F1F0-ATPase). This mechanism controls the entry of H+ ions and maintaining a more basic cytoplasmic pH when compared to the extracellular medium. Furthermore, the F-ATPase system has dual role in the acid tolerance of cells and eliminating protons in certain circumstances it generates ATP (energy) for the growth and persistence of bacterial species (Lemos & Burne, 2008). Studies have reported that the F-ATPase activity in *Bifidobacterium animalis* and *Bifidobacterium animalis* subsp *lactis* increases with environmental acidification (Gibson & Wang, 1994; Sánchez, de los Reyes-Gavilán, & Margolles, 2006). Matsumoto, Ohishi, & Benno (2004) evaluated the aciduricity and F-ATPase activity of *B. lactis* (2 strains), *B. animalis* (4 strains), *B. bifidum* (4 strains). *B. breve*, *B. infantis*, *B. catenulatum*, *B. longum*, *B. pseudocatenulatum* and *B. adolescentis* and demonstrated that *B. lactis* and *B. animalis* were able to survive in an acidic environment (they were stable at pH 3-5 for 3 h). They reported that this ability was related to the species and not the strain and was associated with increased F-ATPase activity. In contrast with the current study findings, Matsumoto, Ohishi, & Benno (2004) reported that *B. longum* was not acid tolerant, with reduced growth by about 3-fold at log10 after exposure to pH 5 for 3 h.

Other ATP-independent mechanisms have been studied in *S. mutans*, *S. gordonii*, *S. salivarius* and *L. casei*. The membrane composition may be altered in the presence of acids, increasing the levels of long-chain monounsaturated fatty acids, allowing the species to become more resistant to acids (Fozo & Quivey, 2004; Fozo, Kajfasz, & Quivey, 2004). The cell membrane of *B. animalis* contains large amounts of

these fatty acids (Ruiz, Sánchez, Ruas-Madiedo, de Los Reyes-Gavilán, & Margolles, 2007), suggesting these bifidobacteria may also display this type of acid tolerance.

Most species of bifidobacteria can degrade many carbohydrates, including glucose, fructose, mannose, N-acetylglucosamine and sorbitol. Many of these species have glycosidases. Transporters and metabolic enzymes to ferment sugars, even those sugars that are not absorbed by humans or animals (Fushinobu, 2010), which likely contribute to their survival and proliferation in hostile environments. In addition to sugar degradation, microbial coaggregation is essential for biofilm development, characterized by the specific recognition and adhesion of genetically different microorganisms in order to promote organization and cell-cell interactions to increase the resistance of the individual species or the biofilm as a whole (Kolenbrander, Palmer, Periasamy, & Jakubovics, 2010). Few studies have evaluated the coaggregation of *S. mutans* and species of bifidobacteria. The first study to assess the interaction between species of oral and intestinal microbiota was carried out by Ledder, Timperley, Friswell, Macfarlane, & McBain (2008). Those authors found strong coaggregation between *Fusobacterium nucleatum* and six of the nine evaluated oral species and only one coaggregation with an intestinal bacterium, *B. adolescentis*. In that study, *S. mutans* had better interactions with *L. rhamnosus* and minimum coaggregation with intestinal bacteria. In the present study, coaggregation was not evaluated; however, the increasing biomass of the biofilm suggests an interaction among the species. Overall, the combination of *S. mutans* 3VF2 or *S. mutans* 3VF2 and *S. sobrinus* with *B. longum* and *A. israelii* showed the highest values of biofilm biomass. Nagaoka *et al.,* (2008) evaluated the coaggregation and adherence of *B. adolescentis* and some oral bacterial species, such as *A. naeslundii*, *S. mitis*, *S. sanguinis* and *F. nucleatum*. Their results showed that *B. adolescentis* had better coaggregation with *F. nucleatum* and that adherence to the hydroxyapatite discs was only possible through intermediate oral species such as *F. nucleatum*. Haukioja *et al.,* (2006) evaluated the adhesion of bifidobacteria and lactobacilli to hydroxyapatite discs coated or not coated with saliva. Those authors found that the studied bacteria exhibited low adhesion with or without the presence of saliva, and that this ability was improved when the species had coaggregated with *F. nucleatum.*

Bifidobacteria and lactobacilli have a low capacity to adhere to tooth structure by themselves (Haukioja *et al.*, 2006; Nagaoka *et al.*, 2008), so tests inducing initial

caries lesions (enamel demineralization) were carried out in the present study using species of lactobacilli and bifidobacteria combined with *S. mutans* and/or *S. sobrinus*. The dual-species biofilms of *S. mutans* with *B. longum*, *L. casei*, *B. animalis* or *S. sobrinus* and the multi-species biofilms of *B. animalis* and *A. israelii* with *S. mutans* and *S. sobrinus* caused the highest loss of surface hardness, however there was no difference in comparison with the other species tested, except for *B. lactis*. A study comparing different combinations of *S. mutans* with *L. casei* and *L. acidophilus* and the impact on the non-cavitated enamel lesions induction was recently developed by De Campos *et al.*, (2015). Their results showed that dual combination of *S. mutans* and *L. casei* or triple combination of *S. mutans, L. casei* and *L. acidophilus* promoted the highest loss in enamel surface hardness. The depth of lesions was analyzed using polarized light microscopy and erosive lesions developed in enamel after 20 days of cariogenic challenge. The results of the present study are consistent with those results, showing that the combination of *L. casei* and *S. mutans* caused a greater enamel demineralization compared to other species tested. Studies evaluating the impact of the biofilm formed by *Bifidobacterium* species and *S. mutans* on demineralization of dental enamel have not yet been found, making comparisons with the present results difficult.

It may be concluded that *B. animalis* and *B. longum* have acidogenic/aciduric potential and ability to form biofilms similar to cariogenic bacteria. They were able to induce enamel demineralization when associated either with *S. mutans* or in combination with *S. mutans* and *S. sobrinus*.

Competing interests

The authors declare that there are no conflicts of interest.

Ethical approval

The experimental procedures described here were approved by the Ethics Committee of Araçatuba Dental School - UNESP (protocol 197/2013).

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Table 1. Biofilm formation. Absorbance values (550nm) obtained after 48h of bacterial biofilm formation.

Monospecies	Mean/SD	Dual-species	Mean/SD	Multi-species	Mean/SD
B. longum	[A.a] 0.286/0.138	*3VF2 + B. longum*	[A.B.b] 1.720/0.230	*3VF2+ S.sob.+ B. longum*	[A.c] 2.043/0.406
B. animalis	[A.a] 0.296/0.075	*3VF2 + B. animalis*	[C.b] 0.962/0.148	*3VF2 + S.sob.+ B. animalis*	[A.B.c] 1.771/0.540
B. lactis	[A.a] 0.352/0.179	*3VF2 + B. lactis*	[B.D.b] 1.479/0.311	*3VF2 + S. sob. + B. lactis*	[A.B.b] 1.642/0.375
B. dentium	[A.a] 0.281/0.033	*3VF2 + B. dentium*	[A.B.D.b] 1.553/0.104	*3VF2 + S.sob. + B. dentium*	[B.b] 1.423/0.200
L. casei	[A.a] 0.232/0.093	*3VF2 + L. casei*	[C.D.b] 1.138/0.339	*3VF2+ S.sob.+ L. casei*	[A.B.c] 1.633/0.319
L. acidophilus	[A.a] 0.220/0.044	*3VF2 + L. acidophilus*	[B.C.D.b] 1.391/0.315	*3VF2 + S.sob.+ L. acidophilus*	[A.c] 1.922/0.474
A. israelii	[A.a] 0.567/0.152	*3VF2 + A. israelii*	[A.b] 2.04/0.600	*3VF2+ S.sob+ A. israelii*	[A.b] 2.103/0.397
S. sobrinus	[B.a] 1.948/0.504	*3VF2 + S. sobrinus*	[A.a] 2.054/0.567		
S. mutans ATCC	[A] 0.416/0.166				
S. mutans 3VF2	[B] 1.71/0.236				

[A] Different upper case letters show statistical difference among the groups, considering each condition of biofilm (mono. dual or multi-species), according to ANOVA and Tukey tests.
[a] Different lower case letters show statistical difference between each species combined (dual or multi-species) or not (monospecies), according to ANOVA and Tukey tests.
S. sob. – S. sobrinus
3VF2 – S. mutans 3VF2.

