Carolina Simonetti Lodi

AVALIAÇÃO DO POTENCIAL CARIOGÊNICO DE LEITES FERMENTADOS CONTENDO PROBIÓTICOS

Araçatuba – SP
2011

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Tese apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, para obtenção de título de Doutor em Odontopediatria.

Orientadora: Profª Drª Cleide Cristina Rodrigues Martinhon
Co-orientador: Prof. Adj. Alberto Carlos Botazzo Delbem

ARAÇATUBA - SP
2011

Catalogação na Publicação (CIP)

Serviço Técnico de Biblioteca e Documentação – FOA / UNESP

Lodi, Carolina Simonetti.

L823a Avaliação do potencial cariogênico de leites fermentados contendo probióticos / Carolina Simonetti Lodi. - Araçatuba : [s.n.], 2011

115 f. : il. ; tab. + 1 CD-ROM

Tese (Doutorado) – Universidade Estadual Paulista, Faculdade de Odontologia

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1. Produtos fermentados do leite 2. Probióticos 3. Cárie dentária 4. Desmineralização do dente 5. Streptococcus mutans 6. Lactobacillus

Black D27

CDD 617.645

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Associações	CROSP - Conselho Regional de Odontologia de São Paulo SBPqO - Sociedade Brasileira de Pesquisa Odontológica IADR – International Association for Dental Research

Dedicatória

Dedico este trabalho,

Aos meus pais **ROBERTO** e **VERA**,

Pela confiança que depositam em mim... Exemplos de dedicação, honestidade, simplicidade, felicidade e amor. Agradeço por todos os momentos em que podemos estar juntos e pelas palavras de conforto que sempre trazem segurança e tranqüilidade.

Nada teria acontecido se eu não tivesse o apoio constante de vocês. Muitas vezes não fisicamente, mas em pensamentos e palavras;

A vocês dedico este trabalho, pois sem vocês eu nada seria.

Amo vocês!

Agradecimentos Especiais

À Deus,

Presente em todos os momentos da minha vida, protegendo-me e guiando meus passos nesse longo caminho rumo a grandes realizações. Devo a Ele todos os momentos de alegria e sucesso até aqui conquistado.

Muito obrigado pela companhia!

À Roberta e Rodrigo,
minha irmã e meu irmão,

Agradeço à Deus e meus pais por me concederem a bênção de ter dois irmãos.

Pessoas que fazem meu coração sorrir...

...Que não encontro todos os dias, mas que tenho a chance de dizer agora...

Para mim o que importa não é o que eu tenho na vida, mas quem eu tenho na vida... Por isso...

Guardo vocês dentro do meu coração....

Amo vocês!

Muito obrigado!

Ao João Eduardo,
Meu marido...

"Quando encontrar alguém e esse alguém fizer seu coração parar de funcionar por alguns segundos, preste atenção: pode ser a pessoa mais importante da sua vida.

Se os olhares se cruzarem e, neste momento, houver o mesmo brilho intenso entre eles, fique alerta: pode ser a pessoa que você está esperando desde o dia em que nasceu.

Se o toque dos lábios for intenso, se o beijo for apaixonante, e os olhos se encherem d'água neste momento, perceba: existe algo mágico entre vocês.

Se o primeiro e o último pensamento do seu dia for essa pessoa, se a vontade de ficar juntos chegar a apertar o coração, agradeça: Deus te mandou um presente."

Se um dia tiverem que pedir perdão um ao outro por algum motivo e, em troca, receber um abraço, um sorriso, um afago nos cabelos e os gestos valerem mais que mil palavras, entregue-se: vocês foram feitos um pro outro.

Se por algum motivo você estiver triste, se a vida te deu uma rasteira e a outra pessoa sofrer o seu sofrimento, chorar as suas lágrimas e enxugá-las com ternura, que coisa maravilhosa: você poderá contar com ela em qualquer momento de sua vida.

Se você tiver a certeza que vai ver a outra envelhecendo e, mesmo assim, tiver a convicção que vai continuar sendo louco por ela...

...é o amor que chegou na sua vida."

Carlos Drummond de Andrade

Agradeço à Deus todos os dias por ter colocado você em minha vida....

Obrigada pelo companheirismo e apoio constante.

Amo muito você!

À minha orientadora,
Profª. Drª. Cleide Cristina Rodrigues Martinhon,

Agradecer é admitir que houve um momento em que se precisou de alguém; é reconhecer que ninguém jamais poderá lograr para si o dom de ser auto-suficiente. Ninguém e nada cresce sozinho; sempre é preciso um olhar de apoio, uma palavra de incentivo, um gesto de compreensão, uma atitude de amor...

Obrigada pela paciência, compreensão e carinho que dedicou à mim e a este trabalho. Pela confiança que depositou em mim e pela amizade eterna...

Minha imensa gratidão.

Ao meu co-orientador,
Prof. Dr. Alberto Carlos Botazzo Delbem,

Admiro sua competência e dedicação ao trabalho e à pesquisa.

Obrigada pelas orientações sábias e práticas. Pela ajuda, disponibilidade e pela companhia agradável em vários momentos.

Aos Prof. Dr. Carlos Gonzalez Cabezas e Profª Margherita Fontana,

Toda minha admiração. Agradeço por toda a sabedoria transmitida durante minha estada em Ann Arbor e por todos os momentos de alegria e descontração.

Thank you very much!

Ao meu avô Raphael,

Pela presença constante em minha vida e pelas palavras sempre doces e carinhosas.

Pela maneira invejável que se dedica aos seus filhos e netos.

Amo você.

À Mariana, Donizeti, Alessandra e Marcelo,

Meus cunhados e irmãos de coração...

Pela convivência sempre alegre e feliz.

Vocês também são minha família.

Amo vocês.

À aluna Lidiane Viana,

Pela ajuda indispensável no desenvolvimento da parte experimental deste trabalho, pelos momentos alegres e pela amizade.

Muito Obrigada!

À Susan Flannagan,

Obrigada pelas orientações sábias, pela ajuda, disponibilidade, amizade e pela companhia sempre agradável em Ann Arbor.

Thank you very much!

Agradecimentos

Faculdade de Odontologia de Araçatuba, na pessoa dos professores Dr. Pedro Felício Estrada Bernabé, digníssimo Diretor e Dra. Ana Maria Pires Soubhia, digníssima Vice-Diretora.

Ao Curso de Pós-Graduação em Odontopediatria da Faculdade de Odontologia de Araçatuba –UNESP, na pessoa do coordenador Prof. Dr. Alberto Carlos Botazzo Delbem.

Aos docentes da Disciplina de Odontopediatria da Faculdade de Odontologia de Araçatuba - UNESP, Prof. Dr. Alberto Carlos Botazzo Delbem, Prof. Dr. Célio Percinoto, Prof. Dr. Robson Frederico Cunha, Profª. Drª. Rosângela dos Santos Nery, Profª. Drª. Sandra M. H. C. Ávila de Aguiar pelo carinho e atenção em todos os momentos.

Aos funcionários da Disciplina de Odontopediatria, Maria dos Santos Ferreira Fernandes e Mário Luis da Silva, pela amizade, auxílio indispensável e convivência tão especiais.

Aos queridos amigos da minha turma de Doutorado, Adelisa, Renata e Vanessa, pelos anos de convivência, amizade e respeito.

À todos os colegas do curso de Pós-Graduação em Odontopediatria (Mestrado e Doutorado) da Faculdade de Odontologia de Araçatuba – UNESP, pela convivência carinhosa.

Aos amigos do Laboratório de pesquisa do Departamento de Odontopediatria pela convivência durante todo o doutorado: Adelisa, Amanda, Ana Paula, Ana Elisa, Ana Carolina (Tuca), Carla, Dani Câmara, Dani Oliveira, Emilene, Eliana Takeshita, Gabriel, Jackeline, José Antonio, Isabelle, Lidiane, Marcelle, Maria Daniela, Marcelo, Michele.

Aos amigos da Universidade de Michigan – Ann Arbor: Chip, Matt, Susan, Letícia, Renata, Sudha, Tatiana Botero, Zhihong, Kristy, Zhaocheng, Atsushi pela amizade e pelos momentos de descontração.

Aos funcionários da Biblioteca da Faculdade de Odontologia de Araçatuba - UNESP, Ana Cláudia, Cláudio, Ivone, Izamar, Luzia, Maria Cláudia e Marina pela atenção e disponibilidade com que sempre me receberam.

Aos funcionários da Seção de Pós-Graduação da Faculdade de Odontologia de Araçatuba - UNESP, pelo profissionalismo, carinho e paciência.

Aos demais professores e funcionários da Faculdade de Odontologia de Araçatuba – UNESP.

À Fernanda Brighenti, por ter colaborado em todos os momentos da realização deste trabalho e pela amizade.

À Prof. Drª Cristiane Ito por me dar a oportunidade de acompanhar os trabalhos do laboratório e assim aprender as metodologias necessárias para o desenvolvimento do meu trabalho.

Aos voluntário deste trabalho, Dani Oliveira, Fernanda, Isabelle, Jackeline, José Antônio, Lidiane, Marcelle, Marcelo, Marchelo e Michele pela imensa colaboração e compreensão durante a realização deste trabalho.

Às professoras e amigas do curso de Especialização do HRAC-USP, que de alguma forma contribuíram para que eu chegasse até aqui e que apesar da distância continuam guardadas na lembrança e no coração.

Ao diretor do hemocentro de Araçatuba, Dr. Wolney Gois Barreto, e aos funcionários pela coleta e doação das bolsas de sangue.

Ao Frigorífico Friboi, em especial, ao veterinário Henrique Borges, pela atenção e por ter permitido a coleta dos dentes bovinos.

À Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo apoio financeiro para a realização do Curso de Doutorado.

A todos aqueles que, direta ou indiretamente, contribuíram para a realização deste trabalho,

...Meus sinceros agradecimentos.

Posso ter defeitos, viver ansioso e ficar irritado algumas vezes mas não esqueço de que minha vida é a maior empresa do mundo, e posso evitar que ela vá à falência.

Ser feliz não é ter um céu sem tempestade, caminhos sem acidentes, trabalhos sem fadigas, relacionamentos sem desilusões.

Ser feliz é encontrar força no perdão, esperança nas batalhas, segurança no palco do medo, amor nos desencontros.

Ser feliz não é apenas valorizar o sorriso, mas refletir sobre a tristeza. Não é apenas comemorar o sucesso, mas aprender lições nos fracassos. Não é apenas ter júbilo nos aplausos, mas encontrar alegria no anonimato.

Ser feliz é reconhecer que vale a pena viver apesar de todos os desafios, incompreensões e períodos de crise.

Ser feliz é deixar de ser vítima dos problemas e se tornar um autor da própria história. É atravessar desertos fora de si, mas ser capaz de encontrar um oásis no recôndito da sua alma. É agradecer a Deus a cada manhã pelo milagre da vida.

Ser feliz é não ter medo dos próprios sentimentos. É saber falar de si mesmo. É ter coragem para ouvir um "não". É ter segurança para receber uma crítica, mesmo que injusta.

E, quando você errar o caminho, recomece. Pois assim você descobrirá que ser feliz não é ter uma vida perfeita. Mas usar as lágrimas para irrigar a tolerância. Usar as perdas para refinar a paciência. Usar as falhas para lapidar o prazer. Usar os obstáculos para abrir as janelas da inteligência.

Pedras no caminho?
Guardo todas, um dia vou construir um castelo…

Fernando Pessoa

Epígrafe

Resumo

Lodi CS. Avaliação do potencial cariogênico de leites fermentados contendo probióticos. [Tese]. Araçatuba: Universidade Estadual Paulista; 2011.

Um número crescente de produtos contendo probióticos está disponível no mercado e vem sendo utilizados pelos consumidores. Diante disso, o objetivo deste trabalho foi avaliar *in situ*, *in vivo* e *in vitro* a relação da bactéria probiótica com a cárie dentária. No estudo *in situ* investigou-se a cariogenicidade do leite fermentado contendo probióticos através da quantificação dos açúcares totais e redutores presente no produto, análise do seu efeito na desmineralização do esmalte dental bovino, análise microbiológica da saliva antes e após o período experimental, análise microbiológica e quantificação dos carboidratos álcalis-solúveis presente no biofilme. Para isso, dez voluntários utilizaram um dispositivos contendo 4 blocos de esmalte dental bovino. O experimento consistiu de 3 etapas de 14 dias cada onde os voluntários gotejaram solução de sacarose 20% ou a solução de tratamento (Tratamento A - Yakult® ou Tratamento B - Batavito®) 8X/dia. Decorrido o período experimental, o biofilme e a saliva foram analisados quanto a quantidade de microrganismos totais (MT), *Streptococcus* totais (ST) e *Streptococcus* do grupo *mutans* (SM), *Lactobacillus* (L). Para os dados de dureza foram calculados a porcentagem de variação de dureza superficial e a perda integrada de dureza de subsuperfície. Após o tratamento B foi observado menor quantidade de MT no biofilme quando comparado com o tratamento A, mas não diferiu da solução de sacarose 20%. Na saliva, o tratamento com solução de sacarose 20% diminuiu a quantidade de MT e ST, e aumento a quantidade de SM. O tratamento A provocou uma diminuição na quantidade de MT, ST e SM e o tratamento B diminuiu a quantidade de MT. Para os dados de dureza, foi observado que no tratamento B maior dureza superficial final, menor porcentagem de variação de dureza de superfície e menor perda mineral. No estudo *in vivo*, investigou-se a capacidade do leite fermentado contendo probióticos de alterar a microbiota bucal após a sua ingestão. Para isso, 10 voluntários ingeriram 1 frasco de leite fermentado por dia (leite

fermentado A – Yakult®, leite fermentado B – Batavito®). O experimento consistiu de 2 etapas (Tratamento C – Yakult® e tratamento D – Batavito®) de 14 dias cada. As amostras de saliva foram coletadas no início e final de cada fase para avaliar a contagem de MT, ST e SM e L. Após o tratamento D foi observado uma diminuição de todos os microrganismos avaliados. E por fim, no estudo *in vitro*, foi avaliada a capacidade da bactéria probiótica em prevenir o desenvolvimento da lesão de cárie utilizando biofilme multiespécie. O estudo foi dividido em 4 grupos onde os blocos de esmalte dental bovino foram inoculados com uma mistura em quantidades iguais de culturas overnight de *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus gordonii*, *Actinomyces naeslundii* e um *Lactobacillus* que foi alterado em cada grupo: G1 - *Lactobacillus casei*; G2 - *Lactobacillus rhamnosus* GG; G3 - *Lactobacillus reuteri* e G4 - *Lactobacillus casei* Shirota. Todos os grupos foram expostos 3X/dia durante 30 minutos ao TSBS (caldo triptona de soja) suplementado com sacarose a 5% e a uma solução de saliva artificial contendo flúor o restante do dia, durante 7 dias. Ao final do período o biofilme foi analisado quanto à quantidade de MT, ST, SM e L. Os blocos foram avaliados em relação à porcentagem de variação de dureza de superfície, área e profundidade da lesão. Foi observado maior quantidade de ST e SM no G3. O G3 apresentou dureza final maior quando comparado com os outros grupos e menor porcentagem de alteração de dureza de superfície quando comparados com os grupos G1 e G4.

