JULIANA NUNES BOTELHO

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Universidade Estadual de Campinas

Faculdade de Odontologia de Piracicaba

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Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Dentistry, in the Cariology area.

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Odontologia, área de Cariologia.

Orientador: Prof. Dr. Jaime Aparecido Cury

Este exemplar corresponde à versão final da tese defendida pela aluna Juliana Nunes Botelho e orientada pelo Prof. Dr. Jaime Aparecido Cury.

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ABSTRACT

Sucrose is the most cariogenic dietary carbohydrate while starch is considered non-cariogenic for enamel and slightly cariogenic for dentine. The combination starch and sucrose (starch+sucrose) has been considered more cariogenic than sucrose alone but this subject remains debatable. Also, the effect of fluoride toothpaste on the cariogenicity of this combination is unknown. The aims of this study were to evaluate: (i) the effect of starch+sucrose on enamel and dentine demineralization using an *S. mutans* biofilm model modified by adding human saliva to simulate amylase action; (ii) the in situ effect of fluoride toothpaste (FT) containing 1100 µg F/g on dentine demineralization progression; and (iii) the in situ effect of fluoride on the cariogenic potential of starch+sucrose on enamel and dentine demineralization. In vitro, *S. mutans* biofilms were grown on enamel and root dentine slabs for 5 and 4 days, respectively, in a saliva-containing medium and exposed to the following treatment: 1% starch; 10% sucrose; or starch+sucrose (8x/day). Biofilms were then analyzed for their biochemical and microbiological compositions, and dental demineralization was evaluated. Biofilms exposed to starch+sucrose were more acidogenic ($p < 0.0001$) and caused higher demineralization ($p < 0.0001$) on either enamel or dentine than those exposed to each carbohydrate alone. The in situ effect of FT on dentine demineralization was tested in a pilot crossover study, in which 10% sucrose was applied extra-orally to the slabs 8x/day in 2 phases of 14 days each. At days 10 and 14 of each phase, dentine demineralization was evaluated. The effect of toothpaste was significant ($p<0.0001$), but the effect of time was not ($p>0.05$). The results suggest that FT at 1100 µg F/g can reduce dentine demineralization even under high cariogenic challenges - biofilm accumulation and sugar exposure. The in situ effect of the treatments (water, 2% starch, 10% sucrose and starch+sucrose) and that of the toothpastes (non-FT and FT) were tested in a crossover, single-blind and split-mouth study conducted in 4 phases of 14 days each. The volunteers used two of the treatments 8 times/day and one of the toothpastes 3 times/day. The effect of the factors (toothpaste and treatments) was significant

($p<0.05$) for enamel and dentine, but not ($p>0.05$) for the interaction. The findings suggest that, regardless of the cariogenic challenge provoked by the different sources of the dietary sugars tested, fluoride toothpaste is effective in reducing enamel and dentine demineralization. In conclusion, the results suggest that starch may enhance the cariogenic potential of sucrose and fluoride from toothpaste reduces enamel and dentine demineralization caused by the combination of these carbohydrates.

Key Words: Dental caries. Dietary carbohydrates. Diet, cariogenic. Alpha-Amylase. Biofilms.

RESUMO

Sacarose é o carboidrato mais cariogênico da dieta e o amido é considerado não cariogênico para esmalte e moderadamente cariogênico para dentina. Por outro lado, a combinação de amido e sacarose (amido+sacarose) tem sido considerada mais cariogênica que apenas sacarose, mas esse ainda é um assunto em debate. Além do mais, o efeito de dentifrício fluoretado na cariogenicidade dessa combinação é desconhecido. Assim, com o objetivo de estudar esse assunto três experimentos foram conduzidos: (i) o primeiro avaliou efeito de amido+sacarose na desmineralização de esmalte e dentina, usando um modelo de biofilme de *S. mutans* modificado pela adição de saliva para simular a ação da amilase, (ii) o segundo avaliou in situ o efeito de dentifrício contendo 1.100 µg F/g (DF) na progressão da desmineralização de dentina radicular, e o terceiro (iii) avaliou in situ o efeito do fluoreto no potencial cariogênico de amido+sacarose na desmineralização de esmalte e dentina. In vitro, biofilmes de *S. mutans* foram formados sobre blocos de esmalte e dentina radicular, por 5 e 4 dias respectivamente, em meio de cultura contendo saliva e expostos a um dos seguintes tratamentos: amido a 1%, sacarose a 10% ou de sua combinação (8x/dia). Os biofilmes foram analisados quanto às suas composições bioquímicas e microbiológicas, e a desmineralização dos blocos foi avaliada. Biofilmes expostos à combinação foram mais acidogênicos ($p<0,0001$) e provocaram maior desmineralização ($p<0,0001$) em esmalte e dentina que o efeito dos carboidratos isolados. In situ, o efeito do DF foi testado em um estudo piloto, cruzado no qual sacarose a 10% foi aplicada extraoralmente 8x/dia em 2 fases de 14 dias. Após 10 e 14 dias em cada fase, a desmineralização da dentina foi avaliada. O efeito do dentifrício foi significativo ($p<0,0001$), mas o efeito do tempo não ($p>0,05$). Esses resultados sugerem que DF com 1.100 µg F/g é capaz de diminuir a cárie dentinária mesmo sob alto desafio cariogênico de acúmulo de biofilme e exposição à sacarose. In situ, o efeito dos tratamentos (água, amido a 2%, sacarose a 10% e amido+sacarose) e o efeito do dentifrício (não fluoretado e fluoretado) foram

testados em um estudo cruzado, cego, boca-dividida em 4 fases de 14 dias. Os voluntários usaram dois dos tratamentos 8x/dia e um dos dentifrícios 3x/dia. O efeito dos fatores (dentifrício e tratamentos) foram significativos ($p<0,05$) para esmalte e dentina, mas a interação não ($p>0,05$). Os resultados sugerem que, independente do desafio cariogênico provocado pelos diferentes carboidratos da dieta testados, o dentifrício fluoretado é efetivo na redução da desmineralização de esmalte e dentina. Em conclusão, os resultados sugerem que amido deve aumentar o potencial cariogênico da sacarose mas que fluoreto de dentifrício é capaz de reduzir a desmineralização tanto do esmalte quanto da dentina provocada pela combinação desses carboidratos.

Palavras-chave: Cárie dentária. Carboidratos da dieta. Dieta cariogênica. Alfa-Amilase. Biofilmes.

SUMÁRIO

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INTRODUÇÃO

A cárie é uma doença biofilme-açúcar dependente (Fejerskov, 2004), caracterizada por uma destruição progressiva e localizada da parte mineral de esmalte e dentina pelos ácidos produzidos pelo biofilme dental acumulado (fator necessário) exposto frequentemente aos carboidratos da dieta (fator determinante negativo).

A dissolução da parte mineral pode ocorrer em diferentes velocidades dependendo do substrato dentário. A perda mineral na dentina é duas vezes e meia mais rápida quando comparada ao esmalte (Ögaard et al., 1988), e tem sido explicada por: a) seu maior pH crítico desmineralizante (Hoppenbrouwers et al., 1987; Shellis, 2010); b) conter menores cristais e maior conteúdo de carbonato (Nyvad e Fejerskov, 1982, Hoppenbrouwers et al., 1987; Wefel, 1994); c) maior porcentagem de matriz orgânica (Kawasaki e Featherstone, 1997); e d) maior permeabilidade (ten Cate et al., 1998). Assim, após a metabolização de carboidratos fermentáveis pelos microrganismos do biofilme promovendo a queda do pH a valores inferiores ao crítico para o esmalte (pH ≈ 5,5), já ocorrerá a dissolução mineral da dentina a qual se inicia antes (pH ≈ 6,5) e se estende por maior período de tempo (Curzon e Preston, 2004).

Independente do substrato dental, o frequente consumo de carboidratos fermentáveis é o fator determinante para o desenvolvimento da cárie, e dentre os açúcares da dieta, a sacarose é considerada o carboidrato mais cariogênico (Paes Leme et al., 2006, como revisão), pois além de fermentável, é o único substrato para a síntese de polissacarídeos extracelulares (PECs) pelos microrganismos do biofilme dental (Rölla, 1989; Cury et al., 2000). Os PECs provocam alterações qualitativas na matriz do biofilme formado (Dibdin e Shellis, 1988), explicando a maior cariogenicidade da sacarose comparada aos outros açúcares fermentáveis (Cury et al., 2000).

Entre os demais carboidratos, o amido é o principal componente da dieta humana (Lingström et al., 2000), e é considerado como não cariogênico para o esmalte (Firestone et al., 1982; Ribeiro et al., 2005) ou de baixa cariogenicidade

para a dentina (Lingström et al., 1994) quando usado como única fonte de carboidrato (Sheiham, 2001). O amido cozido (solúvel), contido em diversos alimentos, causa uma maior desmineralização do esmalte quando comparado ao amido cru (Brudevold et al., 1985). Apesar disso, esse amido processado (solúvel) causa quedas de pH menores do que aquelas observadas para a sacarose (Neff, 1967; Lingström et al., 1989; Ribeiro et al., 2005). Entretanto, devido ao maior pH crítico desmineralizante da dentina (Hoppenbrouwers et al., 1987; Shellis, 2010), essa queda do pH pode resultar na dissolução mineral da dentina quando do metabolismo microbiano do amido solúvel consumido (Lingström et al., 1989; Aires et al., 2008).

Porém, na dieta contemporânea, o amido é consumido simultaneamente ou intercalado com a sacarose (Lingström et al., 2000, como revisão), e essa associação poderia ser potencialmente mais cariogênica que o efeito isolado da sacarose (Bowen e Koo, 2011, como revisão), pois os produtos da hidrólise do amido (maltose, maltotriose e dextrinas de baixo peso molecular) podem ser fermentados a ácidos pelos microrganismos do biofilme (Clarkson et al., 1987), provocando a desmineralização dentária. Além disso, a presença desses hidrolisados resulta na produção de polissacarídeos estruturalmente diferenciados em relação àqueles produzidos pela metabolização apenas da sacarose (Vacca-Smith et al., 1996; Xiao e Koo, 2010), tornando o biofilme mais cariogênico. O potencial cariogênico da combinação de amido e sacarose (amido+sacarose) tem sido estudado por diferentes modelos experimentais, incluindo estudos in vitro (Duarte et al., 2008; Thurnheer et al., 2008), estudos de cárie em esmalte em ratos (Firestone et al., 1982; Mundorff-Shrestha et al., 1994; Thurnheer et al., 2008), e estudos in situ (Ribeiro et al., 2005; Aires et al., 2008).

É importante considerar que estudos in vitro que avaliem a cariogenicidade de produtos amiláceos, devem conter a amilase, pois esta enzima é requerida para a metabolização do amido na cavidade bucal (Scannapieco et al., 1993), sendo responsável por aproximadamente 75% da atividade de amilase do biofilme (Fiehn e Moe, 1983). Estudos in vitro foram realizados com a presença de

amilase salivar e avaliaram a composição de biofilmes de *Streptococcus mutans* formados sobre discos de hidroxiapatita quando expostos a amido+sacarose. Esses trabalhos mostraram que a presença da combinação aumentou a expressão gênica (gene da *gtfB*), acidogenicidade e produção de PECs na matriz do biofilme (Duarte et al., 2008; Klein et al., 2009). As alterações na composição e conformação estrutural do biofilme (formação de microcolônias) provavelmente resultaram no aumento da sua coesividade e integridade estrutural (Xiao e Koo, 2010), demonstrando que a combinação influencia a composição, estrutura e propriedades da matriz do biofilme (Bowen e Koo, 2011, como revisão).

A avaliação do efeito cariogênico da dieta composta por amido+sacrose também foi avaliada em estudos de cárie experimental em ratos (Firestone et al., 1982; Mundorff-Shrestha et al., 1994). Eles mostraram que uma maior incidência de lesões de cárie foi constatada para a dieta que continha a associação de sacarose com hidrolisados de amido quando comparada à sacarose isoladamente.

Esse efeito sinérgico de amido+sacarose foi confirmado por um estudo in situ que avaliou a desmineralização do esmalte decíduo (Ribeiro et al., 2005). Os biofilmes formados sobre o esmalte expostos à combinação dos carboidratos foram mais cariogênicos que aqueles formados quando expostos apenas à sacarose, levando a maiores perdas minerais (Ribeiro et al., 2005).