Table 2. Virulence factors comparisons among the groups of microorganisms

		Streptococcus	*Lactobacillus*	*Bifidobacterium*
Acidogenicity* (AUC)		32.58 (2.13)[A]	52.06(6.74)[A]	46.29 (7.41)[A]
Aciduricity (% bacterial growth)		62.33(7.35)[a.b]	64.07(5.95)[b]	40.30(5.42)[a]
Biofilm formation (Abs values – 550nm)	**Mono-species**	1.35(0.03)[A]	0.31(0.03)[B]	0.30(0.02)[B]
	Dual-species	-	1.22(0.05)[A]	1.41(0.06)[A]
	Multi-species	-	1.78(0.07)[A]	1.70(0.06)[A]
Enamel demineralization (%SHL)	**Dual-species**	-	-69.91(4.44)[A]	-71.75(2.35)[A]
	Multi-species	-	-72.47(1.77)[A]	-73.61(2.40)[A]

* Means (Standard Error of Means)

[A] Different upper case letters show statistical difference among the groups. according to ANOVA and Tukey (acidogenicity/mono-species biofilm) or unpaired *t*-Student tests (dual/multi-species biofilm).

[a] Different lower case letters show statistical difference among the groups. according to Kruskal-Wallis and Mann-Whitney tests (aciduricity).

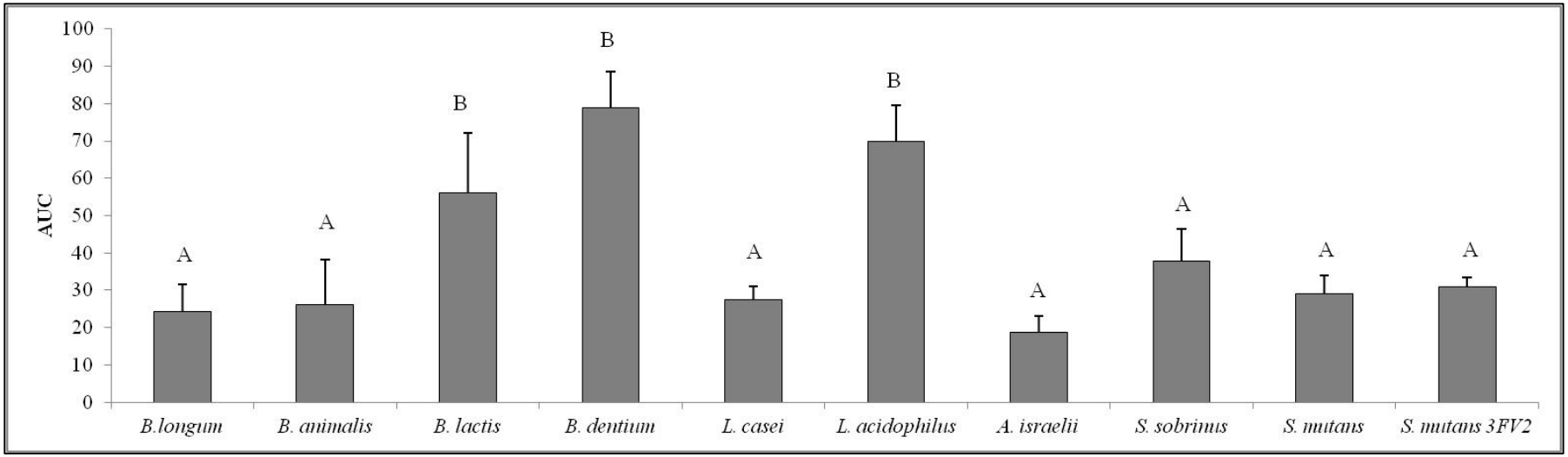

Figure 1. Acidogenicity (acid production). Means (standard deviations) of area under the curve (AUC) of pH values obtained for bacterial strains during pH drop.

[A]Different upper case letters show statistical difference among bacterial species, according to ANOVA followed by Tukey tests ($p<0.05$).

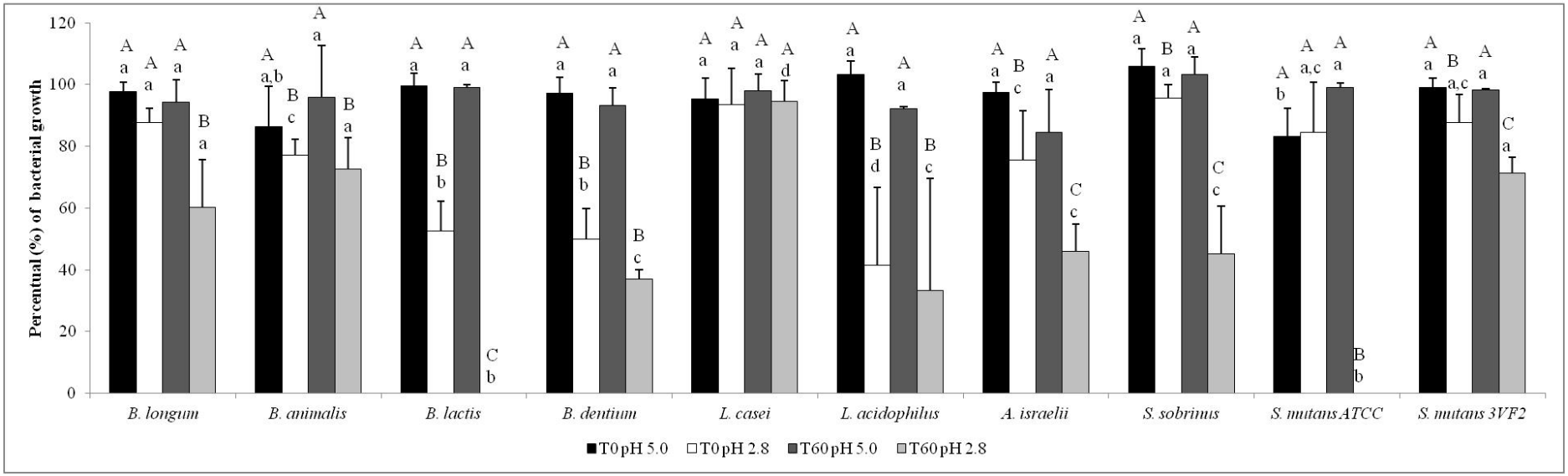

Figure 2. Aciduricity (acid tolerance). Means (standard deviations) of percentage (%) of bacterial growth obtained after baseline (Time 0) and 60 min (Time 60) of exposition to glycine buffer in pH 5.0 or pH. 2.8 in relation to pH 7.0.

[A]Different upper case letters show statistical difference within each tested strain for both time of exposure (T0 and T60) and pH (5.0 and 2.8), according to ANOVA and Tukey tests ($p<0.05$).

[a]Different lower case letters show statistical difference among bacterial strains, considering pH and time separately, according to ANOVA and Tukey tests($p<0.05$).

