CAROLINA PATRÍCIA AIRES

EFEITO *IN VITRO* E *IN SITU* DO AMIDO E SUA COMBINAÇÃO COM SACAROSE NA COMPOSIÇÃO DO BIOFILME E NA DESMINERALIZAÇÃO *IN SITU* DA DENTINA RADICULAR

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do Título de Doutor em Odontologia, área de concentração em Cariologia.

Orientador: Prof Dr Jaime Aparecido Cury
Co-orientador: Prof Dr Hyun Koo

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RESUMO

Sacarose é o mais cariogênico dos carboidratos da dieta e há evidências que sua cariogenicidade aumenta quando associada ao amido. Isso seria explicado pelas mudanças na matriz do biofilme formado, pois de acordo com estudo *in vitro* um "novo" polissacarídeo extracelular (EPS) é sintetizado pela glicosiltransferase (GTF) B de *S. mutans* a partir de sacarose e hidrolisados do amido. Entretanto, isso precisa ser confirmado em biofilmes, pois o efeito desse "novo" polissacarídeo pode não ser evidenciado quando da ação simultânea das diferentes GTFs de *S. mutans* e de outras bactérias. Em acréscimo, a maior cariogenicidade da associação amido-sacarose é conhecida para o esmalte, mas não para dentina. Na realidade, não há evidência nem mesmo se amido isoladamente seria cariogênico para dentina e cárie radicular é uma preocupação atual considerando o declínio de cárie, maior retenção de dentes e o aumento da expectativa de vida das populações. Assim, o objetivo da presente tese foi estudar a influência da associação amido-sacarose em biofilmes *in vitro* e *in situ*. *In vitro*, biofilmes de *S. mutans* foram formados sobre discos de hidroxiapatita na presença de amido, sacarose ou associações. *In situ*, a cariogenicidade da associação amido-sacarose foi testada através de um estudo cruzado e em cada fase blocos de dentina radicular foram submetidos extraoralmente a tratamentos com soluções de amido, sacarose ou suas combinações. Os biofilmes formados *in vitro* e *in situ* foram analisados quanto às suas composições bioquímicas e microbiológicas, enquanto que cárie dentinária foi avaliada somente *in situ*. O biofilme de *S. mutans* formado *in vitro* na presença da associação amido-sacarose mostrou composição distinta em EPS em relação àquele formado na presença dos açúcares isolados, porém o mesmo não foi evidenciado *in situ*. Quanto à expressão dos genes *gtfBCD*, resultados preliminares promissores foram encontrados mas aparentemente distintos para as condições *in vitro* e *in situ*. Amido se mostrou levemente cariogênico para dentina em comparação com sacarose, mas a associação amido-sacarose não foi mais cariogênica que sacarose isolada. Conclui-se que embora haja evidências que a associação amido-sacarose possa aumentar a cariogenicidade do biofilme formado, isso não foi confirmado *in situ* para dentina radicular, pois a associação não se mostrou mais cariogênica que sacarose isolada.

Palavras-chave: biofilme, amido, sacarose, dentina, desmineralização

ABSTRACT

Sucrose is the most cariogenic of the dietary carbohydrates but there is evidence that its cariogenicity is enhanced when used in combination with starch. This could be explained by changes in biofilm matrix, since an *in vitro* study showed that a novel extracellular polysaccharide (EPS) is synthesized by glicosiltransferase (GTF) B of *S. mutans* in presence of sucrose and starch hydrolysates. However, this should be confirmed in biofilms since the effect of this novel polysaccharide could not be evidenced when different GTFs from *S. mutans* and from other bacteria are synthesized simultaneously. In addition, the cariogenicity of starch-sucrose combination is well established for enamel but not for dentine. Also, evidence is still lacking on the cariogenicity of starch for root dentine, and root caries is a current concern considering the decline of caries, higher tooth retention and the increase of the life expectancy for populations. Thus, the aim of this study was to evaluate the influence of starch, sucrose and their combinations in *in vitro* and *in situ* biofilms. *In vitro*, *S. mutans* biofilms were formed on hydroxyapatite discs in presence of starch, sucrose or their combination. *In situ,* the cariogenicity of starch-sucrose association was tested by a crossover study and during each phase root dentine slabs were submitted extraorally to solutions of starch, sucrose or starch-sucrose association. *In vitro* and *in situ* biofilms were analyzed for biochemical and microbiological composition, and root dentine caries was evaluated *in situ*. *In vitro* biofilms formed in presence of starch-sucrose association showed distinct EPS composition in relation to those formed in the presence of isolated sugars, but the same trend was not evidenced *in situ*. In relation to gtfBCD expression, promising preliminary results were found, but they were distinct for *in vitro* and *in situ* studies. Starch was moderately cariogenic for dentine in comparison to sucrose but starch-sucrose association was not more cariogenic than sucrose alone. In conclusion, although starch-sucrose association could enhance the cariogenicity of the biofilm formed, this trend was not confirmed *in situ* for root dentine since the association was not more cariogenic than sucrose alone.

Key-words: biofilm, starch, sucrose, dentine, demineralization

SUMÁRIO

INTRODUÇÃO GERAL

Cárie pode ser atualmente conceituada como uma doença biofilme-açúcar dependente e o conhecimento da importância dos carboidratos da dieta na manifestação clínica da doença tem evoluído dos estudos populacionais aos moleculares.

Entre os carboidratos (açúcares) da dieta, sacarose é considerada o mais cariogênico (Paes Leme *et al*., 2006, como revisão), entretanto isso só foi demonstrado de forma insofismável quando seu poder cariogênico foi experimentalmente comprovado em comparação com seus monossacarídeos componentes (Cury *et al.,* 2000). Assim, sacarose é tão fermentável a ácidos quanto glicose e frutose (Tenuta *et al.,* 2006), entretanto ela é o único carboidrato da dieta usado para formar um biofilme tendo uma matriz rica em polissacarídeos extracelulares (PEC) tipo glucano (Rölla *et al*., 1985; Cury *et al.,* 2000).

Assim, no contexto atual de cárie como doença biofilme-açúcar dependente, precisa ficar bem clara a implicação da dieta. No passado recente, todo açúcar que fosse fermentável seria considerado cariogênico, pois os sinais das lesões da doença eram considerados como simples conseqüência da dissolução acumulativa dos minerais dos dentes pelos ácidos de origem bacteriana. Essa maneira simplista mudou drasticamente com os conceitos de que o pH decorrente da fermentação dos açúcares também é relevante para a formação de um biofilme mais cariogênico pela seleção de bactérias tipo estreptococos mutans e lactobacilos que sobrevivem e predominam num meio ácido (Marsh, 2003). Em acréscimo, a quantidade e tipo de nutriente são relevantes no tipo de biofilme formado (Bowden & Li, 1997).

Nesse aspecto, quando sacarose é metabolizada pelas bactérias dos biofilmes dentais, o pH resultante não só seleciona no biofilme as bactérias mais cariogênicas (Bradshaw & Marsh, 1998) como o ácido produzido é suficiente para desmineralizar, não só a dentina (Aires *et al*., 2002) como o esmalte de dentes permanentes (Cury *et al*., 2000) ou decíduo (Ribeiro *et al.,* 2005). Entretanto, acidogenicidade pode diferenciar a sacarose de outros açúcares menos fermentáveis como lactose (Aires *et al*., 2002) amido (Ribeiro *et al.,* 2005), mas não a diferencia de glicose e frutose (Tenuta *et al* 2006; Vale *et al.,* 2007). Por outro lado, quando sacarose é hidrolisada por enzimas bacterianas chamadas de glicosiltransferases (GTF), enquanto uma das unidades desse dissacarídeo é fermentada a

ácidos promovendo queda de pH no biofilme, a outra é polimerizada como PECs tipo glucano ou frutano (Hamada & Slade, 1980; Rölla *et al*., 1985). Os PECs tipo glucano mudam a estrutura e propriedades dos biofilme formados e os insolúveis conferem maior porosidade ao biofilme dental formado (Dibdin & Shellis, 1988), pois permitem a penetração de substratos acidogênicos para as camadas mais internas do biofilme e, conseqüentemente produção de ácidos próximo à estrutura dental (Zero *et al*., 1986). Assim, considerando que a solubilidade da estrutura dental aumenta com o decréscimo de pH (Dawes, 2003), a formação de um biofilme dental poroso seria um fator determinante da cárie dental.

Os PECs são considerados um dos principais fatores de virulência de bactérias cariogênicas, como o *Streptococcus mutans* (Hamada & Slade, 1980; Yamashita *et al*., 1993; Bowen, 2002). Os *Streptococcus mutans* produzem no mínimo 3 GTFs: GTFB, GTFD e GTFC. A GTFB sintetiza a partir de sacarose PECs insolúveis tipo glucano com ligações predominantemente α-(1→3), enquanto a GTFD é responsável pela produção de PECs solúveis com ligações α-(1→6) e a GTFC produz uma mistura de PECs insolúveis e solúveis (Hanada & Kuramitsu, 1989). Os PECs solúveis atuam como reserva energética e podem ser metabolizados pelas bactérias do biofilme, enquanto os PEC insolúveis estão relacionados com a aderência de bactérias à estrutura dental e com a aderência de bactérias entre si, o que contribui para a formação e acúmulo de biofilme (Jenkinson & Lamont, 1997). A importância de cada um destes polissacarídeos foi demonstrada em um modelo de estudo animal utilizando mutantes de *Streptococcus mutans* (Yamashita *et al*., 1993). Estes autores verificaram que cepas mutantes que não apresentavam os genes para a síntese de PEC insolúvel, genes *gtf*B e *gtf*C, exibiram níveis reduzidos de lesão de cárie em ratos se comparados àquelas apresentadas pelas cepas selvagens. Evidências para a associação entre PEC insolúvel e cárie tem sido encontradas (Cury *et al.,* 2000; Mattos-Granner *et al,* 2000; Nobre dos Santos *et al*., 2002).

A relação entre sacarose e cárie dental tem sido extensivamente estudada em condições experimentais controladas avaliando a composição do biofilme formado (Paes Leme *et al.,* 2006 como revisão; Ccahuana-Vásquez *et al*., 2007; Vale *et al*.,2007). Esses estudos sobre o efeito da freqüência e concentração da sacarose na estrutura da matriz do

biofilme necessariamente deverão chegar ao nível molecular avaliando *in vivo* a expressão dos gens responsáveis pela síntese da matriz rica em PECs. Nesse aspecto, a literatura mostra resultados *in vitro* conflitantes sobre o efeito de açúcares na expressão das *gtfs*, ativando (Li & Burne 2001) ou reprimindo (Fujiwara *et al*., 2002; Shemesh *et al*., 2007). Em acréscimo, os limitados dados existentes devem ser conseqüência da dificuldade de extrair RNA de biofilmes apresentando uma matriz rica em PECs (Cury & Koo, 2007; Cury *et al*., 2008).

Por outro lado, o amido não é substrato para a formação de PEC tipo glucano e em acréscimo é fermentado lentamente pelo biofilme dental gerando uma queda de pH não tão acentuada quando comparado com sacarose (Jensen & Schachtele, 1983; Birkhed e Skude, 1978; Ribeiro *et al.,* 2005). Esse pequeno decréscimo de pH explica resultados em animais (Hefti & Schmid, 1979), estudo controlado em humanos (Gustafsson *et al*., 1954), dados epidemiológicos (Newbrun *et al*., 1980) e experimentos *in situ* (Lingström *et al*., 1994) porque esse carboidrato da dieta não tem sido considerado cariogênico para o esmalte de dentes permanentes ou mesmo decíduo (Ribeiro *et al*., 2005). Entretanto, o pH crítico para desmineralização da dentina (~6,5) é maior que do esmalte (~5,5) (Hoppenbrouwers *et al*., 1987) e assim uma queda de pH não tão acentuada pode levar ao desenvolvimento de lesões de cárie dentinária. Por outro lado, isso ainda não foi comprovado de forma incontestável, pois os resultados do único trabalho experimental realizado não foram convincentes (Lingström *et al*., 1994).