Por outro lado, a maior cariogenicidade da associação desses carboidratos não foi mostrada por dois estudos subsequentes, sendo o primeiro com biofilme multiespécie formado sobre blocos de esmalte bovino, e o outro avaliando cárie experimental em ratos (Thurnheer et al., 2008). Com relação à dentina radicular, no único estudo in situ feito, uma tendência que a combinação amido+sacarose poderia ser mais cariogênica que apenas a sacarose foi observada (Aires et al., 2008).

As bases biológicas explicando como o amido poderia aumentar o potencial cariogênico da sacarose foram revisadas recentemente (Bowen e Koo, 2011). As diferenças na desmineralização dentária encontrada in situ (Ribeiro et al., 2005; Aires et al., 2008) podem ter sido influenciadas pelo uso de diferentes

substratos dentais (esmalte e dentina) provenientes de diferentes fontes (humanos ou bovinos) e do uso de dentifrício fluoretado. É importante ressaltar que a cinética de progressão da cárie é diferente em esmalte e dentina (Ögaard et al., 1988), assim como o efeito de dentifrício fluoretado em reduzir a cárie nesses substratos também pode ser diferente.

É conhecido que dentifrício fluoretado contendo uma concentração de pelo menos 1.000 µg F/g, é necessário para um efeito anticárie no esmalte (Marinho et al., 2003; Walsh et al., 2010). Tem sido mostrado experimentalmente que dentifrício contendo 1.100 µg F/g é capaz de reduzir a desmineralização do esmalte quando da exposição à sacarose (Duggal et al., 2001; Paes Leme et al., 2004; Ccahuana-Vásquez et al., 2007; Cenci et al., 2008). Entretanto, há poucos estudos sobre o efeito de dentifrício contendo 1.100 µg F/g na desmineralização de dentina. No único estudo clínico controlado que avaliou o efeito dessa concentração na dentina radicular, Jensen e Kohout (1988) mostraram que ele foi capaz de reduzir a incidência de cárie em comparação ao placebo. Além disso, estudos in vitro usando um modelo de ciclagem de pH (Dunipace et al., 1994) e in situ quando da exposição à sacarose (Vale et al., 2011; Kusano et al., 2011) mostraram que o dentifrício de 1.100 µg F/g tem efeito na redução da desmineralização e na progressão da lesão em dentina comparado ao controle negativo. Contudo, em nenhum dos estudos realizados até o presente momento, a capacidade de dentifrício contendo 1.100 µg F/g em controlar a cariogenicidade da combinação amido+sacarose em esmalte e dentina foi avaliada.

Assim, com o objetivo de avaliar o potencial cariogênico da combinação amido+sacarose e o efeito de fluoreto na redução da desmineralização de esmalte e dentina foram feitos três trabalhos: no primeiro (capítulo 1) foi avaliado o efeito de amido+sacarose em um modelo de biofilme de *S. mutans* modificado pela adição de saliva para simular a ação da amilase; no segundo (capítulo 2) foi avaliado in situ o efeito de dentifrício de 1.100 µg F/g na progressão da desmineralização da dentina radicular e no terceiro (capítulo 3) foi avaliado in situ o efeito do fluoreto no potencial cariogênico de amido+sacarose em esmalte e dentina radicular.

CAPÍTULO 1*

Effect of the combination of starch and sucrose on *S. mutans* biofilm composition, and on enamel and root dentine demineralization

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Declaration of interests

The authors of the present study declare that there are no conflicts of interest in relation to this work.

ABSTRACT

Sucrose is the most cariogenic dietary carbohydrate while starch is considered non-cariogenic for enamel and moderately cariogenic for dentine. However, cariogenicity of starch and sucrose combined remains unclear. The aim of this study was to evaluate the effect of the combination of these sugars on *Streptococcus mutans* biofilm composition and on enamel and dentine demineralization. Biofilms of *S. mutans* UA159 were grown on saliva-coated enamel and dentine slabs in culture medium. They were exposed (8 times/day) to the following treatments: 0.9% NaCl (negative control); 1% starch; 10% sucrose; or 1% starch and 10% sucrose (starch+sucrose). To simulate the effect of human salivary amylase on the metabolization of starch, the biofilms were pretreated with saliva before each treatment and saliva was also added to the culture medium. The acidogenicity of the biofilm was estimated evaluating twice/day the culture medium pH. After 4 (dentine) and 5 days (enamel) of growth, biofilms (n = 9) were individually collected and biomass, viable microorganisms, intracellular polysaccharide, and soluble and insoluble extracellular polysaccharides (IEPS) were quantified. Dentine and enamel demineralization was assessed by percentage of surface hardness loss (%SHL). Biofilms exposed to the combination starch+sucrose were more acidogenic and caused higher demineralization ($p < 0.0001$) on either enamel or dentine than those exposed to each carbohydrate alone. IEPS formation on dentine exposed to starch+sucrose was significantly higher ($p < 0.0001$), when compared to the other groups. The findings suggest that starch increases the cariogenic potential of sucrose.

INTRODUCTION

Dental caries is a sugar-biofilm dependent disease [Fejerskov, 2004] and sucrose is the most cariogenic dietary carbohydrate [Paes Leme et al., 2006]. Starch, a major source of dietary carbohydrate, is considered non or slightly cariogenic when used as the sole dietary carbohydrate source [Sheiham, 2001]. However, currently, starch is consumed simultaneously or interspersed with sucrose [Lingström et al., 2000], and this combination could influence the biofilm composition, modulating the pathogenesis of dental caries [Bowen and Koo, 2011].

The increased cariogenic potential of starch and sucrose combined (starch+sucrose) has been explained by the fact that these two carbohydrates in the presence of the respective enzymes, salivary α-amylase and glycosyltransferases, enhance the formation of highly insoluble extracellular polysaccharides and structurally change the biofilm matrix. This would result in the accumulation of strong cohesive and adherent biofilms on dental surfaces [Bowen and Koo, 2011]. The cariogenic potential of these sugars combination was suggested by in vitro studies evaluating *S. mutans* biofilm composition formed on hydroxyapatite discs [Duarte et al. 2008; Klein et al., 2009]. Further, the combination of starch+sucrose caused higher enamel caries in rats [Firestone et al., 1982; Mundorff-Shrestha et al., 1994] and induced in situ higher demineralization on deciduous enamel [Ribeiro et al., 2005] than sucrose. However, the greater cariogenicity of starch+sucrose was not confirmed by two subsequent studies, one using a multispecies biofilm model formed on enamel slabs, and in other evaluating caries in rats [Thurnheer et al., 2008]. Moreover, regarding root dentine, the combination of starch+sucrose was not significantly more cariogenic than sucrose when evaluated in situ [Aires et al., 2008].

These inconsistencies could be explained by the way starch is decomposed in the mouth. Salivary amylase is required to metabolyze starch [Scannapieco et al., 1993], and it is responsible for approximately 75% of the total amylase activity in biofilm [Fiehn and Moe, 1983]. Therefore, to evaluate the cariogenic potential of starch+sucrose, in the present study we used a validated *S. mutans* biofilm model

[Ccahuana-Vásquez and Cury, 2010] which was previously tested to evaluate the cariogenicity of milk [Muñoz-Sandoval et al.; Giacaman et al., 2012]. This model was modified by addition of saliva to simulate the key role of salivary amylase on starch metabolization. Also, this model simulates the “fast and famine” exposure to dietary sugars to which dental biofilm is daily subjected in the mouth.

MATERIAL AND METHODS

Experimental design

Independent studies were conducted using slabs of bovine enamel or dentine. *S. mutans* UA159 biofilms were grown on these slabs using a validated model [Ccahuana-Vásquez and Cury, 2010] modified to simulate the action of salivary amylase. They were grown in ultrafiltered (10-kDa-cutoff membrane; Prep/Scale; Millipore, MA, USA) buffered tryptone-yeast extract broth (UTYEB) and they were exposed 8x/day to the one of the following treatments: 0.9% NaCl; 1% starch; 10% sucrose; and 1% starch + 10% sucrose (starch+sucrose). Each experiment was carried out three times, each in triplicate (n = 9). To simulate the effect of salivary amylase, saliva was added to culture medium and the biofilms were also pretreated with saliva before exposing to the treatments described above. The culture media was changed 2x/day, at beginning and end of the treatments (Fig. 1), and its pH was determined as indicator of biofilm acidogenicity. After 4 days for dentine and 5 days for enamel, the biomass (dry weight), viable bacteria and polysaccharide composition of biofilm samples were determined. Demineralization occurred on enamel and dentine slabs was assessed by percentage of surface hardness loss. For statistical analysis each biofilm/slab was considered as an experimental unit, being the data for enamel and dentine analyzed independently.

Enamel and dentin slabs preparation

Flattened and polished enamel and root dentine slabs (4x7x1 mm) were obtained from the bovine incisors [Ccahuana-Vásquez and Cury, 2010]. Baseline

surface hardness (SH) of the slabs was measured using a Knoop microhardness tester coupled to FM-ARS 900 software (Future-Tech FM, Kawasaki, Kanagawa, Japan) by making 3 indentations, spaced 100 μm from each other, using loads of 50 g for enamel and 5 g for dentine for 5 seconds. Slabs with SH 323.1 ± 8.7 kg/mm² and 40.5 ± 2.0 kg/mm² for enamel and dentine, respectively, were used in the study, after sterilization with ethylene oxide.

Saliva collection and preparation

Whole saliva was collected on ice from two healthy volunteers (22 and 24 years old) who chewed paraffin film (Parafilm M; American Can Co, Neenah, WI, USA). They had not used in the past 3 months antimicrobials, mouth rinses or any other medication known to affect salivary composition and flow and who provided written informed consent previously approved by the Research and Ethics Committee of Piracicaba Dental School (Protocol No. 104/2011).

Saliva was used: 1) to form acquired pellicle on enamel and dentine surfaces; 2) to pretreat the slabs before treatments; and 3) as an additive to the culture medium where the biofilms were grown. Saliva collection was daily made in the morning before any meal and in the afternoon after 2 h fasting. For acquired pellicle formation, saliva was diluted 1:1 with adsorption buffer and supplemented with the protease inhibitor phenylmethylsulfonyl-fluoride (PMSF, 1.0 mmol/L, final concentration) [Koo et al., 2000], and then centrifuged at 3.800 *g* for 10 min at 4ºC. Saliva used to pretreat the biofilms and that added to culture medium was daily collected and immediately centrifuged as described above. Both supernatant was collected and individually filtered (FILTERMAX 0.2 μm Vacuum System, TPP, St. Louis, MO, USA). The clarified filtered-sterilized saliva was added to the culture medium in the proportion of 1:10 (v/v). That used to pretreat the biofilm was diluted 1:1 (v/v) with 0.9% NaCl.

S. mutans *biofilm growth*

For acquired pellicle formation, slabs were maintained in a 24-wells plate and incubated with filtered saliva in an orbital shaker at 60 rpm, at 37°C for 30 min. The

slabs coated by human salivary pellicle were individually positioned in a new 24-well plate containing 2.0 mL of *S. mutans* UA159 inoculum (OD 1.6 at 600 nm) prepared in a proportion of 1:500 in UTYEB supplemented with 1% sucrose. After 8 h at 37ºC and 10% CO_2, the slabs were transferred to another plate where they were immersed in 2.0 mL UTYEB containing 0.1 mM glucose (basal salivary concentration) and 10% saliva.

After 24 h of biofilm growth, slabs were daily submitted 8x/day to the treatments, during 3 days for dentine and 4 days for enamel. Culture medium was changed twice, before the first and after the last exposure to the treatments of the day. The pH of each changed medium was measured as indicator of biofilm acidogenicity.

***Treatments* (Fig. 1)**

The biofilms on the enamel and dentine slabs were individually exposed to the treatments 8x/day at defined times (9:00, 10:30, 12:00, 13:00, 14:30, 16:00, 17:00 and 18:30 h). Before each treatment, slabs were taken out from the UTYEB medium containing 0.1 mM glucose and 10% saliva and they were transferred to a new plate containing saliva for the pretreatment. After 1 min, they were transferred to another plate containing the treatments (0.9% NaCl; 1% starch; 10% sucrose; or 1% starch + 10% sucrose). After 3 min, slabs were washed 3 times in 0.9% NaCl and were returned to the culture plate containing the medium described above.