Com base nos resultados obtidos e considerando as limitações dos modelos utilizados, pode-se concluir nos estudos *in situ* e *in vivo* que o leite fermentado contendo probiótico foi capaz de promover alterações na microbiota bucal além de desmineralização no esmalte dental; e o Batavito® foi o leite fermentado que menos favoreceu o desenvolvimento da lesão de cárie; e no estudo *in vitro* que apenas o grupo contendo a bactéria probiótica *L. reuteri* foi capaz de reduzir a perda mineral.

Abstract

Lodi CS. Probiotic-containing fermented milk cariogenicity evaluation. [Thesis]. Araçatuba: Univ. Estadual Paulista; 2011.

Probiotics are live microorganisms, which when administered in adequate amounts, confer a health benefit on the host. An increasing number of probiotic-containing products are available, and these products have been orally consumed. However, the objective of this study was to evaluate *in situ*, *in vivo* and *in vitro* the relation between probiotic bacteria and dental caries. In the *in situ* and *in vivo* study it was evaluate the effect of 2 probiotic-containing fermented milk on biofilm and saliva microorganisms and on enamel surface. The *in situ* study was performed in 3 phases: 20% sucrose, treatment A (Yakult®) and treatment B (Batavito®). Salivary microorganisms were counted at baseline and after and biofilm was analyzed just after the trial period. *In vivo* study was performed in 2 phases: treatment C (Yakult®) and treatment D (Batavito®). The saliva was collected at baseline and at the end of the trial period for microbiological analysis. In the *in situ* study, biofilm data showed less total microorganisms (TM) after treatment B than treatment A but similar to 20% sucrose. In the saliva, 20% sucrose decreased TM and total streptococci (TS), and increased mutans streptococci (MS). Treatment A significantly decreased TM, TS and MS. Treatment B decreased TM. It was observed less MS in treatment B when the final data were compared among the treatments. Treatment B differed from the other treatments in relation to final microhardness, percentage change of surface hardness and integrated loss of subsurface hardness ($p<0.05$). *In vivo* study showed that just treatment D decreased all microorganisms. It was observed higher lactobacilli (L) in treatment D when the baseline data were compared among the groups. In the *in vitro* study, it was determined the ability of probiotic bacteria to prevent primary caries development using a multi-species biofilm and microbial artificial-mouth model. Four groups were inoculated with mixed overnight cultures of *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus gordonii*, *Actinomyces naeslundii* and one *Lactobacillus* strain that was changed for each group: G1 - *Lactobacillus casei*, G2 - *Lactobacillus rhamnosus* GG, G3 - *Lactobacillus*

reuteri and G4 - *Lactobacillus casei* Shirota. All groups were exposed to circulating trypticase soy broth supplemented with 5% sucrose (TSBS), 30 min, 3 times per day and a mineral wash solution for the rest of the day during 7 days. At the end of the study, biofilm bacterial colonies counts, surface microhardness change, caries lesion area and lesion depth were determined. There were no significant differences in the number of TM and L among the groups. However, TS and MS were significantly higher in the G3 compared to the other groups. The drainage fluid pH and caries vessel fluid pH at the end of the experiment were very similar for all groups. There were no significant difference in the initial microhardness, lesion area and lesion depth among the groups. But, the G3 had significantly higher final microhardness than the other groups, and a significantly smaller surface hardness change than the G1 and G4 groups. Based on these results and considering the limitations of the models used, it was possible to conclude in the *in situ* and *in vivo* study that probiotic-containing fermented milks were able to promote changes in the oral microbiota and demineralization in enamel; and Batavito® was the fermented milk that less favored the caries lesion development; and in the *in vitro* study that only the *L. reuteri* mix-culture biofilm group had a reduced mineral loss as measured by surface microhardness.

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Lista de Abreviaturas

ATCC = American Type Culture Collection

BHI = Brain heart infusion

°C = Graus Celsius

CFU = Colony-forming units

dNTP = Desoxirribonucleotídeos trifosfatados

EPS = Extracellular Polysaccharide

g = Gramas

h = Horas

kb = Kilo base (= 1000 base pair)

Kg/mm^2 = Kilogramas por milímetro quadrado

KHN = Knoop hardness number

ΔKHN = Integrated loss of subsurface hardeness

KCl = Cloreto de potássio

K_2HPO_4 = Fosfato de potássio dibásico

KH_2PO_4 = Fosfato de potássio monobásico

L = Lactobacillus

Log = Logarítimo

min = Minutos

mg = Miligramas

$MgCl_2$ = Cloreto de magnésio

mL = Mililitros

mm = Milímetros

μL = microlitros

mol L^{-1} = Mol por litro (Molar)

MRS = Man, Rogosa and Sharpe

MS = Mutans streptococci

MT = Micorganismo total

MW = Mineral washing

NaCl = Cloreto de sódio

nm = nanometro

PBS = Phosphate buffered saline

PCR = Polymerase Chain Reaction

pH = Potencial hidrogeniônico

p = Significância

ppm = Partes por milhão

s = segundos

SD = Standard deviation

SM = *Streptococcus* do grupo *mutans*

SMH = Surface microhardness

SMHi = Initial surface microhardness

SMHf = Final surface microhardness

%SMH = Percentage change in surface microhardness

ST = *Streptococcus* total

TBE buffer = Tris/Borate/EDTA buffer

TM = Total microorganisms

TS = Total streptococci

TSBS = Caldo triptona de soja suplementado com 5% de sacarose

UFC = Unidades formadoras de colônias

U = Units

V = volt

W = Watt

Xg = Times gravitation

SUMÁRIO

Introdução Geral

Introdução Geral

Probiótico é definido pela Organização Mundial da Saúde como sendo microrganismos vivos que quando administrados em quantidades adequadas conferem benefícios à saúde do hospedeiro [Food and Agriculture Organization of the United Nations, World Health Organization, 2001]. As espécies mais comumente utilizadas e pesquisadas pertencem ao gênero dos *Lactobacillus* e *Bifidobacterium* [Ouwehand et al., 2002], microrganismos estes comumente encontrados na cavidade bucal, inclusive nas lesões de cárie [Chhour et al., 2005].

O conhecimento atual a respeito do importante papel da microflora intestinal conduziu a estratégias para promover saúde através da manipulação dessa comunidade microbiana [Fooks e Gibson, 2002]. A ação do probiótico no trato gastrointestinal é baseada na aderência da bactéria probiótica à mucosa intestinal, inibindo dessa forma a adesão de patógenos. Semelhantemente na cavidade oral, os probióticos devem aderir aos tecidos dentais como parte do biofilme e competir com o crescimento de bactérias cariogênicas ou patógenos peridontais [Comelli et al, 2002].

A produção eficiente de ácidos orgânicos, que é uma característica comum tanto dos *Lactobacillus* como dos *Bifidobacterium* pode ser prejudicial à saúde bucal. Matsumoto et al. [2005], avaliaram a cariogenicidade da bactéria probiótica *Lactobacillus salivarius* em ratos e puderam observar que a inoculação destes microrganismos promoveu um incremento da atividade cariogênica aumentando a aderências dos *S. muttans* à superfície dental. Por outro lado, esses mesmos microrganismos têm sido relacionados com benefícios à saúde bucal, como a produção de substâncias inibitórias do crescimento de *Streptococcus sobrinus* [Meurman et al, 1995; Meurman, 2005; Meurman e Stamatova, 2007; Yli-Knuuttila et al, 2006], bem como a redução do risco de cárie em crianças de 3-4 anos de idade após a ingestão de leite contendo probiótico [Näse et al, 2001]. Além disso, o consumo de iogurte contendo *Lactobacillus reuteri* reduziu ligeiramente a contagem de *Streptococcus mutans* bucal [Nikawa et al, 2004]. Resultados similares também foram obtidos com

cepas de *Bifidobacterium* ou *Lactobacillus reuteri* [Çaglar et al, 2005; 2006; 2007; 2008]. Já os resultados de Montalto et al. [2004] demonstraram que não houve nenhum aumento na contagem de *Streptococcus mutans* quando se administrou uma mistura de *Lactobacillus* probióticos na forma de cápsulas ou líquida.

A sobrevivência das bactérias probióticas no produto alimentício é de fundamental importância, devendo o alimento conter pelo menos uma população de 10^7 UFC/g de bactérias probióticas viáveis no momento da compra do produto. Esta concentração é recomendada por alguns autores [Rybka e Fleet, 1997; Vinderola e Reinheimer, 2000]. Entretanto, tem sido proposto que a dose mínima diária de cultura probiótica considerada terapêutica seja de 10^8 e 10^9 UFC, o que corresponde ao consumo diário de 100 g de um produto contendo 10^6 a 10^7 UFC/g [Lee e Salminen, 1995; Blanchette et al, 1996]. Tem sido mostrado que a rotina de consumo [Cabana et al, 2006] e o processamento do alimento pode comprometer a viabilidade da bactéria probiótica e interferir no benefício terapêutico advindo desse consumo [Twetman e Stecksén-Blicks, 2008].

Para que o probiótico seja capaz de exercer um efeito anticariogênico, primeiramente a bactéria deve ser capaz de aderir à superfície dental; segundo, ela deve se tornar parte do biofilme dental; e finalmente, ela deve competir com as bactérias cariogênicas reduzindo o nível de colonização destas [Comelli et al, 2002].

Além dos gêneros bacterianos, a maioria dos leites fermentados contém açúcares e pH ácido que podem facilitar a aderência de microrganismos patogênicos à superfície dentária e consequentemente o desenvolvimento de cárie [Matsumoto et al, 2005], por isso alguns veículos como leites, iogurtes, queijos, gomas de mascar e comprimidos [Çaglar et al, 2005; 2006; 2007; 2008] têm sido estudados na tentativa de se estabelecer a maneira mais eficaz de se administrar os probióticos.

Twentman e Stecksén-Blicks [2008] realizaram um trabalho de revisão de literatura mostrando a escassez de estudos que relacionam a ação dos probióticos e a saúde bucal. Foram encontrados apenas 14 trabalhos com esse

enfoque, mas todos avaliaram a instalação da bactéria probiótica ou a diminuição/aumento dos microrganismos patogênicos após a ingestão de produtos contendo probióticos em amostras de saliva, não sendo encontrados trabalhos que avaliaram a instalação desses microorganismos no biofilme e segundo Meurman [2005] amostras de saliva podem subestimar a verdadeira situação do biofilme.

Em estudo realizado por Lodi et al [2010a, 2010b] foi analisado o potencial cariogênico de leites fermentados contendo probióticos (Parmalat®-uva, Chamyto®, Paulista®, Batavito®, Yakult®, Vigor Club®). O estudo consistiu de 2 etapas, sendo primeiramente realizado um estudo *in vitro* [Lodi et al. 2010a] para analisar algumas propriedades desses produtos como pH, capacidade tampão, conteúdo de flúor, cálcio e fósforo. Ficou demonstrado que todos os produtos possuem pH baixo e capacidade tampão elevada, características estas que podem auxiliar na desmineralização do esmalte dental. Além disso, pode ser observado também a presença dos íons flúor, cálcio e fósforo nos leites fermentados possibilitando uma diminuição no potencial cariogênico dos produtos.

Posteriormente, foram selecionadas 2 marcas dentre as citadas anteriormente (Yakult®, Batavito®) para a realização de um estudo *in situ* onde foram avaliados a concentração dos íons flúor, cálcio, fósforo e carboidratos álcalis-solúveis presentes no biofilme dental, bem como a desmineralização do esmalte dental bovino através do teste de microdureza superficial [Lodi et al., 2010b]. Ficou demonstrado que ambos os produtos promoveram queda no pH do biofilme dental e desmineralização na superfície do esmalte dental bovino, embora o tratamento com o leite fermentado Batavito® tenha apresentado perda mineral significantemente menor quando comparado com os outros grupos (Yakult® e Sacarose 20%), bem como, a maior concentração de íons flúor, cálcio e fósforo e menor concentração de carboidratos álcalis-solúveis no biofilme dental. Neste estudo não pode ser determinado o motivo de uma perda mineral menor após a utilização de uma determinada marca de leite fermentado (Batavito®), podendo a menor desmineralização dental estar relacionada com o

tipo e a quantidade de bactéria probiótica e a quantidade de carboidratos presente nos produtos.

Por outro lado, acredita-se que quanto mais cedo ocorrer à colonização bucal por bactérias probióticas maior é seu efeito em longo prazo [Twentman e Stecksén-Blicks, 2008], portanto, diante da escassez de trabalhos relacionando probióticos e saúde bucal, o objetivo deste trabalho foi avaliar *in situ*, *in vivo* e *in vitro* a relação da bactéria probiótica com a cárie dentária a fim de elucidar se a indicação do consumo precoce desses produtos poderá trazer prejuízos à saúde bucal das crianças.

Capítulo 1

Probiotic-containing fermented milk effects on biofilm, oral microbiota, and dental enamel

Abstract

The aim of this study was to evaluate the effect of 2 probiotic-containing fermented milks on biofilm and salivary microorganisms and on enamel surface. *In situ* study was performed in 3 phases: 20% sucrose, treatment A (Yakult®), and treatment B (Batavito®). Salivary microorganisms were counted at baseline and after, and biofilm was analyzed just after the trial period. *In vivo* study was performed in 2 phases: treatment C (Yakult®) and treatment D (Batavito®). Saliva was collected at baseline and at the end of the trial period for microbiological analysis. In the *in situ* study, biofilm data showed less total microorganisms (TM) after treatment B than after treatment A, but similar to 20% sucrose. In saliva, 20% sucrose decreased TM and total streptococci (TS) and increased mutans streptococci (MS). Treatment A significantly decreased TM, TS, and MS. Treatment B decreased TM. It was observed less MS in treatment B when the final data were compared among the treatments. Treatment B differed from the other treatments in relation to the final microhardness, percentage change in the surface hardness, and integrated loss of subsurface hardness ($p < 0.05$). *In vivo* study showed that just treatment D decreased all microorganisms. It was observed higher lactobacilli (L) in treatment D when the baseline data were compared among the groups. In conclusion, probiotic-containing fermented milks were able to promote changes in the biofilm, oral microbiota, and demineralization of the enamel; Batavito® was the fermented milk that less favored the caries lesion development.