A menor cariogenicidade do amido em relação à sacarose pode também ser devido ao seu alto peso molecular, pois moléculas com tamanho ≥10 kD se difundem com dificuldade pela matriz de biofilmes ricos em PECs (Thurnheer *et al*., 2003). Isso seria uma explicação para o baixo potencial cariogênico do amido em relação à carboidratos de baixo peso molecular, como a sacarose (Guggenheim *et al*. 2004). Entretanto, o amido é facilmente degradado pela amilase salivar (Karn & Malacinski, 1978) e compostos de menor peso molecular, chamados de hidrolisados de amido, podem ser fermentados a ácidos por bactérias orais (Clarkson *et al*., 1987). Esses fatores explicariam porque a queda de pH encontrada no biofilme dental não é tão acentuada.

Por outro lado, os hidrolisados de amido podem atuar como aceptores para a formação de PECs (Vacca-Smith *et al*., 1996) quando sacarose sofre ação das GTFs, agindo como substratos para receber as unidades de glicose transferidas. Em acréscimo, um aumento na aderência de *S. mutans* e *Actinomyces* à hidroxiapatita, além de aumento na da síntese de PEC insolúvel pela glicosiltransferase tipo B (GTF B) tem sido observado na presença de hidrolisados de amido e sacarose (Vacca-Smith *et al*., 1996). Desta forma, na presença de hidrolisados de amido e sacarose, haveria a formação de um "novo" polissacarídeo (Vacca-Smith *et al*., 1996) que poderia conferir ao biofilme maiores propriedades cariogênicas que aquelas apresentadas por um biofilme formado na presença de sacarose isolada.

Assim, o efeito da associação amido-sacarose no desenvolvimento de cárie dental tem sido estudado porque amido é comumente usado na idade moderna juntamente com sacarose. Ratos infectados com cepas de *Streptococcus mutans* e *Actinomyces naeslundi* apresentaram maior incidência de lesões de cárie dental para o grupo amido-sacarose que para sacarose isoladamente (Firestone *et al*., 1982). Em trabalho experimental *in situ* foi observado efeito sinérgico da associação amido-sacarose na desmineralização do esmalte de dentes decíduos, pois enquanto amido não foi cariogênico a associação provocou maior desmineralização que sacarose isolada (Ribeiro *et al*., 2005).

Por outro lado, os mecanismos pelos quais a associação amido-sacarose pode ser potencialmente mais cariogênica que a sacarose ainda não foram totalmente explorados. A sugestão de que os hidrolisados de amido favorecem a formação de um "novo" polissacarídeo (Kopec *et al.,* 1997), promovem maior aderência bacteriana e maior formação de PEC na presença de sacarose foi testada *in vitro* com enzimas purificadas (Vacca-Smith *et al*., 1996). Entretanto, os polissacarídeos sintetizados por enzimas purificadas possuem propriedades distintas daqueles formados por uma mistura de GTFs (Inoue *et al*., 1982), como no caso de biofilme.

Desta forma, o objetivo do primeiro trabalho (capítulo 1) foi avaliar o efeito da associação amido-sacarose em biofilme de *S. mutans* e o do segundo (capítulo 2) foi ampliar o estudo avaliando *in situ* o biofilme formado, e ao mesmo tempo estimar seu efeito na desmineralização da dentina radicular.

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Influences of starch and sucrose on *Streptococcus mutans* biofilms

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Introduction: The combination of starch and sucrose has been shown to be potentially more cariogenic than either alone. The aim of this study was to examine the influence of starch and sucrose, alone or in combinations, on formation, polysaccharide composition, gene expression, and acidogenicity of *Streptococcus mutans* biofilms.
Methods: *S. mutans* UA159 biofilms were formed on saliva-coated hydroxyapatite (sHA) disks in batch culture for 5 days in the presence of 1% (weight/volume) starch, 1% sucrose, 1% starch plus 1% sucrose, 1% starch plus 0.5% fructose plus 0.5% glucose, or 1% sucrose plus 1% glucose.
Results: Amylase activity from sHA disks was detected up to 48 h, thereby increasing the availability of reducing sugars and acidogenicity in the early stages of biofilm development. *S. mutans* grown in the presence of sucrose alone or in combinations formed well-defined and tightly adherent biofilms comprised of mostly water-insoluble polysaccharides (INS); in contrast, the presence of starch or starch + glucose + fructose resulted in little biofilm formation with minimal amounts of INS. However, the combination of starch + sucrose produced biofilms with more biomass and acidogenicity, and a higher content of INS than those grown in sucrose or sucrose + glucose ($P < 0.05$). The INS extracted from biofilms formed in the presence of starch + sucrose displayed a higher percentage of 3-linked branching (3,4-, 3,6-, and 3,4,6-linked glucose) compared to those from biofilms grown in sucrose or sucrose + glucose. Furthermore, biofilms grown in starch + sucrose expressed significantly higher levels of *gtfB* messenger RNA levels than sucrose-grown or sucrose + glucose-grown biofilms ($P < 0.05$).
Conclusion: The combination of starch and sucrose has profound effects not only on the composition and structure of the polysaccharide matrix but also on gene expression of *S. mutans* within biofilms, which may enhance the cariogenic potential of dental biofilms.

Key words: amylase; biofilms; starch; *Streptococcus mutans*

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Dental caries results from the interaction of specific bacteria with constituents of the diet within dental biofilms known as plaque. Sucrose is considered to be the 'arch criminal' from the dietary perspective, because it is fermentable, and serves as a substrate for the synthesis of polysaccharides in dental biofilms (3, 27). In addition, starches are also an important source of fermentable carbohydrates, and are usually consumed simultaneously or interspersed with sucrose (24); starch is considered non-cariogenic or slightly cariogenic when used as the sole source of carbohydrate in the diet (24). However, combinations of starch and sucrose are potentially more cariogenic than either carbohydrate alone (2, 13, 29).

Streptococcus mutans is regarded as the primary microbial culprit of dental caries; this bacterium synthesizes extracellular polysaccharides, mostly glucans, from sucrose (and may also use starch hydrolysates as acceptors); it is acidogenic and acid-tolerant, which are critical virulence properties involved in the pathogenesis of dental caries in animals and humans (25, 28, 33, 37). Glucans promote the accumulation of microorganisms on the tooth surface, and contribute to the

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establishment of the extracellular polysaccharide (EPS) matrix, which provides bulk and structural integrity for dental biofilms, and serve as a reserve source of energy (3). The formation of EPS matrix by *S. mutans* involves the interaction of at least three glucosyltransferases (GTFs) and an endodextranase, which participate in the synthesis and degradation of glucans; these enzymes are products of the *gtfB*, *gtfC*, *gtfD*, and *dexA* genes (15, 22). *S. mutans* synthesizes glucans directly from sucrose, but not from undigested starch. However, starches can be digested by salivary α-amylases to maltose, maltodextrins, and other oligosaccharides, some of which can be acceptors during glucan synthesis (14, 36).

Enzymatically active α-amylase and GTFs have been identified in salivary pellicles formed *in vitro* and *in vivo* (1, 23, 32, 35). Furthermore, starch hydrolysates produced by salivary α-amylase bound to saliva-coated hydroxyapatite (sHA) increased the synthesis of glucans from sucrose by surface-adsorbed GTF B; the hydrolysates also affected the structure and bacterial binding sites of the glucans (36). Moreover, maltose and maltodextrins from starch hydrolysis can be metabolized into acids by mutans streptococci (6). Clearly, starch could enhance the cariogenic potential of sucrose, as indicated by previous *in vivo* and *in situ* studies (2, 14, 29); the interaction of sucrose and starch through GTF enzymes and amylase adsorbed on the tooth surface may modulate *in situ* the development of cariogenic biofilms by influencing the synthesis of the EPS at structural and molecular levels, and the availability of fermentable carbohydrates for acid production.

Thus, the explanation for the greater cariogenicity of the dietary combination of starch and sucrose may be associated with biochemical and structural changes in the biofilms. In this study, we investigated whether combinations of starch and sucrose in the presence of surface-adsorbed salivary amylase and *S. mutans*, influence biofilm formation by affecting the synthesis and structure of EPS, and expression of the *gtfB*, *gtfC*, *gtfD*, and *dexA* genes using our sHA disk biofilm model (19).

Materials and methods

Amylase activity of salivary pellicle

Hydroxyapatite discs (Clarkson Chromatography Products, Inc., South Williamsport, PA; surface area 2.7 ± 2 cm^2) were coated with filter-sterilized, GTF-free, clarified human whole saliva (10, 19). The levels of amylase in saliva were unaffected by filtration, as determined experimentally by immunodetection and direct enzyme assay as described elsewhere (35). The sHA was incubated in ultrafiltered (Amicon 10 kDa molecular weight cut-off membrane; Millipore Co., Billerica, MA) buffered tryptone yeast-extract broth (pH 7.0) containing 1% starch (soluble starch −80% amylopectin and 20% amylose; Sigma Chemical Company, St Louis, MO) at 37°C and 5% CO_2 for 5 days; neither bacteria nor saliva was added to this solution. The 1% starch solution was replaced daily until the fifth day of the experimental period (120 h). Amylase activity was determined by measuring the amount of reducing sugars (4) released into the solution at different time-points to determine whether the surface-adsorbed amylase remain active on the HA surface over time.

Biofilm preparation and analysis

Biofilms of *S. mutans* UA159 (ATCC 700610) were formed on sHA disks placed in a vertical position using a disk holder (see Fig. 1) in batch cultures at 37°C in 5% CO_2 for 5 days (19). The biofilms were grown in buffered tryptone yeast-extract broth containing: (i) 1% starch, (ii) 1% sucrose; (iii) 1% starch + 1% sucrose; (iv) 1% starch + 0.5% glucose + 0.5% fructose; or (v) 1% sucrose + 1% glucose. The culture medium was replaced daily; pH values and amounts of reducing sugars and total carbohydrates in the medium were measured daily after the first 24 h of incubation. At the end of the experimental period (120-h-old biofilms), the biofilms were dip-washed three times, and then gently swirled in physiological saline to remove loosely adherent material. The biofilms were placed in 5 ml sterile saline solution, and the hydroxyapatite surfaces were gently scraped with a sterile spatula to harvest adherent cells. The removed biofilms were subjected to sonication using three 30-s pulses at an output of 7 W (Branson Sonifier 150; Branson Ultrasonics, Danbury, CT). The homogenized suspension was used for dry weight, total protein, and polysaccharide analyses. For the dry weight determination, three volumes of cold ethanol (−20°C) were added to 1 ml biofilm suspension, and the resulting precipitate was collected (10,000 ***g*** for 10 min at 4°C). The supernatant was discarded, and the pellet was washed twice with cold ethanol, and then lyophilized and weighed. Total protein in the biofilm suspension was determined by acid digestion followed by ninhydrin assay (26). The polysaccharide composition (extracellular water-soluble and insoluble, and intracellular polysaccharide) was determined by colorimetric assays as detailed by Koo et al. (18); the polysaccharide content was expressed per mg of dry weight or protein. Briefly, an aliquot (4 ml) of the suspension was centrifuged at 10,000 ***g*** for 10 min at 4°C. The supernatant was collected and the biofilm pellet was resuspended and washed in the same volume of water; this procedure was repeated twice. All the supernatants were pooled and three volumes of cold ethanol were added, and the resulting precipitate was collected. The precipitate, or water-soluble polysaccharides, were collected by centrifugation and washed three times with cold ethanol and resuspended in 1 ml MilliQH_2O; the total amount of carbohydrate was determined

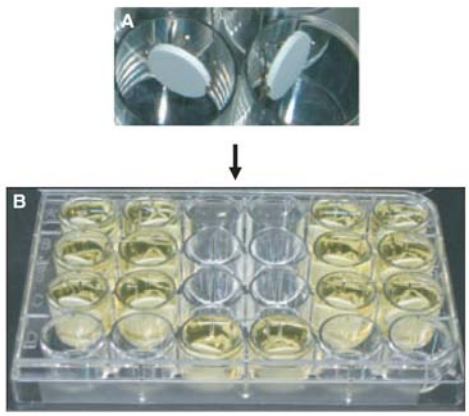

Fig. 1. Saliva-coated hydroxyapatite (sHA) biofilm model. (A) sHA disks placed in a vertical position; (B) biofilms forming in a 24-well plate.

by the phenol–sulfuric method (11). The biofilm pellet was dried in a Speed Vac concentrator and used for determination of: (i) extracellular insoluble polysaccharides; and (ii) intracellular iodophilic polysaccharides. The insoluble polysaccharides were extracted using 1 M NaOH (1 mg biofilm dry weight/0.3 ml of 1 M NaOH) under agitation for 2 h at 37°C. The supernatant was collected by centrifugation, and precipitated with three volumes of cold ethanol. The precipitate was washed three times with cold ethanol and resuspended in 1 ml 1 M NaOH; the total amount of carbohydrate was determined by the phenol–sulfuric method (11). The intracellular iodophilic polysaccharides were extracted with hot 5.3 M KOH (0.8 mg of biofilm dry weight/ml KOH) and quantified using 0.2% I_2/2% KI solution as described by DiPersio et al. (9).