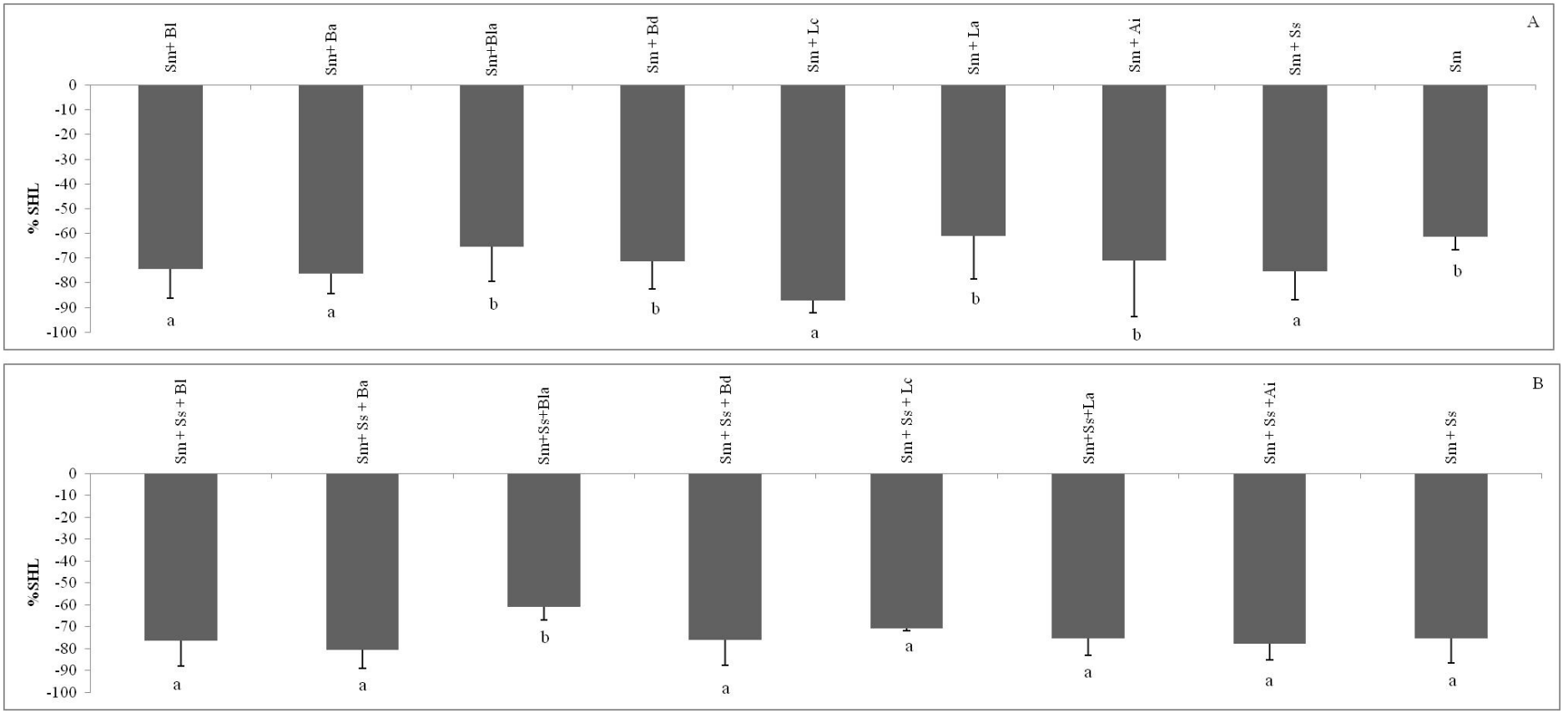

Figure 3. Enamel demineralization. Percentage of surface hardness loss (%SHL) after 7 days of **(A)** dual-species and **(B)** multi-species biofilm exposure. Sm: *S. mutans 3VF2*, Ss: *S. sobrinus*, Bl: *B. longum*, Ba: *B. animalis*, Bla: *B.lactis*, Bd: *B. dentium*, Lc: *L. casei*, La: *L. acidophilus*, Ai: *Aisraelii*.[a] Different letters show statistical difference among the groups, according to ANOVA and Tukey tests.

Capítulo 3

Capítulo 3

Molecular analysis of the in vitro antagonist growth effect of bifidobacteria on the biofilm of periodontal pathogens*

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Running title: Antagonism of bifidobacteria on periodontopathogens

Significance and impact of the study

This study is the first to demonstrate that some *Bifidobacterium* species either alone or in combination can cause an antagonistic effect toward periodontal pathogens. Such results therefore underline the promising therapeutic potential of these bifidobacteria for the prevention and/or coadjuvant treatment of periodontal diseases.

*Manuscript prepared according to instructions of the journal "*Letters in Applied Microbiology"* (ANEXO F)

Abstract

Microbiota from subgingival biofilm plays a pivotal role in the development of periodontal diseases. In virtue of the disadvantages of prolonged antibiotic use, supplements containing probiotics have recently been addressed for periodontal therapies. This study aimed to investigate the *in vitro* antagonistic growth effect of some species of bifidobacteria, either alone or in combination, on the biofilm of periodontal bacteria. Two putative periodontopathogens, *Porphyromonas gingivalis and Fusobacterium nucleatum* and one "beneficial" bacteria of the subgingival biofilm, *Streptococcus oralis*, were studied. These strains were incubated in microplates to form biofilm in the presence of *Bifidobacterium longum, Bifidobacterium lactis, and Bifidobacterium infantis*, either alone or in combination, for 24, 72 and 168 hours. The total counts of each bacterium after incubation were analyzed by checkerboard DNA-DNA hybridization. The results showed that *B. infantis* and *B. lactis* demonstrated the best effect against the growth of *F. nucleatum* (24h and 72h) and *P. gingivalis* (168h). All the double combinations of bifidobacteria tested demonstrated an inhibitory effect against *F. nucleatum* (72h) and *P. gingivalis* (168h) and did not affect *S. oralis* growth. Some species of bifidobacteria, either alone or in combination, were capable of inhibiting specific periodontal pathogens, minimally affecting the growth of normal microbiota.

Keywords: Periodontal disease, biofilm, *Porphyromonas, Fusobacterium, Bifidobacterium*, probiotics.

Introduction

The oral cavity is a very complex ecosystem composed of more than 700 bacterial species (Paster et al. 2006). Periodontitis, one of the most prevalent oral diseases, occurs as a consequence of an imbalance between this ecosystem and the immune system, reducing the indigenous microbiota and favoring the overgrowth of pathogenic strains. *Fusobacterium nucleatum* and *Porphyromonas gingivalis* are Gram-negative anaerobe bacteria which harbor the subgingival biofilm and have been associated with chronic and acute periodontal diseases (Moore and Moore 1994; Ximenez-Fyvie et al. 2000). *F. nucleatum* plays an important role in the development and maturation of biofilm for its ability to coaggregate with early colonizers, such as *Streptococcus oralis, S. mitis, S. sanguinis* and other streptococci as well as late pathogenic colonizers, such as *Prevotella* and *Porphyromonas* species (Bradshaw et al. 1998). *P. gingivalis* has an arsenal of virulence factors which lead to breakdown of periodontal tissues through direct activities of proteases and by inducing high levels of proinflammatory mediators (Kadowaki et al. 2000; Bodet et al. 2006).

Systemic or local antibiotic periodontal therapy is usually administered as an adjunctive to dental scaling and root planning for reducing bacterial counts and consequently potentiating the effects of traditional periodontal therapy and preventing the recurrence of infection (Greenstein and Polson 1998; Mariotti and Monroe 1998). However, the use of systemic antibiotics has some disadvantages including adverse drug reactions, the risk of development of bacterial resistance, uncertain patient compliance and lower concentration of the drug at subgingival sites (Feres et al. 2000). In addition, some individuals with periodontal disease respond neither to the treatment of scaling and root planning alone, nor to treatment in combination with antibiotic therapy (Wasserman and Hirschfeld 1998).

Recently, researchers have been pointed the use of probiotics as alternative therapy to control periodontal pathogens. The most widely studied probiotic species belong to the genera *Lactobacillus* and *Bifidobacterium.* Several studies have demonstrated the in vitro and in vivo impact of probiotic strains of lactobacilli on the reduction of caries-related bacteria and putative periodontal pathogens (Twetman et al. 2009; Essche et al. 2013, Teughels et al. 2013; Baca-Castañon et al. 2015). However, very few studies have focused on the relationship between species of *Bifidobacterium* and periodontal pathogens, including assays of coadhesion/coaggregation and antagonist effects (Haukioja et al. 2006; Hojo et al. 2007; Nagaoka et al. 2008; Zhu et al. 2010). Zhu et al. (2010) demonstrated that bio-yogurt and some probiotic bacterial species, including *Bifidobacterium*, are capable of inhibiting specific periodontal pathogens. This study aimed to investigate the *in vitro* antagonist growth effect of some probiotic species of bifidobacteria, either alone or in combination, on biofilms of

Fusobacterium nucleatum, *Porphyromonas gingivalis* and *Streptococcus oralis* at different time points, using the checkerboard DNA-DNA hybridization technique (Socransky 1994).

Results and Discussion

Specific bacterial species could persist in the oral environment by virtue of the availability of nutrients and through the influence of a variety of synergistic interactions, such as intergeneric coaggregation and coadhesion of microorganisms forming a dynamic community called biofilm (Socransky and Haffajee 2002). The control of biofilm is important for the prevention of oral diseases, including periodontitis. However, the dense structure of the biofilm limits the diffusion of antibacterial agents and consequently reduces their action (Fux et al. 2005). New concepts in periodontal therapy have been solicited, such as probiotic therapy, as alternatives to the conventional use of antibiotics. Probiotics are capable of adhering to oral surfaces and producing antibacterial substances that can eradicate or inhibit pathogenic bacteria (Samot et al. 2011; 2013). *In vitro* studies are recommended to select the best probiotic strains by their functionally and safety. Firstly, it is necessary that the probiotic presents antagonism activity against the pathogens, as well as the ability to grow, forming biofilms (FAO/WHO 2002).

In the current study, the percentages of *F. nucleatum*, *P. gingivalis* and *S. oralis* combined with *B. longum, B. lactis or B. infantis* are shown in **Figures 1A, 1B and 1C**, respectively. *B. lactis* and *B. infantis* significantly inhibited the *in vitro* growth of *F. nucleatum* after 24, 72 and 168h of incubation. *B. longum* had a similar inhibitory effect on *F. nucleatum* in comparison with the other bifidobacteria after 72h of growth. For *P. gingivalis*, *B. infantis* presented the best inhibitory effect after 24 and 168h of growth. *B. lactis and B. longum* inhibited *P. gingivalis* only after 168h of growth. The growth of *S. oralis*, which is considered as beneficial bacteria related to periodontal health (Tanner et al. 1998), was affected by *B. lactis* and *B. infantis* after24h and by *B. longum* and *B. lactis* after 72h. *B. infantis* did not influence the growth of *S. oralis* after 72 and 168h. No bifidobacteria interfered in *S. oralis* growth after 168h.

