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BÁRBARA EMANOELE COSTA OLIVEIRA

POLISSACARÍDEOS EXTRACELULARES DA MATRIZ DO BIOFILME COMO FONTE DE ENERGIA PARA *STREPTOCOCCUS MUTANS* E EFEITO NA CARIOGENICIDADE DO BIOFILME DENTAL

EXTRACELLULAR POLYSACCHARIDES FROM BIOFILM MATRIX AS ENERGY SOURCE FOR *STREPTOCOCCUS MUTANS* AND EFFECT ON DENTAL BIOFILM CARIOGENICITY

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Orientador: Prof. Dr. ANTÔNIO PEDRO RICOMINI FILHO

Coorientador: Prof. Dr. JAIME APARECIDO CURY

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RESUMO

Polissacarídeos extracelulares (PEC) são sintetizados por enzimas de *Streptococcus mutans* a partir da sacarose, facilitando a aderência microbiana à superfície dental e mudando a estrutura do biofilme, tornando-o mais cariogênico. Tem sido sugerido que PEC poderiam ser utilizados como reserva energética extracelular aumentando a cariogenicidade da sacarose, o que não tem sido experimentalmente comprovado. Este estudo foi conduzido para avaliar se PEC são utilizados por biofilme de *S. mutans* durante período noturno de limitação de carboidratos (jejum), contribuindo com o aumento da desmineralização do esmalte ocorrida durante exposição diária de carboidratos (abundância). O estudo foi *in vitro*, randomizado e cego, utilizando biofilmes de *S. mutans* UA 159, crescidos sobre blocos de esmalte dental bovino que foram selecionados por dureza de superfície (n=12/grupo). Os biofilmes foram cultivados a 37ºC, 10% de CO_2 em meio ultrafiltrado à base de triptona e extrato de levedura contendo 0,1 mM de glicose. Eles foram tratados 8x/dia com glicose 5,25% + frutose 5,25% - acidogênicos e formadores de polissacarídeos intracelulares (PIC); ou sacarose 10% - acidogênica e formadora de PIC e PEC. Essa frequência simulou episódios de abundância e escassez de carboidratos na cavidade bucal. Amostras de biofilmes foram coletadas ao final do 4º dia, após a última exposição diária aos carboidratos (abundância) ou no início do 5º dia, após o período noturno de jejum. Durante o crescimento dos biofilmes, o pH do meio de cultura foi determinado ao início e ao final do dia, sendo o meio armazenado para análises das concentrações de compostos orgânicos e cálcio. Nos biofilmes foram analisados PIC e PEC solúveis e insolúveis, contagem de unidades formadoras de colônia (UFC), peso seco, e suas arquiteturas por microscopia confocal de varredura a laser. A porcentagem de perda de dureza de superfície (%PDS) dos blocos foi calculada. Os dados foram analisados por *Anova two-way* seguido do teste de *Tukey*, ou Teste t (α= 5%). O efeito do fator tipo de carboidrato (Glicose+Frutose ou Sacarose) foi significativo ($p< 0,05$) para as variáveis: polissacarídeos, peso seco, pH e concentração de cálcio do meio de cultura; mas não a condição da coleta (abundância ou jejum) ($p> 0,05$). Maiores quantidades de PIC e PEC solúveis e insolúveis, maior peso de biofilme, menor pH e maiores concentrações de cálcio foram encontrados no grupo sacarose ($p< 0,05$). Ambos os efeitos (carboidrato e condição de coleta) foram significativos em relação à concentração de ácido lático, sendo maior no grupo sacarose ($p< 0,05$). Não houve

efeito do carboidrato e nem do período de coleta quanto à contagem de UFC ($p > 0,05$). O efeito do tipo de carboidrato foi significativo para a %PDS do esmalte ($p < 0,05$), com maior perda no grupo sacarose (40%), mas a desmineralização não aumentou durante o período de jejum ($p > 0,05$). Assim, não foi observado aumento na desmineralização do esmalte no período de escassez de carboidratos, sugerindo que a metabolização de PEC durante o período noturno de jejum poderia não contribuir com o aumento da desmineralização do esmalte ocorrida durante a exposição diária ao carboidrato.

Palavras-Chave: Polissacarídeos, *Streptococcus mutans*, Sacarose, Metabolismo.

ABSTRACT

Extracellular polysaccharides (EPS) are synthesized by *Streptococcus mutans* enzymes from sucrose, facilitating microbial adherence to dental surface and changing the biofilm structure, making it more cariogenic. It has been suggested that EPS could be used as extracellular energy reserve increasing sucrose cariogenicity, which has not been verified experimentally. This study was conducted to evaluate if extracellular polysaccharides (EPS) are used by *Streptococcus mutans (Sm)* biofilm during night starvation, contributing to increase enamel demineralization occurred during daily sugar exposure (abundance). The study was in vitro, randomized and blind, using *S. mutans* UA 159 biofilms, grown on dental enamel blocks that were selected by surface hardness (n=12/group). Biofilms were grown at 37°C, 10% CO_2 in ultrafiltered based tryptone and yeast extract medium containing 0.1 mM glucose. They were treated 8x/day with 5.25% glucose + fructose 5.25% - acidogenic and intracellular polysaccharides formation (IPS); or 10% sucrose - acidogenic and IPS and EPS formation. This frequency simulated abundance and starvation of carbohydrates episodes in the mouth. Biofilm samples were collected at the end of the 4th day, after the last daily exposure to carbohydrates (abundance) or at the beginning of the 5th day after an overnight starvation period. During the biofilm growth, the culture medium pH was determined at the beginning and end of the day, being the medium stored for organic compounds and calcium concentrations analysis. IPS and soluble and insoluble EPS, colony forming units counts (CFU), dry weight, and their architectures by confocal laser scanning were analyzed in biofilms. The percentage of surface hardness loss (%SHL) of enamel slabs was calculated. Data were analyzed by ANOVA two-way followed by Tukey test, or T-test (α = 5%). The factor effect of carbohydrate type (glucose + fructose or sucrose) was significant ($p< 0.05$) for the variables: polysaccharides, dry weight, pH and calcium concentration in the culture medium; but not the harvest condition (abundance or starvation) ($p> 0.05$). Larger amounts of IPS and soluble and insoluble EPS, greater biofilm weight, lower pH and higher calcium concentration were found in the sucrose group ($p< 0.05$). Both effects (carbohydrate and harvest condition) were significant in relation to the concentration of lactic acid, being higher in the sucrose group ($p< 0.05$). There was no effect to the carbohydrate and neither to the harvest period factors in relation to CFU counts ($p> 0.05$). The carbohydrate type effect was significant for enamel %SHL ($p< 0.05$), with greater loss in sucrose group (40%), but the demineralization has not increased during the

starvation period ($p > 0.05$). Thus, there was no increase in enamel demineralization in the carbohydrates starvation period, suggesting that EPS metabolism during the night starvation could not contribute to the increase of enamel demineralization occurred during the daily exposure to carbohydrate.

Keywords: Polysaccharides, *Streptococcus mutans*, Sucrose, Metabolism.

SUMÁRIO

1 INTRODUÇÃO

O biofilme dental é fator necessário para a ocorrência da cárie e a exposição frequente aos carboidratos da dieta é determinante para o desenvolvimento da doença (Fejerskov, 2004). A metabolização dos açúcares pelos micro-organismos do biofilme promove constantes quedas de pH, alterando o equilíbrio local. Dessa maneira, a proliferação de bactérias acidogênicas e acidúricas, tal como *Streptococcus mutans*, é favorecida promovendo a mudança de um biofilme saudável para um biofilme cariogênico (Marsh et al., 2003, 2009).

Quando carboidrato é fornecido em grande quantidade, *S. mutans* é capaz de armazenar o excedente sob a forma de polissacarídeos intracelulares (PIC). Os PIC são polímeros tipo glicogênio (Bramstedt e Lusty, 1968; Zero et al., 1986) e atuam como reserva de energia endógena, importante devido às condições de flutuação de nutrientes que ocorrem na cavidade bucal (Huis in't Veld e Backer Dirks, 1978; Takahashi, 2015). Considerando que o biofilme dental é formado em episódios de "fartura" e "miséria", ou seja, períodos curtos de exposição a carboidrato seguidos por intervalos prolongados de ausência de nutrientes (Carlsson, 1997; Bowden and Hamilton, 1998), essa reserva seria útil para a manutenção do metabolismo bacteriano na restrição de nutrientes (Huis in't Veld e Backer Dirks, 1978; Zero et al., 1986; Bowden and Hamilton, 1998; Zhu et al., 2001). Adicionalmente, se a sacarose estiver disponível, além da produção de ácidos e armazenamento de PIC, ocorrerá a síntese de polissacarídeos extracelulares (PEC), os quais são responsáveis pela maior cariogenicidade do biofilme (Rölla, 1989; Cury et al., 2000; Paes Leme et al., 2006; Aires et al., 2006).