Glycosyl linkage analysis

The extracellular water-soluble and insoluble polysaccharides were extracted as described above, and dissolved in dimethyl sulfoxide (21). For glycosyl linkage analysis, the polysaccharide extracts were methylated by a modification of the method of Ciucanu & Kerek (5) followed by combined gas chromatography/mass spectrometry (GC/MS) analysis as described by York et al. (38). The partially methylated aldital acetates were analyzed on a 30-m Supelco 2330 bonded phase fused silica capillary column by GC/MS using a Hewlett Packard 5890 GC interfaced to a 5970 MSD (mass selective detector, electron impact) as detailed elsewhere (21).

Extraction of RNA and real-time polymerase chain reaction

The RNA extraction and purification, and reverse transcriptase polymerase chain reaction (PCR) conditions and specific primers (for *gtfB*, *gtfC*, *gtfD*, and *dexA*) were similar to those described previously (7, 20). Complementary DNAs (cDNAs) were synthesized using a BioRad iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA). To check for DNA contamination, purified total RNA without reverse transcriptase served as the negative control. The resulting cDNA and negative control were amplified by a MyiQ real-time PCR detection system with iQ SYBR Green supermix (Bio-Rad Laboratories, Inc.) and specific primers. The critical threshold cycle (C_t) was defined as the cycle at which the fluorescence becomes detectable above the background and is inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer set as detailed elsewhere (20). The standard curves were used to transform the C_t values to the relative number of cDNA molecules. Relative expression was calculated by normalizing each gene of interest of the biofilms grown in the presence of various carbohydrates to the 16SrRNA gene (internal control). These values were then compared to those from sucrose-grown biofilms to determine the change in gene expression.

Statistical analyses

An exploratory data analysis was performed to determine the most appropriate statistical test; the assumptions of equality of variances and normal distribution of errors were also checked. The data were then analyzed using analysis of variance, and the *F*-test was used to test any difference among the groups. When significant differences were detected, pairwise comparisons were made between all the groups using Tukey's method to adjust for multiple comparisons. Triplicates from at least three separate experiments were conducted in each of the assays. Statistical software JMP version 3.1 (30) was used to perform the analyses. The level of significance was set at 5%.

Results

The amylase activity of sHA disks in bacteria-free sterile culture medium containing 1% starch was measured daily, and the results are shown in Fig. 2. The salivary amylase adsorbed on the hydroxyapatite surface was active 48 h after salivary pellicle formation, although the enzyme activity declined between 48 and 72 h.

The presence of sucrose alone or in combinations resulted in biofilms displaying four to seven times more biomass, seven to 15 times more total protein, and 10 to 15 times more total EPS than starch or starch + glucose + fructose-grown biofilms (Table 1). However, biofilms of *S. mutans* grown with starch in combination with sucrose exhibited significantly more biomass and total amount of EPS than the biofilms formed in the presence of sucrose, either alone or in combination with glucose ($P < 0.05$).

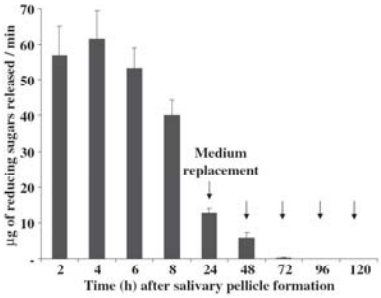

Fig. 2. Amylase activity indicated by the amount of reducing sugars released into the medium during incubation of sHA in 1% starch. The amylase activities at 2, 4, and 6 h after salivary pellicle formation were not significantly different from each other ($n = 12$; $P > 0.05$, ANOVA, comparison for all pairs using Tukey test).

The total amount (in µg/total biofilm dry weight) and content (in µg/mg protein) of extracellular insoluble (INS) and water-soluble (WSP) polysaccharides, and of intracellular polysaccharides (IPS) in biofilms are shown in Fig. 3. Biofilms formed in the presence of sucrose alone or in combinations displayed a significantly higher content of INS than starch or starch + glucose + fructose-grown biofilms ($P < 0.05$). The INS content in starch + sucrose-grown biofilms was significantly higher than that of the sucrose-grown and sucrose + glucose-grown biofilms ($P < 0.05$). On the other hand, starch- or starch + glucose + fructose-grown biofilms were comprised of mostly WSP, and showed higher content of the soluble polysaccharides than biofilms grown in sucrose alone or in combinations ($P < 0.05$). The amount (and content) of

Table 1. Biomass (dry-weight), total amount of protein, and EPS in *Streptococcus mutans* UA159 biofilms formed in the presence of starch and sucrose, alone or in combinations

Experimental groups	Dry-weight (mg)	Total amount of protein (mg)	Total amount of EPS (µg)
Starch	0.75 (0.27)[1]	0.1 (0.08)[1]	88.98 (17.84)[1]
Starch + sucrose	6.25 (0.69)[2]	1.3 (0.12)[2]	1747.99 (146.62)[2]
Sucrose	5.50 (0.45)[3]	1.5 (0.32)[3]	1411.28 (256.45)[3]
Starch + glucose + fructose	1.25 (0.42)[1]	0.2 (0.12)[1]	126.37 (16.58)[4]
Sucrose + glucose	3.92 (0.92)[4]	1.4 (0.21)[2]	850.31 (190.79)[5]

Values (SD, $n = 12$) in the same column followed by the same superscript numbers are not significantly different from each other ($P > 0.05$, ANOVA, comparison for all pairs using Tukey test).

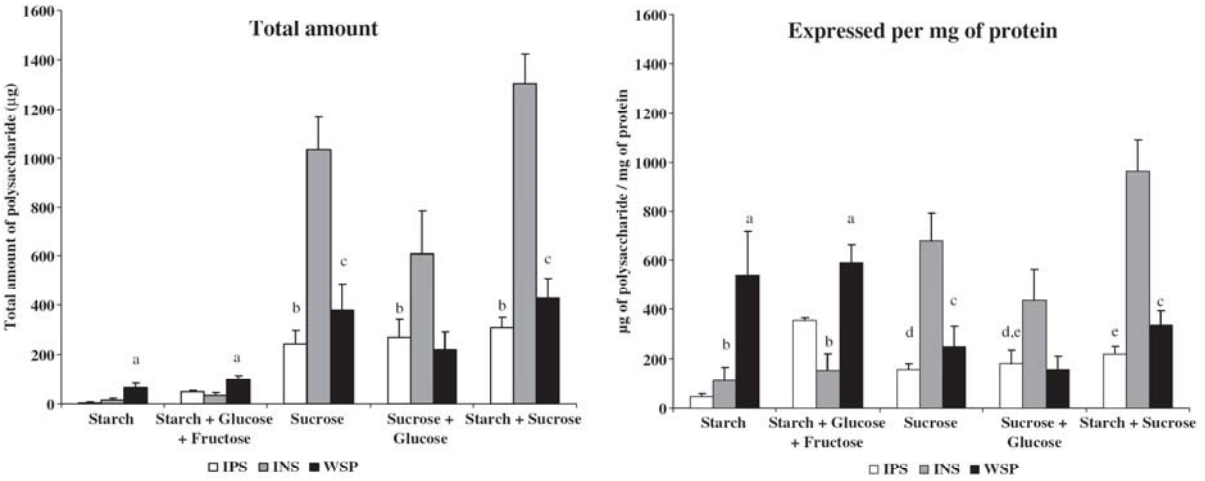

Fig. 3. Total amount (in µg/total biofilm dry-weight) and content (expressed per mg of protein) of intracellular (IPS) and extracellular water-soluble (WSP) and insoluble (INS) polysaccharides in *S. mutans* UA159 biofilms formed in the presence of starch and sucrose, alone or in combinations. Values (SD, $n = 12$) for each type of polysaccharides marked by the same letters are not significantly different from each other ($P > 0.05$, ANOVA, comparison for all pairs using Tukey test).

IPS in biofilms formed with sucrose alone or in combinations was significantly higher than in starch-grown biofilms ($P < 0.05$).

The activity of surface-adsorbed amylase on starch could increase the levels of fermentable carbohydrates in the culture medium, and thereby enhance the acid production by biofilms. Therefore, the reducing sugars and total carbohydrate levels, and the pH of the culture medium surrounding the biofilms were determined daily.

The pH of the culture medium was measured at various time-points during each 24-h incubation period, and the pH-drop curves are illustrated in Fig. 4. Biofilms grown in sucrose, alone or in combinations, rapidly lowered the culture pH to values below 4.5 each day of growth. Biofilms formed in the presence of starch + sucrose displayed the highest rate of acid production during the first 8 h of incubation of early-formed biofilms (between 24 and 48 h) showing significantly lower pH values than starch (t_{28h}, t_{32h}, t_{36h}, and t_{48h}), starch + glucose + fructose (t_{28h}, t_{32h}, and t_{36h}), sucrose (t_{28h} and t_{32h}), and sucrose + glucose (t_{28h}) -grown biofilms ($P < 0.05$). Biofilms formed in starch also lowered the culture pH to 5.3 in the first 48 h. The rate of acid production was slowed after 72 h even though fresh medium was added daily, which is consistent with a decline of amylase activity after 48 h of incubation. Furthermore, biofilms of *S. mutans* grown in starch, either alone or in combinations, displayed elevated levels of reducing sugars in the earlier stages (24–48 h) of biofilm formation (data not shown), which agrees well with the amylase activity data (Fig. 2) and the pH drop curves (Fig. 4).

In an approach to determine whether polysaccharide matrices formed in the presence of different carbohydrates had distinctive structures, the type of glycosyl linkages in WSP and INS extracted from the biofilms were determined (Table 2).

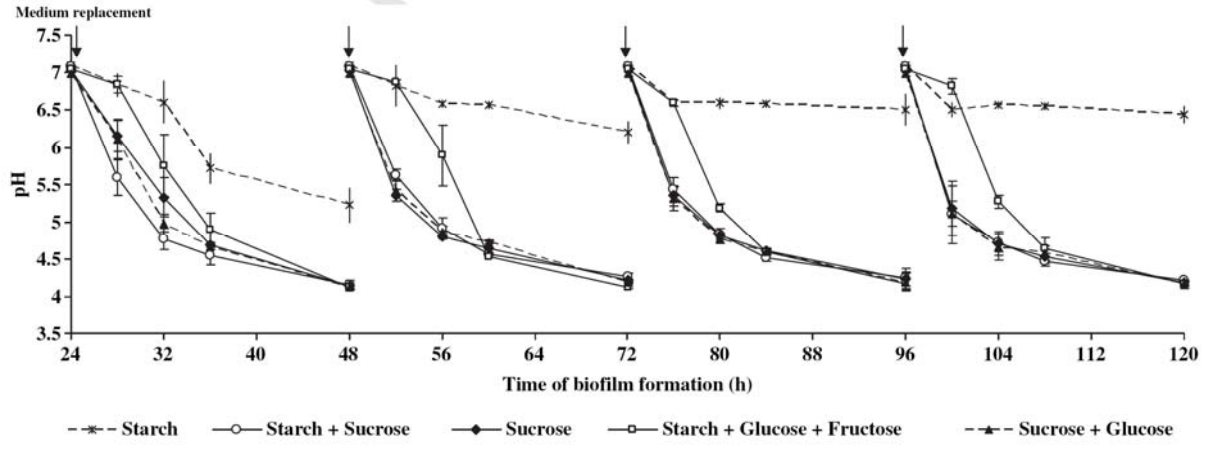

Fig. 4. pH measurements in the culture medium during *S. mutans* biofilm formation. The medium was replaced daily with fresh medium. The pH values ($n = 12$) were determined after 4, 8, 12, and 24 h of incubation; the reducing sugars and total carbohydrate levels ($n = 12$) were measured after a 24-h period of incubation for each day.