Biofilm collection and analysis

After 4 and 5 days of biofilm growth, for dentine and enamel respectively [Muñoz-Sandoval et al.; Giacaman et al., 2012], the slabs were individually washed 3 times in 0.9% NaCl, transferred to microcentrifuge tubes containing 1 mL of 0.9% NaCl and sonicated for 30 s at 7 W (Branson, Sonifier 150, Danbury, CT, USA) to detach the biofilm from the slabs [Ccahuana-Vásquez and Cury, 2010]. Slabs were separated and stored for demineralization analysis, and aliquots of the suspension were analyzed for the following dependent variables:

Biomass. An aliquot of 150 µL of the suspension was centrifuged (10 min at 5.000 g and 4 °C) and the pellet was dried in Speed-Vac concentrator (Savant Instruments, Inc., Hicksville, NY, USA) for 2 h and weighted (± 0.01 mg) to obtain biofilm dry weight, as biomass indicator.

Viable microorganisms. An aliquot of 100 µL of the suspension was serially diluted in 0.09% NaCl and inoculated in triplicate on BHI agar (BD, Sparks, USA) to determine the number of viable microorganisms [Herigstad et al., 2001]. The plates were incubated for 48 h at 37°C, 10% CO_2. Colonies of *S. mutans* were counted and expressed as CFU/mg of biofilm dry weight.

Polysaccharides. An aliquot of 400 µL of the suspension was used to extract and determine the concentration of soluble (SEPS) and insoluble (IEPS) extracellular polysaccharides (EPS), and intracellular polysaccharides (IPS) in the biofilm [Aires et al., 2008]. The results were normalized by biofilm dry weight and expressed as µg/mg of biomass.

Enamel and dentine demineralization

Final SH of the slabs was again measured with 3 indentations 100 µm apart from the initial indentations or in the center of the slabs if the initial indentations were not visible. Baseline and final values were used to obtain %SHL ((Baseline SH value – Final SH value) x 100/ Baseline SH value), which was used as indicator of enamel [Cury et al., 2000] and dentine [Vale et al., 2011] demineralization.

Statistical analysis

The assumptions of equality of variances and normal distribution of errors were checked for all response variables tested, and those that did not satisfy these assumptions were transformed and analyzed by analysis of variance (ANOVA) followed by Tukey's test. Enamel and dentine data were analyzed separately. The SAS software system (version 9.0; SAS Institute Inc., Cary, NC, USA) was used, with a significance level fixed at 5%.

RESULTS

The combination starch+sucrose induced a greater ($p < 0.0001$) decrease in medium pH at 32, 56, 80, and 104 h of biofilm growth, for enamel (Fig. 2a) and at 32, 56 and 80 h for dentine (Fig. 2b), when compared to the other groups.

The biofilms treated with starch+sucrose did not differ from those treated with only sucrose concerning the variables: biomass; viable bacteria; SEPS; and IPS, for either enamel (table 1) or dentine (table 2). The IEPS concentration in biofilms treated with starch+sucrose was higher ($p < 0.0001$) than that found for sucrose alone, for dentine (table 2), but not for enamel (table 1).

Regarding demineralization, starch combined with sucrose caused greater ($p < 0.0001$) %SHL both for enamel and dentine (Fig. 3), when compared to sucrose separately.

DISCUSSION

Starch and sucrose make up the largest proportion of dietary carbohydrates consumed worldwide [Lingström et al., 2000]. While some studies have reported that the combination of starch and sucrose (starch+sucrose) is more cariogenic than sucrose alone [Firestone et al., 1982; Mundorff-Shrestha et al., 1994; Ribeiro et al., 2005], others have not found difference [Aires et al., 2008; Thurnheer et al., 2008].

Our results showed higher demineralization of bovine enamel and dentin when they were exposed to the combination of starch+sucrose than sucrose alone (Fig. 3). With respect to enamel, our results are in agreement with those found in situ for deciduous enamel [Ribeiro et al., 2005]. However, they contrast to in vitro results reported by Thurnheer et al. [2008], using a multispecies biofilm model. Such disagreement could be due to the different biofilm models used and how the biofilm were exposed to carbohydrates. In our study, the biofilm exposure to carbohydrates was intermittent (8x/day), whereas Thurnheer et al. [2008] used continuous feeding exposure of the biofilm to carbohydrate in culture medium. Consequently, the

medium pH was kept at values below 5.0 for all treatments, while in our study it was possible to show differences among the treatments in acidogenicity (Fig. 2).

With regard to dentine, our study showed that the combination starch+sucrose caused greater demineralization than sucrose alone (Fig. 3). These results apparently disagree with those found in situ [Aires et al., 2008] and could not be explained by differences in substrate, since both studies used bovine root dentine. Indeed, in the study by Aires et al. [2008] there was a trend for a greater effect of the combination compared to sucrose, but the difference was not statistically significant. Besides the inherent differences between in vitro and in situ studies considering the control of variables, in the in situ study above, the volunteers were exposed to fluoride from water and dentifrice, which could have masked the cariogenic potential effect of the combination. The present in vitro study was conducted in the absence of fluoride.

The effect of starch+sucrose on the demineralization of both enamel and dentine may be considered synergistic and not simply the sum of the effect of fermentation of 1% starch and 10% sucrose. The data show that starch caused on enamel SHL 4.1% higher than the control, while for sucrose this figure was 29.5% (Fig. 3). If the demineralization was the sum of the effects, a 33.6% greater demineralization would have been expected for the group starch+sucrose compared with the control. However, the effect of the combination was 45.9% higher, strongly suggesting a synergistic effect (1.4 fold). For dentine, the sum of the effect of carbohydrates alone was 47.5% and the combination was 52.1%. The synergistic effect is supported by the statistical analysis done, which showed that the effect of the combination was greater than the effect of carbohydrates separately (Fig. 3).

This synergistic effect of the combination starch+sucrose on enamel and dentine demineralization can be confirmed by the acidogeniciy data found (Fig. 2a, 2b). When the biofilms were exposed to the combination starch+sucrose, the concentration of H^+ in the medium, at the times of 32, 56, 80 and 104 h of biofilm growth, was higher than that found when they were exposed to the carbohydrates separately. For example, at time 56 h, the H^+ concentration for the groups treated

with starch or sucrose was 1.2 x 10^{-7} and 74.2 x 10^{-7} M, respectively. However, the value found for the group treated with starch+sucrose was 200.9 x 10^{-7} M, which is 2.7-fold higher than the sum of the effect of the starch and sucrose.

The explanation for this synergistic effect was not the aim of the present study, but it could be the result of the increased starch degradation by amylase to fermentable products by *S. mutans*. It is known that the action of this enzyme is essential for starch to be metabolized by bacteria present in biofilm [Scannapieco et al., 1993], mainly *S. mutans,* which do not have amylolytic activity [Edwardsson, 1968]. This enzyme is found in acquired pellicle [Hannig et al., 2004] and in biofilm matrix [Fiehn and Moe, 1983]. In our biofilm model, the action of this enzyme in both sites was provided by pretreatment of dental substrates with saliva and by the presence of saliva in the culture medium in which biofilms were grown (see M&M). Also, it is well known that any carbohydrate to be fermented by bacteria biofilm, must first diffuse into the biofilm matrix followed by transference to bacteria cytoplasm. However, this process is hampered when starch is used as the carbohydrate source for the biofilm bacteria metabolism, since its diffusion into the biofilm is limited due to its high molecular weight [Thurnheer et al., 2003]. Also, in the biofilm matrix it must first be degraded in products than can be transported into bacteria [Webb et al., 2008]. However, this diffusion can be facilitated by the effect of sucrose and starch on the matrix of biofilm formed [Koo and Bowen, 2011].

Thus, the concentration of insoluble extracellular polysaccharides (IEPS) in the biofilm exposed to starch+sucrose was greater than that found in the biofilm exposed to starch alone (tables 1, 2). When compared to the group treated only with sucrose, starch+sucrose showed a significantly higher concentration of IEPS, in the biofilms grown on dentine (table 2). It is well known that sucrose changes the biofilm matrix composition [Paes Leme et al., 2006] and the biofilm becomes more porous [Dibdin and Shellis, 1988]. It has also been suggested that extracellular polysaccharides produced by sucrose in the presence of starch hydrolysates have a differentiated structure, which could explain the higher cariogenicity of the combination of starch+sucrose when compared to sucrose [Vacca-Smith et al.,

1996; Xiao and Koo, 2010]. These results could be explained by an increased starch diffusion into the biofilm exposed to starch+sucrose. Once inside the biofilm, the starch might be hydrolyzed by amylase to products that can be fermented by *S. mutans*.

However, in the present study the increased cariogenicity found for the combination of starch+sucrose, when compared to sucrose, could be due to some uncontrolled factor. The lower pH observed in the medium where biofilms treated with starch+sucrose were maintained, when compared to sucrose treatment (Fig. 1a, 1b), could be due to a contamination of the medium by sugars even after the three washing made with 0.9% NaCl, mainly considering the high viscosity of starch. We checked this possibility and it seems to be irrelevant because the residual concentration of sugar found in the medium was very low (0.03%). Indeed, the medium pH found after the 8^{th} exposure to the treatments, when the biofilms were immediately transferred to fresh medium and maintained overnight in fresh medium (times 24, 48, 72 and 96 h), did not show difference among the sucrose and starch+sucrose groups.

Another limitation of the present study was the use of biofilm model of *S. mutans*, a bacterium that is unable to metabolize starch [Edwardsson, 1968]. Therefore, using human saliva, we improved our model allowing starch degradation by salivary amylase. Starch was degraded by salivary amylase to products fermentable by *S. mutans,* what was confirmed by the acidogenicity of the culture medium (Fig. 2a, 2b) and by the demineralization of enamel and dentine found for the groups treated with starch (Fig. 3).

In conclusion, using *S. mutans* biofilm model we showed that starch increases the cariogenic potential of sucrose. However, this biofilm model only simulated degradation of starch by salivary amylase, since this bacterium is unable to metabolize starch. Also, *S. mutans* has no sites for amylase binding, which is important in the cariogenicity of the starch. Therefore, further studies are needed to investigate the cariogenicity of starchy products and particularly the combination of starch+sucrose using more specific biofilm model. This model should include *S.*

mutans, the most cariogenic bacteria, and other bacteria that are able to metabolize starch and to adsorb salivary amylase. Studies in this direction are being conducted with 3-species biofilm composed by *A. naeslundii, S. gordonii* and *S. mutans*.

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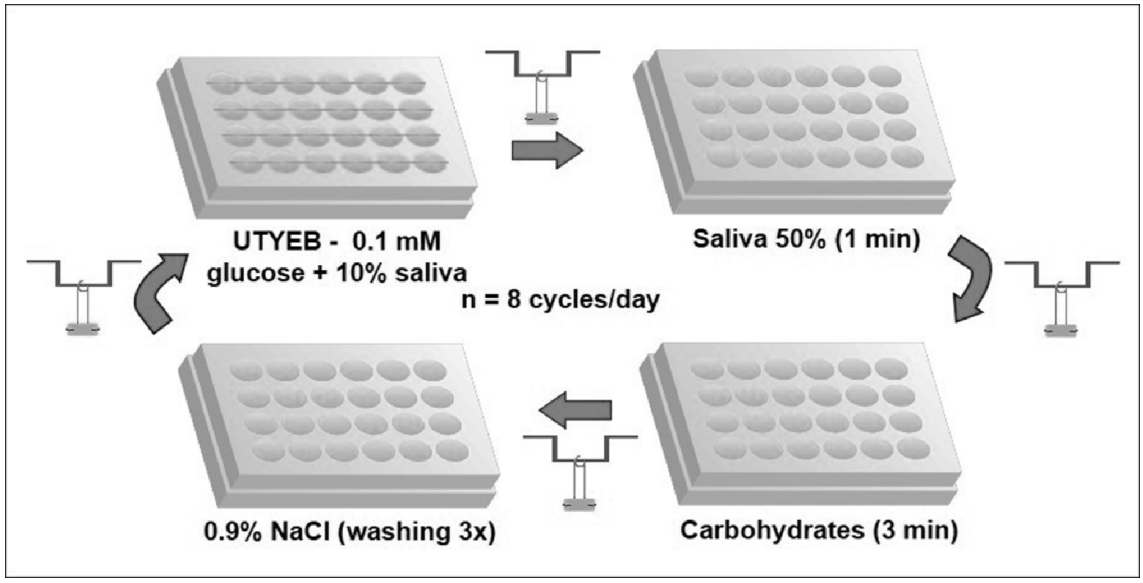

Fig. 1. Diagram of the daily treatments of the biofilms formed on slabs of enamel or dentine ( ), which were made 8 times/day (9:00, 10:30, 12:00, 13:00, 14:30, 16:00, 17:00 and 18:30 h). The medium was changed twice/day, at beginning of the treatments (9:00 h) and in the end (18:30 h).