Polissacarídeos extracelulares compõem a matriz do biofilme e são sintetizados a partir da quebra da molécula de sacarose por enzimas extracelulares produzidas por *S. mutans*, conhecidas como frutosil (FTF) e glucosiltransferases (GTF) (Bowen & Koo, 2011). As FTFs utilizam a frutose da molécula de sacarose para síntese de frutanos, que são compostos solúveis formados por inúmeras moléculas de frutose unidas por ligações β (2→1) ou β (2→6), liberando glicose para produção de ácidos (Colby e Russel, 1997). Por outro lado, três GTFs distintas são produzidas por *S. mutans* e são responsáveis pela síntese de glucanos a partir da molécula de glicose proveniente da sacarose, liberando frutose para produção de ácidos. A GTF

tipo B produz glucanos insolúveis, compostos por ligações α (1 → 3) em sua cadeia principal, capazes de se adsorver a outros micro-organismos tornando-os produtores de glucanos. A GTF D sintetiza glucanos solúveis, cuja cadeia principal é composta por ligações tipo α (1 → 6) (Aires et al., 2011; Bowen e Koo, 2011), enquanto a GTF C tem afinidade à película salivar e consegue sintetizar um polímero misto com características solúveis e insolúveis (Bowen e Koo, 2011). Os polímeros formados diferem quanto à sua estrutura e função, aumentando a aderência e agregação bacteriana (Bowen e Koo, 2011), estruturação (Xiao et al., 2012; Koo et al., 2013) e porosidade do biofilme dental (Dibdin e Shellis, 1988).

Adicionalmente, tem sido sugerido que os PEC poderiam ser metabolizados pelos micro-organismos do biofilme em períodos de ausência de nutrientes (Wood, 1967; DaCosta e Guibbons, 1968), uma vez que além das transferases, *S. mutans* possuem enzimas tipo hidrolases. Sendo assim, frutanases (FruA) hidrolisariam ligações tipo β (2→1) ou β (2→6) presentes nos frutanos (Walker et al., 1983), enquanto as dextranases (DexA), hidrolisariam ligações α (1 → 6) presentes nos dextranos e os monossacarídeos resultantes (glicose e frutose) seriam utilizados para manutenção do metabolismo bacteriano. Essa metabolização possibilitaria uma contínua produção de ácidos e como consequência, o desafio cariogênico seria prolongado (Wood, 1967; DaCosta e Guibbons, 1968; Colby e Russel, 1997). Clinicamente, isso seria importante principalmente durante o período noturno, no qual o fluxo salivar diminui e o *clearance* é reduzido (Dawes, 1972).

Em estudo realizado com células planctônicas, foi observado que a indução de FruA ocorreu somente na presença de levanos, inulina ou sacarose. A atuação das frutanases ocorreria nas moléculas terminais de frutose dos frutanos, liberando frutose no biofilme dental continuamente (DaCosta e Gibbons, 1968). Além disso, a maior parte das FruA foram encontradas aderidas à superfície celular, o que agilizaria a metabolização desses polissacarídeos e consequente produção de ácidos (DaCosta e Gibbons, 1968; Walker et al., 1983). Em relação à degradação de glucanos, maior expressão do gene relacionado à enzima DexA foi verificada em biofilme formado por 120 h utilizando sacarose como fonte de energia, sugerindo que biofilmes maduros poderiam ser mais metabolicamente ativos e persistentes (Klein et al., 2009). Um estudo in vitro demostrou que PIC conferiram vantagem para a persistência bacteriana de células planctônicas crescidas com glicose, mas não quando biofilme foi cultivado

na presença de sacarose, que é substrato para síntese PEC. Na ausência de FruA, o metabolismo bacteriano não foi alterado após restrição de nutrientes, o que poderia ser um reflexo da utilização de outras fontes de energia (Busuioc et al., 2009). Estudos de caracterização de polissacarídeo tem demonstrado que PEC insolúveis não são exclusivamente formados por ligações tipo α (1 → 3) (Hayacibara et al., 2004; Aires et al., 2001), possuindo, portanto, ramificações com ligações solúveis que também poderiam ser hidrolisadas.

Embora estudos indiquem que PEC podem ser metabolizados (Wood, 1967; DaCosta & Gibbons, 1968; Walker, 1983; Whiting et al., 1993), dados avaliando essa condição em biofilme são escassos. Um estudo que utilizou biofilme humano acumulado por 24 h concluiu que parte dos PEC solúveis produzidos poderiam ser metabolizados, mas o período de coleta das amostras e a exposição ao carboidrato não foram padronizados (Wood, 1967). A utilização de células e enzimas dispersas em solução também é limitante, uma vez que não apresentam o mesmo comportamento de enzimas adsorvidas e células organizadas em biofilmes (Kopec et al., 1997; Busuioc et al., 2009; Bowen & Koo 2011). Outro aspecto a ser considerado, é o cultivo de biofilme sob exposição constante ao carboidrato em alta concentração. Esta condição não mimetiza o que ocorre no ambiente oral, e células submetidas a períodos de restrição de nutrientes parecem ser mais persistentes e metabolicamente ativas que aquelas que não sofrem restrição (Huis in't Veld e Backer Dirks, 1978; Zhu e al., 2001).

A análise da utilização de PEC por *S. mutans* em períodos de escassez de carboidratos poderia responder se a metabolização ocorre e se seria suficiente para produzir danos significantes ao substrato dental, em virtude do aumento da cariogenicidade do biofilme. Considerando que a metabolização de PEC não foi avaliada simulando as condições de flutuação de nutrientes que ocorrem na cavidade oral e, tendo em vista que células planctônicas não se comportam da mesma maneira que células organizadas em biofilmes, o papel energético que os PEC desempenham na cariogenicidade do biofilme dental não tem sido bem esclarecido. Sendo assim, o objetivo deste estudo foi avaliar a utilização de polissacarídeos extracelulares como fonte de energia em período de jejum na ausência de carboidrato e sua influência na desmineralização do esmalte dental.

2 ARTIGO

Biofilm polysaccharides degradation during starvation and enamel demineralization

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Biofilm polysaccharides degradation during starvation and enamel demineralization

ABSTRACT

This study was conducted to evaluate if extracellular polysaccharides (EPS) are used by *Streptococcus mutans (Sm)* biofilm during night starvation, contributing to increase enamel demineralization occurred during daily sugar exposure. *Sm* biofilms were formed during 5 days on bovine enamel slabs of known surface hardness (SH). The biofilms were exposed to sucrose 10% or glucose 5.25% + fructose 5.25%, carbohydrates that differ on EPS formation. The biofilms were treated with the carbohydrates 8x/day but were maintained in starvation during the night. Biofilm samples were harvested during two times, on the end of the 4th day and in the morning of the 5th day, conditions of sugar abundance and starvation, respectively. The slabs were also collected to evaluate the percentage of SH loss. The biofilms were analyzed for EPS soluble and insoluble and intracellular polysaccharides, viable bacteria and biofilm architecture. pH, calcium and acids concentration were determined in the culture medium. The data were analyzed by two-way ANOVA followed by Tukey's test or t-Test. The effect of the factor carbohydrate treatment was significant ($p<0.05$) but not the harvest condition ($p>0.05$). Larger amounts of EPS soluble and insoluble were formed in sucrose than glucose+fructose group ($p<0.05$), but was no observed their metabolization during starvation time ($p>0.05$). Greater enamel %SHL was found for sucrose than glucose+fructose group ($p<0.05$) but the demineralization did not increase during starvation ($p>0.05$). In conclusion, the findings suggest that EPS metabolization during night starvation do not contributed to increase enamel demineralization occurred during the daily abundance of sugar.

Keywords: Polysaccharides, *Streptococcus mutans*, Sucrose, Metabolism.

Introduction

Dental caries is a biofilm-sugar related disease that depends on biofilm accumulation on tooth surface and its frequent exposure to dietary carbohydrates (Fejerskov, 2004). The cariogenic biofilm forms and grows on dental surfaces in a dynamic condition in which the exposure to dietary carbohydrates occurs in an intermittent manner (Marsh, 2003). During the day, the biofilm is frequently exposed to short periods of great amount of carbohydrates, followed by long periods of non-exposure between the meals and overnight. These episodes are known as "feast" and "famine" periods and they are determinants for bacterial metabolism and biofilm growth (Carlsson, 1997; Bowden and Hamilton, 1998; Ccahuana-Vásquez & Cury, 2010). In “feast” periods, bacteria such as *S. mutans* are able to store the excess of available carbohydrate as intracellular polysaccharides (IPS), which act as a reserve energy source for “famine” periods (Busuioc et al, 2009; Takahashi, 2015). In addition to storing energy as IPS, enzymes glucosyl- and fructosyltranferases produced by *S. mutans* synthesize extracellular polysaccharides (EPS) when sucrose is the available carbohydrate (Rölla, 1989; Bowen, 2002; Paes Leme et al., 2006).