Key words: Cultured milk products; Probiotics; Tooth demineralization; Dental caries; *Streptococcus mutans*; *Lactobacillus*

*Capítulo escrito de acordo com as instruções do periódico Caries Research (ANEXO B)

Introduction

Limitations in traditional disease management strategies have been overcome, and a number of researchers are developing probiotic methods to treat the dental caries [Anderson and Shi, 2006]. Research focusing on probiotics has progressed considerably in the last 20 years, and significant advances have been made in the selection and characterization of specific probiotic cultures and the substantiation of health claims relating to their consumption [Teughels et al., 2008].

The mechanism of probiotic action in the oral cavity is not fully understood but is commonly explained by a combination of local and systemic immune responses as well as non-immunologic defense mechanisms [Çaglar et al., 2005a; Meurman and Stamatova, 2007]. The principal health-promoting effects are ascribed to the enhancement of the mucosal immune defense and macrophage activity as well as elevations in the number of killer cells, T-cells, and interferons [Fuller and Gibson, 1997]. To be effective against oral infections, probiotic bacteria need to adhere to the oral mucosa and dental tissues as part of the biofilm and compete with the growth of dental pathogens [Comelli et al., 2002]. The most widely used species belong to the genera *Lactobacillus* and *Bifidobacterium* once these organisms are already produced in the dairy industry and because they are very rarely implicated in infections of humans [Teughels et al., 2008].

Probiotics are provided into food items in 1 out of 4 basic ways: as a culture concentrate added to beverages (such as fruit juices); inoculated into prebiotic fibers, which promote the probiotic bacterial growth; inoculated into milk and milk-based foods (such as milk drinks, yoghurt, and cheese); and as lyophilized cells packaged as dietary supplements (tablets, chewing gums, and straws). The archetypical probiotic food is yoghurt, and the daily consumption of dairy products seems to be the most natural way to ingest probiotic bacteria [Çaglar et al., 2005a]. Another advantage is that milk products contain basic nutrients for the growing child; they are also considered safe for the teeth with

possible beneficial effects on the salivary microbial composition and inhibition of caries development due to their natural content of casein, calcium, and phosphorous [Levine, 2001; Petti et al., 2001].

However, the best vehicle for probiotic delivery has yet to be identified. Çaglar et al. [2006] investigated the effect of the probiotic bacterium *L. reuteri* ATCC 55730 on the levels of salivary mutans streptococci and lactobacilli in young adults, when ingested via 2 different non-dairy delivery systems (straws and tablets). A significant reduction in the mutans streptococci levels was recorded after the ingestion of the probiotic bacteria via both straws and tablets, which was in contrast to the placebo controls. Another study [Montalto et al., 2004] evaluated whether there was any difference between taking probiotic lactobacilli in the liquid form or in capsules on *S. mutans* counts in a 45-day double-blind, randomized, placebo-controlled intervention study. The oral administration of the probiotic strains significantly increased the salivary counts of the lactobacilli. The effect occurred irrespective of whether the lactobacilli were administered in liquid or in capsule form, indicating that probiotics ingested in capsule form might result in a temporary increase in oral lactobacilli.

Recently, our research group evaluated some properties of some fermented milk brands [Lodi et al., 2010a] and their effect on biofilm inorganic composition [Lodi et al., 2010b]. It was observed that although all the products were milk-based beverages and presented in their composition fluoride, calcium, and phosphate, they promoted caries lesion in bovine enamel blocks [Lodi et al., 2010a; Lodi et al., 2010b]. In this context, the aim of this study was evaluate the effect of the short-term using of 2 brands of probiotic-containing fermented milks on biofilm, salivary microorganisms, and dental enamel.

Material and Methods

Selection of the Fermented Milks

The fermented milks used in this study were Yakult® (fermented milk A) and Batavito® (fermented milk B) that have fluoride, calcium, and phosphate in their composition [Lodi et al., 2010a] also contains probiotic bacteria.

According to the manufacturers, Yakult® contains in its composition a single probiotic bacteria *L. casei* Shirota, and Batavito® a mix of 4 probiotic bacteria (*L. acidophilus*, bifidobacteria, *Streptococcus salivarius thermophilus*, and *L. paracasei*).

Fermented Milk Total and Reducing Sugar Analysis

The determination of total (alkali-soluble carbohydrates) and reducing sugars was done using the colorimetric spectrophotometer (HITACHI U-2000, Hitachi High-Technologies Corporation, Toronto, ON, Canada), as described, respectively, by Dubois et al. [Dubois et al., 1956] and Somogyi-Nelson [Nelson, 1944].

Fermented Milk Microbial Analysis

Both the fermented milks used in this study were serial tenfold diluted in phosphate-buffered saline (PBS) and double plated in Brain Heart Infusion agar (Himedia, Himedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India), Mitis Salivarius agar (Himedia, Himedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India), Mitis Salivarius Sucrose Bacitracin agar (Sigma-Aldrich Co., St. Louis, MO, USA), and Rogosa agar (Himedia, Himedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India) to analyze total microorganisms (TM), total streptococci (TS), mutans streptococci (MS), and lactobacilli (L), respectively. The plates for TM, TS, and MS were incubated at 37°C for 48 h in an anaerobic chamber. The plates for L were incubated aerobically at 37°C for 72 h. Colonies were counted with a colony counter (CP 600 Plus, Phoenix Indútria e Comércio de Equipamentos Científicos Ltda., Araraquara, SP, Brazil). The identification of the mutans streptococci colonies was performed using morphocellular (Gram staining) and morphocolonial analysis. The results were

expressed as colony-forming units by milligrams of fermented milk (CFU/mg of fermented milk).

Enamel Block Preparation and Analysis

Enamel blocks measuring 4 × 4 × 2 mm were obtained from bovine incisor teeth previously stored in 2% formaldehyde solution (pH 7.0) for 1 month [White and Featherstone, 1987]. They had their surfaces serially polished, and the selected blocks were divided in agreement with the average of hardness (358.8 to 395.0 Kg/mm^2) of the total blocks and the trust interval. Surface enamel microhardness (SMH) measurements were made using a Shimadzu HMV-2000 hardness tester (Shimadzu Corp., Kyoto, Japan). For baseline SMH (SMHi), 5 indentations spaced 100 µm from each other were made (25 g load, 10 s) in the center of the enamel block [Vieira et al., 2005].

After the trial, SMH was again measured (SMHf). Five indentations spaced 100 µm from each other and from the baseline were made. The percentage change in SMH (%SMH) was calculated [%SMH = 100 (SMHf - SMHi)/SMHi]. The cross-sectional microhardness was measured using a Micromet 5114 hardness tester (Buehler Ltd., Lake Bluff, IL, USA). The test was performed by sectioning the blocks longitudinally through the center. One of the halves was embedded in acrylic resin with the cut face exposed and gradually polished. One row of 14 indentations was made at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 µm) from the outer enamel surface under a 5 g load for 10 s. The integrated area above the curve (cross-sectional profiles of hardness into the enamel), using the hardness values (KHN), was calculated by the trapezoidal rule (GraphPad Prism, version 3.02) in each depth (mm) from the lesion up to sound enamel. This value was subtracted from the integrated area of sound enamel, to obtain the integrated area of the subsurface regions in enamel, which was named as the integrated loss of subsurface hardness (ΔKHN; kg/mm^2/mm) [Spiguel et al., 2009].

In Situ Experiment

This study was previously approved by the local Human Ethical Committee (FOA-UNESP, protocol # 2008-01519). Ten healthy volunteers aged 21–32 years were selected, and they agreed and signed the informed consent. The *in situ* study involved a blind crossover design performed in 3 phases: 20% sucrose, Yakult® (treatment A), and Batavito® (treatment B). One week before the experiment beginning and during the whole experiment, the volunteers used non-fluoridated dentifrice. Each volunteer wore acrylic palatal devices containing 4 enamel bovine blocks covered by a plastic mesh to enable dental biofilm accumulation and to protect it from disturbance [Cury et al., 2000]. Besides that, 1 mm of space was left between the enamel and the plastic mesh.

Before starting the experiment, saliva was collected from each volunteer for the initial microbiological analysis. In each phase, 8 times a day, volunteers removed the appliance from the oral cavity and dripped two drops the following treatment solutions on the enamel bovine blocks: 20% sucrose solution (Control), Yakult® (treatment A), or Batavito® (treatment B). Five minutes after the application, the device was replaced in the mouth. After the trial period (14 days), saliva was collected again and also the formed biofilm for microbiological analysis. A washout period of 7 days was established between each phase. No restriction was made regarding the volunteers' diet, but they were instructed to remove the appliance during meals and oral hygiene [Cury et al., 2000]. They were not allowed to use any antimicrobial or fluoride product during the experiment.

In Vivo Experiment

This study was previously approved by the local Human Ethical Committee (FOA-UNESP, protocol # 2008-01519). Ten healthy volunteers aged 21–32 years were selected, and they agreed and signed the informed consent. The *in vivo* study involved a blind crossover design performed in 2 phases: Yakult® (treatment C) and Batavito® (treatment D). Each volunteer drank 1 bottle of fermented milk per day for 14 days.). One week before the experiment

beginning and during the whole experiment, the volunteers used non-fluoridated dentifrice. A washout period of 7 days was established between each phase. The only restriction made regarding the volunteers' diet was no intake other source of bacteria probiotic. Saliva was collected at baseline and at the end of each phase for microbiological analysis.

Dental Biofilm Microbial Analysis

At the end of the in situ experiment, the volunteers returned the palatal appliances. The plastic mesh was removed, and the biofilm was harvested and weighed. Around 5 mg of the biofilm was resuspended in phosphate-buffered saline (PBS: 8.0 g NaCl, 0.2 g KCl, 1.0 g K_2HPO_4, and 0.2 g KH_2PO_4 per liter, adjusted to pH 7.4; 1 mL/mg of biofilm) and sonicated on ice in an ultrasonic cell disruptor (XL; Misonix Inc., Farmingdale, NY, USA) for 6 × 9.9 s; amplitude 90%, and 40 W of power [Bowen, 1986]. The suspensions were serial tenfold diluted in PBS and double plated as previously described for the fermented milk microbial analysis of TM, TS, MS, and L.

The remaining biofilm was dried with phosphorus pentoxide (Vetec Química Fina Ltda., Duque de Caxias, RJ, Brazil) for 12 h at room temperature. Insoluble extracellular polysaccharides (EPS) were extracted by adding 1.0 mol L^{-1} NaOH (10 µL/mg dry weight) to the biofilm. The samples were vortexed for 1 min, and after 3 h under agitation at room temperature, they were centrifuged (1 min; 11,000 *Xg* at room temperature) [Nobre dos Santos et al., 2002]. Supernatants were precipitated with 75% cooled ethanol overnight, centrifuged, and resuspended in 1.0 mol L^{-1} NaOH [Ccahuana-Vásquez et al., 2007]. Carbohydrate analysis was done by the phenol-sulfuric acid procedure [Dubois et al., 1956]. The results were expressed as mg/g dry weight.

Saliva Microbial Analysis

Saliva samples from each volunteer were performed at baseline and at the end of each phase by oral rinses with phosphate-buffered saline (PBS; 0.1 M; pH 7.2) for 1 min [Samaranayake et al., 1986]. The samples were centrifuged for

10 min at 8,000 X*g*, the supernatant discarded, and 2.5 mL of PBS added to the pellet. The suspensions were serial tenfold diluted in PBS and double plated as previously described for the fermented milk microbial analysis of TM, TS, MS, and L. The results were expressed as colony-forming units by milliliters of saliva (CFU/mL).

Statistical Analysis

Each volunteer was considered as "n" in all the analysis. The null hypothesis tested was that the fermented milk would not promote changes in the biofilm, saliva, and demineralization of the dental enamel. Statistical analysis was carried out using BioEstat Version 5.0. Data from CFU were logarithmically transformed prior to analysis. Mean and SD for each measured parameter (CFU in the biofilm, saliva, and fermented milk; EPS in the biofilm; total and reducing sugars in the fermented milk; SMHi, SMHf, %SH, and ΔKHN in the enamel block) were calculated for each group. These data were analyzed using a single-factor analysis of variance model (ANOVA). Multiple comparisons were conducted using the Tukey or Bonferroni test when significant effects ($p < 0.05$) were detected. Data were analyzed using a Kruskal-Wallis one-way analysis when they were not normally distributed or variances were not equal. Data (means) from microorganisms in the saliva before and after each trial phase were submitted to a paired *t*-test procedure. The significance limit was set at 5%.

Results

Fermented Milk Total and Reducing Sugar Analysis

The means of total sugar in 20% sucrose, fermented milk A, and fermented milk B were 155.67 mg/mL, 158.49 mg/mL, and 191.33 mg/mL, respectively, and it could be observed statistical differences among the groups (Table 1).

The means of reducing sugar in 20% sucrose, fermented milk A, and fermented milk B were 0.15 mg/mL, 9.83 mg/mL, and 0.83 mg/mL,

respectively, and it could be observed statistical differences among the groups (Table 1).

Fermented Milk Microbial Analysis

In fermented milk A, it was observed 9.05 ± 0.06 log CFU/mg TM, 2.10 ± 0.12 log CFU/mg TS, and 8.72 ± 0.07 log CFU/mg (5.2×10^8 UFC/mL) L. In fermented milk B, it was observed 7.93 ± 0.07 log CFU/mg TM, 7.21 ± 0.59 log CFU/mg TS, and 7.24 ± 0.20 log CFU/mg (1.7×10^7 UFC/mL) L. Both the fermented milks did not show MS when plated (Table 1).

Fermented milk A showed higher concentrations of TM and L and a lower concentration of TS than fermented milk B, and these differences were statistically significant ($p < 0.01$).