Table 2. Percentage of glycosyl linkages of water-soluble (WSP) and insoluble (INS) polysaccharides extracted from *S. mutans* biofilms grown in the presence of starch and sucrose, alone or in combinations

	Starch		Starch + sucrose		Sucrose		Starch + glucose + fructose		Sucrose + glucose	
Glycosyl residue	WSP	INS	WSP	INS	WSP	INS	WSP	INS	WSP	INS
3-linked glucose	–	n/d	±	+++	++	+++	–	n/d	++	+++
4-linked glucose	+++++	n/d	+++++	+	+++	+++	+++++	n/d	++	++++
6-linked glucose	–	n/d	+	+++	+++	++	–	n/d	++	++
3,4-linked glucose	–	n/d	–	±	–	–	–	n/d	–	–
3,6-linked glucose	–	n/d	±	++	++	+	–	n/d	+	+
3,4,6-linked glucose	–	n/d	–	±	–	–	–	n/d	–	–

n/d, not determined.
–, 0–1%; ±, 1–4%; +, 5–9%; ++, 10–19%; +++, 20–29%; ++++, 30–59%; +++++, ≥ 60% or more.

Major structural differences were observed in WSP and INS from biofilms grown with starch or sucrose, alone or in combinations. Soluble polysaccharides from starch + sucrose-grown biofilms displayed higher percentages of 4-linked glucose and less 3-, 6-, and 3,6-linked glucose than those from biofilms grown in sucrose or sucrose + glucose. In contrast, the INS from starch + sucrose biofilms showed higher levels of 3-linked branching (3,4-, 3,6-, and 3,4,6-linked glucose) and considerably less 4-linked glucose than the sucrose-based biofilms. The WSP of starch-grown and starch + glucose + fructose-grown biofilms were comprised predominantly of 4-linked glucose.

Lastly, the expression of *gtfB*, *gtfC*, *gtfD*, and *dexA* in *S. mutans* biofilms grown in starch or sucrose, alone or in combinations, was determined by real-time reverse transcription PCR. Overall, the expression of *gtfB* messenger RNA (mRNA) in biofilms formed with starch + sucrose was significantly increased (25–40%) whereas *gtfD* mRNA levels were decreased (20–30%) when compared with sucrose and sucrose + glucose-grown biofilms ($P < 0.05$); *gtfC* and *dexA* expression was also decreased in starch + sucrose-grown biofilms but the differences were not statistically significant ($P > 0.05$). The gene expression of starch- and starch + glucose + fructose-grown biofilms was not determined because of minimal biofilm formation (and poor RNA yield).

Discussion

The results of this study showed that the combination of starch and sucrose exposed to surface-adsorbed salivary amylase and *S. mutans* clearly influenced the formation and acidogenicity of biofilms by at least four routes: (i) enhanced the total biomass and the content of extracellular insoluble polysaccharides, (ii) synthesized a structurally distinctive EPS matrix, (iii) enhanced acid production in the early stages of biofilm formation, and (iv) affected the expression of specific genes involved in EPS matrix formation (e.g. *gtfB*). Our monospecies biofilm model is advantageous in examining specific actions of carbohydrates on *S. mutans* physiology and genetics, especially on the glucan-mediated processes involved in the formation of the polysaccharide matrix in biofilm, although it does not mimic the complex microbial community found in dental plaque.

S. mutans growing in the presence of sucrose alone or in combinations formed a well-defined, firmly adherent, and highly acidogenic biofilm on the surface of sHA comprised mostly of insoluble polysaccharides containing 3-, 4-, and 6-linked glucose; which agrees well with the glycosyl linkage profile of insoluble glucans synthesized by surface-adsorbed streptococcal glucosyltransferases (17, 21). In contrast, the presence of starch alone or in combination with glucose and fructose resulted in little (and loosely attached) biofilm formation displaying predominantly soluble polysaccharides with 1,4-linked glucose, which suggests that starch and its hydrolysates might be incorporated on to the sHA surface (36). The inability of *S. mutans* to form adherent and established biofilms on the surface of sHA in the presence of starch or starch + glucose + fructose may be related to a lack of insoluble polysaccharide synthesis because insoluble glucans are essential in providing structural integrity and bulk to biofilms (3). However, the combination of starch and sucrose enhanced the acidogenicity of early-formed biofilms (up to 48 h, when salivary amylase is active), and more importantly increased the production of insoluble EPS by *S. mutans* within biofilms when compared to sucrose alone or sucrose with glucose. The starch hydrolysates released by the action of surface-adsorbed amylase combined with sucrose in the medium enhanced the extracellular and intracellular sugar metabolism by *S. mutans* at the pellicle–biofilm interface by providing oligosaccharides to serve as acceptors in glucan synthesis by glucosyltransferases (14, 36), and fermentable carbohydrates for acid production (6). These effects would certainly increase the biofilm accumulation on the tooth surface and accelerate the breakdown of microbial homeostasis in dental plaque (3, 27). Interestingly, the addition of excess glucose in the sucrose medium resulted in less biomass and EPS content than biofilms formed with sucrose alone; an observation consistent with previous studies showing that *S. mutans* growing with glucose in excess diminished the synthesis of extracellular polysaccharides and repressed the sugar uptake by the phosphotransferase system (12, 16). The intracellular polysaccharide accumulation was not markedly affected whether biofilms were grown in the presence of sucrose alone or in combinations, although the IPS content in starch + sucrose-grown and sucrose + glucose-grown biofilms was slightly higher than in sucrose-grown biofilms.

Furthermore, biofilms grown in the presence of starch + sucrose resulted in a structurally distinct EPS matrix when compared to those formed in sucrose or sucrose + glucose. It is noteworthy that the presence of starch + sucrose resulted in insoluble polysaccharides comprised predominantly of $1 \rightarrow 3$ and $1 \rightarrow 6$ linkages, and higher percentages of branch points from 3,4-, 3,6-, and 3,4,6-linked glucose than those from sucrose- or sucrose + glucose-grown biofilms. It is apparent that the presence of oligosaccharides from starch hydrolysis is contributing to the insolubilization of exopolysaccharide matrix in starch + sucrose-grown biofilms by (i) enhancing the content of insoluble polysaccharides containing (ii) a higher percentage of insoluble 3-linked branching. The higher content of insoluble polysaccharides in starch + sucrose-grown

biofilm matrix can be explained by previous observations that starch hydrolysates in combination with sucrose increased the synthesis of insoluble glucans, and also affected the structure of glucans synthesized by surface-adsorbed GTF B, resulting in enhanced *S. mutans* binding compared to those formed with sucrose alone (21, 36); this enhancement may be associated with changes in the binding sites of the modified glucans (21, 36). Clearly, the influence of the oligosaccharides on the GTF B activity plays a critical role in changing the physical and biochemical properties of the biofilms matrix, and thereby influencing its cariogenic properties. However, in biofilms, the formation and maturation of the EPS matrix is a result of a dynamic interaction of all of the three GTFs acting in concert and is influenced by an endodextranase produced simultaneously by *S. mutans* (15, 17, 22). The presence of starch in combination with sucrose may be modulating all of the enzymes responsible for synthesis and degradation concomitantly, resulting in a structurally distinct matrix. Further studies shall elucidate how starch and sucrose influence the synthesis and degradation of glucans concomitantly during the EPS matrix development at molecular and structural levels.

A recent *in situ* study showed that a combination of 2% starch + 10% sucrose was potentially more cariogenic than 10% sucrose alone, despite the total amounts of EPS in the matrices of the biofilms being similar to each other (29). Although higher levels of acidogenic and aciduric bacteria, such as *Lactobacilli*, were found in the biofilms, the enhanced cariogenicity of starch + sucrose may be also explained by the structural differences of the EPS matrix between starch + sucrose-grown biofilms and those formed in sucrose alone. The structural changes in the matrix may affect the diffusion properties, bacterial binding sites, physical integrity, and architecture of the biofilms (8, 21, 34, 36, 39). It is therefore feasible that such changes in the EPS matrix of biofilms could modulate the pathogenesis of dental caries, substantiating the concept that there is a starch hydrolysate contribution to the formation of cariogenic dental plaque. The exact mechanisms by which the structural changes enhance the virulence of the biofilms need further elucidation.

Lastly, we examined the expression profile of the genes encoding the synthesis (*gtfB*, *gtfC*, *gtfD*) and degradation (*dexA*) of glucans by *S. mutans* within biofilms, in an attempt to explain the structural differences of the EPS matrix observed between starch + sucrose-grown and sucrose-grown biofilms. Our data indicate that biofilms formed in starch + sucrose expressed significantly higher levels of *gtfB* mRNA and less *gtfD* mRNA than those formed in sucrose. It is noteworthy that expression of *gtfC* and *dexA* was decreased in starch + sucrose biofilms (although the differences were not statistically significant), indicating an overall effect of induction of *gtfB*. This observation could explain, in part, the differences observed in the structure of the EPS matrix, e.g. higher percentage of 3-linked branching in insoluble polysaccharide and less 6-linked glucose in the soluble polysaccharide matrix of starch + sucrose biofilms. Furthermore, *gtfB* is a critical virulence gene associated with the pathogenesis of dental caries (37); *S. mutans* treated with therapeutic agents that repress the expression of *gtfB*, or mutant strain of this organism defective in *gtfB*, are far less cariogenic than untreated or parent strains *in vivo* (19, 20, 37). Thus, the presence of a combination of starch and sucrose would result in a more virulent (cariogenic) biofilm. We are currently pursuing detailed gene expression profiling at different stages of biofilm formation to better understand the molecular mechanisms involved in the EPS synthesis in the presence of starch and sucrose.

Our data offer, in part at least, an explanation for why starch and sucrose combinations are potentially more cariogenic than either alone; and furthermore illustrate that composition of the diet can influence the virulence traits of the oral pathogen *S. mutans*. Clearly, surface-adsorbed α-amylase may have an additional role in dental biofilm formation other than promoting specific bacterial adhesion (31) by contributing directly with the synthesis of a structurally distinct extracellular polysaccharide matrix and by enhancing the expression of *gtfB*. Further studies using additional microorganisms that bind amylase in a multispecies biofilm model shall elucidate even further the role of starch and sucrose in the virulence of cariogenic biofilms.

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EFFECT OF STARCH AND SUCROSE ON DENTAL BIOFILM FORMED AND ON ROOT DENTINE DEMINERALIZATION

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Running Title: Effect of starch and sucrose on dentine *in situ*
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DECLARATIONS

1- All authors have made substantive contribution to this study and/or manuscript, and all have reviewed the final paper prior to its submission.