EPS, produced from sucrose, contribute to microbial attachment and shifts on matrix tridimensional organization, mainly enhancing its porosity (Dibdin & Shellis, 1988; Bowen & Koo, 2011; Koo et al., 2013). This structural change favors acid diffusion through the biofilm and pH fall next to the tooth surface, increasing enamel demineralization (Cury et al., 2000). Additionally, it has been suggested that biofilm cariogenicity could be also risen by degradation of the EPS matrix. Thus, soluble glucans and fructans could be degraded by specific hydrolases, releasing glucose and fructose to acid production. This phenomenon would happen in "famine" periods and would act as exogenous energy source to the bacterial metabolism maintenance (Wood, 1967; Whiting et al., 1993; Colby and Russell, 1997; Paes Leme, 2006). Clinically, the acid produced in a non-removed biofilm could extend the mineral loss especially at night time period in which salivary flow rate is lower and the clearance is decreased (Dawes, 1972).

Although there are studies that indicate that EPS can be metabolized (Wood, 1967; DaCosta & Gibbons, 1968; Walker, 1983; Whitting et al., 1993), there is no data evaluating this condition in biofilms. The use of planktonic cells and enzymes dispersed in solution does not represent the same behavior as cells organized in

biofilm and adsorbed enzymes (Kopec, et al., 1997; Busuioc et al., 2009; Bowen & Koo 2011), which could modify this property. Another aspect to consider is the growth of biofilms under constant exposure to high concentrations of carbohydrate, since this condition does not mimic the oral environment and cells undergone starvation seems to be more persistent and metabolically active than those grown without restriction of nutrients (Huis in't Veld & Backer Dirks, 1978; Zhu at al., 2001). In addition, the effect of EPS degradation on enamel demineralization has not been shown.

The assessment of the extracellular polysaccharides utilization by *S. mutans* in starvation period could answer if the metabolization would occur and whether it would be of magnitude to produce significant damage to the tooth. Thus, the aim of this study was to evaluate the degradation of extracellular polysaccharides as energy source during overnight starvation period when no carbohydrate is available and its consequent effect on enamel demineralization.

Material and methods

Experimental design

An *in vitro*, randomized and blinded factorial (2x2) study, approved by the local Research and Ethics Committee (142/2014), was conducted using a validated cariogenic biofilm model (Ccahuana-Vásquez and Cury, 2010). The factors under study were type of carbohydrate (sucrose or a mixture of glucose + fructose) to feed the biofilms during the day and time of biofilm harvest (at the end of the day after the last sugar treatment, condition of sugar abundance or after overnight starvation), generating 4 experimental groups: (1) glucose + fructose "abundance", (2) glucose + fructose "starvation", (3) sucrose "abundance", and (4) sucrose "starvation". Sucrose and glucose + fructose are acidogenic carbohydrates and are stored as IPS, but only sucrose is substrate for EPS formation. *S. mutans* UA159 biofilms were formed on bovine enamel slabs, selected by surface hardness and randomized into the experimental groups (n=12). The slabs were coated by saliva and the biofilms were grown in ultrafiltered tryptone-yeast extract broth (UTYEB). The biofilms were exposed 8 times a day to 5.25% glucose + 5.25% fructose solution or 10% sucrose solution. The culture medium was changed twice daily and the pH was measured. Intracellular and extracellular polysaccharides (soluble and insoluble), biofilm dry weight and the

number of viable cells were analyzed. Acids and the calcium concentration present in the medium were determined. Enamel demineralization was calculated by percentage of surface hardness loss (%SHL). Confocal laser scanning microscopy was used to visualize the biofilm organization. The hypothesis under study was that EPS are metabolized during night starvation, increasing enamel demineralization occurred during daily sucrose exposure. Three independent experiments were carried out (n=4) and the data were statistically analyzed according to the factorial design of this study, considering enamel slab as a statistical block (n=12).

Enamel slabs preparation

Enamel slabs (4 x 7 x 1 mm) were obtained from bovine incisor teeth. The crowns were sectioned using a low-speed diamond blade to obtain enamel slabs. The surfaces were ground and polished using aluminum oxide abrasive papers (#400, #600 and #1200) and 1 µm diamond paste in a grinder machine (Phoenix Beta, Buehler, USA). The surface hardness (SH) was determined using a Knoop indenter (Future-Tech FM, Kawasaki, Japan) in which three indentations spaced 100 µm from each other were performed with 50 g load for 5 s on the polished surface center. The slabs with intra-variability higher than 10% were excluded and the selected slabs were randomized into the groups. The slabs were placed in 24-well culture plates, in vertical position using a metallic holder, and submitted to sterilization by exposure to ethylene oxide.

Biofilm model

The biofilm model was adapted from Ccahuana-Vasquez and Cury's model (Ccahuana-Vásquez and Cury, 2010). *S. mutans* UA159 reference strain (Ajdic et al., 2002) was used in the experiment and cultures stored at -80°C were first grown on Columbia blood agar (CBA). To inoculum preparation, *S. mutans* colonies were transferred to UTYEB broth supplemented with 1% glucose and incubated overnight at 37°C, 10% CO_2 (Ccahuana-Vasquez and Cury, 2010). The cells were centrifuged, washed with saline solution, and resuspended in fresh UTYEB. The cell suspension was standardized in a spectrophotometer at OD_{600} of 1.6 ± 0.5 to obtain the bacterial inoculum. Prior to cell adhesion phase, the slabs were immersed in filtered fresh stimulated human saliva to acquired pellicle formation at 37°C, 10% CO_2, during 30 min (Koo et al., 2003; Lemos et al., 2010). Saliva-coated slabs were transferred to the

wells containing 2 mL of fresh UTYEB with 1% glucose and the bacterial inoculum (1:500 v/v) and incubated for 8 h to promote initial adhesion of the microorganisms. Only in the adhesion phase, the UTYEB pH 7.0 was strongly buffered (10x higher the usual phosphate concentration) to avoid pH fall and enamel demineralization during this phase. After adhesion, the slabs were transferred to fresh UTYEB pH 7.0 with 0.1 mM glucose basal concentration and incubated overnight at 37°C, 10% CO_2 for 16 h. At beginning of 2nd day, the biofilms were exposed to episodes of "feast" and "famine" comprised of 8 daily exposures to carbohydrate solutions: 10% sucrose or a combination of glucose 5.25% and fructose 5.25% at predetermined times (08:00, 09:30, 11:00, 12:00, 13:30, 15:00, 16:00 and 17:30 h) for 3 min (Fernandez et al., 2015). After the cariogenic challenge, enamel slabs were rinsed 3 times in NaCl 0.9% solution, and replaced in the culture plate. The culture medium was changed twice daily, before the first challenge and after the last one. The pH was evaluated in each change and the medium of each well was stored individually in microcentrifuge tubes at -80°C for later assessment of acids and calcium concentration. At the end of the 4th day of biofilm formation, right after the last treatment, the biofilms were rinsed and remained during 10 min in fresh saline solution as standardized in a pilot study to avoid overestimation in the polysaccharides analysis. Half of slabs with biofilm formed was collect for "abundance" condition evaluation. The other half was placed in fresh culture medium without glucose basal concentration, and at beginning of the 5th day, the biofilm was collected in "starvation" condition, after approximately 16 h of fasting. The harvest was realized by sonication as described by Ccahuana-Vasquez and Cury (2010). The enamel slabs were separated to evaluate the demineralization provoked and the suspension was used for analyses of the biofilm.

Biofilm analyses

An aliquot of 100 µL of the biofilm suspension was ten-fold serially diluted in saline solution until 1:10^7. Two drops of 20 µL of each dilution was plated on Todd-Hewitt broth (THB) plus agar and incubated at 37°C, 10% CO_2 for 48 h and the counts of the colony forming unit (CFU) were performed with the aid of a stereoscopic microscope (Tenuta et al., 2006; Ccahuana-Vasquez and Cury, 2010). The extraction of S-EPS, I-EPS and IPS was performed as described by Aires et al. (2008) from an aliquot of 400 µL of the sonicated biofilm suspension. The amount of total carbohydrates was quantified by the phenol sulfuric method (Dubois et al., 1956) using

glucose as standard. Another aliquot of 400 µL of the sonicated biofilm suspension was added in pre-weigh microcentrifuge tubes to perform the biofilm dry weight analysis. The dry weight was determined by the difference between the final and initial weight of the microcentrifuge tubes (Koo et al., 2003; Ccahuana-Vasquez and Cury, 2010).