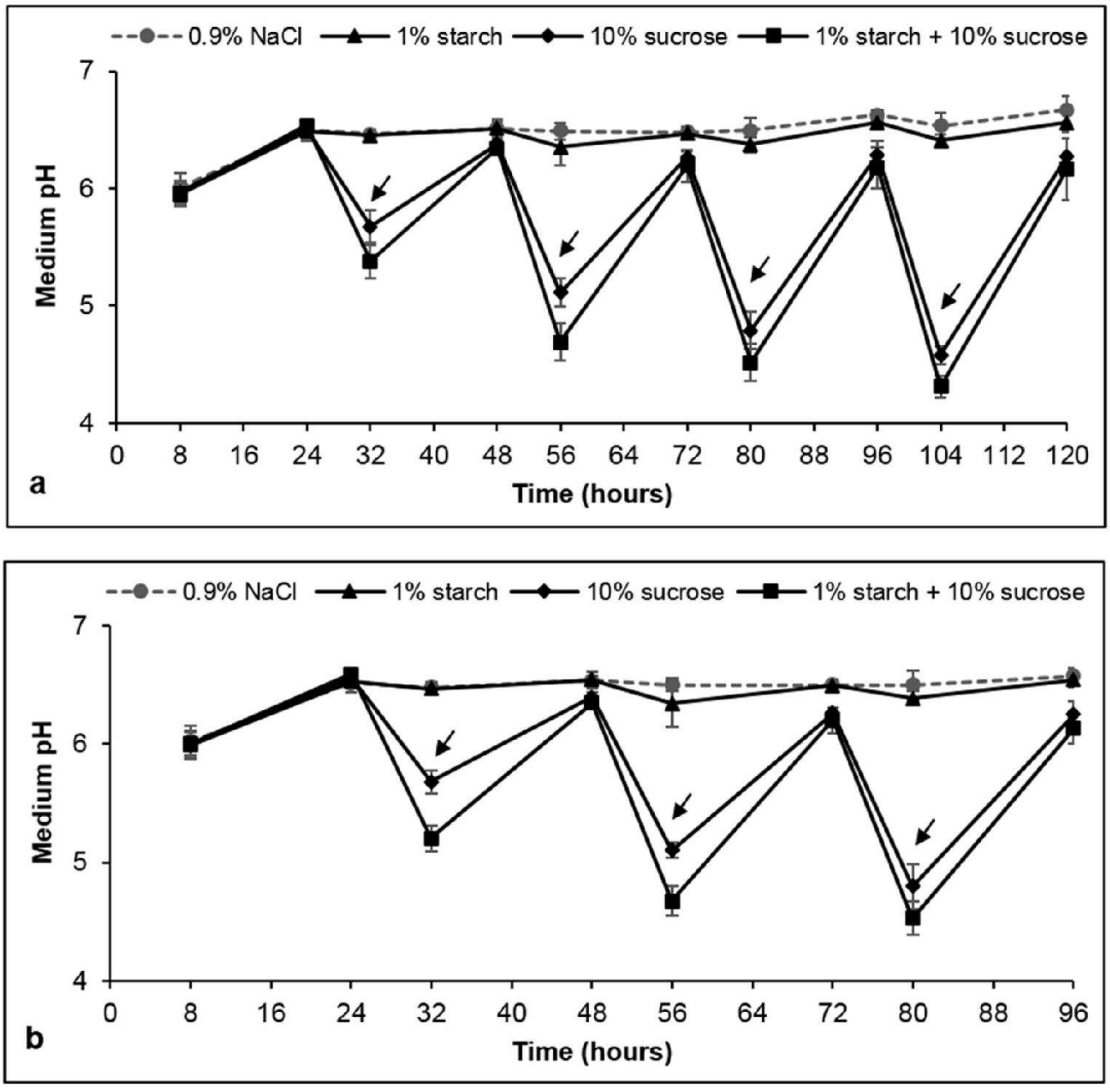

Fig. 2. Acidogenicity of biofilm (medium pH) formed on enamel (a) and dentine (b) slabs (mean ± SD, n = 9) according to the time (h) and treatments (0.9% NaCl; 1% starch; 10% sucrose; 1% starch + 10% sucrose). Arrows indicate significant differences between starch+sucrose and sucrose group ($p < 0.0001$).

Table 1. Biofilm composition formed on enamel according to treatments (mean ± SD, n = 9).

Treatament	Dependent variables				
	Biomass (mg)*	**Viable bacteria** (CFU/mg dry weight) x 10^{9}*	**SEPS** (µg/mg dry weight)*	**IEPS** (µg/mg dry weight)*	**IPS** (µg/mg dry weight)*
0.9% NaCl	0.5 ± 0.3^{A}	1.9 ± 1.6^{A}	3.1 ± 1.0^{A}	2.5 ± 1.2^{A}	2.6 ± 2.0^{A}
1% starch	0.5 ± 0.2^{A}	1.9 ± 0.9^{A}	3.01 ± 1.2^{A}	2.7 ± 1.1^{A}	4.1 ± 2.0^{A}
10% sucrose	1.8 ± 0.4^{B}	1.8 ± 0.4^{A}	2.21 ± 0.9^{A}	23.1 ± 13.3^{B}	2.8 ± 1.2^{A}
1% starch + 10% sucrose	1.7 ± 0.5^{B}	1.8 ± 1.1^{A}	2.20 ± 0.5^{A}	26.7 ± 14.7^{B}	4.1 ± 1.2^{A}

SEPS=soluble extracellular polysaccharides; IEPS=insoluble extracellular polysaccharides; IPS=intracellular polysaccharides. *For statistical analysis, biomass was transformed in square root; Viable bacteria was transformed to $(X)^{-2}$; SEPS, IEPS and IPS were transformed in $\log_{10}$ (X); %SHL was transformed in square root. Within columns, distinct letters indicate significant differences among treatment groups ($p < 0.0001$).

Table 2. Biofilm composition formed on dentine according to treatments (mean ± SD, n = 9).

Treatament	Dependent variables				
	Biomass (mg)*	**Viable bacteria** (CFU/mg dry weight) x 10^{9}*	**SEPS** (µg/mg dry weight)*	**IEPS** (µg/mg dry weight)*	**IPS** (µg/mg dry weight)
0.9% NaCl	0.6 ± 0.2^{A}	1.3 ± 0.6^{A}	3.2 ± 2.7^{A}	2.7.± 1.3^{A}	5.2 ± 2.1^{A}**
1% starch	0.6 ± 0.2^{A}	2.8 ± 1.0^{B}	3.4 ± 1.9^{A}	5.7 ± 3.5^{A}	5.9 ± 1.5^{A}
10% sucrose	1.3 ± 0.4^{B}	2.6 ± 0.8^{B}	3.1 ± 1.5^{A}	20.3 ± 3.8^{B}	4.3 ± 1.3^{A}
1% starch + 10% sucrose	1.2 ± 0.4^{B}	2.0 ± 0.3^{B}	2.5 ± 1.2^{A}	28.7 ± 7.0^{C}	5.3 ± 1.5^{A}

SEPS=soluble extracellular polysaccharides; IEPS=insoluble extracellular polysaccharides; IPS=intracellular polysaccharides. *For statistical analysis, biomass and IEPS were transformed by the $\log_{10}$ (X); Viable bacteria was transformed by $(X)^{-2}$; SEPS was transformed by the square root. **One value indicated by the SAS Software as an outlier was removed: 11.36. Within columns, distinct letters indicate significant differences among treatment groups ($p < 0.0001$).

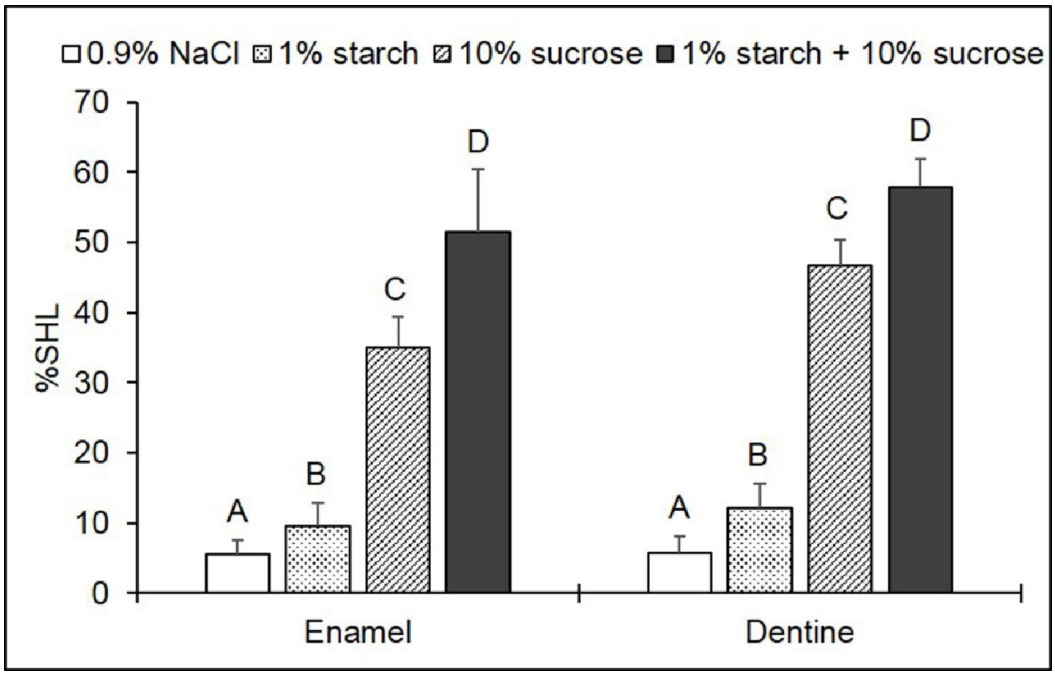

Fig. 3. Percentage of surface hardness loss (%SHL) in enamel and dentine slabs according to the treatments of the biofilms (mean ± SD, n = 9). Different letters indicate significant differences ($p < 0.0001$) among treatments (within the dental substrates). For statistical analysis, %SHL for enamel was transformed in square root.

CAPÍTULO 2*

The effect of fluoride toothpaste on root dentine demineralization progression: a pilot study

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ABSTRACT

The anticaries effect of fluoride (F) toothpaste containing 1100 µg F/g reducing enamel demineralization is well established but its effect on dentine is few explored. Furthermore, it has been shown that toothpaste containing high F concentration is necessary to remineralize root dentine lesions, suggesting that the concentration of

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1100 µg F/g would not be enough to reduce root dentine demineralization mainly when dentine is subjected to a high cariogenic challenge. Thus, the aim of this pilot study was to evaluate in situ the effect of F toothpaste, at concentration of 1,100 µg F/g, on dentine demineralization. In a crossover and double-blind study, conducted in two steps of 14 days, six volunteers wore palatal appliance containing four slabs of bovine root dentine whose surface hardness (SH) was previously determined, to which 10% sucrose solution was applied extra-orally 8x/day. Three times a day, volunteers used non-F toothpaste (negative control) or F toothpaste (1100 µg F/g, NaF/SiO_2). On the 10^{th} and 14^{th} days of each phase two slabs were collected and SH was again determined. Dentine demineralization was assessed by percentage of SH loss (%SHL). The effect of toothpaste was significant, showing lower %SHL for F toothpaste group (42.0 ± 9.7) compared with non-F (62.0 ± 6.4) ($p < 0.0001$), but the effect of time was not significant ($p > 0.05$). This pilot study suggests that F toothpaste at 1100 µg F/g is able to decrease dentine caries even under a high cariogenic challenge of biofilm accumulation and sugar exposure.

DESCRIPTORS: Dentin. Sucrose. Fluorides. Biofilms. Dental caries.

INTRODUCTION

Over the last decades, the significant increase in longevity and the reduction of enamel caries prevalence around the world have raised the concern about caries prevention in the elderly, who will have a large amount of root dentine exposed in their mouths. Therefore, since dentine is more susceptible to caries than enamel[1,2], the concern about the prevention of root caries is increasing[3].