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

ABSTRACT

It has been observed that the combination of starch with sucrose is more cariogenic to enamel than sucrose alone but this effect on dentine demineralization and on the dental biofilm formed has not been totally explored. A crossover and blind study was conducted in four steps of 14 days each, during which 11 volunteers wore palatal appliance containing 10 slabs of root dentine to which the following treatments were applied extraorally: 2% starch gel-like solution (starch group); 10% sucrose solution (sucrose group); a solution containing 2% starch and 10% sucrose (starch+sucrose group), or 2% starch solution followed by 10% sucrose solution (starch→sucrose group). On the 14th day of each phase the biofilms were collected for biochemical and microbiological analyses, and dentine demineralization was assessed by microhardness. A higher demineralization was found in dentine exposed to sucrose and starch sucrose combinations than to starch alone ($p<0.05$) but the sucrose-containing groups did not differ significantly from each other ($p>0.05$). The amount and percentage of soluble/insoluble extracellular polysaccharides in the biofilm formed in presence of starch differed ($p<0.05$) from those formed in presence of sucrose and combinations of starch with sucrose but these did not differ statistically ($p>0.05$). Furthermore, RNA were successfully isolated and purified from *in situ* biofilms and only biofilms formed in response to sucrose and starch sucrose combinations showed detectable levels of *gtfB* and *gtfC* mRNA. The findings suggest that the combination of starch with sucrose may not be more cariogenic to dentine than sucrose alone.

INTRODUCTION

Sucrose is considered the most cariogenic dietary carbohydrate and this is partly attributed to its role as a substrate for the synthesis of extracellular glucans [Rölla *et al*., 1985]. Starch is also an important source of fermentable carbohydrate, which is consumed simultaneously or interspersed with sucrose [Lingström *et al*., 2000]. However, while starch is considered non-cariogenic or slightly cariogenic when used as the sole source of carbohydrate in the diet [Lingström *et al*., 2000] the combination of starch and sucrose is considered potentially more cariogenic than either carbohydrate alone [Bowen *et al*., 1980; Firestone *et al*., 1982]. Indeed, an *in situ* study showed evidence that the combination of starch with sucrose is more cariogenic for deciduous enamel than sucrose alone [Ribeiro *et al*., 2005].

The enhanced cariogenic potential of the starch-sucrose combination may be attributed to starch hydrolysates, the result of starch digestion by α-amylases. Some of these digestion products can act as acceptors during polysaccharides synthesis, which would increase the synthesis of insoluble glucans by glucosyltransferase-B *in vitro* [Vacca-Smith *et al*., 1996]. Also, starch-sucrose combination showed effects on the composition and structure of the polysaccharide matrix, and also enhanced the acidogenicity of *Streptococcus mutans* biofilm [Kopec et al., 1997; Duarte *et al*., 2007].

However, these promising in vitro findings suggesting that starch would increase the cariogenic potential of biofilm formed in presence of sucrose should be tested *in vivo*. Furthermore, the cariogenic potential of this association for dentine has not been totally explored. Thus, the aim of this study was to evaluate in situ the effect of the combination of starch with sucrose on dental biofilm formed and on dentine demineralization.

MATERIALS AND METHODS

Ethical aspects and volunteers

This study was approved by the Research and Ethics Committee of FOP/UNICAMP (protocol no. 31/2004), and volunteers signed an informed, written consent to participate (Resolution Nº 196 of the National Health Council, Health Ministry, Brasília, DF, 10/03/1996).

Eleven volunteers (20–34 years old), who fulfilled inclusion criteria (normal salivary flow rate, good general and oral health, ability to comply with the experimental protocol, not having used antibiotics during the 2 months prior to the study, not using fixed or removable orthodontic device) were selected to participate in the study.

Experimental design

The study used a single-blind, crossover design, conducted in four phases of 14 days each, during which 11 volunteers wore palatal appliances containing 10 slabs of root dentine with known surface microhardness. The following treatments were applied extraorally onto the slabs, 8 times per day: 2% starch solution (starch group); 10% sucrose solution (sucrose group); a solution containing 2% starch and 10% sucrose (starch+sucrose group), and 2% starch and 10% sucrose solution used interspersed (starch→sucrose group). The sequence of treatments used by each volunteer was randomly determined using a computer-generated randomization list. At the end, all volunteers had used all treatments. The sample size was determined on the basis of our previous findings using the same experimental protocol, expecting a statistical power of 0.8. On the day 14 of each phase, the biofilms were collected for biochemical and microbiological analyses, and dentine blocks were evaluated for mineral loss by surface and cross-sectional microhardness. This study was blind only with respect to the examiners, since the volunteers could differentiate the taste and viscosity of the solutions tested. For all determinations, the volunteer was considered as the experimental unit.

Root dentin slabs and palatal appliance preparation

Root dentine slabs (4 x 4 x 2 mm) were prepared from bovine incisor teeth as previously described [Hara *et al*., 2003]. The slabs were randomly divided into four groups, according to the treatments. The slabs were immersed in artificial saliva in order to minimize further ionic changes between the slab and the oral environment [Hara *et al*., 2003 for details]. The surface microhardness of the dentine slabs were determined by making 3 indentations, spaced 100 µm from each other, using a Knoop indenter with a 5 g load for 5 s and a Future-Tech FM microhardness tester coupled to software FM-ARS 900. Slabs with discrepant microhardness values were excluded from the sample and a total of 560 slabs presenting a mean hardness of 38.0 kg/mm^2 (SD=6.0) were selected. Acrylic

palatal appliances were made for each volunteer with ten sites (five in each side of the appliance) for slabs positioning. In order to allow plaque accumulation and to protect dentin surface from mechanical disturbance, plastic meshes were fixed over the cavities containing the slabs, leaving a 1-mm space on the slab surface [Hara *et al*., 2003 for details].

Treatments

The concentration of 10% sucrose used in the present experiment is usually found in soft drinks. The 2% starch concentration was based on the maximum solubility (gel-like) of the starch used (Soluble starch - 80% amylopectin and 20% amylose; Sigma Chemical Company, St. Louis, MO, USA). Also, Ribeiro *et al*. [2005] used the same concentrations in the evaluation of the effect of these carbohydrates in enamel caries.

During the 14 days of each experimental phase, eight times per day, volunteers removed the appliance from the oral cavity, dripped one drop of the treatment solutions on each dental slab, and after 5 min the appliance was replaced in the mouth. For the interspersed use of starch and sucrose (group starch→sucrose), volunteers first dripped one drop of starch solution and after 5 min replaced the appliance in the mouth. Five minutes later, considered enough time to starch hydrolysis [Mörmann and Mühlemann, 1981], the appliances were removed and the sucrose solution was dripped, as described earlier. A wash-out interval of 7 days was established between the experimental phases.

Considering the crossover design of this study, no restriction was made with regards to the volunteer's diet, but they were instructed to remove the appliances during meals [Cury *et al*., 2000]. Throughout the entire experiment, volunteers used a dentifrice containing 1100 µg F/g (NaF) and silica as abrasive, consumed water optimally fluoridated (0.7 mg F/L) and received instructions as previously described [Cury *et al*., 2000].

Microbiological and polysaccharide analysis

On the day 14 of each experimental phase, approximately 10 h after the last exposure to treatments or toothbrushing, the biofilm formed on one side of the appliance was collected for biochemical and microbiological analyses. The protocol used was previously standardized when different parameters of sonication were tested to determine viable bacteria biomass and to extract extra and intracellular polysaccharides [Aires *et al*.,

2007]. Thus, at least, 5 mg of dental biofilm was weighed to ± 0.01 mg (Analytical Plus AP 250D, Ohaus Corp., Florham Park, N.J., USA) in sterile microcentrifuge tubes, suspended in 1 mL of 0.9% NaCl solution and sonicated at 7 watts for 60s using Digital Sonifier® Unit, model S-150D (Branson Ultrasonics Corporation, Danbury, CT, USA). From this suspension, aliquots of 250 and 400 µL were collected for microbiological and polysaccharyde analysis, respectively (Figure 1).

***Microbiological analysis**:* An aliquot of 50 µL of the sonicated suspension was diluted in 0.9% NaCl and serial decimal dilutions were inoculated in duplicate by the drop-counting technique in the following culture media: blood agar, for total microbiota; mitis salivarius agar (MSA) for total streptococci, mitis salivarius agar plus 0.2 units of bacitracin/mL and 15% of sucrose (MSB), for mutans streptococci group [Gold *et al*., 1973]; Rogosa SL agar, for lactobacillus; and CFAT medium [Zylber and Jordan, 1982], for actinomyces. The plates were incubated in 10% CO_2 at 37º C for 48 h (blood agar, MSA, MSB, Rogosa) or 72 h (CFAT). The colony-forming units (CFU) were counted and the results expressed in CFU/mg dental biofilm wet weight and in percentage of total streptococci (% TS), mutans streptococci (% MS), lactobacilli (% LB), and actinomyces (% AC) in relation to total microorganisms.

***Polysaccharide extraction**:* An aliquot of 400 µL of sonicated biofilm suspension was centrifuged at 10.000 *g* for 5 min at 4 ºC. The supernatant containing soluble EPS extracted was collected and transferred to another tube named SEPS. The pellet was washed with 100 µL of saline solution, centrifuged and the supernatant added to the tube SEPS, to which 3 volumes of cold ethanol were added. To the pellet, 400 µL of 1M NaOH were added for insoluble EPS extraction [Cury *et al.,* 1997]. The tube was vortexed, agitated 15 min, centrifuged and the supernatant was transferred to another tube named IEPS. The pellet was washed with 100 µL of 1M NaOH, centrifuged and the supernatant was added to the tube IEPS to which 3 volumes of cold ethanol were added. To the microcentrifuge tube containing the residual pellet, 400 µL of 1M NaOH were added for intracellular polysaccharide (IP) extraction [Tenuta *et al*., 2006]. This tube was vortexed, heated 15 min at 100 ^{o}C, centrifuged and the supernatant was transferred to another tube named IP. The

pellet was washed and IP extracted was recovered as described for insoluble EPS extraction.

The tubes containing cold ethanol plus SEPS, IESP or IP were kept for 30 min at –20º C, centrifuged and the pellets were washed twice with 70 % cold ethanol. The polysaccharides precipitated were resuspended in 250 µL of 1 M NaOH and total carbohydrate was estimated by phenol sulphuric method [Dubois *et al*., 1956] (Figure 1), using glucose used as standard. The results were normalized by wet weight of biofilm.

Real-time quantitative RT-PCR

On the day 14 of each experimental phase, after collecting the biofilm formed on one side of the appliance, the volunteers dripped one drop of their respective treatment solution on dental slabs in the opposite side, and after 5 min the appliance was replaced in the mouth. Thirty minutes later, enough time to promote *gtfBC* expression [Li and Burne, 2001], the biofilm was collected, weighed and kept in RNA*Later*® solution for further analysis. Three pools of biofilm from the same volunteers for each treatment were submitted to RNA extraction, since according to a pilot study around 100 mg of biofilm wet weight formed in presence of sucrose was necessary to extract enough RNA for gene expression analysis by Real-time quantitative reverse transcription (RT)-PCR. The RNA*Later* solution was carefully removed and the pellets were resuspended with cold phosphate-buffered saline and pooled in one microcentrifuge tube. The RNA extraction and purification were performed according to Cury and Koo [2007] but the washing-sonication steps were made using 1.0 mL of phosphate-buffered saline. Also, the homogenization in NAES was made by sonication using one 10-s pulse at 5 watts. The quantification of RNA extracted and its purification were made essentially as described by Cury and Koo [2007].

Real-time quantitative reverse transcription (RT)-PCR was performed to evaluate gene expression of *gtfBCD*. One microgram of RNA (RNA Integrity Number-RIN between 6.4-7.4 as determined by Agilent 2100 electrophoresis bioanalyzer) and specific primers (Fujiwara *et al.* 2002) were used to generate cDNA (Superscript III Reverse Transcriptase Invitrogen). For each sample, a mock reaction without addition of reverse transcriptase was performed (negative RT control) to check for DNA contamination. The resulting cDNAs and negative controls were amplified by a MyiQ real-time PCR detection system with iQ

SYBR Green supermix (Bio-Rad Laboratories, Inc., CA, USA) and specific primers for *gtfB, gtfC* and *gtfD* as described elsewhere (Koo *et al.,* 2006). Standard curves for each gene were prepared as described elsewhere (Yin *et al.*, 2001, Koo *et al*., 2006). Total cDNA abundance was normalized using *S. mutans* specific16S rRNA gene as a housekeeping control [Koo et al., 2006].