Culture medium analysis

The pH of the culture medium was evaluated twice a day at each medium change as an indicator of biofilm acidogenicity. The pH measurement was performed using a pH microelectrode (Accumet; Cole-Parmer, USA) coupled to a pH meter (Procyon SA-720, Olímpia, Brazil) calibrated with pH standards of 4.0 and 7.0, done directly inside the wells, just after the medium change. The calcium concentration in the medium collected during the biofilm development was analyzed by colorimetric method using Arsenazo III (Vogel et al., 1983). It was used 10 µL of each sample in 96-well plates and the measurement was done in a microplate spectrophotometer reader (Multiskan, Thermo Scientific) at a wavelength of 650 nm (Fernández et al., 2016). The quantification of organic acids in culture medium from the 4th and 5th days was performed by High Performance Liquid Chromatography (Alliance 2795, Waters, USA) using a refractive index detector and an Aminex HP-87H column (Bio-Rad Laboratories, USA) (Carvalho-Netto et al., 2015). Standard curves of lactic, acetic and formic acids, and ethanol were used to calculate the concentration in the samples. Triplicates of two independent experiments were used (n=6).

Enamel demineralization assessment

After biofilm removal, the slabs were separated to evaluate the enamel demineralization. The SH was again performed by three indentations separated by 100 µm from the initial SH measurement and the percentage of surface hardness loss (% SML) was calculated as follows: (baseline SH - SH after assay × 100)/baseline SH (Cury et al., 2000).

Confocal laser scanning microscopy (CLSM)

To visualize the biofilm organization under glucose + fructose or sucrose exposure, additional enamel slabs were used for biofilm formation in each group (n=2). The extracellular matrix was labeled during the biofilm development using Alexa Fluor

647-dextran conjugate (molecular weight, 10,000; Excitation 650 nm/ Emission 668 nm; Thermo Scientific, USA) (Xiao and Koo, 2010). The *Sm* cells staining with SYTO-9 green fluorescent nucleic acid (Excitation 485 nm/ Emission 498 nm; Thermo Scientific, USA) were performed at the end of the biofilm development, on the 4th day. Enamel slabs were immersed in saline solution 0.9% with 2.5 µM SYTO-9 and incubated under protection from light for 20 min. The images were obtained in a DMI 6000 CS inverted microscope coupled to TCS SP5 computer-operated confocal laser scanning system (Leica Microsystems CMS, Mannheim, Germany) using 40x oil immersion objective (numeric aperture 1.25). An ar-ion laser tuned at 488 nm and a He-Ne laser at 633 nm was used for excitation. A series of images were obtained at 1 µM distance in the Z axis from the five different sites of the enamel slab surface (Pereira-Cenci et al., 2008). Three-dimensional reconstructions were performed using the software Image J (Hartig, 2013) in order to visualize the organization of biofilms.

Statistical analysis

Data were analyzed by two-way ANOVA, considering the factors carbohydrate (glucose + fructose or sucrose) and biofilm harvest time ("abundance" or "starvation" condition) under study, followed by Tukey's HSD Test. Assumptions of homogeneity of variances and normal distribution of errors were checked for all response variables tested using the Kolmogorov-Smirnov test. Data that violated the assumptions were transformed to square-root (biofilm dry weight and %SHL) and to $\log_{10}$ (CFU counts, CFU counts/mg biofilm dry weight, polysaccharides, organic compounds) before they were analyzed. The calcium concentration and pH in the culture medium data were analyzed by t-Test, being calcium values transformed to their inverse. The statistical analysis was done using SAS software (SAS Institute Inc., version 8.01, Cary, N.C., USA) employing a significance level fixed at 5%.

Results

In relation to polysaccharides, the statistical analyses showed significant difference for the factor carbohydrate, however no difference were observed for the time of harvest and for the interaction effect between the two factors (carbohydrate and time of harvest) for the variables: polysaccharides, biofilm dry weight, CFU by dry weight and %SHL. The amounts of intracellular (IPS), and soluble (S-EPS) and

insoluble (I-EPS) extracellular polysaccharides in biofilms formed under daily exposure to sucrose were higher, when compared to those found in biofilms exposed to glucose + fructose ($p < 0.05$). Also, the amount of I-EPS was higher under sucrose exposure compared with glucose + fructose ($p < 0.05$). The factor harvest condition was no significant ($p > 0.05$) (Fig. 1).

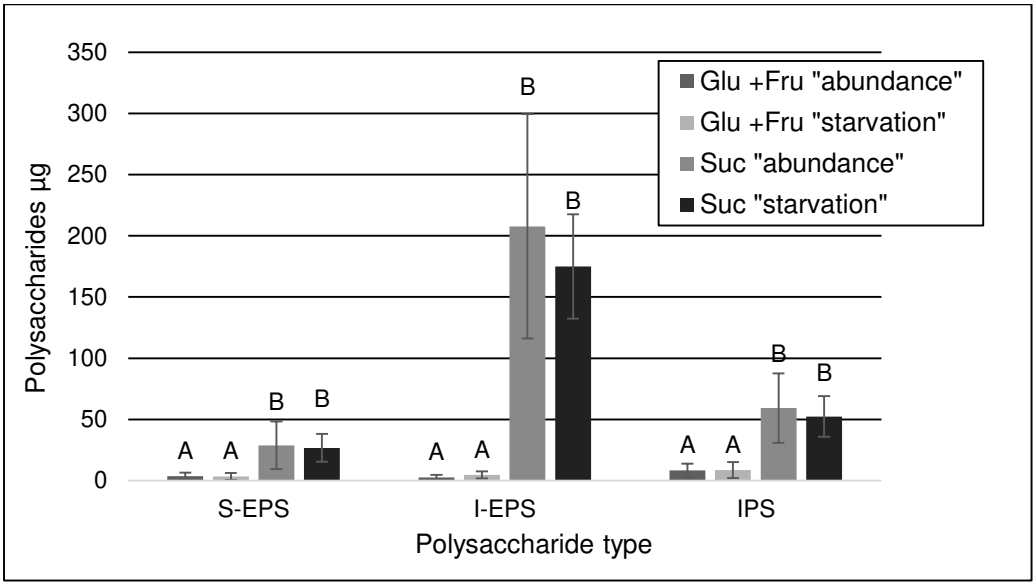

Figure 1: Amounts (µg) of extracellular polysaccharides, soluble (S-EPS) and insoluble (I-EPS) and intracellular polysaccharides (IPS) according to biofilm treatments (Glucose + Fructose or Sucrose) and time of harvest ("abundance" or "starvation"). Distinct capital letters indicate significant statistically differences among groups for each polysaccharide type ($p < 0.05$). (Mean ± SD; n = 12).

The biofilm dry weight was much higher when the biofilm was formed under sucrose exposure than those formed with glucose + fructose ($p < 0.05$). On the other hand, there was no effect to the carbohydrate and neither to the harvest period factors in relation to CFU counts ($p > 0.05$) (Table 1). When CFU counts data were normalized by biofilm dry weight, a greater number of viable cells per volume of biofilm was observed into group treated with glucose and fructose ($p < 0.05$) (Table 1). CLSM images showed extracellular polysaccharides (red) and *S. mutans* cells (green), synthesized in biofilms exposed to sucrose (Fig. 2). In glucose + fructose group, only *S. mutans* cells (green) was visualized. It was also possible to observe that biofilm under sucrose treatment presented higher volume of biomass due biofilm height (Fig. 2), which is in accordance to EPS analysis (Fig.1) and biofilm dry weight (Table 1).

Table 1. Mean ± SD of biofilm dry weight (mg), CFU counts (Log_{10}), CFU counts /mg biofilm dry weight (Log_{10}/mg) and %SHL according to carbohydrates exposure and time of harvest. (n=12; p< 0.05).