Very few studies have investigated the competition between *Bifidobacterium* and periodontal pathogens (Zhu et al. 2010). In vitro antagonist interactions between probiotics, including *Bifidobacterium*, and periodontal pathogens were demonstrated when probiotics were inoculated first in the culture medium. *Bifidobacterium* inhibited *P. gingivalis, F. nucleatum, A. actinomycentecomitans, P. circumdentaria* and *P. nigrescens* (Zhu et al. 2010). Compared to the current study, all species of bifidobacteria demonstrated an inhibitory effect against periodontal pathogens at the time points evaluated. *B. infantis* and *B. lactis* demonstrated the best effect against *F. nucleatum* and *P. gingivalis* and minimally influenced the growth of *S. oralis*. The

relationship between *B. infantis* and periodontal bacteria has only been investigated by Haukioja et al. (2006) who showed co-adherence between *B. infantis* and *F. nucleatum,* proving that these species could persist in subgingival sites colonized by *F. nucleatum.* Although no study was found showing the antagonist effect of *B. infantis* or *B. lactis* on periodontal pathogens, the inhibitory effect of both bifidobacteria on enteropathogens is well-known, interfering in the adherence of two Gram-negative bacteria; *Escherichia coli* and *Salmonella enteric*, to intestinal cells (Bernet et al. 1993; Candela et al. 2008). The effect of the administration of *B. animalis* subsp. *Lactis* BB-12 (*B. lactis*) on the reduction of mutans streptococci and lactobacilli counts has been previously demonstrated, indicating them as alternative antimicrobial agents for caries prevention (Caglar et al. 2008; Taipale et al. 2012).

The percentages of *F. nucleatum, P. gingivalis* and *S. oralis* in double or triple combination with the bifidobacteria species are presented in **Figures 2A, 2B and 2C**. After 24h, no combination had an effect on the growth of *F. nucleatum*, except for the triple combination *B. longum* + *B. lactis* + *B. infantis*. In contrast, all combinations similarly reduced the growth of *F. nucleatum* after 72h. After 168h, *F. nucleatum* growth was not affected by any combination of bifidobacteria. For *P. gingivalis,* only the combinations *B. longum* + *B. lactis* and *B. longum* + *B. lactis* + *B. infantis* inhibited growth after 72h. All combinations significantly reduced the growth of *P. gingivalis* after 168h. None of the combinations affected the growth of *S. oralis*, except for the triple combination of bifidobacteria after 24 and 72h.

Specific probiotic combinations are able to enhance the inhibition of pathogens when compared to single probiotic strains (Collado et al. 2006). This study showed that all the double combinations of bifidobacteria tested had an inhibitory effect against *F. nucleatum* (after 72h) and *P. gingivalis* (after 168h) and did not affect *S. oralis* growth. Combinations of *Lactobacillus* and *Bifidobacterium* strains have been studied for prevention of oral diseases, such as dental caries (Singh et al., 2011) and periodontal diseases (Toiviainen et al., 2005). However, no study was found for the combinations of various species of *Bifidobacterium*. Considering the effects on periodontal health, the combination of *L. rhamnosus* and *B. animalis* subsp. *lactis*BB-12 demonstrated significant reductions in clinical parameters, without affecting the composition of the oral microbiota (Toiviainen et al. 2015). Probiotics can produce lactic acid, hydrogen peroxide, bacteriocins or other antimicrobial substances either alone or in combination and could inhibit pathogens (Samot and Badet 2013). A study demonstrated that *B. adolescentis* and *B. longum* decreased vitamin K concentration and inhibited the growth of *P. gingivalis*. Both bifidobacteria and *P. gingivalis* require vitamin K for their growth and probably compete for its acquisition in the oral cavity (Hojo et al. 2007). Another explanation for the reduction in periodontal pathogens is the fact that bifidobacteria produces organic acids, such as lactic acid,

which causes damage to Gram-negative bacteria by disrupting the outer membrane (Alakomi et al. 2000) or through its chelanting capacity (Presser et al. 1997).

In vitro biofilm models have their limitations and cannot completely reproduce the complexity of the oral environment. However, they have certain advantages, such as not having the ethical conflicts of clinical studies and being able to be used to analyze a variety of important in vivo processes in a highly reproducible manner (Schlafer et al. 2011). The limited number of studies evaluating antagonism among probiotic bacteria, especially bifidobacteria and periodontal pathogens limit comparisons with the current results. In addition, differences in methodologies used for evaluating the antagonism make the comparisons inappropriate (Essche et al. 2013). This study concluded that *B. longum, B. lactis* and *B. infantis,* either alone or in combination, can cause an antagonistic effect toward periodontopathogens and these results underline the promising therapeutic potential of these bifidobacteria for the prevention and/or coadjuvant treatment of periodontal diseases.

Material and methods

Bacterial strains

All reagents and salts were purchased from Sigma Aldrich (St. Louis, MO, USA) and culture mediums from Difco Laboratories (Kansas City, MO, USA). *Fusobacterium nucleatum* subsp. *nucleatum* (ATCC 25585), *Porphyromonas gingivalis* (33277) and *Streptococcus oralis* were grown in enriched media with Brain Heart Infusion Agar (BHI) (26 g/L) supplemented with Trypticase Soy Agar (TSA) (20 g/L), Yeast extract (10 g/L), 5 μg/mL hemin, 0.3 μg/mL menadione and 5% defibrinated sheep blood, while *Bifidobacterium longum* subsp. *longum* (ATCC 15707), *Bifidobacterium longum* subsp. *infantis* (ATCC 15697) and *Bifidobacterium animalis* subsp. *Lactis* (ATCC 27673) were grown in MRS broth and 0.05% L-cysteine. All species were incubated at 35^{o}C under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2) and pure cultures of each strain, confirmed by the Gram's method, were used in all the experiments (Almaguer-Flores et al., 2012).

Biofilm assays

Bacterial growth from 24 to 72h cultures of each strain was harvested in enriched mycoplasma broth-media (5 μg/ml hemin and 0.3 μg/ml menadione) and the optical density (OD) in each tube was adjusted to 1 at 600 nm in a spectrophotometer. a total of 108 cells/ml suspension of each reference strain was added either alone or in combination (Table 1) in equal volumes to 12-well plates in a total volume of 2 mL. Next the plates with the pure and mixed cultures of the bacterial strains were incubated for 24, 72 and 168 hours at 35°C under anaerobic conditions. All experiments were performed in triplicate (Almaguer-Flores et al. 2012).

After incubation, 100 µL of the bacterial suspension of each well were placed in individual microtubes with 100 µL of 0.5 M NaOH and were mixed by vortexing. Bacterial species were quantified using the checkerboard DNA-DNA hybridization technique as previously described (Socransky et al. 1994; Almaguer-Flores et al. 2012). Briefly, three to seven-day cultures of the reference strains (Table 1) were harvested and the cells were placed in tubes containing 1 mL of TE buffer, washed twice and lysed at 37°C for 1 hour with either 10% sodium dodecyl sulfate (SDS) plus proteinase K (20 mg ml^{-1}) for Gram-negative strains or lysozyme (15 mg/mL) plus achromopeptidase (5 mg ml^{-1}) for Gram-positive strains. DNA from the cultures was isolated and purified according to a previous methodology (Smith et al. 1989). Whole-genomic DNA probes were prepared by labeling 1mg DNA with digoxigenin (Roche Diagnostics, Mannheim, Germany) using a random primer technique (Feinberg and Vogelstein 1983). Hybridizing against individual pure cultures adjusted to 10^4 to 10^7 cells was performed to test the specificity and sensitivity of DNA probes.

Prior to checkerboard DNA-DNA hybridization, each sample was boiled for 10 minutes and neutralized with 800 µL 5M ammonium acetate. DNA from each sample either alone or in combination (Table 1) was then placed into individual lanes (Minislot-30, Immunetics, Inc., Cambridge, MA), concentrated onto a 15 cm x 15 cm positively charged nylon membrane (Roche Diagnostics) and fixed to the membrane by cross-linking under ultraviolet light. The membranes were pre-hybridized at 42°C for 2 hours in 50% formamide, 5 x standard saline citrate (SSC), 5 x Denhardt's solution, 25mM sodium phosphate (pH 6.5), and 0.5 mg ml^{-1} yeast RNA (Roche Diagnostics). Each membrane was placed in a second device (Miniblotter-45, Immunetics) with the sample lanes rotated 90° to the channels of the apparatus. The probes were diluted to ~20 ng ml^{-1} in hybridization solution, placed in individual channels of the device and hybridized overnight at 42°C. Probes were hybridized in sets of 6 consecutive channels, leaving empty channels (hybridization solution only) to allow noise and background correction of signals. The membranes were washed twice at high stringency for 20 minutes at 68° C in phosphate buffer (0.1 x SSC and 0.1% SDS). Membranes were blocked by 1 h incubation in maleate buffer (100 mM maleic acid and 150 mM NaCl, pH 7.5) containing 1% casein. Hybrids were detected by exposing the membranes to a 1:50,000 dilution of anti-digoxigen in antibody conjugated to alkaline phosphatase for 30 minutes (Engler-Blum, et al. 1993). Signals were detected using a chemiluminescent agent (CDP-Star, Roche Diagnostics) for 30 minutes at room temperature and exposed to films in autoradiographic cassettes for 30 minutes. Films were then photographed using a digital photodocumentation system (DigiDoc, BioRad Laboratories, Hercules, CA.) and signals detected with specialized software (Quantity One, BioRad Laboratories). Results were adjusted by subtracting the average plus two standard deviations of

the noise and background detected in the empty lanes, and converted to absolute counts by comparison with the standards on the membrane. Failure to detect a signal was recorded as zero.