Enamel Analysis

The means and standard deviations (SD) of the 3 groups analyzed in this study are shown in Table 2. The initial microhardness ($p = 0.853$) showed no statistically significant difference among the groups. Treatment B differed from the other groups in relation to the final microhardness, percentage of surface hardness change, and integrated loss of subsurface hardness ($p < 0.05$). It was not observed statistical difference in relation to the variables analyzed between treatment A and 20% sucrose ($p > 0.05$).

In Situ Experiment

By comparing biofilm data, it was observed lower TM in treatment B when compared with treatment A, but no statistical difference was observed between treatment B and 20% sucrose. TS, MS, and L did not show statistical difference among the groups (Table 3).

In saliva analysis, when the baseline and final data of the microorganisms were compared in each treatment, it was observed that TM and TS decreased, and MS increased after the use of 20% sucrose solution; these changes were statistically significant, but L did not show statistical difference at

the end of this phase (Table 4). Treatment A showed a significant decrease in TM, TS, and MS; however, L did not show statistical difference after this trial phase (Table 4). TM was the only one that showed a significant decrease in treatment B. However, TS and MS decreased and L increased with that treatment, but these changes did not show statistical differences (Table 4).

It was observed similar amount of all microorganisms in saliva when compared the baseline data of the 3 treatment ($p > 0.05$). Regarding the final data of the microbial count in the saliva, it was observed a lower MS in treatment B, and this data was statistically significant when compared with the other treatments (Table 5).

The EPS concentration in the biofilm did not show statistical difference between the treatments. The 20% sucrose and treatment A showed 13.84 mg/mg of dry biofilm, and 12.35 mg/mg of dry biofilm for treatment B.

In Vivo Experiment

Treatment C did not change the concentration of any microorganism when it was compared baseline and final data. After treatment D, it was observed a statistically significant decrease in all the microorganisms analyzed (Table 6).

It was observed similar TM, TS, and MS in saliva when compared at the beginning of the 2 treatments, but the amount of L was higher at the beginning of treatment D, and this difference was statistically significant. When compared the final data of the 2 treatment it was not observed statistical difference between the groups.

Discussion

The effects of probiotics on oral health are a relatively new research area, but the concept of probiotics being beneficial from a dental point of view may appear controversial.

In our *in vivo* study, it was used probiotic-containing fermented milks available in the market, which were orally administrated, allowing direct

contact with oral tissues. Although fermented milks were not developed with the purpose of preventing dental caries with the promotion of changes on oral microbiota, it was observed that the fermented milk B decreased salivary count of all the microorganisms investigated after 2 weeks using. These results were similar to the previously reported intakes [Çaglar et al., 2006; Näse et al., 2001; Ahola et al., 2002; Nikawa et al., 2004]. It was also observed that fermented milk A showed a tendency to increase salivary count of TS, MS, and L at the end of the trial, but the difference was not statistically significant.

Some studies evaluating the effect of lactobacilli-derived probiotics on mutans streptococci reported significant reductions of salivary mutans streptococci immediately after the termination of daily intakes [Çaglar et al., 2006; Näse et al., 2001; Ahola et al., 2002; Nikawa et al., 2004]. The post-treatment levels decreasing were not directly dependent on the daily administration vehicle, which was milk, cheese, yoghurt, lozenges, or straws prepared with freeze-dried strains. Çaglar et al. [2006] investigated whether slowly melting tablets would allow a more thorough contact between the probiotic bacteria and the oral environment compared with the direct swallowing pattern from the straw and concluded that both the regimes equally reduced the prevalence of salivary mutans streptococci after 2 weeks of use.

Conflicting findings were reported by Montalto et al. [2004] that evaluated the administration of probiotic lactobacilli in liquid and capsule ways in order to determine the role of direct contact with the oral tissues. Interestingly, it was found that both ways of administration significantly increased the salivary lactobacilli counts, while the levels of mutans streptococci were not significantly modified by the intervention [Montalto et al., 2004]. Some studies have indicated that direct contact with the oral tissues is not a prerequisite for a beneficial effect, and a pure systemic administration of a probiotic could enhance lactobacilli proliferation in the oral cavity [Çaglar et al., 2006; Montalto et al., 2004; Ahola et al., 2002].

Differences among different strains and strains of the same species are probably the reason for the conflicting results of probiotic efficacy that were reported in the early studies [Twetman and Stecksén-Blicks, 2008]. Today, most of the research is carried out with well-defined, dairy-based live lactobacilli strains. In this study, fermented milk A contains in its composition a single probiotic bacteria *L. casei* Shirota, and fermented milk B a mix of 4 probiotic bacteria (*L. acidophilus*, bifidobacteria, *S. salivarius thermophilus*, and *L. paracasei*), according to the manufacturers. Another interesting point is that although fermented milk A (5.2×10^8 UFC/mL) showed a higher amount of probiotic L in its composition than the fermented milk B (1.7×10^7 UFC/mL), fermented milk The best results after the consumption of fermented milk B could be explained by this difference in their probiotic-containing once the simultaneous application of different probiotics can affect the balance of the oral ecosystem in a possible additive, cumulative, or competitive modes of action [Meurman and Stamatova, 2007].

Saliva samples may underestimate the true situation of the oral biofilm [Meurman, 2005]. Moreover, it is difficult to obtain biofilm collected *in vivo* due to the need of oral hygiene restrictions. Thus, we performed also an *in situ* study to investigate the effects of probiotic-containing fermented milks on biofilm.

Treatment B showed lower concentrations of TM in the biofilm when compared with the other groups (20% sucrose and treatment A). However TS, MS and L did not show statistically significant difference among the groups that could be explained by the optimal dose needed for pathogenic bacteria suppression has not been achieved during the experimental phases once the volunteers just dripped the fermented milks on enamel blocks. Lee and Salminen [1995] suggested a intake of 100 g of 10^6 to 10^7 of a containing-probiotic product as a optimal dose; but Çaglar et al. [2008] showed in their study significant mutans streptococci reduction with a low-amount consumption of probiotic bacteria with the use of only 53 g of ice cream with 1

× 10^7 CFU per gram ingested daily. On the other hand, microorganism reduction was also achieved with 200 g of yogurt containing 2 × 10^8 CFU per gram in their former study [Çaglar et al., 2005b].

The use of both the fermented milks in the *in situ* study showed a decrease of all the microorganisms in the salivary count when compared the baseline and final data, except L in treatment B that showed an increase in its amount. The salivary microorganism reduction called our attention to the safety model for volunteers' oral health and to permit us to study the biofilm artificially formed in the oral cavity. The reduction on the salivary counts may be due to the use of the appliance that could increase the salivary flow. On the other hand, the differences between the probiotic bacteria in the fermented milks analyzed could not be responsible for this reduction once they were just dripped on the enamel block, and the amount of fermented milk used was too low to interfere with the oral microbiota.

The microhardness test was used to evaluate the changes in the enamel surface and demineralization area in the *in situ* study. Treatment B differed from the other groups in relation to the final microhardness, percentage of surface hardness change, and integrated loss of subsurface hardness. Both the products used in this study are based-milk and contain in their composition fluoride, calcium, and phosphate [Lodi et al., 2010a]. These ions should confer an enamel protective effect, but in both the treatments, it was observed that enamel demineralization may be due to the presence of sucrose on their composition.

Sucrose is considered the most cariogenic factor from the dietary because of its fermentability and it works as a substrate for the synthesis of polysaccharides in dental biofilm [Bowen, 2002]. To try understanding the results described above, we investigated the amount of total and reducing sugars in both the fermented milks and the EPS concentration in the biofilm. Both the fermented milks presenting reducing and total sugars and EPS were observed in the biofilm formed after their use, which could explain the

demineralization observed. Fermented milk A showed a lower amount of total sugar and a higher amount of reducing sugar when compared with fermented milk B, which does not match the lower mineral loss observed after treatment B, inferring that probiotic bacteria could really have had some protective effect on dental caries development.

In conclusion, the findings of the present study demonstrated that probiotic-containing fermented milks were able to promote changes in the biofilm, oral microbiota and demineralization of the enamel surface; collectively, the data showed that Batavito® was the treatment that less favored the caries lesion development. More systematic studies and randomized controlled trials are needed for finding out the best probiotic strains, daily doses, and probiotic vehicles for a promising and safe perspective to oral health.

Acknowledgments

The authors would like to express their gratitude to the volunteers for their valuable participation. We also thank Maria dos Santos Fernandes for the laboratorial assistance in this study. Conceived and designed the experiment: CSL, FLB, ACBD, and CCRM. Performed the experiments: CSL and LV. Analyzed the data: CSL and ACBD. Wrote the paper: CSL, ACBD, and CCRM.

Table 1. Variables analyzed in the treatment solutions

Variables	20% Sucrose	Fermented Milk A	Fermented Milk B
Total Sugar[1]	155.67[a] (1.0)	158.4[b] (1.4)	191.33[c] (0.1)
Reducing Sugar[1]	0.15[a] (0.004)	9.83[b] (0.50)	0.83[c] (0.167)
TM[2]	N/A	9.05[a] (0.06)	7.93[b] (0.07)
TS[2]	N/A	2.10[a] (0.12)	7.21[b] (0.59)
MS[2]	N/A	N/G	N/G
L[2]	N/A	8.72[a] (0.07)	7.24[b] (0.20)

[a,b]Means (SD) followed by distinct letters are significantly different according to each variable

[1]Total and reducing sugar were expressed in mg/mL

[2]TM (total microorganisms), TS (total streptococci), MS (mutans streptococci) and L (lactobacilli) were expressed in log CFU/mL

N/A Variable not analyzed

N/G No microorganism growth

Table 2. Mean and standard deviation (SD) of the variables analyzed in the enamel surface according to the treatments

Variables (n=10)	20%Sucrose	Treatment A	Treatment B	Significance (p)
SMHi[1]	377.1[a] (0.7)	377.3[a] (0.4)	377.2[a] (0.4)	= 0.853
SMHf[2]	76.0[a] (66.8)	127.0[a] (74.8)	189.6[b] (67.0)	< 0.05
%SMH[3]	-79.8[a] (17.8)	-66.3[a] (19.8)	-49.7[b] (17.8)	< 0.05
ΔKHN[4]	9961.9[a] (1468.2)	8940.9[a] (2800.3)	4063.5[b] (1697.2)	< 0.05

[a,b]Means (SD) followed by distinct letters are significantly different according to each variable

[1]Initial microhardness (Hardness Knoop)

[2]Final microhardness (Hardness Knoop)

[3]Percentage of subsurface hardness change (Hardness Knoop)

[4]Integrated loss of subsurface hardenss (Hardness Knoop)

Table 3. The distribution of microorganisms in the biofilm (log CFU/mg) after 14 days according to the treatments (in situ study)

Variables (n=10)	20% Sucrose	Treatment A	Treatment B
TM[1]	6.87[a,b] (0.49)	7.04[a] (0.24)	6.66[b] (0.47)
TS[2]	5.73[a] (0.53)	5.81[a] (0.87)	5.71[a] (0.65)
MS[3]	3.24[a] (1.38)	2.99[a] (0.99)	3.14[a] (1.22)
L[4]	5.19[a] (1.97)	5.55[a](0.75)	5.18[a] (1.67)

[a,b]Means (SD) followed by distinct letters are significantly different according to each variable
[1]Total microorganisms
[2]Total streptococci
[3]Mutans streptococci
[4]Lactobacilli

Table 4. The distribution of microorganisms in saliva (log CFU/mL) at the baseline and after 14 days according to the treatments (in situ study)

Variables (n=10)	20% Sucrose		Treatment A		Treatment B	
	Baseline	14 days	Baseline	14 days	Baseline	14 days
TM	6.82[a] (0.69)	6.12[b] (1.0)	6.96[a] (0.79)	6.33[b](0.71)	6.81[a] (1.15)	5.87[b] (1.17)
TS	6.20[a] (0.72)	5.52[b] (0.89)	6.38[a] (0.80)	5.75[b] (0.72)	6.17[a] (1.04)	5.59[a] (0.92)
MS	2.89[a] (1.08)	3.95[b] (0.91)	3.67[a] (1.60)	3.28[b] (1.03)	3.51[a](0.83)	2.72[a] (1.24)
L	4.06[a] (0.95)	3.90[a] (0.82)	4.00[a] (0.79)	3.57[a] (0.78)	3.97[a] (1.24)	4.01[a] (2.10)

[a,b]Means (SD) followed by distinct letters are significantly different according to each variable
[1]Total microorganisms
[2]Total streptococci
[3]Mutans streptococci
[4]Lactobacilli

Table 5. The distribution of microorganisms in saliva (log CFU/mL) after 14 days according to the treatments (in situ study)

Variables (n=10)	20%Sucrose	Treatment A	Treatment B
	14 days	14 days	14 days
TM	6.12[a] (1.0)	6.33[a] (0.71)	5.87[a] (1.17)
TS	5.52[a] (0.89)	5.75[a] (0.72)	5.59[a] (0.92)
MS	3.95[a] (0.91)	3.28[a] (1.03)	2.72[b] (1.24)
L	3.90[a] (0.82)	3.57[a] (0.78)	4.01[a] (2.10)

[a,b]Means (SD) followed by distinct letters are significantly different according to each variable
[1]Total microorganisms
[2]Total streptococci
[3]Mutans streptococci
[4]Lactobacilli

Table 6. The distribution of microorganisms in saliva (log CFU/mL) at the baseline and after 14 days according to the treatments (in vivo study)

Variables	Treatment C		Treatment D	
(n=10)	Baseline	14 days	Baseline	14 days
TM	6.74[a] (1.25)	6.70[a] (1.15)	6.96[a] (0.79)	6.05[b] (1.28)
TS	6.04[a] (0.97)	6.19[a] (1.10)	6.48[a] (0.83)	5.41[b] (0.94)
MS	3.48[a] (0.94)	3.74[a] (1.21)	4.17[a] (0.62)	3.18[b] (1.17)
L	3.11[a] (0.79)	3.57[a] (1.49)	4.01[a] (093)	3.02[b] (0.77)

[a,b]Means (SD) followed by distinct letters are significantly different according to each variable
[1]Total microorganisms
[2]Total streptococci
[3]Mutans streptococci
[4]Lactobacilli

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Capítulo 2

In vitro evaluation of probiotic bacteria efficacy in preventing primary caries development using a multi-species microbial artificial-mouth model

Abstract

The aim of this study was to determine the ability of probiotic bacteria to prevent primary caries development using a multi-species biofilm and microbial artificial-mouth model. Four groups were inoculated with mixed overnight cultures of *Streptococcus mutans, Streptococcus salivarius, Streptococcus gordonii, Actinomyces naeslundii* and 1 Lactobacillus strain that was changed for each group: G1 - *Lactobacillus casei*, G2 - *Lactobacillus rhamnosus* GG, G3 - *Lactobacillus reuteri* and G4 - *Lactobacillus casei* Shirota. All groups were exposed to circulating trypticase soy broth supplemented with 5% sucrose, 30 min, 3 times per day and a mineral wash solution for the rest of the day during 7 days. At the end of the study, biofilm bacterial colony counts, surface microhardness change, caries lesion area and lesion depth were determined. There were no significant differences in the amount of total microorganisms and lactobacilli among the groups. However, total streptococci and mutans streptococci were significantly higher in the G3 compared to the other groups. The drainage fluid pH and caries vessel fluid pH at the end of the experiment was very similar for all groups. There was no significant difference in the initial microhardness, lesion area and lesion depth among the groups. But, the G3 had significantly higher final surface microhardness than the other groups, and a significantly smaller surface hardness change than the G1 and G4 groups. In conclusion, under the conditions of this study only the *L. reuteri* mix-culture biofilm group (G3) had a reduced mineral loss as measured by surface microhardness.