Fluoride (F) toothpaste is widely used and it has been considered responsible for the caries decline occurred in developed countries in the 1980s[4] and in developing ones in the 1990s[5]. The anticaries effect of F toothpaste containing 1100 µg F/g (ppm F) reducing enamel caries is well established[6,7] but its effect on dentine has not been extensively studied[8,9].

Furthermore, it has been shown that toothpaste containing a high F concentration is necessary to remineralize root dentine lesions[10,11,12,13], and the effect of F in decreasing demineralization has been little explored[14,15,16]. Considering that the critical pH for the dentine dissolution is about 6.8 to 6.0, a slight decrease in the pH of the biofilm fluid will lead to demineralization[17]. However, if F is present in biofilm fluid, and the pH is not lower than 4.5, fluorapatite will be formed as long as hydroxyapatite is dissolved[18]. This reduction in demineralization is not able to avoid loss of part of the minerals, but a higher availability of F might have a better effect. Thus, the concentration of 1100 µg F/g would not be enough to reduce dentine demineralization if this dental tissue is exposed to a high cariogenic challenge by biofilm accumulation and sugar exposure over time.

The aim of this pilot study was thus to evaluate *in situ* the effect of 1100 µg F/g toothpaste on the progression of root dentine demineralization.

MATERIAL AND METHODS

Ethical aspects and volunteers

This pilot study was approved by the Research and Ethics Committee of Piracicaba Dental School (Protocol No. 104/2011), and volunteers signed an informed, written consent to participate (Resolution No. 196 of the *Conselho Nacional de Saúde, Ministério da Saúde - MS*, Brasília, Brazil, 10/03/1996).

Six volunteers (21–35 years old), who fulfilled inclusion criteria (normal salivary flow rate, good general and oral health with no active caries lesions or periodontal treatment needs, ability to comply with the experimental protocol, not having used antibiotics during the 2 months prior to the study and not using fixed or removable orthodontic devices) were selected to participate in the study. The volunteers selected had the mean decayed, missing and filled tooth surfaces index (DMFS) of 9.5 ± 10.03.

Experimental design

The study used a double-blind, crossover design, and was conducted in two phases of 14 days each, during which six volunteers wore palatal appliances containing four slabs of bovine root dentine with known surface hardness (SH)[19]. A 10% sucrose solution, prepared by the researchers, was provided for the volunteers. This solution was applied extra-orally to the slabs eight times per day as a cariogenic challenge. Three times a day, the volunteers brushed their teeth and the appliance with a non-F toothpaste (negative control) or F toothpaste (1100 µg F/g, NaF/silica-based) (the dentifrice formulations were prepared by Colgate/Palmolive, São Bernardo do Campo, Brazil). On the 10th and 14th days of each phase, two slabs were collected and evaluated for mineral loss by SH. The sequence of toothpaste used by each volunteer was randomly assigned and, after the two phases, all volunteers had used the two treatments. For all determinations, the volunteer was considered the experimental unit.

Dentine slabs and palatal appliance preparation

The root dentine slabs (4 x 4 x 2 mm) were prepared from bovine incisor teeth as previously described[19]. The artificial saliva, prepared by the researchers, contained 1.5 mM Ca, 0.9 mM P, 150 mM KCl, 0.1 M Tris, pH 7.0[20]. The slabs were immersed in this solution for 24 h, to allow dentine mineral gain and minimize further ionic changes when exposed to saliva *in situ*[21]. The SH of the dentine slabs was determined by making 3 indentations, spaced 100 µm apart, using a Knoop indenter with a 5 g load for 5 s and a microhardness tester coupled to FM-ARS 900 software (Future-Tech Corp., Kawasaki, Japan). Before performing the dentine hardness measurements, the slabs were allowed to dry for at least 30 minutes to minimize the interference of dentine dehydration with the measurements[8]. Forty-eight slabs with a mean hardness of 43.2 kg/mm^2 (SD 4.4) were selected and were randomly divided into two groups of 24 specimens each, according to the toothpaste treatments. Acrylic palatal appliances were made for each volunteer with four positions (two in each side of the appliance) for slabs. Plastic meshes were fixed over the cavities to

protect the dentine slab surfaces from mechanical attrition, leaving a 1 mm space for biofilm accumulation (see Hara *et al.*[19] for details).

Treatments

During the lead-in and washout periods of 7 days each, the volunteers brushed their teeth with non-F toothpaste. Volunteers lived in an optimally fluoridated city (0.6-0.8 mg F/L for the region) and received instructions as previously described[22]. Considering the crossover design of this study, no restriction was made with regards to the volunteers' diet, except that they were instructed to avoid F-rich foods containing bioavailable F, such as tea. They also received oral and written information to refrain from using any antibacterial substance.

To provide a cariogenic challenge, during the 14 days of each experimental phase, 8 times per day (8:00, 9:00, 10:00, 11:00, 14:00, 15:30, 17:00, 19:00 h), volunteers removed the appliance from the oral cavity and applied one drop of the 10% sucrose solution on each dental slab. After 5 minutes, the device was re-inserted in the mouth.

Throughout the experiment, volunteers brushed their teeth and the device after the main mealtimes, 3 times a day (7:30, 12:30, 20:00 h), with the toothpaste assigned for each phase. The device was removed, volunteers brushed their teeth and the appliance with the assigned toothpaste, taking care to only apply the slurry formed on the slabs area of the device. The device was then washed gently in tap water and re-inserted in the mouth. The volunteers were instructed to wear the intraoral devices throughout the 14 experimental days, removing them only for oral hygiene and during meals.

Dentine Analysis

On the 10^{th} and 14^{th} days of each phase, two slabs were removed from the devices and the SH was measured again. One row of three adjacent indentations spaced 100 µm apart was made 100 µm from the three baseline measurements. The mean values of the three baseline indentations and the three measurements

after treatments were then averaged, and the percentage of SH loss (%SHL) was calculated according to the following equation:

%SH = (*baseline* SH – SH *after* in situ *test*) × 100/*baseline* SH

SH loss was used as indicator of dentine demineralization[8,22]. The results of the two dentine slabs for each volunteer subjected to each treatment were averaged and analyzed statistically (n = 6). The SH was used to estimate demineralization of root dentine because there is a high correlation with mineral loss assessed by transverse microradiography[8].

Statistical Analysis

A factorial 2 x 2 was considered for the statistical analyses, and the factors under evaluation were:

- toothpaste at 2 levels (fluoridated and non-fluoridated) and
- time at 2 levels (10^{th} and 14^{th} days).

Volunteers were considered as statistical blocks. The assumption of equality of variances and normal distribution of errors was checked. The variables were analyzed by two-way analysis of variance (ANOVA). The analysis was conducted with SAS 9.0 software (SAS Institute, Cary, USA), with a significance level fixed at $p < 0.05$.

RESULTS

The effect of the factor dentifrice was significant ($p < 0.001$, Figure 1), showing lower %SHL for the group treated with F toothpaste (42.0 ± 9.7) when compared with the control (62.0 ± 6.4) group. However, the effect of time was not significant ($p > 0.05$).

DISCUSSION

The effect of F toothpaste in reducing enamel caries, provided the concentration is 1000 µg F/g or higher, is based in evidence[7]. Considering that

dentine is more caries-susceptible than enamel and demineralizes 2.5 faster than in enamel,[23] it is possible that a concentration of 1100 µg F/g might not be high enough to control root dentine caries. In fact, there is some evidence that toothpaste containing 5000 µg F/g is more effective in repairing root dentine caries than one containing 1100 µg F/g[11,12].

In the present *in situ* study, simulating *in vivo* conditions of biofilm accumulation and high exposure to sucrose, the toothpaste containing 1100 µg F/g significantly reduced ($p < 0.05$) root dentine demineralization compared with the placebo dentifrice. The 32% of reduction found is in agreement with *in vitro* findings,[24] but was lower than that found *in* situ (47%)[8], which might be explained by differences in the duration of both studies.

The effect of the 1100 µg F/g toothpaste in reducing root dentine demineralization by 32% was accomplished by using toothpaste 3 times/day. This value is very close to that found by Kusano *et al*.,[9] when a 1100 µg F/g toothpaste was use only once a day but at night. The data suggest that when brushing at night is performed daily, the other two brushing episodes (in the morning and after lunch) are less important. The percentage of reduction of dentine demineralization found in the present study was lower than the 67% of reduction of root caries found *in vivo* by Jensen and Kohout[25] with elderly people living in a non-fluoridated community. The high cariogenic challenge of our *in situ* study and the effect of optimally fluoridated water, attenuating the strength of F-toothpaste use, may partially explain the smaller effect observed.

We also evaluated the effect of the time and tested whether the experimental phases lasting for 10 or 14 days would demonstrate the caries progression. Nevertheless, our findings showed that the %SHL were similar in both periods for the F toothpaste and control groups. This may be explained by the fact that dentine demineralizes faster than enamel, and that demineralization is very fast in the first week and progresses slower after that, as a result of the presence of demineralized organic matrix acting as a barrier between biofilm and dentine surface.[23] In this context, the period between the two evaluations might not be long

enough to show the possible differences in SH or, perhaps, progression of the lesion could not be measured because the outer surface layer may have reached equilibrium with the biofilm fluids and progression would be observed in the body of the lesion.

These results showed that although the 1100 µg F/g toothpaste could not avoid dentine demineralization, it was able to reduce it even under a high cariogenic challenge of biofilm accumulation and sugar exposure. Moreover, this is a pilot study with six volunteers, and other *in situ* studies with more volunteers may be necessary, followed by clinical trials with elder populations to assess the initiation and progression of dentine caries lesions while using toothpastes with different F concentrations.

CONCLUSION

In summary, this pilot *in situ* study suggests that a 1100 µg F/g toothpaste is able to decrease root dentine caries.

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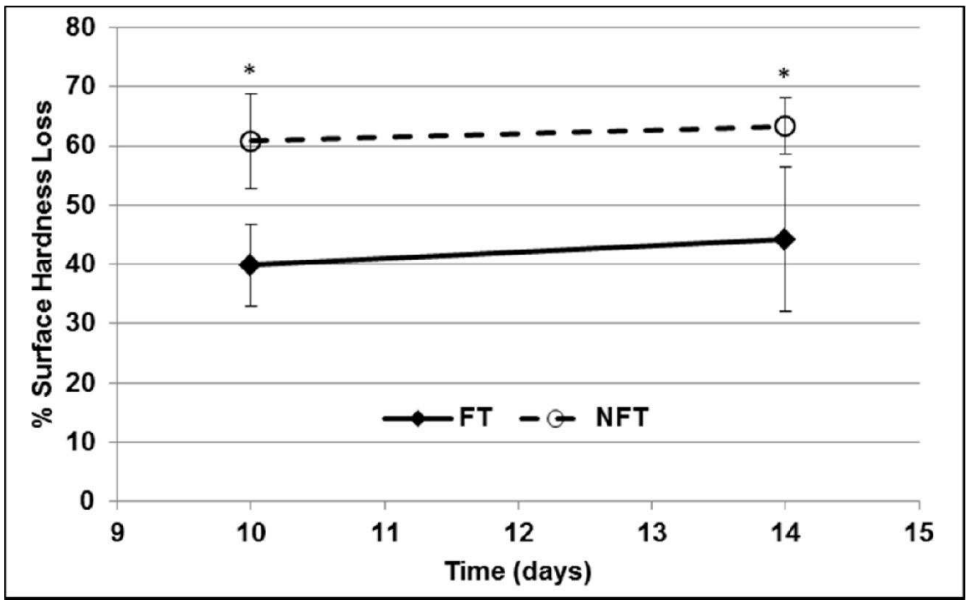

Figure 1 – Means of dentine surface hardness loss according to toothpaste treatment (bars denote standard deviations; n = 6; FT – fluoride toothpaste; NFT – non-fluoride toothpaste; *Illustrates statistically significant difference among toothpaste treatments [$p < 0.001$] in the different time point evaluated).