Data analysis

The absolute counts of each bacterium in combination were transformed into percentages of growth (proportion), based on the total number of bacteria which grew alone (considered as 100% of growth). All results were submitted to statistical analysis at 5% significance using ANOVA and Tukey's tests.

Acknowledgments

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Conflict of interest

No conflict of interest is declared.

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Table 1. Experimental groups for testing the antagonism of bifidobacteria on periodontal bacteria.

Single species of bifidobacteria		
F. nucleatum	*P. gingivalis*	*S. oralis*
F. nucleatum + B. longum	*P. gingivalis + B. longum*	*S. oralis + B. longum*
F. nucleatum + B. lactis	*P. gingivalis + B. lactis*	*S. oralis + B. lactis*
F. nucleatum + B. infantis	*P. gingivalis + B. infantis*	*S. oralis + B. infantis*
Double or triple combinations of bifidobacteria		
F. nucleatum + B. longum + B. lactis + B. infantis	*P. gingivalis + B. longum + B. lactis + B. infantis*	*S. oralis + B. longum + B. lactis + B. infantis*
F. nucleatum + B. longum + B. lactis	*P. gingivalis + B. longum + B. Lactis*	*S. oralis + B. longum + B. lactis*
F. nucleatum + B. longum + B. infantis	*P. gingivalis + B. longum + B. infantis*	*S. oralis + B. longum + B. infantis*
F. nucleatum + B. lactis + B. B. infantis	*P. gingivalis + B. lactis + B. B. infantis*	*S. oralis + B. lactis + B. B. infantis*

Legends of figures

Figure 1. Antagonism of single species of bifidobacteria on *F. nucleatum* (A), *P. gingivalis* (B) and *S. oralis* (C) biofilms.

[a]Different lowercase letters show statistical difference among the groups of bifidobacteria, according to ANOVA and Tukey tests.

*Statistical difference between 24 and 72h, considering each group separately, according to ANOVA and Tukey tests.

‡Statistical difference between 24 and 168h, considering each group separately, according to ANOVA and Tukey tests.

¥ Statistical difference between 72 and 168h, considering each group separately, according to ANOVA and Tukey tests.

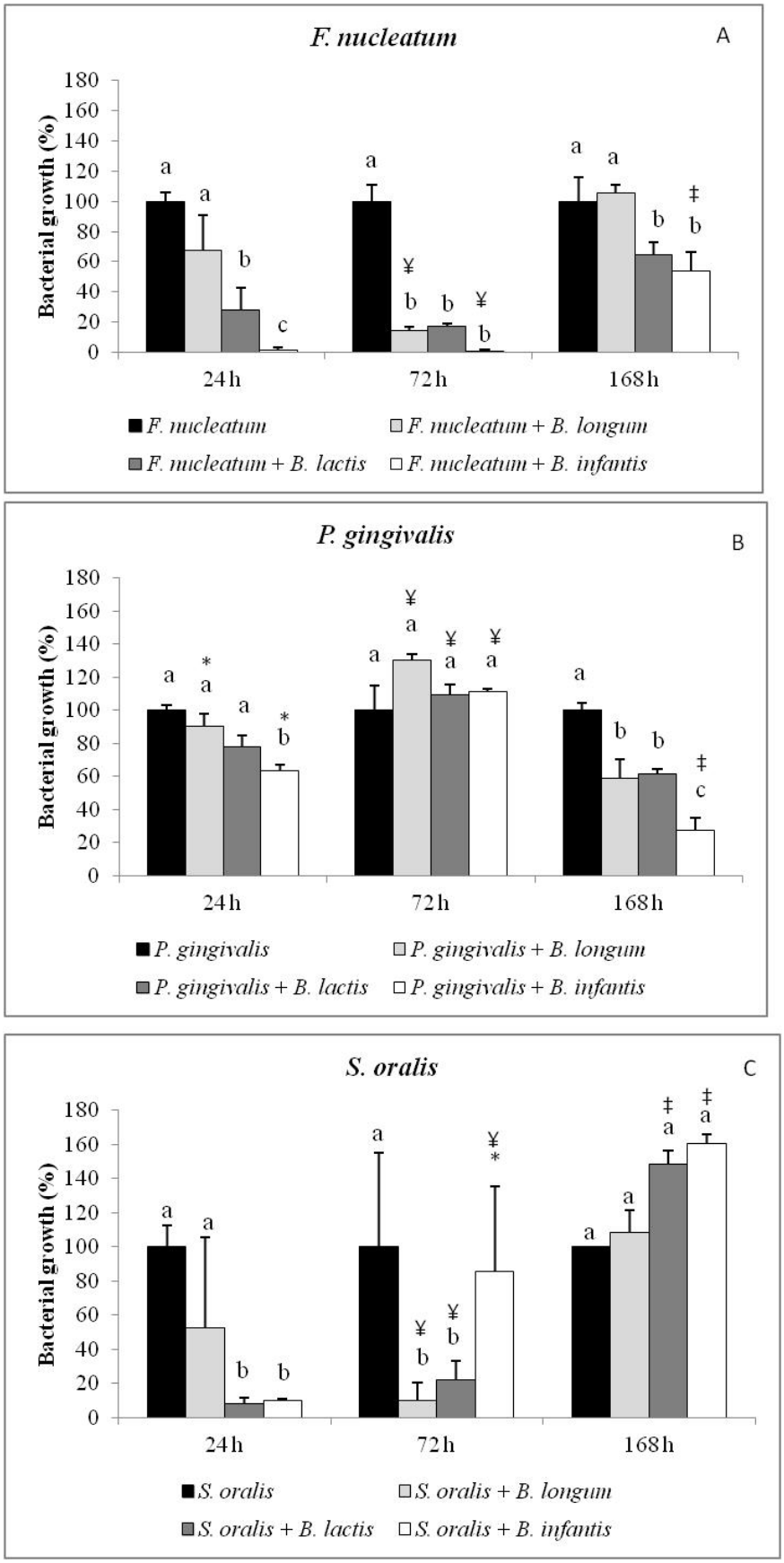

F. nucleatum
A
Bacterial growth (%)
180
160
140
120
100
80
60
40
20
0
24h
72h
168h
F. nucleatum
F. nucleatum + B. longum
F. nucleatum + B. lactis
F. nucleatum + B. infantis
P. gingivalis
B
Bacterial growth (%)
24h
72h
168h
P. gingivalis
P. gingivalis + B. longum
P. gingivalis + B. lactis
P. gingivalis + B. infantis
S. oralis
C
Bacterial growth (%)
24h
72h
168h
S. oralis
S. oralis + B. longum
S. oralis + B. lactis
S. oralis + B. infantis

Figure 1

Figure 2. Antagonism of the combinations of bifidobacteria on *F. nucleatum* (A), *P. gingivalis* (B) and *S. oralis* (C) biofilms.

[a] Different lowercase letters show statistical difference among the groups of bifidobacteria, according to ANOVA and Tukey tests.

* Statistical difference between 24 and 72h, considering each group separately, according to ANOVA and Tukey tests.

‡Statistical difference between 24 and 168h, considering each group separately, according to ANOVA and Tukey tests.

¥ Statistical difference between 72 and 168h, considering each group separately, according to ANOVA and Tukey tests.