Key words: Probiotics; Dental caries; *Streptococcus mutans*; *Lactobacillus*

*Capítulo escrito de acordo com as instruções do periódico Caries Research (ANEXO B)

Introduction

The fact that caries is a bacterially mediated process has been known for a long time. Since then, scientific results have refined the process of caries development to a multifaceted disease process. Host, bacteria and nutrients are required to foment the production of organic acids and the subsequent demineralization activity [Keyes, 1968]. According to this model, all three elements must be present for disease initiation and progression; the removal of any one element leads to the interception of the disease process [Anderson and Shi, 2006]. The production of organic acids from dietary sugars is a key factor in the caries process. Low pH generated from acids challenges the homeostasis in the oral microbial community with a selection towards bacteria that induce caries lesions [Bradshaw and Marsh, 1998; Marsh, 2003].

Although fluoride and other preventive efforts have led to a dramatic decline in dental caries, the ability to control the actual infection has been limited [Rolla and Ogaard, 1991; Reich, 2001; Kargul et al., 2003]. To overcome the limitations of the traditional disease management strategies, a number of researchers are developing probiotic methods to treat the cariogenic biofilm by interfering with the oral colonization of cariogenic pathogens [Teughels et al., 2008].

Probiotic supplements are generally regarded as safe because the microorganisms they contain are identical to those found in the human gastrointestinal and vaginal microflora and they are very rarely implicated in human infections [Twetman and Stecksen-Blick, 2008]. The use of probiotic strains has emerged as an alternative way of combating oral bacterial infections in analogy with other parts of the gastrointestinal tract [Meurman, 2005]. The most abundantly used probiotic strains are of the genus lactobacillus, even as they may play a role in the maintenance of the microecological balance in the oral cavity [Kõll-Klais et al., 2005; Simark-Mattsson et al., 2007].

Although probiotics are administered orally by ingestion, so far the studies on the impact of these microorganisms in oral health are scarce

[Axelsson et al., 1989] and their effects on oral microbial ecology remain unknown. The lactobacillus group contains homofermentative and heterofermentative species and all of them are aciduric. Several strains produce low-molecular-weight antimicrobial substances with an inhibitory activity against a wide range of bacterial species, including oral streptococci [Kõll-Klais et al., 2005; Axelsson et al., 1989; Ishihara et al., 1985; Meurman et al., 1995; Silva etal., 1987]. Recent studies with probiotic strains *Lactobacillus rhamnosus* GG and *Lactobacillus reuteri* SD2112 (ATCC55730) have demonstrated that these strains can interfere with oral mutans streptococci colonization [Meurman et al., 1995; Ahola et al., 2002; Caglar et al., 2006; Hatakka et al., 2001; Näse et al., 2001; Nikawa et al., 2004].

Among the various intestinal probiotic lactobacillus strains, *L. casei* Shirota has been intensively studied and used in fermented milk products for more than 70 years [Fujimoto et al., 2008]. Its various benefits include improving the balance of intestinal microbes and volatile fatty acids in the gastrointestinal environment, antitumor action, activation of the immune system, and antimicrobial activity [Asahara et al., 2001; Kanazawa et al., 2005; Matsuzaki et al., 2004; Nagao et al., 2000; Spanhaak et al., 1998]. However, to date, the number of studies that have been conducted relating these microorganisms with oral health are limited [Lima et al, 2005; Lodi et al., 2010a; Lodi et al., 2010b]. The aim of this study was to compare the ability of three probiotic bacteria (*L. rhamnosus* GG, *L. reuteri* SD2112 (ATCC55730), and *L. casei* Shirota) to prevent primary caries development using a multi-species biofilm and microbial artificial-mouth model [Fontana et al., 1996a].

Materials and Methods

Specimen Preparation

Enamel specimens were created from bovine permanent incisor teeth stored in 0.1% thymol solution. Teeth were selected based on size and quality of the enamel. Teeth with signs of demineralization, fractures, or other defects were not used for the study. Teeth were sectioned with an SBT Model 650 low-

speed saw to obtain 3 x 3 mm specimens. These specimens were ground and polished (Spectrum System 1000, LECO Corporation, St. Joseph, Michigan, USA) using an ascending grit series of silicon carbide paper to a P4000-grit paper followed by a cloth with 1-µm diamond polishing suspension to create highly polished plano-parallel specimens. Final specimens were evaluated at high magnification for any obvious cracks or other flaws in the enamel surface.

Specimens were individually mounted on the ends of acrylic rods using cyanoacrylate (Loctite Super Glue, Henkel Consumer Adhesives Inc, Avon, Ohio, USA). The lateral sides of each specimen were covered with an acid-resistant varnish (Nailwear Nail enamel, Avon Prods Inc, New York, New York, USA) allowing only the enamel surface to be exposed. The teeth were sterilized with ethylene oxide prior to treatment.

Baseline Sound Surface Enamel Microhardness Analysis and Specimen Selection

Specimens were mounted on acrylic blocks for baseline-sound microhardness analysis (Microhardness Tester LM 247AT, LECO Corporation, St Joseph, Michigan, USA). Four baseline indentations spaced 200 µm from each other were placed with a Vickers diamond under a 200 g load for 15 s near the center area of the sound specimen. The average microhardness of the 4 indentations on the surface of each specimen was determined. Only specimens with a surface microhardness (SMH) within the range between 300 and 380 Kg/mm^2 were accepted for the study.

Bacterial Strains and Culture Conditions

The following reference strains were used: *Streptococcus mutans* TH16, *Streptococcus salivarius* (ATCC 13419), *Streptococcus gordonii*, *Actinomyces naeslundii* (ATCC 19039) and *Lactobacillus casei* (ATCC 393). The probiotics used were *Lactobacillus rhamnosus* GG (ATCC 53103), *Lactobacillus reuteri* SD 2112 (ATCC 55730), and *Lactobacillus casei* Shirota isolated from Yakult fermented milk (Table 1). *S. mutans*, *S. salivarius*, *S. gordonii* and *A. naeslundii* were cultered in Brain Heart Infusion (BHI) (Becton, Dickinson and Co., Sparks, MD, USA)

agar at 37°C for 1 or 2 days. *L. casei*, *L. rhamnosus* GG, *L. reuteri* and *L. casei* Shirota were cultured in MRS agar (Becton, Dickinson and Co., Sparks, MD, USA) at 37°C for 2 days.

Identification by DNA Sequencing of Lactobacillus casei Shirota isolated from Yakult

Genomic bacterial DNA was extracted using the MasterPure Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's recommendations, with the following modification. At the time of addition of the Ready-Lyse lysozyme, 5 U of mutanolysin (Sigma-Aldrich Co.) and 100 U of achromopeptidase (Sigma-Aldrich Co.) were also added, followed by incubation at 37°C for 30 min. PCR amplification was performed (Veriti 96-well Thermal Cycler, Applied Biosystems, Foster City, CA, USA). Each reaction mixture (50 µl) contained 5 µL of template DNA, 1 µL of dNTP 10 mM (Invitrogen, Carlsbad, CA, USA), 5 µL 10X Buffer (Invitrogen), 2 µL $MgCl_2$ 50 mM (Invitrogen), 0.5 µL Platinum Taq DNA polymerase (Invitrogen), 34.5 µL of water, 1 µL of primer LcSl (5'-CTCAAAGCCGTGACGGTC-3') and 1 µL of primer LcSr (5'-CACTAGGATTATTAGCACCACGT-3'). Custom primers, based on Fujimoto et al. [2008], were purchased from Invitrogen. The amplification program consisted of 95°C for 1 min, followed by 35 cycles of 95°C for 15 s, 50°C for 15 s, and 72°C for 30 s, and finally 72°C for 7 min. Amplification of the expected 0.5 kb PCR product was verified by electrophoresis at 100 V in a 3.0% agarose gel in TBE buffer (Invitrogen).

The PCR product was purified using a Qiaquick PCR Purification Kit (Qiagen Inc, Valencia, CA, USA) and sequenced using primers LcSl and LcSr at the University of Michigan DNA Sequencing Core facility. Sequences were aligned using the SeqMan Pro module of Lasergene software version 7.1.0 (DNASTAR, Inc.), and compared to the published [Fujimoto et al., 2008] sequence in GenBank, accession #AB246299.

Microbial Model Treatment Regimen

Prior to bacterial inoculation, all specimens were coated with 20 μL of a sterile salivary mix for 20 min at 37˚C. The saliva was collected from three individuals, pooled, diluted 1:10 with PBS, centrifuged (2060*Xg*, 4˚C, 10 min), filtered through a 0.45 μm filter and then through a 0.2 μm filter the day before the experiment and kept at 4˚C. Following salivary pellicle coating, all groups were inoculated once at the beginning of the experiment with mixed overnight cultures of *S. mutans* TH16, *S. salivarius* (ATCC 13419), *S. gordonii*, *A. naeslundii* (ATCC 19039) and *Lactobacillus* (Group 1 - *L. casei* (ATCC 393), Group 2 - *L. rhamnosus* GG (ATCC 53103), Group 3- *L. reuteri* SD 2112 (ATCC 55730), and *L. casei* Shirota from Yakult fermented milk) in trypticase soy broth (Becton, Dickinson and Co., Sparks, MD, USA) supplemented with 5% sucrose (TSBS) (Fisher Scientific, Fair Lawn, New Jersey, USA), at an absorbance of 0.5 at 540 nm each (Spectronic 20D+, Thermo Fisher Scientific, Madison, WI, USA). Following inoculation, specimens were incubated at 37˚C for 2 h to allow the bacteria to adhere to the tooth surface before starting the cycling regimen.

Specimens were exposed, at 37˚C, to circulating TSBS (0.7 ml/min) for 30 min, 3 times per day (3x/day), and to a mineral washing solution [Fontana et al., 1996a] (MW; 0.7 ml/min) containing 0.25 ppm fluoride (Fluoride Standard, Ricca Chemical Company, Arlington, TX, USA) for a total of 22.5 h per day, during the periods without TSBS flow. The circulating fluids were delivered to, and removed from the group-vessels by peristaltic pumps (Pump Diluter Dispenser Wiz, ISCO, Lincoln, NE, USA) regulated by timers (EC72D Two Channel Time Control, Paragon Electrical Products, Mississauga, Ontario, Canada). The TSBS was intended to reproduce nutrient intake 3 times/day, while the MW represents an artificial saliva buffer solution. Every group had its own TSBS bottle. Groups 1 and 2 were connected to one MW bottle, and groups 3 and 4 to another MW bottle. At the end of the study period, the drainage fluids, as well as the fluid remaining in the group's vessel, were monitored for pH (pH meter Accumet model 15 + Accumet combination electrode 13-620-285,

Fisher Scientific, Pittsburgh, PA, USA) in order to document the overall acidic conditions (Table 1).

Post-Treatment Surface Enamel Microhardness Analysis

SMH of the treated specimens was determined after 7 days of incubation in the model. The procedure was identical to the one described previously using a Vickers hardness indenter at a load of 200 g for 15 s. The average specimen microhardness was determined from 4 indentations on the surface of each specimen in proximity to the baseline indents.

Laser Scanning Confocal Microscopy Analysis

Specimens were sectioned across the center of the demineralized/treated area of the specimens using a 3'' X 0.006" thick diamond wafering blade (Mager Scientific Inc, Dexter, MI, USA) in a low speed saw (South Bay Technology Inc, San Clement, CA, USA). Specimens were then stained overnight with a 0.1 mM aqueous solution of Rhodamine B (Rhodamine B 99+% pure, Acros Organics, NJ, USA). The specimens were allowed to air dry before being analyzed with a confocal microscope (Zeiss LSM 510 META Confocal Microscope/Zeiss Axiovert 100M Inverted Microscope, Carl Zeiss MicroImaging, Peabody, MA, USA). Images of the specimens were analyzed to determine the extent of the lesions (LSM Image Browser and Image J software version 1.43u). A 1000 µm long area of the lesion was analyzed per specimen in a defined area. Lesion depth and area were determined [Fontana et al., 1996b].

Microbial Analysis

Three enamel blocks per group were aseptically removed and placed individually in 2 mL of sterile PBS. Then they were vortexed and sonicated (Sonicator Q125, QSonica Manufacturers, Newtown, CT, USA) to disrupt the biofilm from each tooth surface. The dislodged bacteria were serial tenfold diluted and double plated on BHI for total microorganisms (TM), Mitis Salivarius Agar (Becton, Dickinson and Co., Sparks, MD, USA) for total

streptococci (TS), bacitracin (Bacitracin zinc salt, Acros Organics, NJ, USA) sucrose Mitis Salivarius Agar (0.25 U/mL bacitracin and 20% sucrose) for mutans streptococci (MS), and Man Rogosa and Sharpe Agar (MRS) for lactobacilli (L). The plates for TM, TS and MS were incubated under anaerobic conditions with increased carbon dioxide (GasPak EZ sachets, Becton, Dickinson and Co., Sparks, MD, USA) at 37°C for 48 h. The plates for L were incubated aerobically at 37°C for 72 h. The identification of the mutans streptococci colonies was performed using morphocellular (Gram stain) and morphocolonial analysis. The results were expressed as colony-forming units by mg of wet weight biofilm (CFU/mg wet weight).