CAPÍTULO 3*

Fluoride toothpaste effect on the cariogenic potential of the combination of starch and sucrose

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ABSTRACT

Since the effect of fluoride toothpaste on enamel and dentine demineralization provoked by the combination of starch and sucrose (starch+sucrose) is unknown, this crossover, single-blind and *in situ* study was conducted. In 4 phases of 14 days each, the factors evaluated were: treatment at 4 levels (purified water, 2% starch, 10% sucrose and 2% starch + 10% sucrose) and toothpaste at 2 levels (non-fluoride and fluoride toothpaste - 1.100 µg F/g). Two of the treatments were made in each phase (split-mouth design). Fourteen volunteers wore palatal appliances containing on each side, 2 enamel and 2 dentine slabs. In each phase, the volunteers dripped

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8 times/day two of the treatments onto the slabs placed on each side of the appliance and used one of the toothpastes 3 times/day. On the 14th day of each phase, biofilms formed on the dental slabs were collected for biochemical analysis. Enamel and dentine demineralization was assessed by surface and cross-sectional hardness. The effect of the factors (toothpaste and treatments) were statistically significant ($p < 0.05$) for enamel and dentine, but the interaction was not significant ($p > 0.05$). The findings suggest that, regardless the cariogenic challenge provoked by the different sources of the dietary sugars tested, fluoride toothpaste is effective to reduce enamel and dentine demineralization.

Key Words: Biofilms. Dental caries. Dietary carbohydrates. Tooth demineralization.

INTRODUCTION

Dental caries is a sugar-biofilm dependent disease (Fejerskov, 2004) and sucrose is the most cariogenic dietary carbohydrate because, besides being fermented in acids by oral bacteria, it is the only substrate for the synthesis of extracellular glucans in the biofilm matrix (Paes Leme et al., 2006).

Among other dietary carbohydrates, starch is considered non-cariogenic for enamel [Firestone et al., 1982; Ribeiro et al., 2005] or slightly-cariogenic for dentine [Lingström et al., 2000] when used as the only dietary carbohydrate source [Sheiham, 2001]. However, starch is consumed simultaneously or interspersed with sucrose [Lingström et al., 2000] and this association may be potentially more cariogenic than sucrose alone. The quali-quantitative changes in the biofilm matrix would be caused by the simultaneous presence of these two carbohydrates and the action of the enzymes salivary α-amylase and glycosyltransferases (GTFs) [Bowen and Koo, 2011]. First, products of starch degradation by α-amylase can be fermented in acids by oral bacteria [Clarkson et al., 1987] provoking dental demineralization. Also, in the presence of starch hydrolysates and sucrose, polysaccharides structurally different are produced by GTFs than those produced with sucrose alone [Vacca-Smith et al., 1996], making the biofilm more cariogenic.

The higher cariogenic potential of the combination starch+sucrose than sucrose alone was shown in experimental studies on enamel caries in rats [Firestone et al., 1982; Mundorff-Shrestha et al., 1994] and confirmed in an in situ study evaluating the demineralization of deciduous enamel [Ribeiro et al., 2005]. Also, in another in situ study we have found that the combination starch+sucrose could be more cariogenic than sucrose alone [Aires et al., 2008]. The biological basis explaining how starch could enhance the cariogenic potential of sucrose was properly revised [Bowen and Koo, 2011]. Furthermore, using an *S. mutans* biofilm model, we clearly showed that the combination caused higher demineralization in enamel and root dentine than sucrose alone [Botelho et al., forthcoming].

Fluoride from toothpaste is considered very effective to reduce enamel demineralization provoked by sucrose exposure [Duggal et al., 2001; Paes Leme et al., 2004; Ccahuana-Vásquez et al., 2007; Cenci et al., 2008; Botelho et al., forthcoming] but the effect on dentine is still few explored [Kusano et al., 2011; Vale et al., 2011; Botelho et al., forthcoming]. Also, the effect of fluoride on the cariogenicity of the combination of starch+sucrose is unknown.

Therefore, trying to give additional contribution to this subject, in the present in situ study we evaluated the effect of fluoride on the cariogenic potential of the combination starch+sucrose on enamel and dentine.

MATERIAL AND METHODS

Ethical Aspects and Volunteers

This study was approved by the Research and Ethics Committee of Piracicaba Dental School (Protocol No. 104/2011). Volunteers signed an informed, written consent to participate (Resolution No. 466 of the National Health Council, Health Ministry, Brasília, DF, 12/12/2012).

Fourteen volunteers (21–31 years old; DMFS of 6.2 ± 8.7), who fulfilled inclusion criteria (normal salivary flow rate, good general and oral health with no active caries lesions or periodontal treatment needs, not having used antibiotics 2

months prior to this study, and not using fixed or removable orthodontic devices) and followed all recommendations, participated of this study.

Experimental design

The study involved a factorial 2 x 4 design. The factors under evaluation were toothpaste in two levels, non-fluoride toothpaste (NFT) and fluoride toothpaste (FT, 1.100 µg F/g, NaF/silica-based); and treatments in four levels, T1) purified water (control); T2) 2% starch; T3) 10% sucrose; and T4) 2% starch + 10% sucrose (starch+sucrose). During four phases of 14 days each, the volunteers wore acrylic palatal appliances containing two enamel and two root dentine slabs on each side (Fig. 1a). In each phase, one of the toothpastes was used 3 times/day and two of the treatments were made 8 times/day (Fig. 1b), resulting in the following combinations: NFT + T1 and T3; FT + T1 and T3; NFT + T2 and T4; and FT + T2 and T4. The volunteers were randomly assigned to the different combinations (Fig. 2). The use of two treatments (split-mouth design) in the same appliance is supported by previous studies [Cury et al. 2001; Paes Leme et al., 2004; Pecharki et al., 2005; Ribeiro et al., 2005]. After each phase, the biofilms formed on the slabs were collected and pooled for biochemical analysis. Enamel and dentine demineralization was assessed by surface hardness (SH) and cross-sectional hardness, estimating surface hardness loss (%SHL) and the integrated area of hardness loss as a function of lesion depth (ΔS), respectively. For the statistical analysis, each volunteer was considered as an experimental unit (n = 14) and enamel and dentine were independently analyzed. This study was blind only with respect to the examiners, since the volunteers could differentiate the treatments by taste and viscosity of the solutions.

Enamel and dentine slabs and palatal appliance preparation

Enamel and dentine slabs (4 x 4 x 2 mm) were obtained from sound bovine incisors [Hara et al., 2003, for details]. Dentine slabs were immersed in artificial saliva [Delbem and Cury, 2002] for 24 h to minimize further ionic changes

[Aires et al., 2002]. The baseline surface hardness (SH) was determined by making 3 indentations, spaced 100 μm from each other, using a Future-Tech FM hardness tester coupled to FM-ARS 900 software (Future-Tech Corp., Kawasaki, Kanagawa, Japan) with Knoop loads of 50 and 5 g for enamel and dentine, respectively. Before the SH measurements, the dentine slabs were allowed to dry for at least 30 min, a period required to minimize interference with the SH analysis [Vale et al., 2011]. For each phase of the study, enamel and dentine slabs with known SH values were selected, randomly assigned to the treatment groups, and mounted into palatal appliances for each phase of the study, as previously described [Hara et al., 2003, for details]. The plastic meshes were fixed with colorless or red acrylic resin over the cavities containing the slabs (Fig. 1b) indicating where each treatment should be made [Cury et al., 2001; Paes Leme et al., 2004, Pecharki et al., 2005; Ribeiro et al., 2005].

Treatments

During the lead-in and washout periods of 7 days each, the volunteers brushed their natural teeth with NFT. During entire experiment, they drank and consumed foods prepared with optimally fluoridated water (0.6–0.8 mg F/L for the region), and received instructions as previously described [Cury et al., 2000].

The carbohydrate concentration used was the same as that previously described [Ribeiro et al., 2005; Aires et al., 2008]. The starch solution was prepared from soluble starch (80% amylopectin and 20% amylose; Sigma Chemical Co., St. Louis, MO, USA). To prepare 2% starch and 2% starch + 10% sucrose, the suspensions were boiled up to complete dissolution. All solutions were autoclaved and stored at room temperature. Three days a week (Mon, Wed and Fri), the solutions were aseptically transferred to codified vials to be used by the volunteers.

For the carbohydrate treatment (Fig. 3a), the volunteers were instructed to remove the device, drip one drop onto each slab 8 times/day (8:00, 9:00, 10:00, 11:00, 13:30, 15:00, 17:00 and 19:00 h), wait for 5 min, remove the excess fluid with gauze, and re-insert the device in the mouth.

The toothpaste treatment (Fig. 3b) was carried out after the main mealtimes, 3 times a day, when volunteers brushed their teeth and the appliance with the assigned toothpaste, taking care to only apply the slurry formed on the slabs area of the appliance. After that, the device was washed gently in tap water and re-inserted in the mouth.

Polysaccharide analysis

On the 14th day of each phase, approximately 10 h after the last toothbrushing procedure, the biofilms formed on the enamel and dentine slabs of each side of the appliance were homogenized, transferred to sterile microcentrifuge tubes and weighed (± 0.01 mg) (Analytical Plus AP 250D, Ohaus Corp., Florham Park, NJ, USA). Next, 1 mL of 0.9% NaCl solution was added to the tubes, sonicated for 1 min at 7 W (Branson, Sonifier 50, Danbury, CT, USA) [Aires et al., 2008]. An aliquot of 800 µL of this suspension was used to extract and determine the concentration of soluble and insoluble extracellular polysaccharides (EPS) in the biofilm [Aires et al., 2008]. The results were expressed as µg/mg of biofilm wet weight.

Enamel and dentine analysis

After each phase, SH of the slabs was again measured with one row of three adjacent indentations spaced 100 µm apart from the three baseline measurements or in the center of the slabs if the initial indentations were not visible. A mean value from the three indentations was obtained for each slab. A mean value considering two slabs for each dental substrate (enamel and dentine) was used to calculate the percentage of surface hardness loss (%SHL = (Baseline SH value – Final SH value) x 100/ Baseline SH value).

After SH analysis, all the slabs were longitudinally sectioned through the center for cross-sectional hardness determination which was made using a 25 and 5 g load for 5 s, respectively, for enamel and dentine. From the outer surface, three rows of 13 indentations each were made at 10, 20, 30, 40, 50, 60, 80, 100, 120, 140,

160, 180, 200 and 300 μm. The mean values (kg/mm^2) of the three rows at each distance from the surface were then averaged. A mean value considering two slabs for each dental substrate (enamel and dentine) were obtained and the area of hardness loss versus lesion depth (ΔS) was calculated by numerical integration using the trapezoidal rule by the difference between the area under the curve (kg/mm^2 x μm) of the sound substrate (enamel and dentine) minus the area of the demineralized one [Cury et al., 2010].

Statistical Analysis

Data for enamel and dentine were independently analyzed. A factorial 2 x 4 was considered for the statistical analyses, and the factors under evaluation were: toothpaste at 2 levels (NFT and FT), and treatments at 4 levels (water, starch, sucrose and starch+sucrose). For all analyses, the experimental unit considered was the volunteer. The assumptions of equality of variances and normal distribution of errors were checked for all response variables tested, and those that did not satisfy were transformed [Box et al., 2005]. All data were analyzed by ANOVA followed by Tukey's test. The analysis was done with SAS software system (version 9.0; SAS Institute Inc., Cary, NC, USA) with a significance level fixed at 5%.

RESULTS

For polysaccharide concentration (Table 1), was found effect statiscally significant ($p < 0.0001$) only for the factor treatment with carbohydrates. The concentration of soluble and insoluble EPS found in biofilms treated with sucrose and starch+sucrose was higher ($p < 0.05$) than starch and control groups, but did not differ from each other ($p > 0.05$).

The data of enamel demineralization (Table 2) evaluated either by %SHL or ΔS showed statistically significant effect for the factors toothpaste and treatment ($p < 0.05$ and $p < 0.0001$, respectively), but the effect of the interaction was not significant ($p > 0.05$). For %SHL and ΔS, the values were higher ($p < 0.05$) for

sucrose and starch+sucrose groups, which did not differ from each other ($p = 0.2655$ and $p = 0.2527$, respectively). The profile of hardness data at each depth from the dental surface are illustrated in figure 4, for slabs exposed to non-fluoride (a) and fluoride (b) toothpastes.