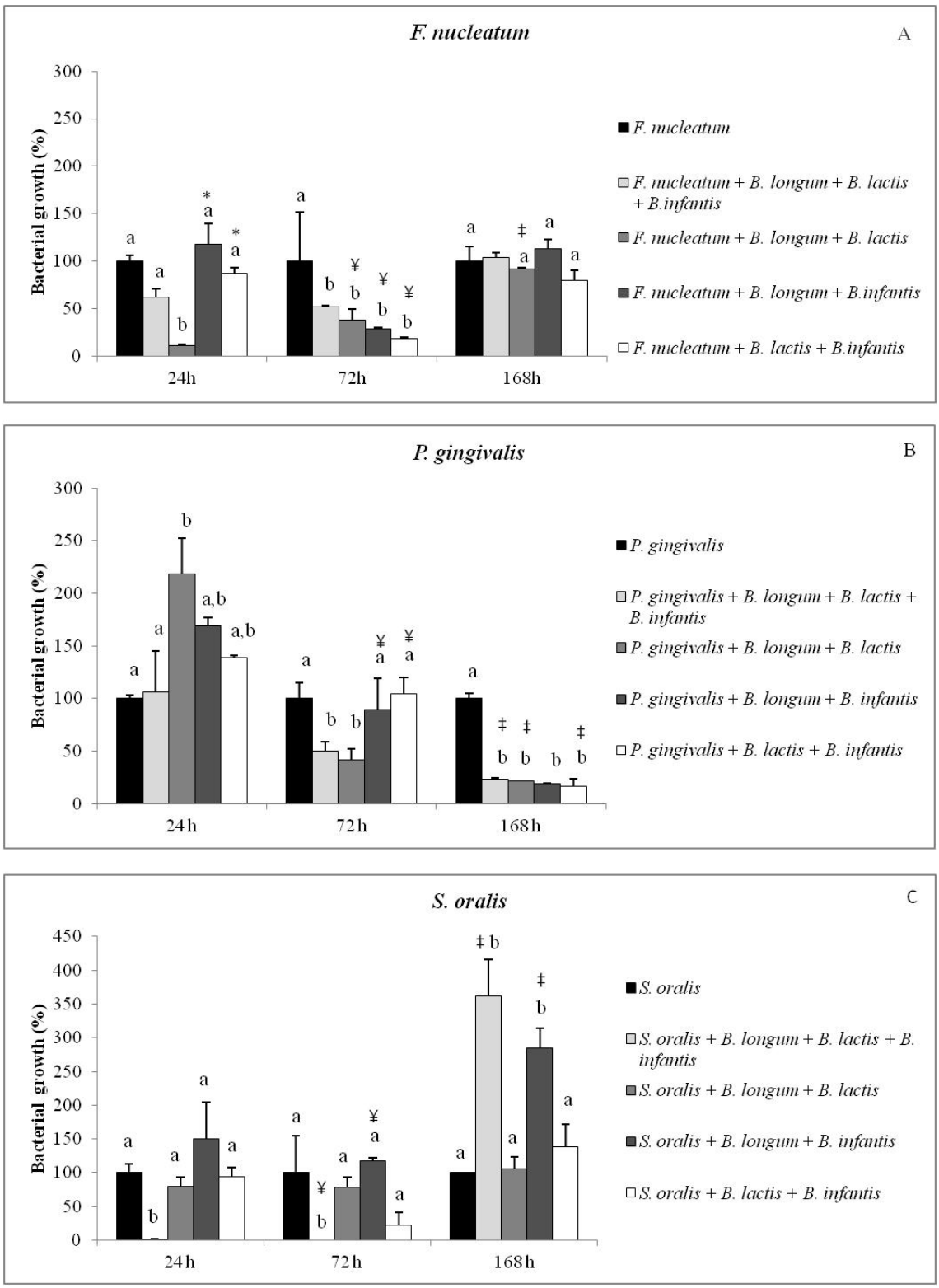

F. nucleatum
A
Bacterial growth (%)
300
250
200
150
100
50
0
24h
72h
168h
F. nucleatum
F. nucleatum + B. longum + B. lactis + B.infantis
F. nucleatum + B. longum + B. lactis
F. nucleatum + B. longum + B.infantis
F. nucleatum + B. lactis + B.infantis
P. gingivalis
B
Bacterial growth (%)
P. gingivalis
P. gingivalis + B. longum + B. lactis + B. infantis
P. gingivalis + B. longum + B. lactis
P. gingivalis + B. longum + B. infantis
P. gingivalis + B. lactis + B. infantis
S. oralis
C
Bacterial growth (%)
450
400
350
300
250
200
150
100
50
0
S. oralis
S. oralis + B. longum + B. lactis + B. infantis
S. oralis + B. longum + B. lactis
S. oralis + B. longum + B. infantis
S. oralis + B. lactis + B. infantis

Figure 2

ANEXO A

Aprovação Comitê de Ética em Humanos

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

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, EUA) and Christine A. Kressirer (Forsyth Institute, EUA) agree with Ethical Principles in Research and was approved by CEP.

Araçatuba, 18 de Agosto de 2013
Araçatuba, August 18th, 2013

Profª Drª ANA CLÁUDIA DE MELO STEVANATO NAKAMUNE
Coordenadora do CEP
CEP Coordinator

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

ANEXO B

Aprovação Comitê de Ética em Animais

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

Comitê de Ética no Uso de Animais (CEUA)
Committee for Ethical Use of Animals (CEUA)

CERTIFICADO

Certificamos que o Projeto **"Detecção molecular e avaliação do potencial cariogênico *in vitro* de bifidobactérias isoladas de crianças com cárie da primeira infância"** sob responsabilidade da Pesquisadora **CRISTIANE DUQUE** e colaboração de Remberto Marcelo Argandoña Valdez e Vanessa Rodrigues dos Santos está de acordo com os Princípios Éticos da Experimentação Animal (COBEA) e foi aprovado pelo CEUA, de acordo com o processo **00197-2013**.

CERTIFICATE

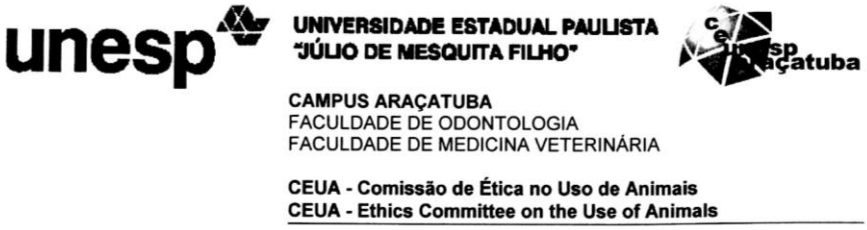
We certify that the research **"Molecular detection and evaluation of *in vitro* cariogenic potencial of bifidobacteria isolated from children with early childhood caries"**, process number **00197-2013**, under responsibility of **CRISTIANE DUQUE** and with collaboration of Remberto Marcelo Argandoña Valdez and Vanessa Rodrigues dos Santos agree with Ethical Principles in Animal Research (COBEA) and was approved by CEUA.

Prof. Dr. Edilson Ervolino
Coordenador da CEUA
CEUA Coordinator

Faculdade de Odontologia de Araçatuba - UNESP
Rua José Bonifácio, 1193 - CEP-16015-050 - Araçatuba – SP
Tel. (18) 3636-3234

ANEXO C

Aprovação do Relatório Final do Comitê de Ética em Animais



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Relatório Final do trabalho intitulado **"Detecção molecular e avaliação do potencial cariogênico in vitro de bifidobactérias isoladas de crianças com cárie da primeira infância"**, Processo FOA nº 2013-00197, sob responsabilidade de Cristiane Duque e colaboração de Remberto Marcelo Argandona Valdez, Vanessa Rodrigues dos Santos, Karina Sampaio Caiaffa, Rodrigo Alex Arthur, Alberto Carlos Botazzo Delbem e Marcelle Danelon foi aprovado pela CEUA em 09 de Dezembro de 2015.

CERTIFICATE

We certify that the study entitled **"Molecular detection and evaluation of in vitro cariogenic potencial of bifidobacteria isolated from children with early childhood caries"**, Protocol FOA nº 2013-00197, under the supervision of Cristiane Duque and collaboration of Remberto Marcelo Argandona Valdez, Vanessa Rodrigues dos Santos, Karina Sampaio Caiaffa, Rodrigo Alex Arthur, Alberto Carlos Botazzo Delbem and Marcelle Danelon had its the Final Report approved by the CEUA on December 09, 2015.

Profa. Adj. Maria Cristina Rosifini Alves Rezende
Vice-Coordenadora da CEUA
CEUA Vice-Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
Rua José Bonifácio, 1193 – Vila Mendonça - CEP: 16015-050 – ARAÇATUBA – SP
Fone (18) 3636-3234 **Email CEUA**: ceua@foa.unesp.br

ANEXO D

Instructions to authors - *International Journal of Paediatric Dentistry*

Author Guidelines

Content of Author Guidelines: 1. General, 2. Ethical Guidelines, 3. Manuscript Submission Procedure, 4. Manuscript Types Accepted, 5. Manuscript Format and Structure, 6. After Acceptance.

Relevant Documents: Sample Manuscript

Useful Websites: Submission Site, Articles published in *International Journal of Paediatric Dentistry*, Author Services, Wiley-Blackwell's Ethical Guidelines, Guidelines for Figures.

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The journal to which you are submitting your manuscript employs a plagiarism detection system. By submitting your manuscript to this journal you accept that your manuscript may be screened for plagiarism against previously published works.