Dentine demineralization

After each phase, four of the ten slabs of each volunteer were randomly removed from the appliances and the surface microhardness (SMH) was again measured. One row of three adjacent indentations spaced by 100 µm 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 surface microhardness change (% SMC) was calculated.

After SMH analysis, the slabs were longitudinally sectioned through the center for cross-sectional microhardness (CSMH) determination which was done according to Hara *et al*. [2003], however the data were expressed in Knoop hardness number (kg/mm^2) since there is discrepancy in the literature to convert the values in mineral volume percent [Featherstone *et al.,* 1983; Kielbassa *et al.* 1999]. Additionally, CSMH of eight slabs of sound dentine was used as a control.

Statistical analysis

The analysis was done with SAS software (SAS Institute Inc., version 8.01, Cary, NC, USA), with a significance level fixed at $p < 0.05$. Volunteers were considered as statistical blocks. The assumption of equality of variances and normal distribution of errors were checked, and data that violated these statistical principles were transformed [Box at al., 1978]. Tukey's test was then used for post-ANOVA comparisons. The % of mutans streptococci and % of lactobacilli were analyzed by the non-parametric Friedman test, since no data transformation was possible. Paired t-test was used to compare surface microhardness before and after the treatments.

RESULTS

The counts of total microorganisms, total streptococci and actinomyces in the biofilms formed did not differ ($p>0.05$) among the treatments (Table 1). However, mutans

streptococci and lactobacilli counts were lower in starch group compared with sucrose and starch→sucrose groups ($p<0.05$) but did not differ from starch+sucrose group ($p>0.05$).

The concentration and percentage of soluble and insoluble EPS (Table 2, Figure 2) in dental biofilm exposed to starch was lower than that found in the other groups ($p<0.05$) but these groups did not differ from each other ($p>0.05$). Regarding IP, the difference among the groups was not significant ($p>0.05$).

The amount of crude RNA extracted (Table 3) was lower in biofilm formed in presence of sucrose ($p<0.05$) compared with the other treatment groups. The yield of purified RNA was higher in starch group in comparison with sucrose ($p<0.05$) but the groups exposed to the combination of starch and sucrose did not differ statistically from the other groups ($p>0.05$). In addition, purified RNA samples (with RIN greater than 6) were selected for *gtfBCD* expression analysis by real-time reverse transcription PCR. Overall only biofilms formed in the presence of sucrose and sucrose/starch combinations displayed detectable levels of *gtfB* and *gtfC* mRNA (but not *gtfD*). None of the *gtf* genes were detected in biofilms formed with starch alone.

Regarding demineralization (Table 4, Figure 4), all treatments significantly decreased dentine SMH ($p<0.05$) but the lowest change was found in starch group ($p<0.05$), while the other groups did not differ significantly from each other ($p>0.05$). These data were confirmed by CSMH and difference between starch and the other groups were consistent up to 30 µm in depth ($p<0.05$).

DISCUSSION

The relation between starch and caries is very complex [Lingström *et al*., 2000] and the present study gives some contribution for its understanding. The findings clearly showed dentine demineralization under biofilm accumulation and frequent exposure to starch (Table 4), giving support to the suggestion that starch products could be cariogenic to dentine [Lingström *et al*., 1994]. It should be emphasized that the cariogenic potential of starch is very low when compared with sucrose and demineralization was limited to approximately 20 µm from dentine surface (Figure 4) while a deepest lesion was found in sucrose groups, giving support to the discussed different effect of these carbohydrate on caries [Sheiham 2001].

However, the synergistic effect between starch and sucrose on enamel demineralization [Bowen *et al*., 1980, Firestone *et al*., 1982, Ribeiro *et al*., 2005] was not evident for dentine since the differences among sucrose, starch+sucrose and starch→sucrose treatment groups (Table 4, Figure 4) were not statistically significant ($p>0.05$). These contrasting data may be explained by differences between enamel and dentine in terms of properties and demineralization progress under biofilm and sugar exposure. Thus, it has been suggested that the 'critical pH' for root dentine is higher (~6.5) than the pH 5.5 estimated for enamel surface [Hoppenbrouwers *et al*., 1987] and when biofilms are exposed to sucrose or starch+sucrose the pH drop would be similar [Ribeiro *et al*., 2005]. Also, demineralization in enamel is roughly linear with time and root dentine demineralizes very fast during the first week and much more slowly there after [Ogaard *et al*., 1988]. Probably, differences could be expected if the experiment time was shorter than 7 days or much longer than 14 days, what should be further evaluated. The contrasting result found with dentine in comparison with deciduous human enamel [Ribeiro *et al*., 2005] should not be attributed to the bovine root dentine used in present study since it does not differ from human dentine in terms of caries progression or inhibition [Hara *et al.,* 2003].

In this study we evaluated the expected caries-enhancing interaction between starch and sugars by the effect on the biofilm formed. The counts of cariogenic bacteria, mutans streptococci and lactobacilli, were higher (Table 1) in biofilm formed under sucrose exposure than under starch alone, in agreement with the different pH drop due to the fermentation of these carbohydrates [Ribeiro *et al*., 2005] and the consequent biofilm enrichment with these aciduric bacteria [Marsh, 2003]. Also, the absence of synergistic or simply additive effect of starch combined with sucrose compared with sucrose on mutans streptococci and lactobacilli agrees with Ribeiro *et al.* [2005], who showed that the combination is not more acidogenic than sucrose alone.

With regard to polysaccharide concentration, sonication was used to separate soluble from insoluble EPS in the biofilm matrix [Aires *et al*., 2007] trying to improve the differentiation of EPS [Ribeiro *et al*., 2005; Pecharki *et al*., 2005]. This approach enabled the differentiation of the percentage of soluble and insoluble EPS from biofilms formed in

presence of starch and sucrose (Table 2, Figure 2). The data confirm our previous *in situ* study using deciduous enamel as tooth substrate for biofilm formation [Ribeiro *et al*., 2005], when the % of insoluble EPS were numerically higher in starch + sucrose combination group than in sucrose group but did not reach statistical significance. Nevertheless, differences in the structure of these polysaccharides should be considered to confirm *in vitro* data [Kopec *et al*., 1997; Duarte *et al*., 2007], deserving further analysis.

The data of RNA from dental biofilm (Table 4) confirm the deleterious effect of matrix EPS [Cury and Koo, 2007; Cury *et al*., 2008] on extraction and purification of RNA from biofilms. Thus, RNA yield from biofilm formed under sucrose was lower than that formed under starch, what should be attributed to the higher concentration of EPS in the sucrose biofilm matrix (Table 2). Also, RNA yield from biofilms formed under the combination of starch and sucrose did not differ ($p>0.05$) from those formed either under starch or under sucrose exposure, suggesting that the matrix of biofilms formed under starch/sugar combination presents some characteristics of the biofilm matrix formed under the isolated exposure of these carbohydrates. Furthermore, selected RNA samples were used to investigate the expression profile of the genes encoding the synthesis of glucans (*gtfBCD*) by *S. mutans* within a multispecies *in situ* biofilm. Only biofilms formed in the presence of sucrose and sucrose/starch combinations displayed detectable levels of *gtfB* and *gtfC* mRNA. Conflicting *in vitro* results have been found about expression of *gtf* genes of *S. mutans* in response to carbohydrates [Li and Burne, 2001; Fujiwara *et al*., 2002; Shemesh *et al*., 2007] and the present study agrees with those showing that sucrose may increase the expression of *gtfBC* genes. This could explain the higher content of EPS in biofilms formed under sucrose exposure compared to starch alone, although lower *S. mutans* cells were found in biofilm of the starch group. It is possible that the amount of sample in this group may have not been sufficient to provide detectable levels of mRNA of the target genes, especially those that are expressed in low abundance such as *gtf* genes.

Although the findings did not show statistical effect of starch combined with sucrose compared with sucrose alone on all parameters evaluated, some trend is noticeable and should be biologically discussed considering future studies. Thus, dentine demineralization and % of insoluble EPS were numerically higher in starch sucrose

combination than in sucrose group. Also, % of soluble EPS was lower and in opposite RNA yield was higher in starch sucrose combination than in sucrose group. The coherence of these data in addition to our recent *in vitro* findings (Duarte et al., 2007) suggest that starch may affect the biofilm matrix formed under sucrose exposure since insoluble EPS is linked to enamel-dentine demineralization [Paes Leme *et al*., 2006] and EPS (sonication-extractable) with the efficiency of RNA extraction from biofilm [Cury & Koo 2007; Cury *et al*., 2008]. Further studies should be conducted to evaluate in more detail the biological effect on the structure and composition of the extracellular matrix of the biofilm formed.

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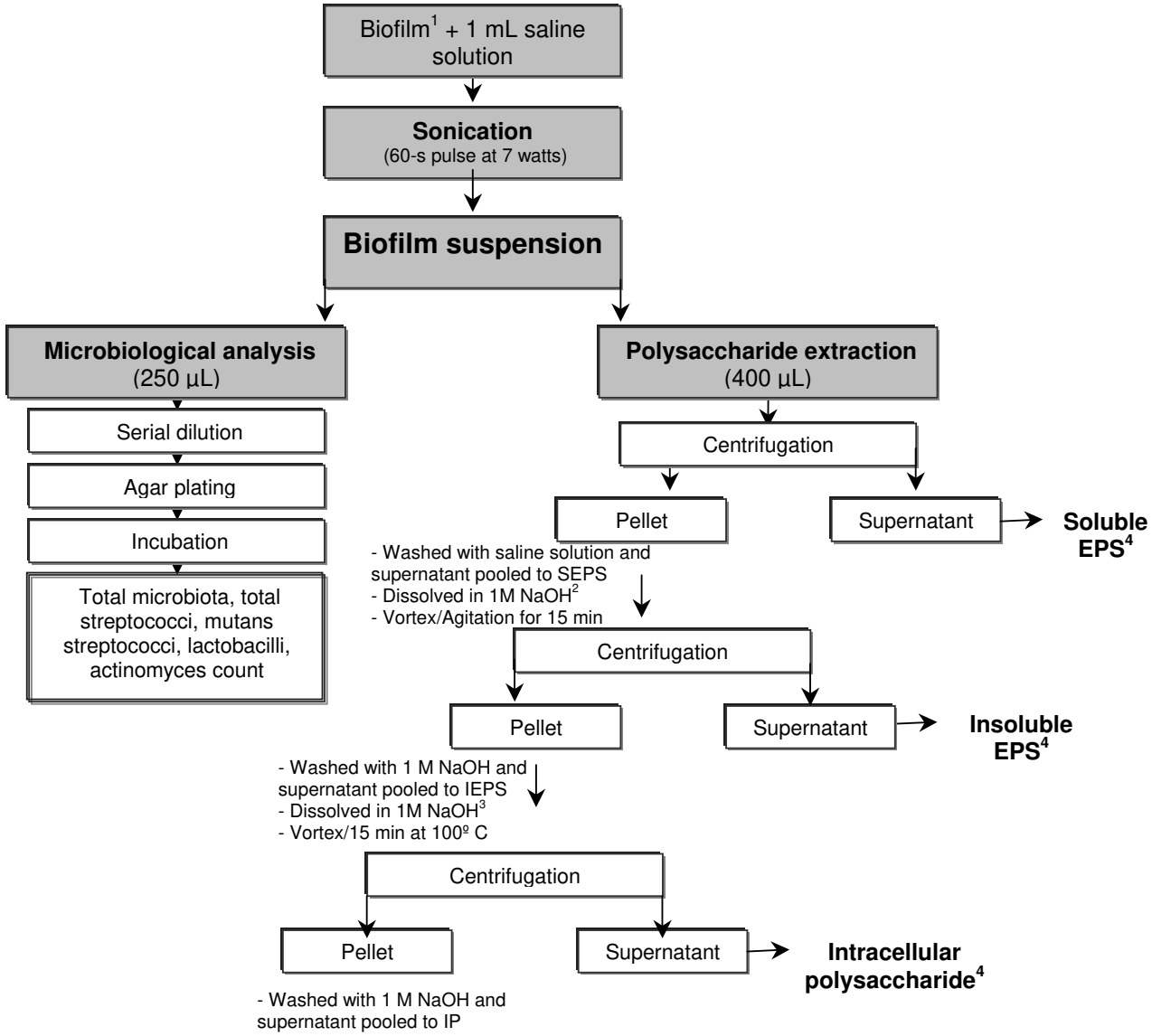

1: At least 5 mg of biofilm wet weight

2: Cury *et al.,* 1997

3: Tenuta *et al.*, 2006

4: Precipitated with 1:3 cold ethanol, centrifuged, washed 2x with 70% cold ethanol, dissolved in 1M NaOH and estimated by phenol sulphuric method (Dubois *et al.*, 1956).