Carbohydrate	Biofilm harvest condition	Analysis				
		Biofilm dry weight (mg)¤	CFU counts (CFU Log_{10}) *	(CFU Log_{10})/mg biofilm dry weight *	%SHL¤	Calcium released from enamel (µg) ¤
Glu + Fru	"abundance"	0.4 ± 0.2 ^{A}	7.4 ± 1.1 ^{A}	12.3 ± 1.1 ^{A}	14.5 ± 7 ^{A}	24.7 ± 14.8 ^{A}
	"starvation"	0.6 ± 0.4 ^{A}	7.8 ± 1.2 ^{A}	15.4 ± 7.5 ^{A}	11.9 ± 5 ^{A}	17.1 ± 10.7 ^{A}
Suc	"abundance"	1.6 ± 0.3 ^{B}	7.6 ± 1.0 ^{A}	4.8 ± .3.0 ^{B}	41.4 ± 7 ^{B}	85.1 ± 29.9 ^{B}
	"starvation"	1.6 ± 0.3 ^{B}	8.3 ± 0.5 ^{A}	5.4 ± 1.2 ^{B}	34.7 ± 8 ^{B}	33.2 ± 24.4 ^{A}

Distinct capital letters indicate significant statistically differences (p < 0.05) among groups for each variable (values within columns).
* The values were transformed by log_{10} to statistical analysis
¤ The values were transformed by square-root

The pH values of culture medium were lower in the group exposed to sucrose. This differentiation was possible to be observed from 56 h, which corresponds to 2nd day of biofilm development, when it started to be mature (Fig. 3). Lower amount of acids was produced by *S. mutans* when biofilm was formed under glucose + fructose exposure, when compared to sucrose (p< 0.05). Among acids, there were effect to the factors carbohydrate and harvest condition (p< 0.05) to lactic acid that was produced in higher quantities for both treatment groups. Moreover, the sucrose group values were 2 times higher than those observed in the group treated with the combined monosaccharides (p< 0.05). Acetic acid was produced in small amounts and only the carbohydrate factor had a significant effect (p< 0.05). Ethanol was also produced as a final compound of the carbohydrate metabolism being significant only to the harvest period factor (p< 0.05) (Fig. 4). In "starvation" condition, acid production was lower for both glucose + fructose and sucrose groups, but it occurred even after all carbohydrate sources were removed (p <0.05). This indicated some metabolization at overnight period ("starvation" condition).

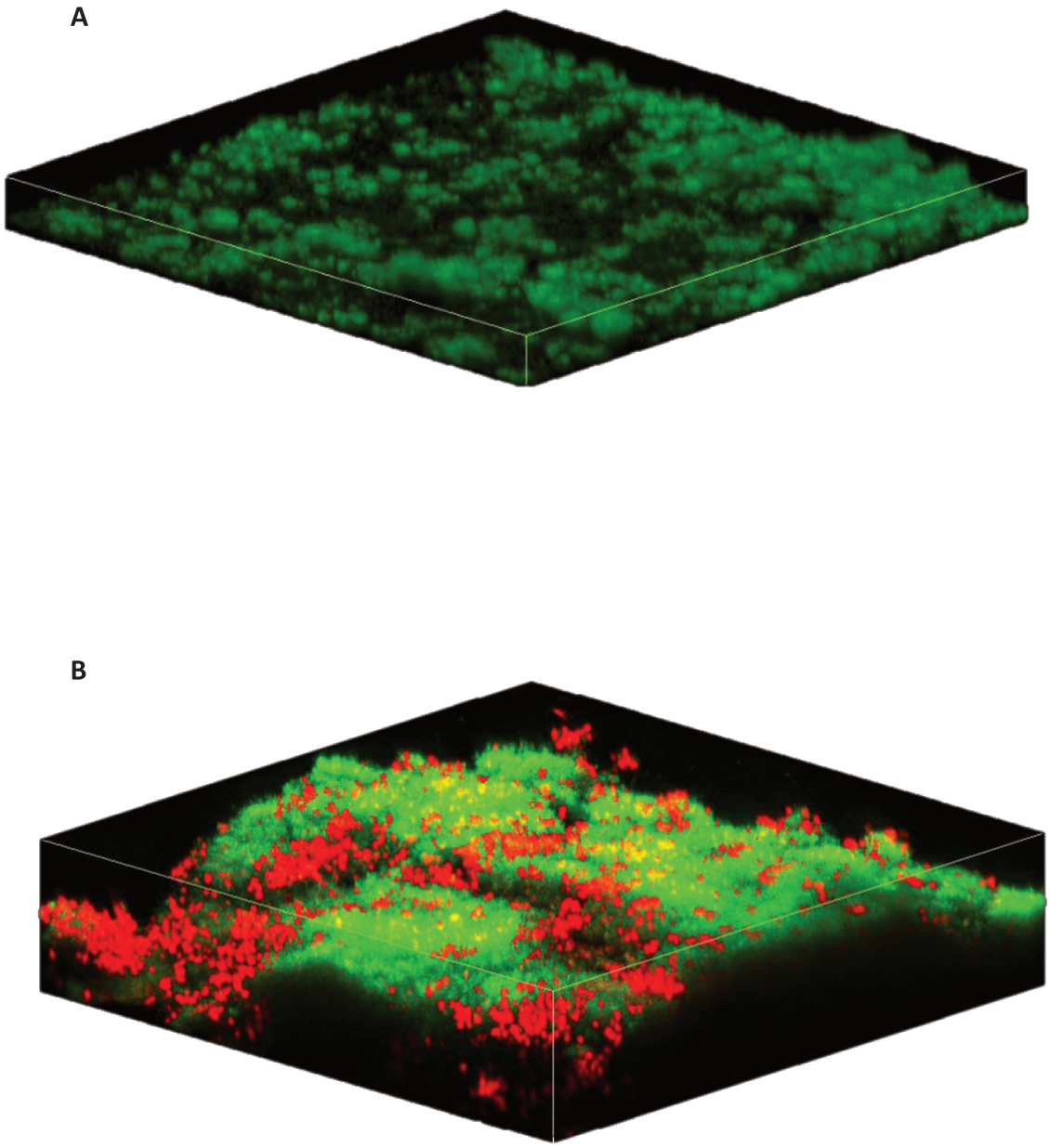

Figure 2. CLSM images showing biofilms formed under exposure to Glucose + Fructose (A) or Sucrose (B) 8x/daily at "abundance" condition (4th day of biofilm development). In green, *S. mutans* cells stained with SYTO 9. In red, extracellular polysaccharides labeled with Alexa Fluor 647 - dextran conjugate. Oil immersion objective of 40x (numeric aperture 1.25).

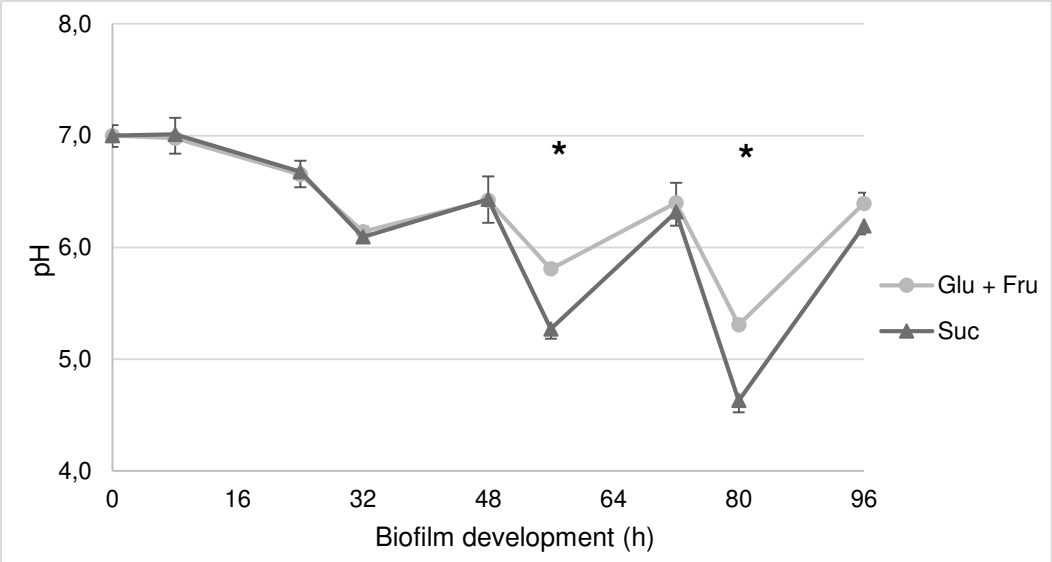

Figure 3. pH values of the culture medium according to biofilm treatments (glucose + fructose or sucrose) and biofilm development time (h) as an indicator of biofilm acidogenicity (Means ± SD; n = 12). Time points at 80 h and 96 h refer to the "abundance" and "starvation" conditions, respectively.