1. GENERAL

International Journal of Paediatric Dentistry publishes papers on all aspects of paediatric dentistry including: growth and development, behaviour management, prevention, restorative treatment and issue relating to medically compromised children or those with disabilities. This peer-reviewed journal features scientific articles, reviews, clinical techniques, brief clinical reports, short communications and abstracts of current paediatric dental research. Analytical studies with a scientific novelty value are preferred to descriptive studies.

Please read the instructions below carefully for details on the submission of manuscripts, the journal's requirements and standards as well as information concerning the procedure after acceptance of a manuscript for publication in *International Journal of Paediatric Dentistry*. Authors are encouraged to visit Wiley-Blackwell Author Services for further information on the preparation and submission of articles and figures.

In June 2007, the Editors gave a presentation on How to write a successful paper for the *International Journal of Paediatric Dentistry.*

2. ETHICAL GUIDELINES

Submission is considered on the conditions that papers are previously unpublished, and are not offered simultaneously elsewhere; that authors have read and approved the content, and all authors have also declared all competing interests; and that the work complies with the Ethical Policies of the Journal and has been conducted under internationally accepted ethical standards after relevant ethical review.

3. CONFLICT OF INTEREST
International Journal of Paediatric Dentistry requires that all authors (both corresponding author and co-authors) disclose any potential sources of conflict of interest. Any interest or relationship, financial or otherwise that might be perceived as influencing an author's objectivity is considered a potential source of conflict of interest. Conflicts of interest must be disclosed when directly relevant or indirectly related to the work that the authors describe in their manuscript. Potential sources of conflict of interest include but are not limited to patent or stock ownership, membership of a company board of directors, membership of an advisory board or committee for a company, and consultancy for or receipt of speaker's fees from a company.

It is the responsibility of the corresponding author to have all authors of a manuscript fill out a conflict of interest disclosure form, and to upload all forms as supplementary material as the manuscript is submitted. Please find the form below:

Conflict of Interest Disclosure Form

(If you encounter problems when accessing the above form, please copy the link and open the form in an Internet Explorer Browser)

4. MANUSCRIPT SUBMISSION PROCEDURE

Articles for the *International Journal of Paediatric Dentistry* should be submitted electronically via an online submission site. Full instructions and support are available on the site and a user ID and password can be obtained on the first visit. Support is available by phone (+1 434 817 2040 ext. 167) or here. If you cannot submit online, please contact Mirlyn Consador in the Editorial Office by e-mail IJPDedoffice@wiley.com.

4.1. Getting Started

Launch your web browser (supported browsers include Internet Explorer 5.5 or higher, Safari 1.2.4, or Firefox 1.0.4 or higher) and go to the journal's online submission site: http://mc.manuscriptcentral.com/ijpd
*Log-in or, if you are a new user, click on 'register here'.
*If you are registering as a new user.
- After clicking on 'Create Account', enter your name and e-mail information and click 'Next'. Your e-mail information is very important.
- Enter your institution and address information as appropriate, and then click 'Next.'
- Enter a user ID and password of your choice (we recommend using your e-mail address as your user ID), and then select your area of expertise. Click 'Finish'.
*If you are already registered, but have forgotten your log in details, enter your e-mail address under 'Password Help'. The system will send you an automatic user ID and a new temporary password.
*Log-in and select 'Author Center'.

4.2. Submitting Your Manuscript

After you have logged into your 'Author Center', submit your manuscript by clicking on the submission link under 'Author Resources'.
* Enter data and answer questions as appropriate.
* You may copy and paste directly from your manuscript and you may upload your pre-prepared covering letter.
Please note that a separate *Title Page* must be submitted as part of the submission process as 'Title Page' and should contain the following:

• Word count (excluding tables)
• Authors' names, professional and academic qualifications, positions and places of work. They must all have actively contributed to the overall design and execution of the study/paper and should be listed in order of importance of their contribution
• Corresponding author address, and telephone and fax numbers and email address

*Click the 'Next' button on each screen to save your work and advance to the next screen.
*You are required to upload your files.
- Click on the 'Browse' button and locate the file on your computer.
- Select the designation of each file in the drop down next to the Browse button.
- When you have selected all files you wish to upload, click the 'Upload Files' button.
* Review your submission (in HTML and PDF format) before completing your submission by sending it to the Journal. Click the 'Submit' button when you are finished reviewing.

4.3. Manuscript Files Accepted

Manuscripts should be uploaded as Word (.doc) or Rich Text Format (.rtf) files (not write-protected) plus separate figure files. GIF, JPEG, PICT or Bitmap files are acceptable for submission, but only high-resolution TIF or EPS files are suitable for printing. The files will be automatically converted to HTML and a PDF document on upload and will be used for the review process. The text file must contain the entire manuscript including title page, abstract, text, references, tables, and figure legends, but no embedded figures. In the text, please reference figures as for instance 'Figure 1', 'Figure 2' to match the tag name you choose for the individual figure files uploaded. Manuscripts should be formatted as described in the Author Guidelines below. Please note that any manuscripts uploaded as Word 2007 (.docx) is now accepted by IPD. As such manuscripts can be submitted in both .doc and .docx file types.

4.4. Review Process

The review process is entirely electronic-based and therefore facilitates faster reviewing of manuscripts. Manuscripts will be reviewed by experts in the field (generally two reviewers), and the Editor-in-Chief makes a final decision. *The International Journal of Paediatric Dentistry* aims to forward reviewers´ comments and to inform the corresponding author of the result of the review process. Manuscripts will be considered for 'fast-track publication' under special circumstances after consultation with the Editor-in-Chief.

4.5. Suggest a Reviewer

International Journal of Paediatric Dentistry attempts to keep the review process as short as possible to enable rapid publication of new scientific data. In order to facilitate this process, please suggest the names and current email addresses of a potential international reviewer whom you consider capable of reviewing your manuscript and their

area of expertise. In addition to your choice the journal editor will choose one or two reviewers as well.

4.6. Suspension of Submission Mid-way in the Submission Process
You may suspend a submission at any phase before clicking the 'Submit' button and save it to submit later. The manuscript can then be located under 'Unsubmitted Manuscripts' and you can click on 'Continue Submission' to continue your submission when you choose to.

4.7. E-mail Confirmation of Submission
After submission you will receive an e-mail to confirm receipt of your manuscript. If you do not receive the confirmation e-mail after 24 hours, please check your e-mail address carefully in the system. If the e-mail address is correct please contact your IT department. The error may be caused by some sort of spam filtering on your e-mail server. Also, the e-mails should be received if the IT department adds our e-mail server (uranus.scholarone.com) to their whitelist.

4.8. Manuscript Status
You can access ScholarOne Manuscripts any time to check your 'Author Center' for the status of your manuscript. The Journal will inform you by e-mail once a decision has been made.

4.9. Submission of Revised Manuscripts
Revised manuscripts must be uploaded within 2 months of authors being notified of conditional acceptance pending satisfactory revision. Locate your manuscript under 'Manuscripts with Decisions' and click on 'Submit a Revision' to submit your revised manuscript. Please remember to delete any old files uploaded when you upload your revised manuscript. All revisions must be accompanied by a cover letter to the editor. The letter must a) detail on a point-by-point basis the author's response to each of the referee's comments, and b) a revised manuscript highlighting exactly what has been changed in the manuscript after revision.

4.10 Online Open
OnlineOpen is available to authors of primary research articles who wish to make their article available to non-subscribers on publication, or whose funding agency requires grantees to archive the final version of their article. With OnlineOpen, the author, the author's funding agency, or the author's institution pays a fee to ensure that the article is made available to non-subscribers upon publication via Wiley Online Library, as well as deposited in the funding agency's preferred archive.

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5. MANUSCRIPT TYPES ACCEPTED

Original Articles: Divided into: Summary, Introduction, Material and methods, Results, Discussion, Bullet points, Acknowledgements, References, Figure legends, Tables and Figures arranged in this order. The summary should be structured using the following subheadings: Background, Hypothesis or Aim, Design, Results, and Conclusions and should be less than 200 words. A brief description, in bullet form, should be included at the end of the paper and should describe Why this paper is important to paediatric dentists.

Review Articles: may be invited by the Editor.

Short Communications: should contain important, new, definitive information of sufficient significance to warrant publication. They should not be divided into different parts and summaries are not required.

Clinical Techniques: This type of publication is best suited to describe significant improvements in clinical practice such as introduction of new technology or practical approaches to recognised clinical challenges.

Brief Clinical Reports/Case Reports: Short papers not exceeding 800 words, including a maximum of three illustrations and five references may be accepted for publication if they serve to promote communication between clinicians and researchers. If the paper describes a genetic disorder, the OMIM unique six-digit number should be provided for online cross reference (Online Mendelian Inheritance in Man).

A paper submitted as a Brief Clinical/Case Report should include the following:

- a short **Introduction** (avoid lengthy reviews of literature);
- the **Case report** itself (a brief description of the patient/s, presenting condition, any special investigations and outcomes);
- a **Discussion** which should highlight specific aspects of the case(s), explain/interpret the main findings and provide a scientific appraisal of any previously reported work in the field.
- Please provide up to 3 bullet points for your manuscript under the heading: 1. Why this clinical report is important to paediatric dentists. Bullet points should be added to the end of your manuscript, before the references.