Figure 1: Microbiological analyses and polysaccharide extraction.

Table 1: Microbiological analysis of dental biofilms according to treatments (Mean ± SD; n = 11).

Treatment groups	MS (CFU/mg biofilm wet weight x 10^2)	% MS	LB (CFU/mg biofilm wet weight x 10^5)	% LB	AT (CFU/mg biofilm wet weight x 10^5)	% AT
Starch	0.6 ± 2.0 ^{A}	0.0007± 0.0021 ^{A}	0.0001 ± 0.0002 ^{A}	0.0001± 0.0001 ^{A}	31.1 ± 56.4 ^{A}	17.5 ± 19.1 ^{A}
Sucrose	7.0 ± 11.5 ^{B}	0.01 ± 0.01 ^{B}	11.78 ± 24.46 ^{B}	8.05 ± 18.03 ^{B}	35.0 ± 61.9 ^{A}	14.4 ± 12.6 ^{A}
Starch+Sucrose	3.2 ± 8.4 ^{AB}	0.02 ± 0.05 ^{AB}	2.53 ± 5.88 ^{AB}	3.96 ± 8.77 ^{AB}	28.6 ± 65.3 ^{A}	13.0 ± 11.3 ^{A}
Starch→Sucrose	9.5 ± 17.0 ^{B}	0.01 ± 0.01 ^{B}	2.56 ± 5.68 ^{B}	2.05 ± 5.07 ^{B}	55.4 ± 108.3 ^{A}	17.2 ± 13.5 ^{A}

MS = Mutans streptococci counts; % MS = percentage of mutans streptococci in relation to total microorganisms (TM); LB = Lactobacilli counts; % LB = percentage of lactobacilli in relation to TM; AT = Actinomyces counts; % AT: percentage of actinomyces in relation to total microorganisms. Treatments whose means are followed by distinct letters differ statistically ($p<0.05$).

Table 2: Polysaccharide analysis of dental biofilms according to treatments (Mean ± SD).

Treatment groups	Extracellular Polysaccharide (µg/mg wet weight)		Intracellular Polysaccharide (µg/mg wet weight)
	Soluble	Insoluble	
Starch	1.2 ± 1.0 ^{A} (n=11)	0.93 ± 0.85 ^{A} (n=10)	1.68 ± 1.09 ^{A} (n=10)
Sucrose	7.63 ± 4.75 ^{B} (n=11)	15.83 ± 11.96 ^{B} (n=11)	2.73 ± 2.60 ^{A} (n=11)
Starch+Sucrose	5.49 ± 4.69 ^{B} (n=11)	16.20 ± 15.47 ^{B} (n=10)	2.48 ± 2.27 ^{A} (n=11)
Starch→Sucrose	4.76 ± 6.75 ^{AB} (n=11)	11.87 ± 12.53 ^{B} (n=11)	2.84 ± 1.98 ^{A} (n=11)

Treatments whose means are followed by distinct letters differ statistically ($p<0.05$).

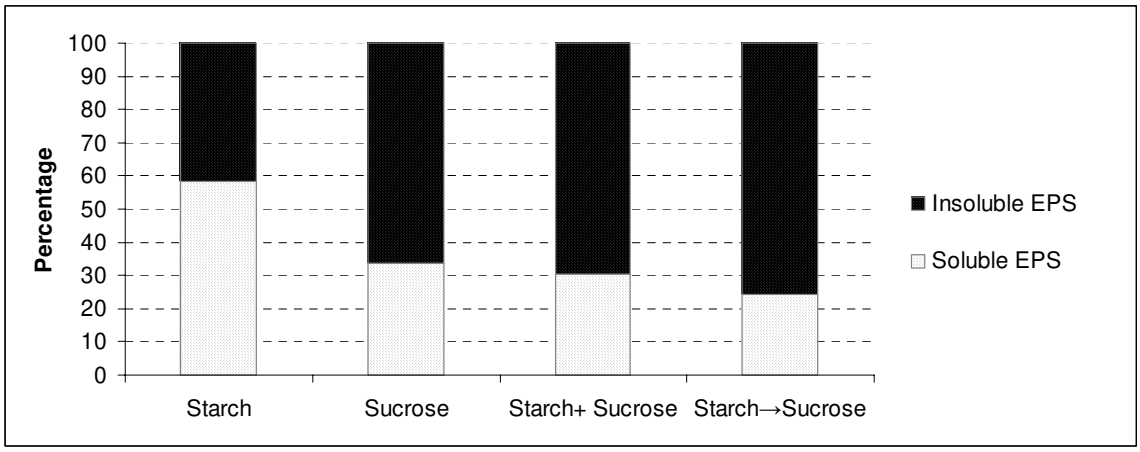

Figure 2: Relative percentage of insoluble and soluble extracellular polysaccharides (EPS) from biofilms according to treatments.

Table 3: Extraction and purification of RNA from biofilms according to treatments (Mean ± SD; n=3 pools).

Treatments groups	**Crude RNA** (µg/mg biofilm wet weight)	**DNA removed** (%)	**Purified RNA** (µg/mg biofilm wet weight)
Starch	1.06 ± 0.11 ^{A}	79.9 ± 3.9 ^{A}	0.21 ± 0.03 ^{A}
Sucrose	0.48 ± 0.13 ^{B}	89.4 ± 4.0 ^{A}	0.05 ± 0.03 ^{B}
Starch+Sucrose	0.74 ± 0.05 ^{C}	85.2 ± 7.8 ^{A}	0.11 ± 0.06 ^{AB}
Starch→Sucrose	0.94 ± 0.14 ^{AC}	86.5 ± 2.7 ^{A}	0.13 ± 0.04 ^{AB}

Treatments whose means are followed by distinct letters differ statistically ($p<0.05$).

Table 4: Mineral loss in dentin surface according to the treatments (Mean ± SD; n=11)

Treatments groups	SMH		% SMC
	Baseline	After treatment	
Starch	38.5 ± 3.5 [a]	30.2 ± 8.0 [b]	-20.3 ± 16.7 [A]
Sucrose	39.2 ± 1.7 [a]	20.4 ± 5.7 [b]	-47.0 ± 8.4 [B]
Starch+Sucrose	39.1 ± 1.6 [a]	18.0 ± 8.0 [b]	-52.6 ± 16.9 [B]
Starch→Sucrose	40.2 ± 1.9 [a]	18.3 ± 6.2 [b]	-52.8 ± 12.0 [B]

SMH: Surface Microhardness; SMC = Surface Microhardness Change.
Means followed by different letters are statistically significant ($p<0.05$). Lower case letters show difference between baseline and after treatment SMH for each treatment and capital letters among the treatments.

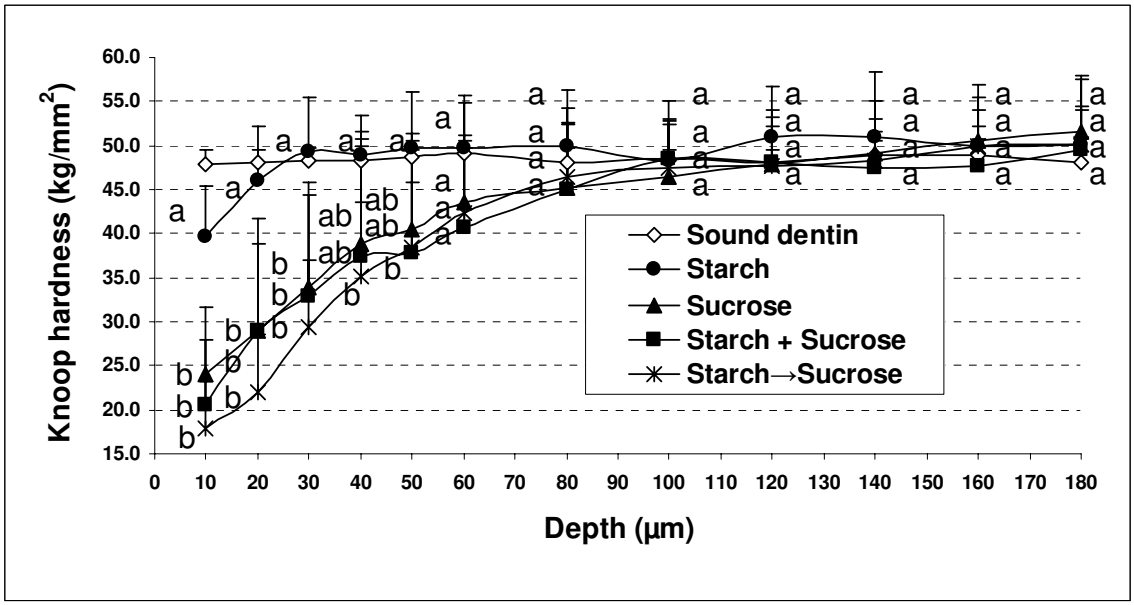

Figure 4. Dentine Knoop hardness (kg/mm^2) according to treatments and the distance (µm) from the surface (Mean; bars denote standard deviation; n=11). Different letters show statistical significance ($p<0.05$) among treatments at each distance.

CONSIDERAÇÕES GERAIS

A matriz de biofilmes tem importância não só estrutural como para a virulência de biofilmes (Branda *et al*., 2005) com destaque para os polissacarídeos extracelulares (PEC) (Sutherland, 2001). A composição da matriz é influenciada pela exposição a carboidratos da dieta e os objetivos dessa tese foram avaliar a importância da associação de amido com sacarose na composição de PEC da matriz do biofilme formado, na regulação da expressão dos gens das enzimas produtoras desses PEC, e no desenvolvimento de cárie em dentina radicular.

O estudo *in vitro* (capítulo 1) mostrou que biofilmes formados na presença de amido-sacarose podem ser potencialmente mais cariogênicos que aqueles formados na presença de sacarose pois apresentaram não só maior concentração de PEC, mas principalmente aumento do insolúvel. Esse aumento de PEC insolúvel poderia ser simplesmente um aumento da atividade da GTFB quando da presença de amido como foi mostrado *in vitro* (Vacca-Smith *et al.,* 1996; Kopec *et al.,* 1997). Entretanto, os dados do estudo 1 mostraram evidências que em acréscimo, a expressão do gene gtf*B* responsável pela síntese desse PEC foi ativada quando da associação de amido-sacarose Além do mais, o estudo 1 com biofilme de *S. mutans* confirmou que os PECs formados na presença de amido-sacarose são estruturalmente diferentes daqueles formados na presença isolada de sacarose ou amido (Vacca-Smith *et al*., 1996). Dessa forma, na presença da associação amido-sacarose além de maior síntese de PEC insolúvel, este PEC foi estruturalmente diferente ("novo" polissacarídeo) se comparado à sacarose. Com estes promissores resultados *in vitro*, a próxima etapa do trabalho foi tentar confirmar isso *in situ* com biofilme complexo (capítulo 2)

O estudo *in situ* simula situações mais próximas daquelas que ocorrem na cavidade bucal e a hipótese levantada pelo estudo *in vitro* do capítulo 1 foi essencial para prosseguir nesta linha. A cariogenicidade da associação amido-sacarose já havia sido testada em animais (Firestone *et al*., 1982) e *in situ* para esmalte (Ribeiro *et al*., 2005). Porém, nesses trabalhos amido e sacarose foram oferecidos juntos e não se considerou a hipótese da necessidade da quebra do amido pela amilase salivar ser precedida da exposição à sacarose em termos dos hidrolisados estarem disponíveis para serem aceptores das unidades de

glicose quando da ação das GTFs na sacarose. Em acréscimo, não era conhecido o efeito de amido ou amido-sacarose em dentina radicular. Desta forma, o delineamento do trabalho do capítulo 2 contemplou um grupo contendo solução de amido separado de solução de sacarose, sendo testado o efeito de amido, sacarose e associações amido-sacarose utilizando dentina radicular como substrato. Porém, o estudo *in situ* (capítulo 2) mostrou que a associação amido-sacarose não foi mais cariogênica que sacarose para dentina radicular. Assim, é possível que as mudanças ocorridas na estrutura do biofilme formado *in situ* não tenham sido suficientes para aumentar sua cariogenicidade, o que pode ser explicado pelas diferenças entre os modelos de estudo.