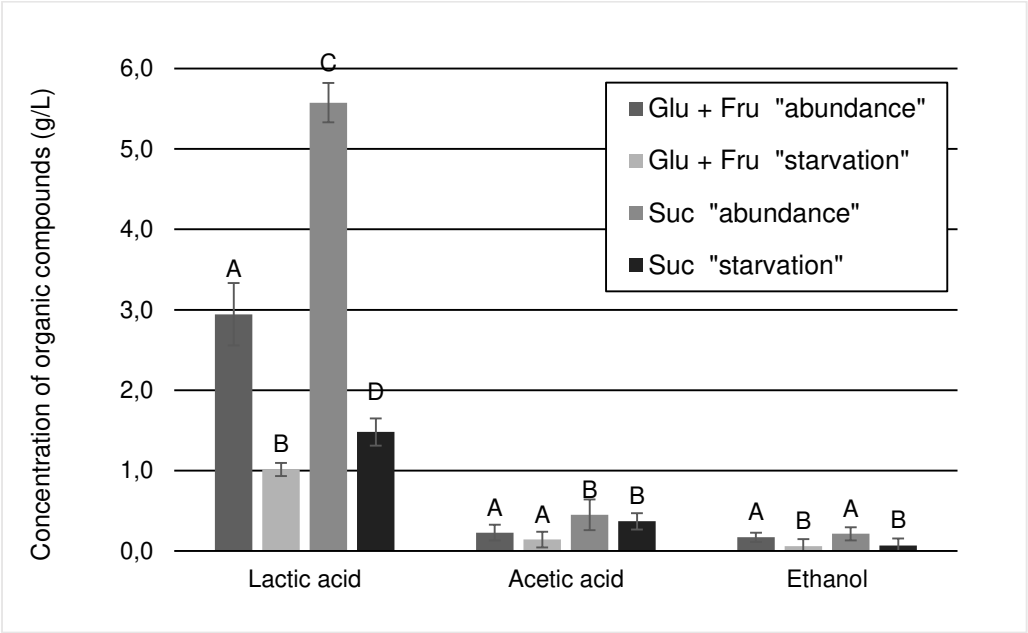

Figure 4: Concentration of organic compounds (g/L) produced by *S. mutans* in the culture medium according to biofilm treatments (glucose + fructose or sucrose) and time of harvest ("abundance" or "starvation"). Distinct capital letters indicate significant statistically differences among groups for each type of acid ($p < 0.05$). (Mean ± SD; n = 6).

The calcium concentration in culture medium during biofilm development was higher in sucrose group ($p< 0.05$). This higher quantity released from enamel was seen from 32 h of biofilm development and became more evident with the biofilm maturation (Fig. 5), mainly at the end of the 4th day ("abundance" condition) ($p <0.05$). The resultant mineral loss of the "abundance and starvation" periods showed larger calcium amount in the culture medium of the biofilm exposed to sucrose and harvested in "abundance" condition ($p< 0.05$) (Table 1). This behavior was consistent with the pH and %SHL data (Table 1). Higher %SHL was observed in the sucrose group enamel slabs, which differed statistically from the group treated with the combined monosaccharides ($p <0.05$). Under sucrose exposure the enamel slabs lost around 30 to 40%, while those exposed to glucose + fructose lost around 15% of the surface hardness. No significant effect was observed in relation to harvest condition to the %SHL data (Table 1).

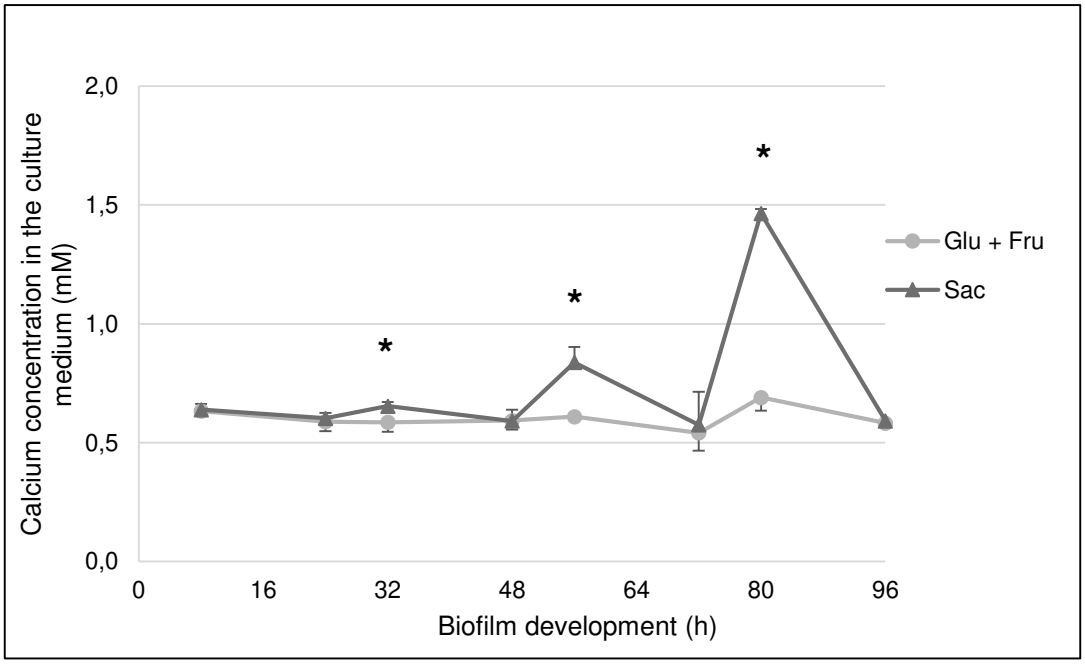

Figure 5. Calcium concentration (mM) in the culture medium according to biofilm treatments and biofilm development time (h) (Mean ± SD, n = 12). Time points at 80 h and 96 h refer to the "abundance" and "starvation" conditions, respectively.

Discussion

The presence of extracellular polysaccharides (EPS) in dental biofilm matrix has been reported as an important virulence factor associated to dental caries development (Rölla, 1989; Cury et al., 2000; Mattos-Graner, et al., 2000; Koo et al., 2013). In this study, it was used a biofilm model in which allowed to simulate the EPS formation and its absence, by sucrose or glucose + fructose exposure, respectively. It was tested if *S. mutans* could use extracellular polysaccharides from biofilm matrix as energy source in periods of absence of fermentable carbohydrates ("starvation") or if this use would be only from intracellular polysaccharides, and if this metabolization could increase the biofilm cariogenicity.

Our results showed that EPS were only synthesized in the presence of sucrose, and that insoluble extracellular polysaccharides (I-EPS) were the most formed, since sucrose is the only carbohydrate that is substrate to synthesis these molecules (Fig. 1) (Rölla, 1989; Paes Leme et al., 2006). It was observed no decrease in the amounts of each type of polysaccharide from the "abundance" to the "starvation" condition in *S. mutans* biofilms formed under sucrose exposure ($p > 0.05$). This indicate that the polysaccharides may have not been metabolized during the starvation time or were metabolized, but in an amount very small. This quantity might not has been detected due its use just to maintain the bacterial basal metabolism, causing no additional damage to the enamel. Furthermore, the enamel demineralization did not increase during starvation ($p > 0.05$).

It has been suggested in literature that EPS from cariogenic biofilm matrix could be used as an extracellular energy source (Wood, 1967; Colby and Russell, 1997). Thus, through the degradation of glucans and fructans by dextranases and fructanases, respectively, microorganisms could maintain their metabolism in "starvation" periods using the monosaccharides glucose and fructose released, prolonging the cariogenic challenge. It was reported an increase in *dexA* gene expression in biofilm formed for 120 h in the presence of sucrose, which could represent glucans degradation (Klein et al., 2009). An *in vitro* study showed that when a biofilm was grown with sucrose there was no IPS utilization, even in absence of fructanases (Busuioc et al., 2009), proposing that other energy sources could be used to bacterial metabolism maintaining.

It is noteworthy that studies performed with biofilms growth under constant exposure to high concentrations of carbohydrate have to be consider. This condition does not mimic that happens into oral environment and cells undergo starvation seem to be more persistent and active metabolically than those grown with nutrients limitation (Zhu at al., 2001). The utilization of planktonic cells is also limiting in this kind of study, since it cells and enzymes dispersed in solution do not have the same behavior as adsorbed enzymes and cells organized into biofilms (Kopec et al., 1997; Bowen & Koo, 2011). Then, this property could be modified when biofilm growth occurs under a dynamic condition of “fest” and “famine” such as into oral cavity. Thus, these could be the reasons they did not found differences between the harvest periods in relation to EPS use and also in relation to IPS metabolization. Another reason could be the break of small fragment ends of the extracellular polysaccharides, which could be metabolized but were not detected by limitation on the technic used.

The biofilm growth under sucrose exposure was more cariogenic due to the great amount of EPS produced ($p<0.05$), and not to the larger number of *S. mutans* cells ($p>0.05$) as already previously reported (Cury et al., 2001; Tenuta et al., 2006). Under this condition, EPS are produced and are able to modify the biofilm architecture (Xiao et al., 2012), representing a great volume in the biofilm and may comprise about 10 to 40 % of the biofilm dry weight (Paes Leme et al., 2006; Bowen and Koo, 2011). In consequence of the higher biofilm matrix quantity, our data showed the biofilm dry weight under sucrose exposure were 3 times higher than those exposed to glucose + fructose (Table 1).