In relation to dentine demineralization (Table 3), the effects of toothpaste and carbohydrate were significant for %SHL and ΔS ($p < 0.05$), with no interaction between these factors ($p > 0.05$). For %SHL, the values for sucrose and starch+sucrose groups were higher ($p < 0.0001$) than the other groups, but did not differ between them ($p = 0.2008$). The %SHL for starch group was higher ($p < 0.0001$) than for the control group. In ΔS analysis, the greatest area of hardness loss was found for starch+sucrose group ($p < 0.05$), and all groups differed among them ($p < 0.05$). The figure 5 illustrates the hardness data at each depth from the dental surface for slabs exposed to non-fluoride (a) and fluoride (b) toothpastes.

DISCUSSION

Starch could increase the cariogenic potential of sucrose because the combination of these dietary carbohydrates modulates the pathogenesis of dental caries [Bowen and Koo, 2011], but the effect of fluoride on demineralization of enamel and dentine has not been explored and it could not be the same considering the different properties of these dental mineral substrates.

Our findings showed that fluoride toothpaste at concentration of 1100 μg/g clearly reduced demineralization either of enamel or dentine (Table 2, 3), regardless the carbohydrates to which the biofilms formed on these dental substrates were exposed. This effect was found either evaluating demineralization on surface (%PDS) or by the area of caries lesion (ΔS). Regarding the effect on enamel (Table 2), the overall results show that fluoride toothpaste reduced enamel demineralization in 39% when the mean of %PDS values for the groups exposed to starch, sucrose and starch+sucrose and treated with fluoride toothpaste are compared with the mean of these groups treated with non-fluoride toothpaste. For

ΔS, the reduction of demineralization was 18%. These percentages of reduction of enamel demineralization by the effect of fluoride toothpaste were of same magnitud irrespective of the cariogenic challenge induced by the different sugars. Thus, although the biofilm exposed to starch+sucrose provoked higher demineralization on enamel than starch and sucrose, the effect of fluoride toothpaste was the same. The effect of fluoride toothpaste on reduction of enamel demineralization induced by sucrose was known [Dugall et al., 2001; Paes Leme et al., 2004; Ccahuana-Vásquez et al., 2007; Cenci et al., 2008] but the present results show that this effect is the same either a less cariogenic carbohydrate as starch is used or a more cariogenic case of starch+sucrose is used. The present findings give support to systematic reviews showing evidence that toothpaste containing 1100 µg F/g is effective to control caries in enamel [Marinho et al., 2003; Walsh et al., 2010] in individuals exposed to different dietary carbohydrates.

About the effect of fluoride toothpaste on dentine, the findings are in agreement with our previous studies showing that toothpaste contaning 1100 µg F/g is effective to reduce dentine demineralization [Hara et al., 2003; Cenci et al., 2008; Vale et al., 2011; Kusano et al., 2011; Botelho et al., forthcoming]. However, the reduction of demineralization found in the present study was lower than that observed for enamel (19% for PDS% and 14% for ΔS). This lower efficacy of fluoride toothpaste on reduction of dentine demineralization compared with the effect in enamel, confirm our previous results found [Kusano et al., 2011] and gives support to the recommendation that a high fluoride toothpaste (5000 µg F/g) should be preferable to control root caries than 1100 µg F/g [Baysan et al., 2001; Ekstrand et al., 2008]. On the other hand, our findings showed that fluoride toothpaste at 1100 µg F/g was effective to reduce equally dentine demineralization, regardless the biofilm was exposed to starch, sucrose or the combination starch+sucrose.

With respect to the effect of the treatments with the carbohydrates on biofilm composition the lower concentrations of extracellular polysaccharides found in starch group compared with sucrose and starch+sucrose (Table 1) agrees with our previous studies [Ribeiro et al., 2005; Aires et al., 2008]. This result gives support

to the lower demineralization found for starch group and confirmes that starch can be considered non-cariogenic for enamel [Firestone et al., 1982; Ribeiro et al., 2005] and sligthly-cariogenic for dentine [Lingström et al., 2000] when it is consumed as the sole carbohydrate source. On the other hand, in agreement with our previous studies, the present findings suggest that, although differences statistically significant were not found for all variables analyzed, starch could increase the cariogenic potential of sucrose. Therefore, the overall data either for enamel or dentine, evaluating demineralization either by surface or cross-sectional hardness (Tables 2 and 3), the profile of caries lesions (Fig. 4, 5) and the biofilm composition (Table 1), all showed a trend that the combination starch+sucrose could be more cariogenic than sucrose alone.

In conclusion, the findings suggest that fluoride toothpaste is effective to reduce enamel or dentine demineralization, regardless the cariogenic challenge provoked by starch, sucrose or the combination starch+sucrose.

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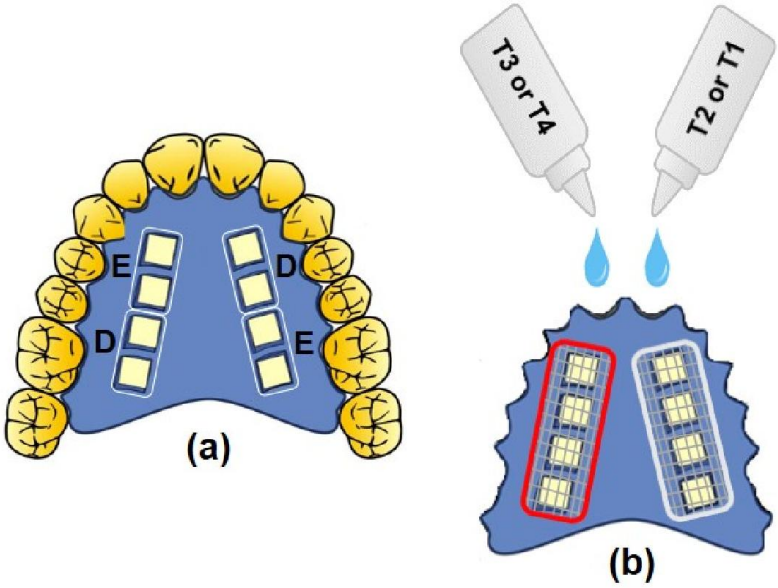

Fig. 1. Schematic illustration of the distribution of slabs in palatal appliance (a) and treatments done by volunteers (b). D: dentine slabs; E: enamel slabs; T1) purified water; T2) 2% starch; T3) 10% sucrose; T4) 2% starch+10% sucrose.

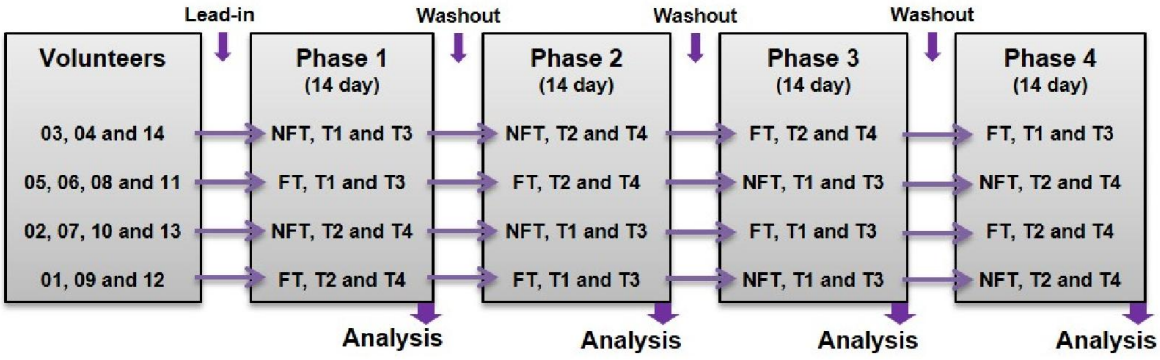

Fig. 2. Schematic illustration of the random distribution of volunteers for toothpastes and treatments. NFT: non-fluoride toothpaste; FT: fluoride toothpaste; T1) purified water; T2) 2% starch; T3) 10% sucrose; T4) 2% starch+10% sucrose.

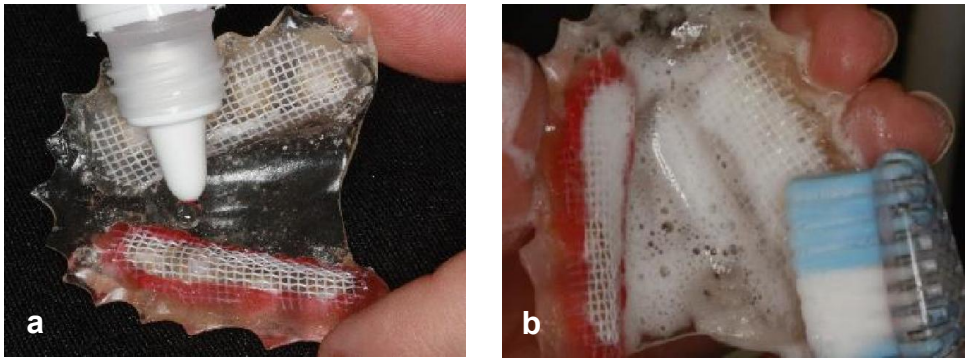

Fig. 3. Extra-oral treatments with solutions 8 times/day (a) and toothpaste use made 3 times/day (b).

Table 1. Means (±SD; n) of extracellular polysaccharide concentration (μg/mg wet weight) in dental biofilms according to the toothpastes and treatments.

Toothpaste groups	Treatment groups	Extracellular polysaccharides (EPS)		
		n	soluble	Insoluble
Non-fluoride	**Purified water**	8	1.3 ± 0.9^{a}	1.4 ± 0.8^{a}
	2% starch	13	1.4 ± 1.0^{a}	2.6 ± 1.8^{a}
	10% sucrose	11	4.5 ± 3.8^{b}	11.7 ± 8.9^{b}
	2% starch + 10% sucrose	13	5.5 ± 5.3^{b}	14.5 ± 8.4^{b}
Fluoride	**Purified water**	11	2.5 ± 2.2^{a}	2.0 ± 1.3^{a}
	2% starch	11	1.9 ± 1.2^{a}	2.6 ± 1.7^{a}
	10% sucrose	14	4.2 ± 2.1^{b}	11.4 ± 6.8^{b}
	2% starch + 10% sucrose	13	3.5 ± 2.4^{b}	14.5 ± 8.4^{b}

For statistical analysis, data of soluble EPS were transformed by the power of $(X)^{0.3}$; insoluble EPS were transformed by the $\log_{10}$ (X). Treatments whose means are followed by distinct letters differ significantly when non-fluoride or fluoride toothpaste was used ($p < 0.05$).

Table 2. Means (± SD) of surface hardness loss (%SHL) and area of hardness loss (ΔS) in enamel slabs according to the toothpastes and treatments (n = 14).

Toothpaste groups*	Treatment groups	%SHL	ΔS
Non-fluoride	**Purified water**	7.6 ± 3.4[a]	4274.5 ± 1284.6[a]
	2% starch	18.5 ± 22.9[b]	5190.8 ± 2347.6[a]
	10% sucrose	38.5 ± 27.1[c]	7856.6 ± 5024.5[b]
	2% starch + 10% sucrose	51.5 ± 21.3[c]	9857.9 ± 4957.3[b]
Fluoride	**Purified water**	5.8 ± 3.5 [a]	3603.6 ± 1606.2[a]
	2% starch	10.9 ± 11.3[b]	4300.4 ± 1628.8[a]
	10% sucrose	23.8 ± 16.9[c]	6502.5 ± 2690.2[b]
	2% starch + 10% sucrose	31.5 ± 16.6[c]	7895.6 ± 3452.6[b]

For statistical analysis, %SHL and ΔS were transformed in $\log_{10}$ (X). *The effect of toothpaste was significant ($p = 0.0023$ and $p = 0.0445$ for %SHL and ΔS, respectively). Treatments whose means are followed by distinct letters differ significantly when non-fluoride or fluoride toothpaste was used ($p < 0.05$).