Statistical Analysis

Mean and variance data for each measured parameter (SMH, confocal lesion depth and area, and CFU) were calculated for each treatment group. Data were logarithmically transformed prior to analysis. These data were analyzed using a single factor analysis of variance model (ANOVA). Where significant ($p < 0.05$) effects were detected, multiple comparisons were conducted using Tukey's procedure. If data were not normally distributed or variances not equal, data were analyzed using a Kruskal-Wallis one-way analysis on ranks. The program Sigma Plot 11.0 was used.

Results

Identification by DNA Sequencing of Lactobacillus casei Shirota isolated from Yakult

DNA sequencing of the strain isolated from Yakult® resulted in 446 bp of double-stranded sequence, all of which agreed with GenBank AB 246299, the published Shirota-specific sequence [Fujimoto et al., 2008].

Microbial Analysis

At the beginning of the experiment the bacterial mixture of all groups was plated to make sure that all groups were inoculated and the bacteria were

viable at the time of inoculation. The results showed that all groups were inoculated with a statistically similar amount of all microorganisms. (Table 2)

After 7 days, when the biofilm was collected, diluted and plated no significant difference was observed in TM ($p = 0.256$) and L ($p = 0.355$) among the groups. However, TS and MS were significantly higher ($p<0.05$) in the *L. reuteri* group (G3) compared to the other groups ($p<0.05$). (Table 3)

pH

The pH of the drainage fluid (G1=3.99; G2=4.19; G3=4.25; G4=4.09) and the pH of the fluid remaining in the caries vessel (G1=3.77; G2=3.93; G3=3.86; G4=3.84) at the end of the experiment was very similar for all groups. All groups had a pH below the critical pH for enamel demineralization ($pH \leq 5.5$).

Caries Lesion Analyses

The mean and standard deviation for the 4 groups analyzed in this study are shown in Table 4. Figure 1 show a representative confocal microscopy images of caries lesions developed in 7 days for each group. The initial microhardness ($p = 0.944$), lesion area ($p = 0.342$) and lesion depth ($p = 0.342$) were not significantly different among the groups. However, the *L. reuteri* group (G3) had significantly higher final microhardness than the other groups, and a significantly smaller surface hardness change than the *L. casei* (G1) and *L. casei* Shirota (G4) groups.

Discussion

It seems easier to affect or change levels of caries-associated bacteria at the time of its colonization compared to later in life when the flora is firmly established. New strategies, such as use of probiotics, could control the microbial activities of the oral biofilm to prevent colonization of selected organisms while supporting growth of other selected ones [Çaglar et al., 2008].

In this experiment, an overnight culture of 5 microorganisms was mixed and subjected to an artificial mouth caries model. The species were chosen to

represent initial colonizers of tooth surface and/or those known to be cariogenic species. Defined species biofilms have the advantage of allowing detailed control of the individual species present [Sissons, 1997]. The ability of mutans streptococci to adhere persistently to teeth is required for promoting lesion development [Lang et al., 2010]. *Streptococcus mutans* and *Lactobacillus casei* were chosen because they have been associated with caries lesion formation and caries lesion progression, respectively [Aas et al., 2005]. *Streptococcus gordonii* is one of the 'viridans streptococci', a heterogeneous group of bacteria which often appear to be pioneer species numerically abundant in human early supragingival dental plaque and usually is associated with tooth surfaces free of caries [Scannapieco, 1994]. *In vivo*, as plaque matures, *Actinomyces* species increase in number, with *A. naeslundii* being associated with some types of caries [Marsh and Bradshaw, 1995].

The early colonizers are of great importance in the successive stages of dental plaque formation, providing sites for attachment, or modifying the environment for subsequent colonizers [Busscher et al., 1992]. Competition between bacterial populations in a community causes a succession of microorganisms and may lead to the dominance of some species. Experiments with mixed continuous cultures using representative oral bacteria indicated that the reduction of culture pH affected bacterial growth rate and subsequently modified bacterial composition. In the continuous cultures, *S. mutans* and *Lactobacillus* dominated the other bacteria, including non-mutans streptococci and *Actinomyces*, at a pH below 5.5 [Bowden and Hamilton,1987; Bradshaw and Marsh, 1998], and the proportions of *S. mutans* and *L. casei* increased gradually after repeated acidification [Bradshaw et al., 1989] or prolonged acidification [McDermid et al., 1986], while those of the other bacteria decreased. It is known that environmental acidification kills bacteria. Svensäter et al. [Svensäter et al., 1997] demonstrated that acidification at pH 2.3-4.5 for 3 h caused cell death of oral bacteria and that the acid-killing efficiency was dependent on bacterial species in the order of *Actinomyces* > non-mutans streptococci > mutans

streptococci > *Lactobacillus*. In our study TM, TS and MS decreased after 7 days when compared with baseline data and the *Lactobacillus* showed similar amount after 7 days. The low pH produced in this caries model could explain the final bacterial data. This acidic condition could have favored *Lactobacillus* strains and injured the non-mutans and mutans streptococci strains how Svensäter et al. showed in their study. When we compare the final colony count among all groups we could observe no statistical difference for TM and L, but G3 (*L. reuteri*) showed a significantly higher amount for TS and MS. Our results are in disagreement with other *in vitro* [Kang et al., 2011; Hasslöf et al., 2010] and clinical studies [Çaglar et al., 2006; Nikawa et al., 2004] that show a decrease in mutans count when *L. reuteri* was used. This microorganism is an obligate heterofermentative resident in the gastrointestinal tracts of humans, and it is reported to produce compounds that exhibit antagonistic activity, such as reuterin [Talarico et al., 1988] and reutericyclin [Ganzle et al., 2000], which are watersoluble, broad-spectrum antimicrobials, effective over a wide pH range, and resistant to proteolytic and lipolytic enzymes [el Ziney and Debevere, 1998]. It is possible that these characteristics may inhibit the streptococci activity, but not kill them. These hypotheses may explain the fact that the *L. reuteri* group (G3), although it had a higher amount of TS and MS, had a higher final microhardness when compared with others group and lower mineral loss when compared with groups 1 and 4.

L. rhamnosus GG (LGG) is a probiotic strain associated with health benefits. *L. rhamnosus* GG may compete with streptococci for oral adherence sites and produce antibacterial substances against mutans streptococci [Meurman et al., 1995]. *L rhamnosus* GG, which itself does not ferment sucrose, is also able to colonize the human mouth and may therefore, to some extent, replace the cariogenic streptococci attached to teeth [Meurman et al., 1994]. In fact, preliminary findings suggest that probiotic-containing milk LGG may reduce the risk of dental caries in children [Ahola et al. 2002; Näse et al., 2001].

In our study, G2 was inoculated with this microorganism, but did not show a decrease in TS and MS when compared with G1 (*L. casei)*.

L. casei Shirota is considered a probiotic due to its ability to modulate the composition and metabolic activity of the intestinal flora [Spanhaak et al., 1998] and improve the human immune system [Nagao et al., 2000]. This microorganism is capable of surviving during the passage through the gastrointestinal tract after consumption, due to its aciduric and acidogenic properties [Yuki et al., 1999]. *L. casei* strain Shirota is one of the most intensively studied probiotics to general health and has been used in fermented milk products for more than 70 years [Fujimoto et al., 2008]. In some countries, such as Brazil and Japan this product is very popular and consumed orally, but studies evaluating the interaction of this microorganisms and oral microbiota are scarce. Lima et al [2005] performed an *in vitro* study comparing the adhesion of 2 probiotic microorganisms (*Lactobacillus casei* Shirota and *Lactobacillus acidophilus*) to an artificial caries model. Their results show that *L. casei* Shirota adhered to artificial carious dentin, and the authors suggested that this microorganism may be related to dental caries. Nevertheless, it was not possible to establish that L. casei *Shirota* participated in caries disease progression or even competed with pathogenic microorganisms. Our results show a trend in that the group that was inoculated with *L. casei* Shirota had a numerically lower amount of all microorganisms tested (TM, ST, MS and L), but presented a trend towards the lower final microhardness and the higher mineral loss. The properties of *L. casei* Shirota mentioned above may potentiate it for activities in low pH conditions in relation to other oral species tested.

The laser scanning confocal microscope was used to measure demineralization area and lesion depth. This method is relatively easy and fast, since it does not require a thin section. In this experiment lesion depth and area did not show any statistical difference among the groups.

The current *in vitro* model included prolonged episodes of low pH due to sucrose refreshment of the medium 3 times per day for one half hour and is

thus an extreme cariogenic situation, unlikely to prevail in the oral cavity, unless in high risk conditions. This severe situation allowed us to assess the cariogenic potential of the probiotic strains tested in a mixed biofilm. It was observed that the *L. reuteri* group (G3) was the one that showed less cariogenic potential under these extreme conditions. However, the potential anticariogenic effect of the probiotic strains tested may have been masked by the severity of the cariogenic situation and could therefore not be ruled out. Additional studies should be performed to elucidate any inhibitory effect of *L. rhamnosus* GG, *L. reuteri* and *L. casei* Shirota on cariogenic potential using a low pH caries model.

In conclusion, under the conditions of this study only the *L. reuteri* mix-culture biofilm group (G3) had a reduced mineral loss as measured by surface microhardness.

Acknowledgments

This research was supported by the University of Michigan and CAPES – PDEE (4835/10-7). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Conceived the experiment: CSL, MF, CGC, SEF, ES. Designed the experiments: CSL, MF, CGC, SEF. Performed the experiments: CSL, MF, CGC and SEF. Analyzed the data: CSL, MF, CGC and SEF. Wrote the paper: CSL, MF, CGC, SEF, ACBD, CCRM and ES. The authors would like to thank Christopher Niquette and Matthew Dery for help with enamel blocks preparation and Dr. J. Christopher Fenno for providing the *S. gordonii* strain.

Table 1. Study design and bacterial strain

Group[a]	Inoculated[b]	Demineralization Time	Treatment Preparation
1	+	7 days	Inocula[c] + L. casei ATCC 393
2	+	7 days	Inocula + L. rhamnosus GG ATCC 53103
3	+	7 days	Inocula + L. reuteri SD 2112 ATCC 55730
4	+	7 days	Inocula + L. casei Shirota (from Yakult)

[a]n=12 enamel blocks/group

[b]Inoculated using mix of bacterial culture at an absorbance each of 0.5 at 540 nm in trypticase soy broth supplemented with 5% sucrose (TSBS)

[c]Inoculum = *Streptococcus mutans* TH16, *Streptococcus salivarius* ATCC 13419, *Streptococcus gordonii* and *Actinomyces naeslundii* ATCC 19039

Table 2. The distribution of microorganisms in the bacterial mixture inoculated in each group

Variables	Groups			
	1	2	3	4
TM[1]	1.0×10^{8a} (1.5×10^{7})	2.6×10^{8a} (6.0×10^{7})	2.1×10^{8a} (2.0×10^{8})	4.0×10^{8a} (4.0×10^{8})
TS[2]	2.0×10^{7a} (1.1×10^{7})	3.3×10^{7a} (1.4×10^{7})	1.1×10^{7a} (1.6×10^{6})	2.4×10^{6a}n(2.7×10^{6})
MS[3]	7.6×10^{5a} (3.3×10^{4})	9.3×10^{6a} (8.5×10^{5})	2.0×10^{6a} (8.0×10^{5})	2.4×10^{6a} (9.5×10^{5})
L[4]	8.9×10^{7a} (1.6×10^{6})	3.8×10^{7a} (2.5×10^{7})	1.8×10^{7a} (1.4×10^{6})	1.3×10^{8a}(1.7×10^{7})

[a,b]Means (SD) followed by distinct letters are significantly different according to each variable
[1]Total microorganisms
[2]Total streptococci
[3]mutans streptococci
[4]Lactobacilli

Table 3. The distribution of microorganisms in the biofilm after 7 days in the artificial caries model

Variables	Groups (n=3)			
	1	2	3	4
TM[1]	$1.5x10^{7a}$ ($1.7x10^{6}$)	$2.0x10^{7a}$ ($7.5x10^{6}$)	$1.8x10^{7a}$ ($6.6x10^{6}$)	$1.2x10^{7a}$ ($2.2x10^{6}$)
TS[2]	$2.6x10^{5a}$($2.5x10^{5}$)	$2.7x10^{5a}$ ($6.1x10^{4}$)	$2.8x10^{6b}$($1.1x10^{6}$)	$1.8x10^{5a}$ ($8.6x10^{4}$)
MS[3]	$1.2x10^{4a}$ ($4.8x10^{3}$)	$1.3x10^{4a}$ ($5.6x10^{3}$)	$1.3x10^{5b}$ ($8.2x10^{4}$)	$1.0x10^{4a}$ ($2.0x10^{3}$)
L[4]	$1.7x10^{7a}$ ($3.0x10^{6}$)	$2.0x10^{7a}$ ($3.2x10^{6}$)	$1.6x10^{7a}$ ($2.2x10^{6}$)	$1.6x10^{7a}$ ($3.8x10^{6}$)

[a,b]Means (SD) followed by distinct letters are significantly different according to each variable
[1]Total microorganisms
[2]Total streptococci
[3]mutans streptococci
[4]Lactobacilli

Table 4. Mean and standard deviation (SD) of the variables analyzed in the enamel surface according to the treatments

Variables	Groups (n=12)			
	1	2	3	4
SMHi[1]	363.3[a] (11.5)	362.2[a] (11.4)	361.5[a] (10.9)	360.1[a] (11.1)
SMHf[2]	50.5[a] (27.3)	67.3[a] (33.5)	163.6[b] (71.5)	39.9[a] (14.7)
%SMH[3]	-86.2[a] (7.4)	-81.5[a,b] (9.3)	-55.0[b] (18.9)	-89.0[a] (3.8)
Lesion area[4]	43692.4[a] (1.6×10^4)	46810.8[a] (1.7×10^4)	35357.9[a] (1.6×10^4)	42494.6[a] (2.2×10^4)
Lesion depth[5]	43.7[a] (16.4)	46.8[a] (17.3)	35.4[a] (16.3)	42.5[a] (12.2)

[a,b]Means (SD) followed by distinct letters are significantly different according to each variable

[1]Initial microhardness (Hardness Vickers)