No caso do modelo *in vitro* (capítulo 1) biofilmes de *S. mutans,* foram formados *in vitro* em condições ideais possibilitando estudar o efeito da associação amido-sacarose na matriz do biofilme formado. Embora essa bactéria seja considerada o principal agente etiológico da cárie dental (Hamada & Slade, 1980) sua porcentagem em biofilme dental é menor que 2% (Nyvad & Kilian, 1987). Particularmente, no presente estudo *in situ* (capítulo 2) ela representava apenas 0,01% do total de bactérias encontradas no biofilme formado quando da exposição à sacarose. Assim, fica difícil comprovar com biofilme complexos (*in situ*) o encontrado com biofilme monoespécie de *S. mutans*, pois as mudanças qualitativas podem ser mascaradas pelas quantitativas. De fato, enquanto no estudo 1 as diferenças entre biofilmes expostos a sacarose ou amido-sacarose atingiram nível de significância estatística, no estudo 2 se limitaram a tendências biológicas as quais devem ser justificadas.

Assim, é bem conhecido que a extração e purificação de RNA de tecidos (Wanqian *et al.,* 2005) ou biofilmes (Keinanen-Toivola *et al*., 2006; Cury *et al.,* 2007; Cury *et al*., 2008) ricos em PEC é dificultada. No estudo 2 a concentração de PEC total foi numericamente maior no biofilme formado quando da associação amido-sacarose seguida de sacarose e amido (amido-sacarose>sacarose>amido) mas o rendimento de RNA foi amido>amido-sacarose>sacarose. Deve ser enaltecido que inclusive há coerência estatística entre a relação inversa da concentração de PEC nos biofilmes de sacarose comparado com amido e o rendimento em RNA. Também os dados sugerem fortemente que o biofilme formado pela exposição a amido-sacarose apresenta características intermediárias àquelas

dos extremos amido e sacarose. Assim, embora os biofilmes de amido-sacarose não tenham diferido estatisticamente de sacarose quanto à concentração de PEC, o rendimento em RNA dos biofilmes formados na presença de amido-sacarose não diferiram estatisticamente nem de amido quanto de sacarose, isto é apresentam características de ambos. Em acréscimo, a desmineralização em dentina e a porcentagem relativa de PEC insolúvel no tratamento amido-sacarose apresentaram valores numericamente maiores se comparados à sacarose. Assim, considerando a relação de PEC insolúvel com cárie dental, estes dados numéricos sugerem uma tendência de maior cariogenicidade da associação amido-sacarose em relação à sacarose. Entretanto, estas modificações não foram suficientes para atingir níveis de significância estatística e deverão ser consideradas em delineamentos de trabalhos futuros. Desse modo, a análise da estrutura química dos PECs formados *in situ* (estudo 2) pode esclarecer e respaldar os dados obtidos *in vitro* (capítulo 1).

Outra diferença relevante entre o estudo *in vitro* e *in situ* que poderia explicar as diferenças encontradas quanto aos PECs diz respeito ao tempo de exposição aos carboidratos. No estudo *in vitro* a concentração de carboidratos é mantida quase que constante durante todo o experimento, enquanto que no estudo *in situ* a concentração sofre influência da depuração salivar. Também no estudo *in vitro* foi simulada uma condição ideal para a atividade amilolítica da amilase salivar podendo ter sido formada uma quantidade suficiente de hidrolisados para servirem de substratos para a síntese do "novo" PEC formado. Outra diferença é que atividade amilolítica *in vitro* deve ser atribuída apenas à atividade da amilase aderida a superfície da HA desde que saliva não foi adicionada ao meio de cultura durante todo o período de formação do biofilme.

Com relação à cariogenicidade do amido, deve ser enfatizado que o estudo *in situ* realizado (capítulo 2) mostrou pela primeira vez de forma convincente que esse carboidrato da dieta pode ser cariogênico para dentina radicular comprovando e compensando as limitações de experimentos anteriores (Lingström *et al.,* 1994). Porém, o efeito foi de menor magnitude (2-3x) em comparação com sacarose o que deve ter relação com as diferenças dos biofilmes formados. Assim, no biofilme formado na presença de amido foram encontradas menores contagens de estreptococos do grupo mutans (11x) e lactobacilos (110.000x), assim como menor concentração de PEC insolúvel (15x).

Merecem destaque os resultados preliminares obtidos sobre a expressão dos genes das enzimas envolvidas com a síntese da matriz dos biofilmes. Estudos dessa natureza são extremamente limitados devido à dificuldade de extrair RNA de biofilmes formados na presença de sacarose (Cury & Koo, 2007). O estudo 2 *in situ* mostrou que é possível quantificar a expressão desses genes de *S. mutans* em biofilmes onde a porcentagem dessas bactérias é menor que 0,1%. Aliada a sensibilidade da análise o estudo 2 mostrou que *in vivo* os genes *gtfB, C e D* são expressos diferentemente quando da exposição a sacarose. Em acréscimo, embora não haja dificuldade da extração de RNA do biofilme formado na presença de amido, não se detectou expressão dos genes *gtfBC e D* no biofilme, resultado à princípio biologicamente coerente porque amido não é substrato para as enzimas produtos desses genes. Entretanto, deve ser considerado que embora a contagem de *S. mutans* no biofilme formado na presença de amido não tenha diferido das associações amido-sacarose, ela foi 10x menor do que aquela encontrada no biofilme formado pela exposição à sacarose.

CONCLUSÃO

Conclui-se que embora o biofilme formado pela exposição a amido-sacarose apresente características de ser mais cariogênico, isso não se refletiu em maior desmineralização da dentina radicular, pois embora a perda de mineral tenha sido maior do que provocada pelo amido ela não foi superior a da sacarose isoladamente.

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* De acordo com a norma da UNICAMP/FOP, baseadas nas normas do International Committee of Medical Journal Editors – Grupo de Vancouver. Abreviatura dos periódicos de acordo com o Medline.

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ANEXO 1: Comprovante de submissão do artigo para a revista Caries Research**.**

De: <r.p.shellis@bristol.ac.uk>
Para: <jcury@fop.unicamp.br>
Enviada em: quinta-feira, 7 de fevereiro de 2008 08:09
Assunto: Ms. No. 200802009, Caries Research

Dear Jaime,

Thank you for submitting your manuscript entitled "EFFECT OF STARCH AND SUCROSE ON DENTAL BIOFILM FORMED AND ON ROOT DENTINE DEMINERALIZATION" to "Caries Research". It will now be submitted to review and we shall inform you as soon as possible of the decision reached by the editorial board. The manuscript reference number is 200802009. Please use this number on all correspondence about the manuscript, which should be sent to the "Caries Research" editorial office at the address listed below.

I have added the CONSORT checklist that you emailed to me. For information regarding the status of your manuscript and for future submissions you can access this system by logging into the journal's online peer review system as follows:

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With kind regards,

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ANEXO 2: Instruções aos voluntários.

USO DO DISPOSITIVO INTRABUCAL

1. O dispositivo deve ser usado durante todo o dia e à noite, inclusive para dormir
2. Quando estiver fora da boca, em nenhum momento o dispositivo deve ser deixado à seco. Guarde-o no porta-aparelho, com uma gaze umedecida em água.
3. Procure evitar que o dispositivo fique fora da boca por um período prolongado, restringindo-se ao tempo necessário para alimentação (1 hora).
4. Não utilize produtos para bochecho ou agentes tópicos de qualquer natureza na cavidade bucal durante a fase experimental
5. Não utilize vitaminas ou suplementos sistêmicos que contenham flúor durante a fase experimental.
6. Não utilize alimentos que possam ser fonte de flúor como chá verde
7. Quando o dentifrício ou a gaze estiver acabando, entre em contato com a pesquisadora para que possam ser repostos.

GOTEJAMENTO DAS SOLUÇÕES

1. Gotejar uma gota da solução que lhe for dada sobre cada bloco dental 8 vezes ao dia nos seguintes horários:

08:00	**15:30**
09:30	**17:30**
11:30	**19:00**
13:30	**21:00**

2. Para gotejar as soluções, remova o dispositivo da boca, seque com gaze a região da telinha (com cuidado) e goteje uma gota da solução indicada sobre cada bloco dental, sem tocar a ponta do conta-gotas no dispositivo para evitar a contaminação da solução. Aguarde 5 minutos, para que a solução se difunda pela placa dental e retorne à cavidade bucal.

3. Se o primeiro gotejamento do dia não puder ser realizado às 08:00 horas, atrase os outros gotejamentos de acordo com o horário do primeiro, com um intervalo mínimo de 1h e30 min entre eles, até totalizar 8 x ao dia.

4. Após o gotejamento da solução, o aparelho deve retornar à boca em 5 minutos.

5. As soluções devem ser trocadas toda segunda, quarta e sexta-feira. Solicitamos que você venha ao laboratório buscar a nova solução nesses dias.

ESCOVAÇÃO

1. Utilize somente o dentifrício fornecido
2. Realize a escovação dos dentes 3 vezes ao dia
3. Sempre que escovar os dentes, escove também o dispositivo intrabucal. A área da telinha não deve ser escovada. Porém, a espuma gerada pela escovação do dispositivo deverá ser trazida sobre ela com a escova. Para esse procedimento, tome cuidado para não retirar a placa.
4. Ao enxaguar o dispositivo, tome cuidado para que jatos de água da torneira não atinjam diretamente a telinha, causando perda da placa acumulada.

Qualquer dúvida, entre em contato pelos telefones:

3433-4851 (casa)
8139-6766 (celular)
2106-5303 (laboratório)

Não deixe de ligar e se necessário, ligue a cobrar.

Obrigada pela colaboração!

ANEXO 3: Certificado do Comitê de Ética.

COMITÊ DE ÉTICA EM PESQUISA

UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

UNICAMP

CERTIFICADO

Certificamos que o Projeto de pesquisa "Efeito de amido e sacarose no biofilme dental formado in situ sobre dentina radicular e no desenvolvimento de cárie", protocolo CEP nº **031/2004**, dos Pesquisadores ***Carolina Patrícia Aires*** e ***Jaime Aparecido Cury***, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde - MS e foi aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Odontologia - UNICAMP.

We certify that the research project "Effect of starch and sucrose on the dental biofilm formed in situ on root dentine and on the development of dental caries", register number **031/2004**, of ***Carolina Patrícia Aires*** and ***Jaime Aparecido Cury***, is in agreement with the recommendations of 196/96 Resolution of the National Health Committee - Brazilian Health Department and was approved by the Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas - UNICAMP.

Piracicaba - SP, Brasil, May 27 2004

Profa. Dra. Cinthia Pereira Machado Tabchoury
Secretaria
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Júnior
Coordenador
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DECLARAÇÃO

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Piracicaba, 10 de Março de 2008.

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