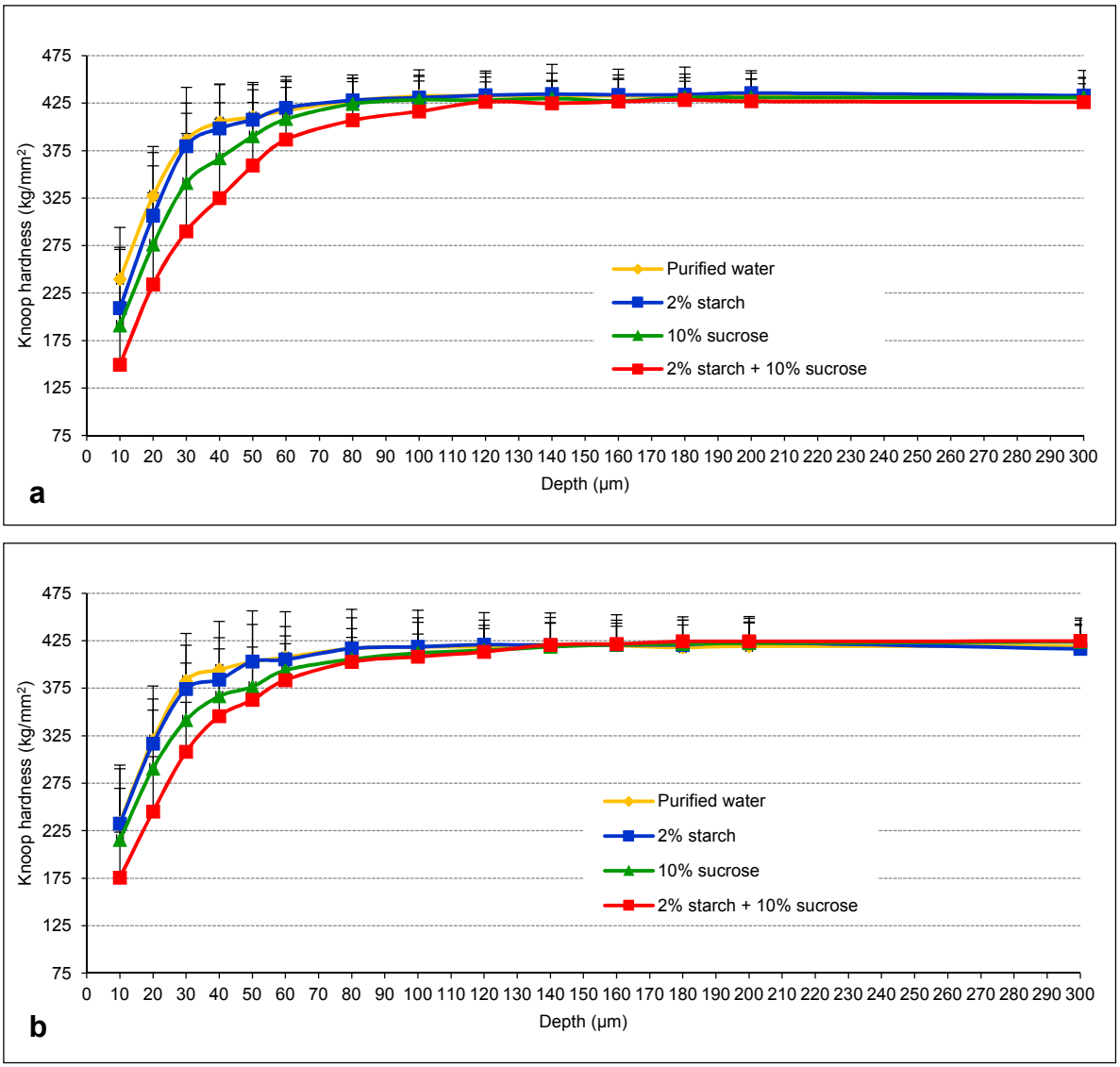

Fig. 4. Means of enamel Knoop hardness (kg/mm^2) for non-fluoride (a) and fluoride (b) toothpaste groups according to the treatments and the distance (µm) from the surface (bars denote SD; n = 14).

Table 3. Means (± SD) of surface hardness loss (%SHL) and area of hardness loss (ΔS) in dentine slabs according to the toothpastes and treatments (n = 14).

Toothpaste groups*	Treatment groups	%SHL	ΔS
Non-fluoride	**Purified water**	17.3 ± 11.1^{a} (n = 13)**	268.1 ± 183.1^{a} (n = 14)
	2% starch	36.9 ± 18.2^{b} (n = 13)**	410.4 ± 250.1^{b} (n = 14)
	10% sucrose	54.3 ± 13.2^{c} (n = 14)	1106.9 ± 703.5^{c} (n = 14)
	2% starch + 10% sucrose	63.5 ± 8.0^{c} (n = 14)	1827.8 ± 592.7^{d} (n = 14)
Fluoride	**Purified water**	13.1 ± 6.6^{a} (n = 14)	216.1 ± 151.4^{a} (n = 14)
	2% starch	31.2 ± 20.4^{b} (n = 14)	348.2 ± 201.5^{b} (n = 14)
	10% sucrose	44.6 ± 11.1^{c} (n = 14)	1168.2 ± 654.2^{c} (n = 14)
	2% starch + 10% sucrose	48.4 ± 11.8^{c} (n = 14)	1343.5 ± 694.8^{d} (n = 14)

For statistical analysis, ΔS was transformed in power of $(X)^{0.2}$. *The effect of toothpaste was significant ($p = 0.0006$ and $p = 0.0591$ for %SHL and ΔS, respectively). Treatments whose means are followed by distinct letters differ significantly when non-fluoride or fluoride toothpaste was used ($p < 0.05$). **Values indicated by the SAS Software as outliers were removed: purified water = 51.3 and 2% starch = 62.3.

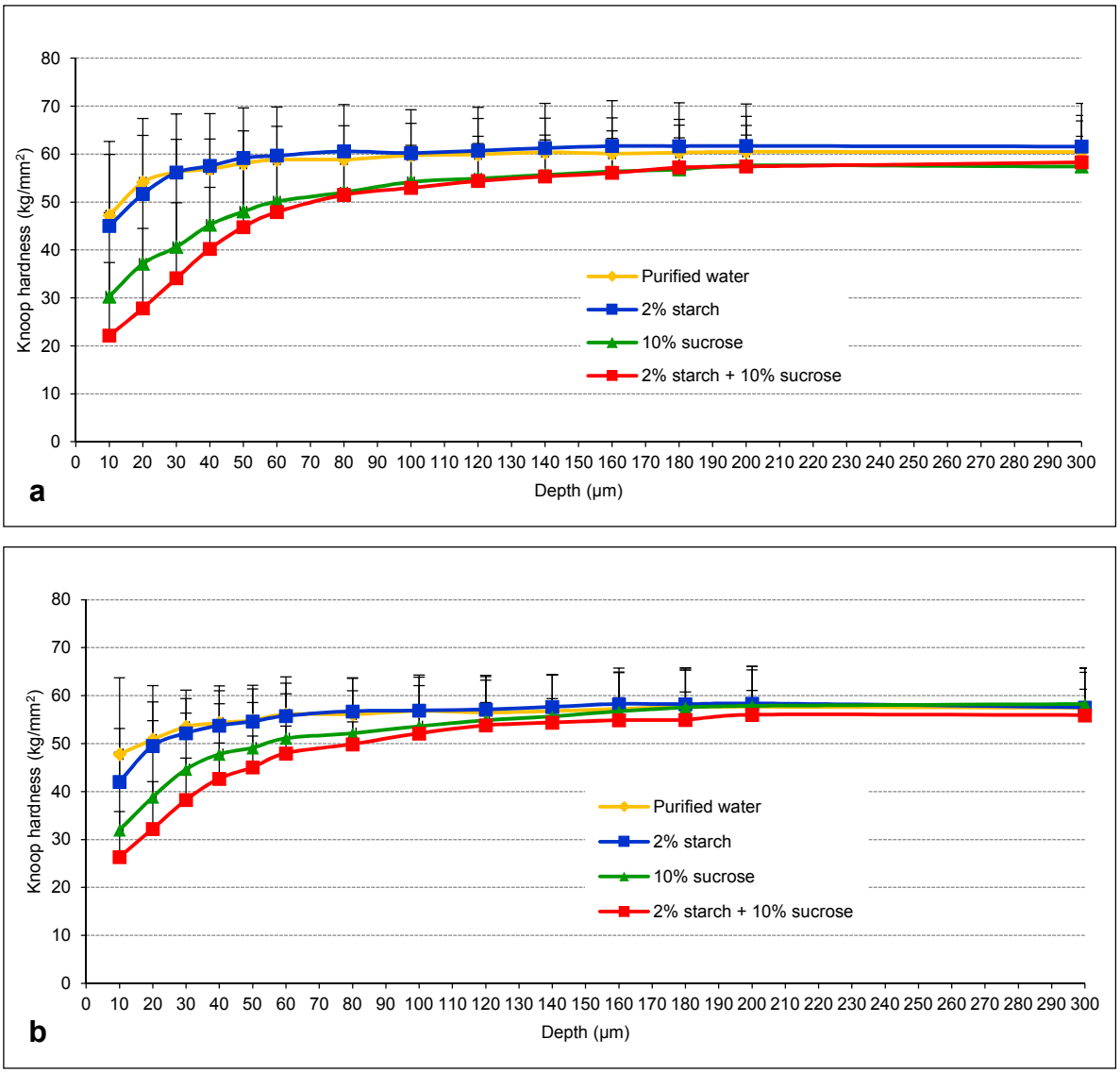

Fig. 5. Means of dentine Knoop hardness (kg/mm^2) for non-fluoride (a) and fluoride (b) toothpaste group according to the treatments and the distance (µm) from the surface (bars denote SD; n = 14).

CONCLUSÃO

Os resultados sugerem que o amido deve aumentar o potencial cariogênico da sacarose, e que dentifrício contendo 1.100 µg F/g é efetivo em reduzir a desmineralização de esmalte e dentina, independente do desafio cariogênico ser provocado por amido, sacarose ou pela combinação desses carboidratos.

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CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Efeito da associação de sacarose e amido na desmineralização do esmalte e da dentina radicular e nas propriedades do biofilme dental formado"**, protocolo nº 104/2011, dos pesquisadores Jaime Aparecido Cury, Altair Antoninha Del Bel Cury, Juliana Nunes Botelho e Lívia Maria Andaló Tenuta, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 23/09/2011.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Effect of sucrose and starch on enamel and dentine demineralization and on biofilm formation and composition"**, register number 104/2011, of Jaime Aparecido Cury, Altair Antoninha Del Bel Cury, Juliana Nunes Botelho and Lívia Maria Andaló Tenuta, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 09/23/2011.

Profa. Dra. Lívia Maria Andaló Tenuta
Secretária
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

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

ANEXO 1 - Certificado do Comitê de Ética em Pesquisa

ANEXO 2 – Confirmação de aceite do artigo

Gmail by Google

Juliana Botelho <jnunesb@gmail.com>

Brazilian Oral Research - Decision on Manuscript ID BOR-2013-0298.R1

sigmarrode@uol.com.br <sigmarrode@uol.com.br> 25 de novembro de 2013 16:51
Para: jnunesb@gmail.com

25-Nov-2013

Dear Miss Botelho:

It is a pleasure to accept your manuscript entitled "(ABOPREV) Effect of fluoride toothpaste on root dentine demineralization progression - A pilot study" in its current form for publication in the Brazilian Oral Research. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of the Brazilian Oral Research, we look forward to your continued contributions to the Journal.

Sincerely,
Prof. Sigmar Rode
Editor-in-Chief, Brazilian Oral Research
sigmarrode@uol.com.br

Associate Editor Comments to Author:

Associate Editor
Comments to the Author:
(There are no comments.)

ANEXO 3 – Confirmação de submissão do artigo

Juliana Botelho <jnunesb@gmail.com>

[Fwd: Caries Research - Manuscript ID 201401021]

jcury@fop.unicamp.br <jcury@fop.unicamp.br> 29 de janeiro de 2014 18:44
Para: jnunesb@gmail.com, mario_villegas@hotmail.com, ptroncoso@alumnos.utalca.cl, rgiacamans@gmail.com

---------- Mensagem Original ----------
Assunto: Caries Research - Manuscript ID 201401021
De: david.beighton@kcl.ac.uk
Data: Qua, Janeiro 29, 2014 5:12 pm
Para: jcury@fop.unicamp.br

29-Jan-2014

Dear Prof. Cury:

Your manuscript entitled "Effect of the combination of starch and sucrose on S. mutans biofilm composition, and on enamel and root dentine demineralization" has been successfully submitted online and is presently being given full consideration for publication in Caries Research.

Your manuscript ID is 201401021.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to Manuscript Central at http://mc.manuscriptcentral.com/cre and edit your user information as appropriate.

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Thank you for submitting your manuscript to Caries Research.

Yours sincerely,

Prof. David Beighton
Caries Research

david.beighton@kcl.ac.uk