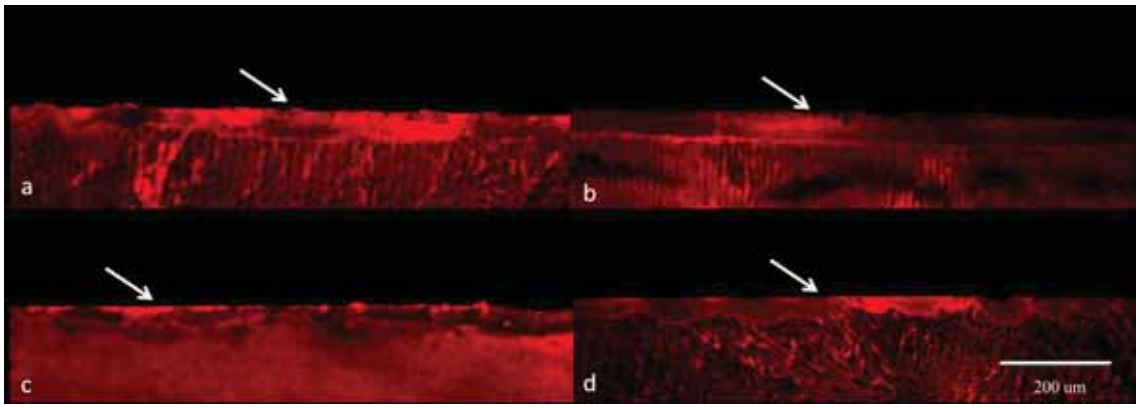
[2]Final microhardness (Hardness Vickers)

[3]Percentage of subsurface hardness change (Hardness Vickers)

[4]Lesion area (μm^2)

[5]Lesion depth (μm)

Figure 1. Representative confocal microscopy images of caries lesions developed after 7 days in the artificial caries model for each group



a = group 1, b = group 2, c = group 3, d = group 4
arrows show enamel caries lesion

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Anexos

ANEXO A

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ANEXO B
Instruções aos autores

Caries Research

Guidelines for Authors
www.karger.com/cre_guidelines

Aims and Scope
Submission
Conditions
Types of Papers
Preparation of Manuscripts
References
Color Illustrations
Supplementary Material
Digital Object Identifier (DOI)
Supplementary Material
Author's ChoiceTM, NIH-Funded Research, Self-Archiving
Page Charges
Proofs
Reprints

Aims and Scope
'Caries Research' is an international journal, the aim of which is to promote research in dental caries and related fields through publication of original research and critical evaluation of research findings. The journal will publish papers on the aetiology, pathogenesis, prevention and clinical control or management of dental caries. Papers on health outcomes related to dental caries are also of interest, as are papers on other disorders of dental hard tissues, such as dental erosion. Aspects of caries beyond the stage where the pulp ceases to be vital are outside the scope of the journal. The journal reviews papers dealing with natural products and other bacterial inhibitors against specific criteria, details of which are available from the Editor.

Submission
Manuscripts written in English should be submitted at Online Manuscript Submission
Should you experience problems with your submission, please contact:

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During the online submission you will be asked to list complete mailing addresses, including e-mail addresses of three potential reviewers for your manuscript.
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Manuscripts should be prepared as a text file plus separate files for illustrations. The text file should contain the following sequence of sections: Title page;

Declaration of interests; Abstract; Introduction; Materials and Methods; Results; Discussion; Acknowledgements; References; Legends; Tables. Each section should start on a new page, except for the body of the paper (Introduction to Acknowledgements), which should be continuous. Submissions which do not conform to these simple guidelines will be returned to the author.

Title page: The first page of each manuscript should show, in order: the title, which should be informative but concise; the authors' names and initials, without degrees or professional status, followed by their institutes; a short title, maximum length 60 characters and spaces, for use as a running head; a list of 3-10 key words, for indexing purposes; the name of the corresponding author and full contact details (postal address, telephone and fax numbers, and e-mail address).
Declaration of Interests: Potential conflicts of interest should be identified for each author or, if there are no such conflicts, this should be stated explicitly. Conflict of interest exists where an author has a personal or financial relationship that might introduce bias or affect their judgement. Examples of situations where conflicts of interest might arise are restrictive conditions in the funding of the research, or if an author or their employer holds patent(s) on a product used in the study, or payment to an investigator from organisations with an interest in the study (including employment, consultancies, honoraria, ownership of shares, travel grant). Investigators should disclose potential conflicts to study participants and should state whether they have done so.

Abstract: The abstract should summarise the contents of the paper in a single paragraph of no more than 250 words (to ensure that the abstract is published in full by on-line services such as PubMed). No attempt should be made to give numerical results in detail. References are not allowed in the abstract.

Introduction: This section should provide a concise summary of the background to the relevant field of research, introduce the specific problem addressed by the study and state the hypotheses to be tested.

Materials and Methods (or Subjects and Methods): All relevant attributes of the material (e.g. tissue, patients or population sample) forming the subject of the research should be provided. Experimental, analytical and statistical methods should be described concisely but in enough detail that others can repeat the work. The name and brief address of the manufacturer or supplier of major equipment should be given.
Statistical methods should be described with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results. When possible, findings should be quantified and appropriate measures of error or uncertainty (such as confidence intervals) given. Sole reliance on statistical hypothesis testing, such as the use of P values, should be avoided. Details about eligibility criteria for subjects, randomization and the number of observations should be included. The computer software and the statistical

methods used should be specified. See Altman et al.: Statistical guidelines for contributors to medical journals [Br Med J 1983;286:1489–93] for further information.

Manuscripts reporting studies on human subjects should include evidence that the research was ethically conducted in accordance with the Declaration of Helsinki (World Medical Association). In particular, there must be a statement in Materials and Methods that the consent of an appropriate ethical committee was obtained prior to the start of the study, and that subjects were volunteers who had given informed, written consent.

Clinical trials should be reported according to the standardised protocol of the CONSORT Statement. The CONSORT checklist must be submitted together with papers reporting clinical trials.

In studies on laboratory animals, the experimental procedures should conform to the principles laid down in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and/or the National Research Council Guide for the Care and Use of Laboratory Animals.

Unless the purpose of a paper is to compare specific systems or products, commercial names of clinical and scientific equipment or techniques should only be -cited, as appropriate, in the 'Materials and Methods' or 'Acknowledgements' sections. Elsewhere in the -manuscript generic terms should be used.

In any manuscript involving microradiography, the following information must be included: the radiation source and filters used and the kV used (this determines the wavelength of radiation and hence the validity of using Angmar's equation).

Manuscripts on experimental enamel caries should show that the lesions retain a relatively well-preserved surface layer, i.e. are not surfacesoftened lesions. Proof of surface integrity can be provided either as illustrations in the paper or as supplementary material for the reviewers. Transverse microradiography, polarized light microscopy of a section immersed in water or backscattered scanning electron microscopy of a polished cross-section can be used to provide the necessary proof. To allow the nature of experimental changes to be assessed, microradiographs or micrographs should be provided to show part of the experimental lesion and the adjacent control (e.g. figure 2 of Zaura et al.: Caries Res 2007;41:489–492). Again, these images can be provided as part of the paper or as supplementary material for review purposes.

Results: Results should be presented without interpretation. The same data should not be presented in both tables and figures. The text should not repeat

numerical data provided in tables or figures but should indicate the most important results and describe relevant trends and patterns.

Discussion: This section has the functions of describing any limitations of material or methods, of interpreting the data and of drawing inferences about the contribution of the study to the wider field of research. There should be no repetition of preceding sections, e.g. reiteration of results or the aim of the research. The discussion should end with a few sentences summarising the conclusions of the study. However, there should not be a separate 'Conclusions' section.

Acknowledgements: Acknowledge the contribution of colleagues (for technical assistance, statistical advice, critical comment etc.) and provide the position(s) of author(s) employed by commercial firms. This section should describe the source(s) of funding that have supported the work inlcuding relevant grant numbers. Please also include this sentence: "The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript." If this statement is not correct, you must describe the role of any sponsors or funders, and amend the sentence as needed. Additionally, the roles of all authors must be described (For example: Conceived and designed the experiments: AA, BB. Performed the clinical examination: AA, CC. Performed the experiments: DD, FF. Analyzed the data: BB, FF. Wrote the paper: AA, CC, FF, EE).

Legends: The table headings should be listed first, followed by the legends for the illustrations.

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Figures with a screen background should not be submitted.
When possible, group several illustrations in one block for reproduction (max. size 180 x 223 mm).

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References
Reference to other publications should give due acknowledgement to previous work; provide the reader with accurate and up-to-date guidance on the field of research under discussion; and provide evidence to support lines of argument. Authors should select references carefully to fulfil these aims without attempting to be comprehensive.
Cited work should already be published or officially accepted for publication. Material submitted for publication but not yet accepted should be cited as 'unpublished results', while unpublished observations communicated to the authors by another should be cited as 'personal communication', with credit in both cases being given to the source of the information. Neither unpublished nor personally communicated material should be included in the list of references. Abstracts more than 2 years old and theses should not be cited without a good reason, which should be explained in the covering letter accompanying the paper.

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One author: [Frostell, 1984] or Frostell [1984].
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(a) Papers published in periodicals: Lussi A, Longbottom C, Gygax M, Braig F: Influence of professional cleaning and drying of occlusal surfaces on laser fluorescence in vivo. Caries Res 2005;39:284-286.

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(c) Monographs: Matthews DE, Farewell VT: Using and Understanding Medical Statistics. Basel, Karger, 1985.

(d) Edited books: DuBois RN: Cyclooxygenase-2 and colorectal cancer; in Dannenberg AJ, DuBois RN (eds): COX-2. Prog Exp Tum Res. Basel, Karger, 2003, vol 37, pp 124-137.

(e) Patents: Diggens AA, Ross JW: Determining ionic species electrochemically. UK Patent Application GB 2 064 131 A, 1980.

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ANEXO C

Comitê de ética

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

COMITÊ DE ÉTICA EM PESQUISA - CEP-

OF. 152/2008
CEP
ACBD/bri.

Araçatuba, 18 de novembro de 2008.

Referência Processo FOA 2008-01519

O Coordenador do Comitê de Ética em Pesquisa desta Unidade, tendo em vista o parecer favorável da relatora que analisou o projeto **"EFEITO DE LEITES FERMENTADOS CONTENDO PROBIÓTICOS NO BIOFILME FORMADO "IN SITU" E NA DESMINERALIZAÇÃO DO ESMALTE DENTAL"** expede o seguinte parecer:

APROVADO:

Informamos a Vossa Senhoria que de acordo com as normas contidas na resolução CNS 215, **deverá ser enviado o relatório final em 13/05/2009.**

Profª. Drª. Alessandra Marcondes Aranega
Vice-Coordenadora do CEP

Ilma Senhora
Drª.CAROLINA SIMONETTI LODI
Araçatuba-SP-

Ciente.De acordo.

/ /

Drª Carolina Simonetti Lodi

Faculdade de Odontologia e Curso de Medicina Veterinária
Rua José Bonifácio, 1193 CEP 16015-050 Araçatuba - SP
Tel (18) 3636-3200 E-mail: [illegible]

ANEXO D

Lista de instruções ao voluntário – Estudo *in situ*

1- Todos os materiais utilizados na pesquisa não acarretam em custo ao voluntário.
2- Os voluntários não deverão utilizar qualquer tipo de produto fluoretado (exceto água) ou anti-séptico bucal uma semana antes e durante todo o experimento.
3- Uma semana antes do experimento e durante todo o experimento os voluntários deverão escovar os dentes com dentifrício não fluoretado fornecido pela pesquisadora.
4- A pesquisa será composta por 3 etapas, com duração de 14 dias cada uma e com intervalo de uma semana entre elas.
5- Os voluntários deverão utilizar o dispositivo bucal durante todo o dia, inclusive para dormir e **DEVERÁ REMOVÊ-LO SOMENTE PARA AS REFEIÇÕES** ocasião em que o dispositivo deverá permanecer na caixa própria para armazená-lo.
6- Evite que o dispositivo fique fora da boca por um período prolongado, restringindo-se ao tempo necessário para cada refeição (40 min).
7- Realize sua higiene bucal normalmente, utilizando o dentifrício fornecido.
8- Os blocos **NÃO** deverão ser escovados. Os voluntários poderão realizar a escovação do dispositivo, **1 VEZ POR DIA**, somente **NA FACE VOLTADA PARA O PALATO** e com o dentifrício fornecido pela pesquisadora.
9- Os voluntários deverão aplicar duas gotas da solução fornecida pela pesquisadora sobre os blocos 8 vezes ao dia, nos seguintes horários: 8:00, 9:30, 11:00, 14:00, 15:30, 17:00, 19:00 e 21:00 horas. O dispositivo deverá ser reposicionado na boca após 5 minutos.
10- No 14º dia o voluntário deverá comparecer no departamento de Odontopediatria, localizado na rua José Bonifácio 1193 para devolver o dispositivo palatino e realizar a coleta da saliva.
11- Quando qualquer material estiver acabando ou sentir algum desconforto na utilização do dispositivo, entrar em contato com a pesquisadora.
12- Favor verificar todos os dias se os fragmentos estão em suas lojas e se a tela protetora continua intacta. Caso não estejam, entrar em contato imediatamente com a pesquisadora.
13- Qualquer dúvida entrar em contato com a pesquisadora (Carolina Lodi) pelo telefone : 3636-3235 (Pediatria) ou (14) 9794-0105.

Resumo das instruções ao voluntário

1- Colocar o dispositivo após a última escovação da noite
2- No dia seguinte (1º dia), iniciar o tratamento com a solução fornecida nos horários pré-estabelecidos: 8:00, 9:30, 11:00, 14:00, 15:30, 17:00, 19:00 e 21:00 horas.
3- Após gotejar a solução de tratamento, esperar 5 minutos para reposicionar o dispositivo na boca.
4- Após as refeições, realizar a higiene bucal com o dentifrício fornecido pela pesquisadora antes de reposicionar o dispositivo.
5- Poderá escovar o dispositivo, 1 vez por dia, somente na face voltada para o palato e com o dentifrício fornecido pela pesquisadora.

Obrigada pela colaboração.

ANEXO E

Lista de instruções ao voluntário – Estudo *in vivo*

1- Todos os materiais utilizados na pesquisa não acarretam em custo ao voluntário.
2- Os voluntários não deverão utilizar qualquer tipo de produto fluoretado (exceto água) ou anti-séptico bucal uma semana antes e durante todo o experimento.
3- Uma semana antes do experimento e durante todo o experimento os voluntários deverão escovar os dentes com dentifrício não fluoretado fornecido pela pesquisadora.
4- A pesquisa será composta por 2 etapas, com duração de 14 dias cada uma e com intervalo de uma semana entre elas.
5- Os voluntários deverão ingerir um frasco do leite fermentado oferecido pela pesquisadora uma vez por dia, pela manhã, durante 14 dias.
6- Realize sua higiene bucal normalmente, utilizando o dentifrício fornecido.
7- Os voluntários não deverão ingerir outra fonte de bactéria probiótica durante o experimento.
8- No 14º dia o voluntário deverá comparecer no departamento de Odontopediatria, localizado na rua José Bonifácio 1193 para coleta da saliva, com jejum de pelo menos 2 horas.
9- Quando qualquer material estiver acabando entrar em contato com a pesquisadora.
10- Qualquer dúvida entrar em contato com a pesquisadora (Carolina Lodi) pelo telefone : 3636-3235 (Pediatria) ou (14) 9794-0105.