The images of biofilms visualized by CLSM showed that the volume of the biofilm formed under glucose + fructose seemed to be lower when compared to the biofilm exposed to sucrose (Fig. 2). Taking together CFU counts normalized by biofilm dry weight and the CLSM images, it is possible to infer that more cells per volume were present in the biofilm exposed to glucose + fructose, because in this condition, no EPS were synthesized. On the other hand, the biofilm exposed to sucrose showed less cells per volume (Table 1), and nevertheless it was more cariogenic, suggesting EPS could determine the bacterial density in the biofilm (Colby and Russell, 1997). The cells dispersed into EPS matrix would be easily exposed to sugars, due to higher porosity of the biofilm grown under sucrose exposure (Dibdin & Shellis, 1988), perhaps, allowing more IPS storage and also acid production when compared with the packed

cells in the biofilm without EPS (glucose + fructose). This could explain the great amount of IPS formed in the group exposed to sucrose (Table 1). Thus, the increase in biofilm cariogenicity can be better understood by changes occurred in matrix composition and structure, and not simply by the higher counts of *Streptococcus mutans* in biofilms (Cury et al., 2001).

Lower pH values of the culture medium were observed for the biofilm exposed to sucrose, even it had been used solutions with equimolar carbohydrates concentration of (Fig. 3). It was already demonstrated that under sucrose exposure, extracellular polysaccharides were able to form compartments within matrix (Xiao et al., 2012). These spaces would favor acids retention, which difficult the neutralization by access restriction to saliva (Xiao et al., 2012). Therefore, the heterogeneous environments within matrix makes the pH variable, since there might have spaces that keep the pH down for longer periods, increasing the cariogenic challenge duration and contributing, together with higher porosity, with mineral loss. After overnight period without any carbohydrate source, a pH drop was verified for both groups, but this values were very similar.

As a result of the metabolism of sugars, there was effect to the main product formed was lactic acid, although acetic acid and ethanol were also found in the culture medium (Fig. 4). When carbohydrate is supplied in excess, the concentration of glucose 6-phosphate are increased, leading to activation of lactate dehydrogenase enzyme, resulting in extensive production of lactic acid and intracellular accumulation of polysaccharides (Huis in ‘t Veld and Backer Dirks, 1978; Takahashi, 2015). The acid produced increases the levels of other metabolic intermediates that inhibit the formation of acetic acid, formic acid and ethanol, by the action of pyruvate-formate lyase (PFL) enzyme. During starvation periods of carbohydrates, lactate dehydrogenase enzyme is inactivated, while the PFL is enabled. By pyruvate degradation, those products that had the formation inhibited, are again produced (Huis in‘t Veld and Backer Dirks, 1978; Takahashi, 2015). Then, glycogen type intracellular polysaccharides are degraded into glucose 1-phosphate, coming back into the glycolytic pathway for energy production (Huis in‘t Veld and Backer Dirks, 1978; Wilson et al., 2010; Takahashi, 2015).

In relation to harvest period effect, it was observed a small quantity of acids in culture medium, indicating some metabolization in starvation overnight period in both

groups ($p < 0.05$). One way to explain this results could be by the final metabolization process after the last carbohydrate exposure, since no difference was found in all polysaccharides type at starvation condition. Moreover, it is possible that some parts of polysaccharides were broken and metabolized only to maintain the basal bacterial metabolism, but is not possible to affirm if were intracellular or extracellular, since no difference was found among groups ($p > 0.05$) (Fig. 1). Finally, EPS may have been used in a small amount that were not accurately detected, due to assay limitations.

The calcium release kinetics was evaluated along the biofilm formation after each “feast” and “famine” episodes (Fig. 5) and the de- and remineralization dynamics could be followed. Besides that, the buffered medium utilization was effective in avoid the mineral loss at the first biofilm formation moment. Higher amount of calcium in the culture medium was observed in group exposes to sucrose when compared to glucose + fructose group. The structural changes on biofilm exposed to sucrose, promote a prolonged contact of the enamel surface with acids produced. The higher porosity (Dibdin and Shellis, 1988) as well as micro-compartments formation within biofilm matrix (Xiao et al., 2012) favor the acid diffusion and pH drops nearby to tooth surface (Dibdin and Shellis, 1988). Consequently, mineral contents are lost in great amount, initially to the biofilm and after, from biofilm to culture medium. Once the demineralization events were higher than those remineralization, the enamel slabs exposed to sucrose 8 times a day lost around 40% of the surface hardness meanwhile glucose + fructose group lost around 14% (Table 1) (Cury et al., 2001). No increasing in the %SHL was found ($p > 0.05$) at starvation condition (Tab. 1). The acids produced in this period (Fig. 4) were not able to increase enamel damage occurred during daily cariogenic challenges (Tab 1). This shown no contribution of the EPS or IPS utilization in the enamel demineralization during carbohydrate night absence ("starvation"), when *S. mutans* biofilm was formed simulating “feast” and “famine” episodes (Fig. 1).

The higher cariogenic potential of the biofilm exposed to sucrose is well established in the literature (Rölla, 1989; Cury et al., 2000, 2001; Paes Leme et al., 2006; Aires et al., 2006; Koo et al., 2013). In the present study, this potential was evidenced by great amounts of EPS, lower pH values of the culture medium, great amounts of acids, and also higher %SHL. Interestingly, it was observed great IPS formation, although have been used solutions in equimolar concentration, and no difference has been found to the CFU counts. These findings lead us to question

whether the larger IPS amount would also be a factor for most sucrose cariogenicity, once the sucrose can be transported intact into the cells, and phosphorylated at membrane (Takahashi, 2015). Then the sucrose-6-phosphate molecule is broken generating fructose (which enters into glycolytic pathway), and glucose (subsequently phosphorylated into G6P) (Russel et al., 1992), which can be converted into G1P that is substrate for the intracellular polysaccharides synthesis (Wilson et al., 2010).

Although it has been observed a slight pH drop and acid production during starvation period, it is not possible to affirm that metabolization was due to EPS degradation and use, since the acid production also occurred in the biofilm formed under glucose + sucrose exposure. Considering that the *Streptococcus mutans* biofilm model used simulates the "feast" and "famine" episodes that occur into the mouth, the EPS metabolism during starvation may not be sufficient to the biofilm cariogenicity increase, but the high frequency of exposure to carbohydrate occurred during the day. In turn, if polysaccharides have been used but in such a quantity that could not be detected, detailed analyzes assessing genes expression related to metabolism and degradation of EPS and IPS could help to strengthen our findings. In summary, the findings suggest that EPS metabolization during night starvation did not contribute to increase enamel demineralization occurred during the daily abundance of sugar.

Acknowledgments

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3 CONCLUSÃO

1. Não foi observado aumento na cariogenicidade do biofilme dental, pela metabolização de PEC durante período de “jejum”
2. Sugere-se que a metabolização de PEC durante período noturno de jejum pode não contribuir para o aumento da desmineralização ocorrida durante exposição diária ao carboidrato.

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* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

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APÊNDICE 1 - *Gráficos referentes aos dados apresentados na Tabela 1 do artigo.*

Fig. 6: % PDS do esmalte de acordo com os carboidratos e período de coleta do biofilme. Letras distintas indicam diferença estatisticamente significativa entre os grupos. (Média ± dp; n = 12; p <0,05)

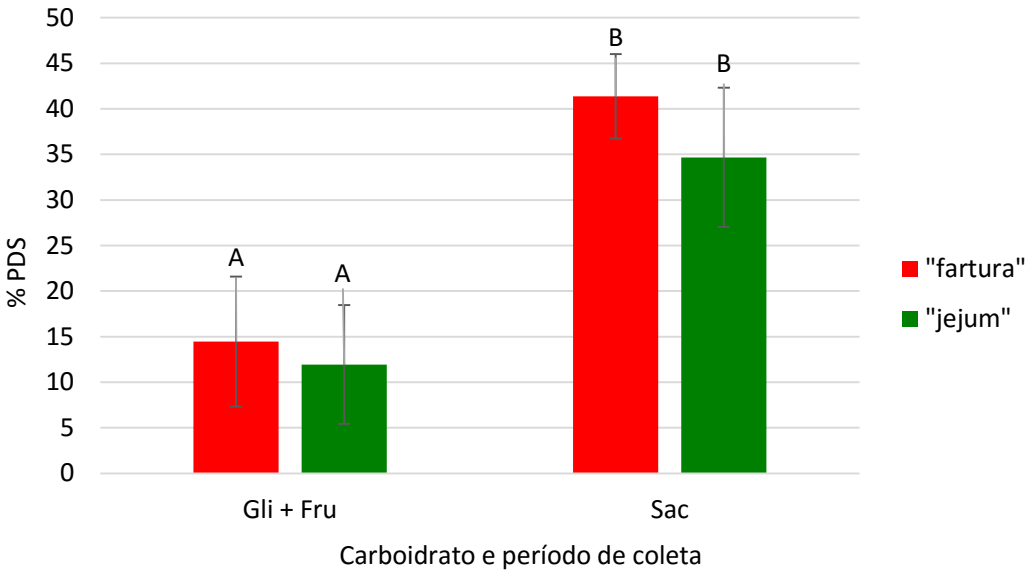


Fig. 7: Contagem de UFC de acordo com os carboidratos e período de coleta do biofilme. Letras iguais indicam não diferença estatisticamente significativa entre os grupos (Média ± dp; n = 12; p > 0,05)