Letters to the Editor: Should be sent directly to the editor for consideration in the journal.

6. MANUSCRIPT FORMAT AND STRUCTURE

6.1. Format

Language: The language of publication is English. UK and US spelling are both acceptable but the spelling must be consistent within the manuscript. The journal's preferred choice is UK spelling. Authors for whom English is a second language must have their manuscript professionally edited by an English speaking person before submission to make sure the English is of high quality. It is preferred that manuscript is professionally edited. A list of independent suppliers of editing services can be found at http://authorservices.wiley.com/bauthor/english_language.asp. 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

6.2. Structure

The whole manuscript should be double-spaced, paginated, and submitted in correct English. The beginning of each paragraph should be properly marked with an indent.

Original Articles (Research Articles): should normally be divided into: Summary, Introduction, Material and methods, Results, Discussion, Bullet points, Acknowledgements, References, Figure legends, Tables and Figures arranged in this order.

Summary should be structured using the following subheadings: Background, Hypothesis or Aim, Design, Results, and Conclusions.

Introduction should be brief and end with a statement of the aim of the study or hypotheses tested. Describe and cite only the most relevant earlier studies. Avoid presentation of an extensive review of the field.

Material and methods should be clearly described and provide enough detail so that the observations can be critically evaluated and, if necessary repeated. Use section subheadings in a logical order to title each category or method. Use this order also in the results section. Authors should have considered the ethical aspects of their research and should ensure that the project was approved by an appropriate ethical committee, which should be stated. Type of statistical analysis must be described clearly and carefully.

(i) Experimental Subjects: Experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included. Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used.

(ii) Clinical trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist should also be included in the submission material.
International Journal of Paediatric Dentistry encourages authors submitting manuscripts reporting from a clinical trial to register the trials in any of the following free, public clinical trials registries: www.clinicaltrials.gov, http://clinicaltrials.ifpma.org/clinicaltrials/, http://isrctn.org/. The clinical trial registration number and name of the trial register will then be published with the paper.

***(iii)DNA Sequences and Crystallographic Structure Determinations*:** Papers reporting protein or DNA sequences and crystallographic structure determinations will not be accepted without a Genbank or Brookhaven accession number, respectively. Other supporting data sets must be made available on the publication date from the authors

directly.

Results should clearly and concisely report the findings, and division using subheadings is encouraged. Double documentation of data in text, tables or figures is not acceptable. Tables and figures should not include data that can be given in the text in one or two sentences.

Discussion section presents the interpretation of the findings. This is the only proper section for subjective comments and reference to previous literature. Avoid repetition of results, do not use subheadings or reference to tables in the results section.

Bullet Points should include one heading:
*Why this paper is important to paediatric dentists.
Please provide maximum 3 bullets per heading.

Review Articles: may be invited by the Editor. Review articles for the *International Journal of Paediatric Dentistry* should include: a) description of search strategy of relevant literature (search terms and databases), b) inclusion criteria (language, type of studies i.e. randomized controlled trial or other, duration of studies and chosen endpoints, c) evaluation of papers and level of evidence. For examples see:
Twetman S, Axelsson S, Dahlgren H et al. Caries-preventive effect of fluoride toothpaste: a systematic review. Acta Odontologica Scandivaica 2003; 61: 347-355.
Paulsson L, Bondemark L, Söderfeldt B. A systematic review of the consequences of premature birth on palatal morphology, dental occlusion, tooth-crown dimensions, and tooth maturity and eruption. Angle Orthodontist 2004; 74: 269-279.

Clinical Techniques: This type of publication is best suited to describe significant improvements in clinical practice such as introduction of new technology or practical approaches to recognised clinical challenges. They should conform to highest scientific and clinical practice standards.

Short Communications: Brief scientific articles or short case reports may be submitted, which should be no longer than three pages of double spaced text, and include a maximum of three ilustrations. They should contain important, new, definitive information of sufficient significance to warrant publication. They should not be divided into different parts and summaries are not required.

Acknowledgements: Under acknowledgements please specify contributors to the article other than the authors accredited. 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 and their location (town, state/county, country) included.

6.3. References
A maximum of 30 references should be numbered consecutively in the order in which they appear in the text (Vancouver System). They should be identified in the text by superscripted Arabic numbers and listed at the end of the paper in numerical order. Identify references in text, tables and legends. Check and ensure that all listed references are cited in the text. Non-refereed material and, if possible, non-English publications should be avoided. Congress abstracts, unaccepted papers, unpublished observations, and personal communications may not be placed in the reference list. References to unpublished findings and to personal communication (provided that explicit consent has been given by the sources) may be inserted in parenthesis in the text. Journal and book references should be set out as in the following examples:

1. Kronfol NM. Perspectives on the health care system of the United Arab Emirates. East Mediter Health J. 1999; 5: 149-167.
2. Ministry of Health, Department of Planning. Annual Statistical Report. Abu Dhabi: Ministry of Health, 2001.
3. Al-Mughery AS, Attwood D, Blinkhorn A. Dental health of 5-year-old children in Abu Dhabi, United Arab Emirates. Community Dent Oral Epidemiol 1991; 19: 308-309.
4. Al-Hosani E, Rugg-Gunn A. Combination of low parental educational attainment and high parental income related to high caries experience in preschool children in Abu Dhabi. Community Dent Oral Epidemiol 1998; 26: 31-36.

If more than 6 authors please, cite the three first and then et al. When citing a web site, list the authors and title if known, then the URL and the date it was accessed (in parenthesis). Include among the references papers accepted but not yet published; designate the journal and add (in press). Please ensure that all journal titles are given in abbreviated form.

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.

6.4. Illustrations and Tables

Tables: should be numbered consecutively with Arabic numerals and should have an explanatory title. Each table should be typed on a separate page with regard to the proportion of the printed column/page and contain only horizontal lines

Figures and illustrations: All figures should be submitted electronically with the manuscript via ScholarOne Manuscripts (formerly known as Manuscript Central). Each figure should have a legend and all legends should be typed together on a separate sheet and numbered accordingly with Arabic numerals. Avoid 3-D bar charts.

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.

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.

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7.1. Copyright

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ANEXO E

Instructions to authors - *Archives of Oral Biology*

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

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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.

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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.
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ANEXO F

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Antibiotic antimicrobial testing and microbial resistance

A number of methods like disc diffusion, Etest, agar dilution, broth microdilution and broth macrodilution, are suitable for *in vitro* antimicrobial susceptibility testing. However, the test used must be performed in accordance with an internationally accepted procedure; for example tests published by the Clinical and Laboratory Standards Institute (CLSI), the British Society for Antimicrobial Chemotherepy (BSAC), the Deutsches Institut fur Normung e.V. (DIN) and the Comite de l'Antibiogramme de la Scoiete Francaise de Microbiologie (CA-SFM). Further guidence and interpretation of MIC 50 and MIC 90 values as well as guidence for the interpretation of multiresistance can be found in Schwarz *et al*. J. Antimicrobial Chemother 2010; 65: 601-604.

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Use 'z' spelling where possible, except analyse, dialyse, hydrolyse, etc.; sulphur, sulphate, etc. When using numbers in the text, one to nine should be written in full and 10 and above should be written as numerals. The Journal uses SI units: g l^{-1} not g/l; d, h, min, s (time units) but week and year in full; mol l^{-1} (not M or N); probability is P; centrifugation conditions relative to gravity (g). Please refer to the Biochemical Journal 'Instructions to Authors' www.biochemj.org/bj/bji2a.htm.

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The Latin binomial name of micro-organisms, plants and animals (other than farm animals) must be given at first mention in the text; thereafter the generic name will be abbreviated in such a way that confusion is avoided when dealing with several genera all beginning with the same letter, viz. *Pseudomonas*, *Proteus*, *Pediococcus*, etc. (see list

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Please click here for a list of abbreviations currently in use for common generic names and for notes on referring to plant pathogenic bacteria.

Gnotobiotic animals

The terminology for describing the environmental status of animals in gnotobiotic experiments has established itself by usage. *Germ-free* implies freedom from any detectable microorganisms or viruses and it is limited by the tests used to detect contaminants. *Conventional animals* have a full complement of associated microbes. *Open conventional animals* are housed in a standard animal house. *Isolator conventional animals* are maintained in isolators and associated with full flora. *Ex-germ-free animals* are those with an associated flora which have become conventional.

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Tests must be presented clearly to allow a reader with access to the data to repeat them. Statistical tests used in the study should be clearly indicated in the Materials and Methods section. It is not necessary to describe every statistical test fully, as long as it is clear from the context what was done. In particular, null hypotheses should be clearly stated.

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