Obrigada pela colaboração.

ANEXO F

Análise de açúcar total dos leites fermentados - Capítulo 1

Método de Dubois

A dosagem dos carboidratos álcalis-solúveis foi realizada conforme descrito por Dubois et al (1956), empregando os seguintes reagentes:

1 – Fenol 5%:
2 – Ácido Sulfúrico concentrado
A reação foi feita de acordo com a tabela abaixo:

	Blank	Padrão	Padrão	Padrão	Padrão	Padrão	Amostra
Água deionizada	0.5 mL	0.4 mL	0.3 mL	0.2 mL	0.1 mL	-	0.3 mL
Solução padrão (Glicose)	-	0.1 mL	0.2 mL	0.3 mL	0.4 mL	0.5 mL	-
Amostra	-	-	-	-	-	-	0.2 mL
Fenol 5%	0.5 mL em todos						
Ac. Sulfúrico	2.5 mL em todos, agitar imediatamente, esperar 20 minutos e ler a 490 nm						

Inicialmente foi colocado água deionizada nos tubos de ensaio de acordo com os volumes descritos na tabela acima. A seguir foram colocados em duplicatas, os padrões de glicose ou as amostras (leite fermentado diluído 1:1000). Então foram adicionados 0.5 mL de fenol 5% em todos os tubos e em seguida colocou-se 2.5 mL de ácido sulfúrico e agitou-se imediatamente. A coloração da reação foi desenvolvida pela adição do ácido sulfúrico concentrado. Após 20 min da colocação do ácido sulfúrico realizou-se a leitura em espectrofotômetro (Cary 50) a 490 nm de absorbância. Esses valores foram anotados, transferidos para uma planilha (programa Excel-Microsoft) e convertidos para mg de glicose.

ANEXO G

Análise de açúcar redutor dos leites fermentados – Capítulo 1

Método de Somoghyi-Nelson

Os açúcares redutores foram determinados pelo método de Somogyi - Nelson utilizando glicose como açúcar padrão (0.05 mg glicose/mL). As amostras de leite fermentado foram diluídas 1:100. Adicionou-se 1 mL de água em cada tubo e em seguida 1 mL do reativo cúprico. A mistura permaneceu 20 minutos em banho fervente. Após esse período foi adicionado 1 mL do reativo arsenomolíbidico e em seguida 10 mL de água qsq. A glicose do leite fermentado reduz o sal de cobre em meio alcalino e à quente. A forma reduzida do sal de cobre atua sobre o reativo arsenomolíbidico, determinando a aparência de uma cor azul, cuja intensidade é proporcional ao teor de glicose da amostra. Para ajuste do espectrofotômetro foi preparado um tubo branco utilizando-se água destilada no lugar do leite fermentado. Realizou-se a leitura em espectrofotômetro (Cary 50) a 500 nm de absorbância. Esses valores foram anotados, transferidos para uma planilha (programa Excel-Microsoft) e convertidos para mg de glicose.

ANEXO H

Preparo e seleção dos blocos de esmalte

Confecção dos blocos de esmalte bovino (4 x 4 x 2 mm)

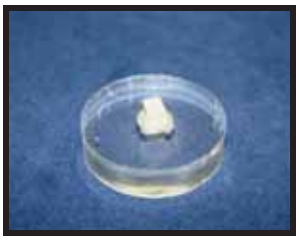
1. Coroa do dente bovino incisivo central inferior, separada da raiz através de disco diamantado de duas faces (KG Sorensen D 91), montado em motor de bancada (Nevoni), mantido sob refrigeração (água destilada/deionizada).

2. Secção da coroa utilizando disco diamantado (série 15 HC Diamond - n. 11-4244 Buehler) separando a superfície vestibular da lingual.

3. Face vestibular fixada na placa de acrílico.

4. Secção da face vestibular no sentido longitudinal, na porção mais plana, utilizando-se 2 discos diamantados (série 15 HC Diamond –n. 11-4243 Buehler), montados em cortadeira sob refrigeração com água destilada/deionizada e separados por um disco espaçador de alumínio com 4 mm de espessura. Em seguida, foi realizado o corte no sentido transversal.

5. Fragmento vestibular do dente bovino, fixado sobre placa de resina. Ao lado, bloco de esmalte dentário.



6. Bloco de esmalte fixado em disco de resina acrílica pré-fabricada (± 3 cm de diâmetro por ± 8 mm de espessura), com auxílio de cera pegajosa (Kota Ind. e Com. LTDA), com a superfície dentinária voltada para cima.

7. Ajuste da dentina para obtenção de superfícies paralelas entre esmalte e dentina, utilizando Politriz APL-4 AROTEC e lixas de granulação 320 (CARBIMET Paper Discs, 30-5108-320, BUEHLER), 2 pesos, durante 20 segundos sob baixa rotação e refrigeração.

8. Blocos fixados com a superfície do esmalte voltada para cima, a qual será polida.

Seqüência do polimento de esmalte:

1. Pedra-pomes, água deionizada e taça de borracha montada em contra-ângulo em baixa-rotação.
2. Na Politriz APL-4 AROTEC - lixa de granulação 600, 800 e 1200 (30 segundos – 2 pesos) e refrigeração a água. Limpeza em lavadora ultrassônica e água destilada/deionizada por 2 minutos, entre cada lixa;
3. Na Politriz APL-4 AROTEC - acabamento final com disco de papel feltro TEXMET 1000 (Buehler Polishing Cloth) (1 minuto – 2 pesos) e suspensão de diamante 1 micron base-água (Buehler);
4. Limpeza em lavadora ultrassônica utilizando solução detergente (Ultramet Sonic Cleaning Solution - Buehler) diluída 20:1 em água destilada/deionizada (2minutos);
5. Lavagem durante 30 segundos com jato de água destilada/deionizada.

ANEXO I

Análise de microdureza – Capítulo 1

1. Microdurômetro Shimadzu Micro Hardness Tester HMV-2.000 (Shimadzu Corporation - Kyoto-Japan), com penetrador tipo Knoop, acoplado ao Software para análise de imagem CAMS-WIN (NewAge Industries, USA).

2. Bloco de esmalte sendo submetido à leitura no microdurômetro, carga estática de 25 gramas e tempo de 10 segundos, para análise da microdureza de superfície.

3. Fotomicrografia das impressões para análise de microdureza de superfície inicial (SMH-inicial), e final (SMH-final) (Aumento: 100x).

ANEXO J

Análise de microdureza em secção longitudinal – Capítulo 1

1. Embutidora metalográfica (AROTEC PRE 30S) – utilizada para inclusão dos blocos de esmalte em 5 gramas de resina acrílica (Buehler Transoptic Powder, Lake Bluff, Illinois, USA), pressão de 150 Kgf/cm2, tempo de aquecimento de 7 minutos e mais 7 minutos de resfriamento. Os blocos foram fixados em posição com cola adesiva (Super Bonder – Loctite).

2. Corpo de prova – plano longitudinal voltado para a superfície da resina acrílica.

3. Microdurômetro Bhulher (Buehler – Lake Bluff, Illinois, USA), com penetrador tipo Knoop, acoplado ao Software para análise de imagem CAMS-WIN (NewAge Industries, USA). Carga estática de 5 gramas e tempo de 10 segundos, para análise da microdureza em secção longitudinal.

4. Fotomicrografia das impressões para análise de microdureza de secção longitudinal nas diferentes distâncias. (Aumento: 100x).

Seqüência do polimento de esmalte:

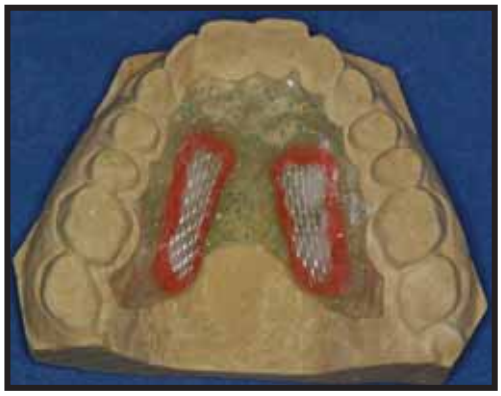
1. Na Politriz APL-4 AROTEC - lixa de granulação 320 (1 minuto – 2 pesos), 600, 800 e 1200 (2minutos – 2 pesos) e refrigeração a água. Limpeza em lavadora ultrassônica e água destilada/deionizada por 2 minutos, entre cada lixa;
2. Na Politriz APL-4 AROTEC - acabamento final com disco de papel feltro MICROCLOTH SUPREME PSA (Buehler Polishing Cloth) (2 minuto – 2 pesos) e suspensão de diamante 1/4 micron base-água (Buehler);
3. Limpeza em lavadora ultrassônica utilizando solução detergente (Ultramet Sonic Cleaning Solution - Buehler) diluída 20:1 em água destilada/deionizada (3minutos);
4. Lavagem durante 30 segundos com jato de água destilada/deionizada.

ANEXO K

Dispositivo palatino – Capítulo 1

1. Dispositivo palatino confeccionado em resina acrílica (Jet – Artigos Odontológico Clássico, São Paulo), com espaço para a inserção dos blocos de esmalte de dente bovino.

2. Os blocos fixados com cera pegajosa e cobertos com tela plástica (peneira grande, ref. 0041, Plásticos Gonçalves Ltda., São Paulo), deixando um espaço (de 1 a 2 mm) entre o bloco e a tela para permitir o acúmulo do biofilme e protegê-lo da perturbação mecânica.

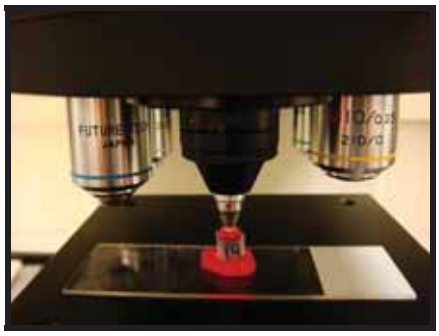


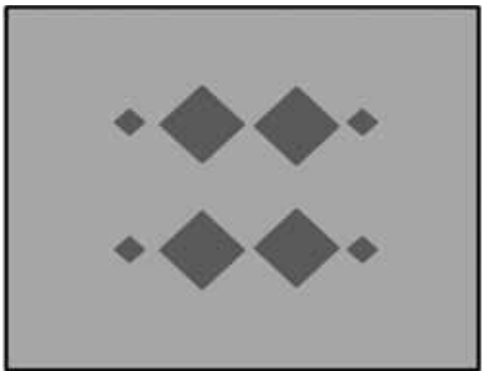
ANEXO L

Análise de microdureza – Capítulo 2

1. Microdurômetro LM 247AT (LECO Corporation, St Joseph, Michigan, USA), com penetrador tipo Vickers, acoplado ao Software para análise de imagem CAMS-WIN (NewAge Industries, USA).

2. Bloco de esmalte sendo submetido à leitura no microdurômetro, carga estática de 200 gramas e tempo de 15 segundos, para análise da microdureza de superfície.



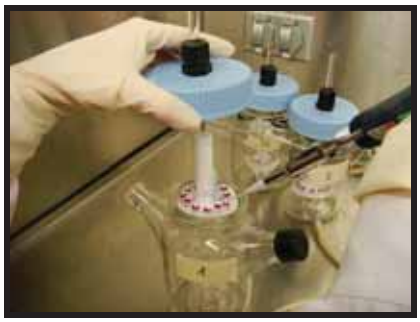


3. Esquema das impressões para análise de microdureza de superfície inicial (SMH-inicial), e final (SMH-final).

ANEXO M

Modelo in vitro de lesão de cárie – Capítulo 2

1. As amostras foram fixadas em hastes de resina e em seguida em disco plástico. Após, o disco foi montado no modelo experimental e esterilizado em óxido de etileno.



2. Com todo material autoclavado, foi aplicado sobre os blocos 20 µL de saliva para formação da película adquirida. Todo procedimento foi realizado dentro do fluxo laminar para evitar contaminação externa.

3. Após 20 min. o inóculo (20 µL) foi aplicado sobre os blocos.

4. Todas as peças foram montadas e levadas à estufa (37°C). Aguardamos 20 min. antes de ligar as bombas para permitir que os microorganismos aderissem à superfície do esmalte.

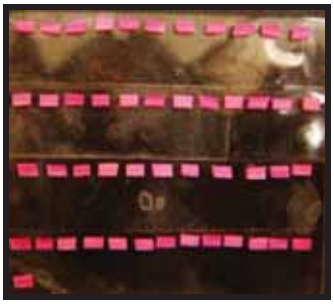
5. Após esse período as bombas eram ligadas e as amostras cobertas com saliva artificial e meio de cultura (TSBS). O meio de cultura era oferecido 3 vezes por dia para simular as refeições diárias.

ANEXO N

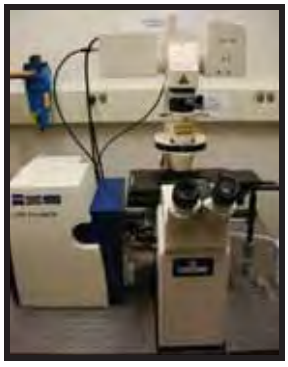
Análise da área e profundidade da lesão: Confocal – Capítulo 2

1. Aplicação de verniz para proteger a superfície do bloco de esmalte que será submetido à análise de confocal.

2. Bloco cortado no sentido longitudinal (0.5 mm espessura) em cortadeira de bancada (SBT Modelo 650) em baixa velocidade.



3. Os blocos foram corados overnight em rodamina B 0.1 mM e fixados em lâminas de vidro.



4. Confocal (Zeiss LSM 510 META Confocal Microscope/Zeiss Axiovert 100M Inverted Microscope, Carl Zeiss MicroImaging, Peabody, MA, USA) acoplado ao Software para análise de imagem LSM Image Browser and Image J software version 1.43u.