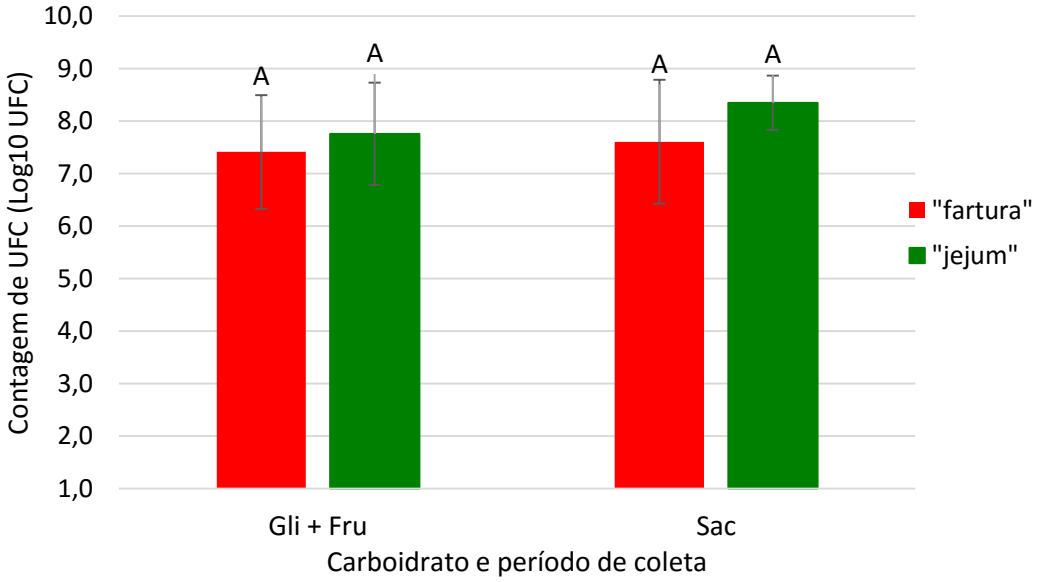


Fig. 8: Peso seco do biofilme de acordo com os carboidratos e período de coleta do biofilme. Letras distintas indicam diferença estatisticamente significativa entre os grupos. (Média ± dp; n = 12; p < 0,05).

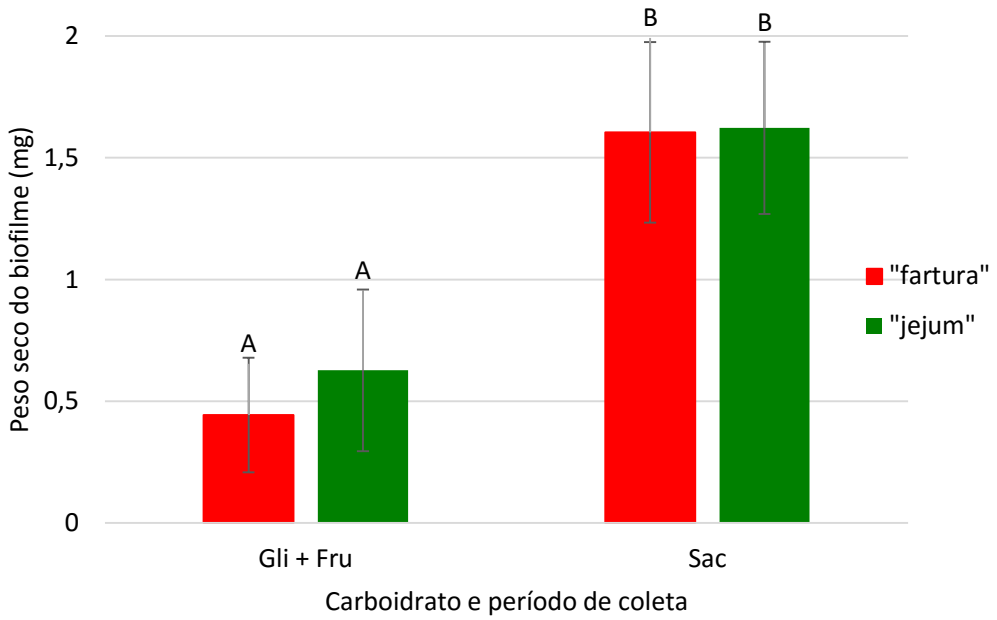
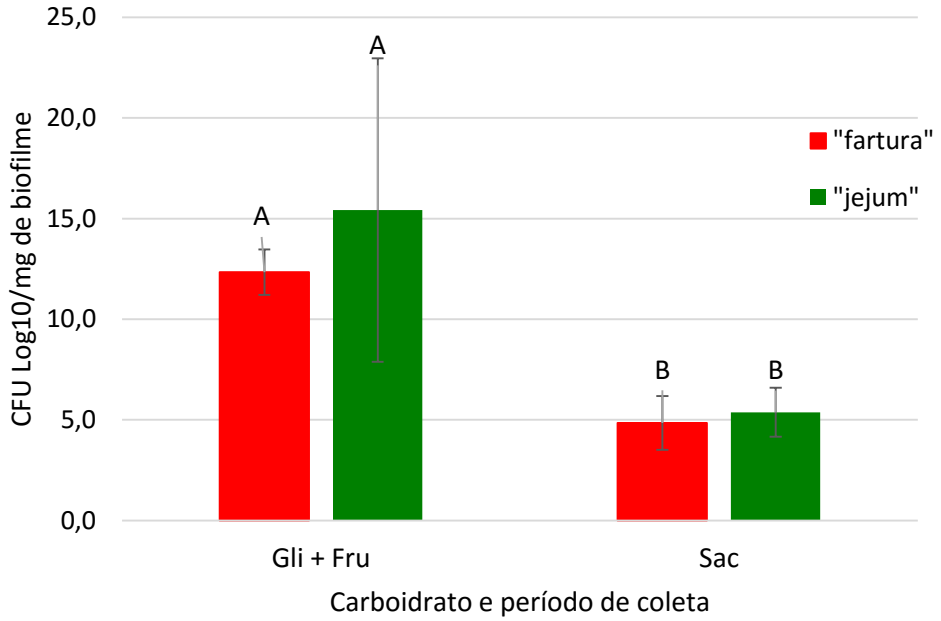


Fig. 9: Contagem de UFC por peso seco do biofilme (Log $_{10}$/mg de biofilme) de acordo com os carboidratos e período de coleta do biofilme. Letras distintas indicam diferença estatisticamente significativa entre os grupos. (Média ± dp; n = 12; p < 0,05).



ANEXO 1: *Comprovante de submissão do artigo*

Caries Research

Submission Confirmation

Print

Thank you for your submission

Submitted to
Caries Research

Manuscript ID
201602030

Title
Extracellular biofilm polysaccharides degradation during starvation and enamel demineralization

Authors
Costa Oliveira , Bárbara
Ricomini Filho, Antônio Pedro
Cury, Jaime

Date Submitted
23-Feb-2016

Author Dashboard

ANEXO 2: *Certificado do Comitê de Ética em Pesquisa da FOP-UNICAMP*

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Papel energético de polissacarídeos intracelulares e extracelulares produzidos por Streptococcus mutans e importância na cariogenicidade do biofilme dental"**, protocolo nº 142/2014, dos pesquisadores Bárbara Emanoele Costa Oliveira, Antonio Pedro Ricomini Filho e Jaime Aparecido Cury, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 16/12/2014.

The Ethics Committee in Research of the Piracicaba Dental School - University of Campinas, certify that the project **"Energetic role of intracellular and extracellular polysaccharides produced by Streptococcus mutans and importance in the cariogenicity of dental biofilm"**, register number 142/2014, of Bárbara Emanoele Costa Oliveira, Antonio Pedro Ricomini Filho and Jaime Aparecido Cury, 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 on Dec 16, 2014.

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

Prof. Dr. Felippe Bevilacqua Prado
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.