UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Desenvolvimento e validação de um modelo de crescimento de biofilmes de *S. mutans* e Estudo do efeito da sacarose na expressão de *gtfBCD* e *dexA* em biofilmes dentais formados in vitro e in situ

Tese de Doutorado apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, como requisito para obtenção do título de Doutor em Odontologia, Área de Cariologia.

Orientador: Prof. Dr. Jaime Aparecido Cury

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RESUMO

Modelos in vitro e in situ tem sido desenvolvidos para o estudo do biofilme dental. Protocolos que simulem episódios de "fartura-miséria" quando açúcares da dieta estão presentes na cavidade bucal e permitam avaliação de cárie dental são necessários. Por outro lado, poucos estudos da expressão gênica de biofilmes formados in situ foram realizados. Assim, este trabalho teve como objetivos: Desenvolver e validar um modelo de formação de biofilme de *S. mutans* que simule episódios de "fartura-miséria" e permita avaliar mudanças no biofilme, e desmineralização do esmalte dental. Também, avaliar o efeito da sacarose na expressão de genes *gtfB, gtfC, gtfD* e *dexA de* biofilme dental formado in vitro e in situ. Para os primeros objetivos, biofilmes de *S. mutans* UA159 cresceram durante 5 dias sobre blocos de esmalte bovino a 37^{o}C, 10% CO_2, em meio de cultura ultrapurificado, sendo avaliado: o efeito de concentração (1 a 20%) de sacarose e da freqüência (0 a 8x/dia) de exposição à sacarose. O efeito da clorexidina (0,012 a 0,12%) 2x/dia e de NaF 0,05% foram testados para validar o modelo. Foram determinadas: viabilidade bacteriana, acidogenicidade do biofilme, biomassa e polissacarídeos; e a desmineralização do esmalte. Para o último objetivo, no estudo in vitro biofilmes de *S. mutans* UA159 cresceram nas mesmas condições acima citadas e foram expostos à sacarose 1% constante (controle) ou sacarose 10% 8x/dia (grupo intermitente) e após 48, 72 e 120 h os biofilmes foram coletados para análise gênica de *gtfB, gtfC, gtfD* e *dexA*. No experimento in situ, um estudo cruzado de 2 fases experimentais de 7 dias foi realizado, com 9 voluntários que usaram um dispositivo palatino contendo 12 blocos de dentina que foram submetidos 8x/dia a soluções de sacarose de 1, 5, 10 e 20%. No final, os biofilmes foram coletados para análise microbiológica, bioquímica e de expressão gênica de *gtfB, gtfC, gtfD* e *dexA*. Os resultados de padronização mostraram que as concentrações de sacarose de 10% e 20% e a frequência 8x/dia provocam mudanças no biofilme e na demineralização similares ao controle. Clorexidina mostrou efeito dose resposta, diminuindo biomassa, viabilidade bacteriana, acidogenicidade do biofilme e demineralização do esmalte, NaF 0,05% não mostrou atividade antimicrobiana, mas apresentou similar efeito a clorexidina 0,12% na redução da desmineralização. Para o último objetivo, os resultados in vitro do grupo intermitente mostraram que a expressão de genes analisados parece ser constante enquanto que no controle a expressão desses genes incrementou-se em função ao tempo. No estudo in situ, o incremento da concentração de sacarose aumentou os valores de peso úmido do

biofilme, lactobacilos e polissacarídeos extracelulares do biofilme. Quanto a expressão gênica, só foi possível quantificar o *gtfB* e não apresentou diferenças entre os grupos. Em conclusão, o modelo in vitro apresenta potencial para avaliar o efeito antimicrobiano de substâncias sobre biofilmes e desmineralização dental. A quantificação da expressão de genes *gtfB, gtfC, gtfD* e *dexA* foi possível in vitro, mas in situ somente a expressão de *gtfB* foi determinada e não parece ser regulada pela concentração de sacarose.
Palavras-chave: Biofilme dental, *S. mutans*, sacarose, expressão gênica

ABSTRACT

In vitro and in situ models have been developed to study dental biofilms. Protocols, that simulate the alternations of "feast or famine" episodes that happen in oral environment in the presence of dietary carbohydrates and allowing dental caries evaluation, are necessaries. On the other hand, few studies about gene expression of dental biofilm formed in situ were performed. Thus, the objectives of this research were: To develop and to validate a model of *S. mutans* biofilm formation simulating 'feast-famine' episodes that allows biofilms and dental demineralization changes assessment. Also, to evaluate the effect of sucrose exposure on expression of *gtfB*, *gtfC*, *gtfD* and *dexA* of in vitro and in situ dental biofilms. For the first objectives, *S. mutans* UA159 biofilms were grown during 5 days on bovine enamel slabs at 37^{o}C, 10% CO_2 in ultra-purified culture media. To develop the model, the effect of sucrose concentration (1 - 20%) 8x/day and frequency (0 - 8x/day) exposure were evaluated and to validate the model, chlorhexidine (CHX) effect (0.012- 0.12%) 2x/day was tested. Bacterial viability, biofilm acidogenicity, biomass and polysaccharides were determined, and enamel demineralization was evaluated by surface hardness loss. To the last objective, for in vitro study, *S. mutans* UA159 biofilms were grown in the same conditions of previous experiments and were exposed to 1% sucrose constantly (control) or 10% sucrose 8x/day (intermittent group) and after 48, 72 and 120 h the biofilms were collected for analysis. For in situ experiment, a crossover study was conducted in two phases of 7 days each, with nine volunteers that wore intraoral palatal appliances containing 12 dental dentin slabs, which were extra orally submitted 8 times/day to sucrose solutions of 1%, 5%, 10% and 20% . On the 7th day, biofilms were collected for analysis. RNA from the in vitro and in situ biofilms were extracted and purified. Gene expression of *gtfB, gtfC, gtfD* and *dexA* were evaluated by real time PCR. In vitro development and validation results showed that 10% and 20% sucrose concentrations and frequency 8x/day provoked biofilm and enamel demineralization changes similar to control group. CHX showed dose-response effect decreasing biomass, bacterial viability and enamel demineralization ($p<0.05$). Also, 0.05% NaF did not show antimicrobial effect but had similar effect than 0.12% CHX decreasing enamel demineralization ($p<0.05$). With regard to gene expression of in vitro experiment, gene expression of intermittent group was constant along the time. In situ results showed that biofilm

wet weight, lactobacillus, extracellular polysaccharides values of the biofilm increased according to the increase of sucrose exposure. No differences for *gtfB* gene expression between the groups were observed ($p<0.05$) and no levels of *gtfC, gtfD* and *dexA* were detected. In conclusion, the model developed and validated has potential to assess substances with antimicrobial effect on biofilm and dental demineralization. Quantification of *gtfB, gtfC, gtfD* and *dexA* gene expression levels were possible for *S. mutans* biofilms in vitro study but only *gtfB* gene expression of in situ study can be determined and was not regulated by sucrose concentration.
Key words: Dental biofilm, *S. mutans*, sucrose, gene expression

SUMÁRIO

INTRODUÇÃO

O biofilme dental é uma complexa comunidade microbiológica embebida em uma matriz extracelular e aderido à superfície dental (Marsh, 2003). Na cavidade bucal, os microrganismos do biofilme dental são expostos a grandes quantidades de carboidratos e alguns destes microrganismos têm a capacidade de metabolizar rapidamente estes carboidratos produzindo ácidos e estocando fontes de energia. Após esta rápida exposição de açúcares, a saliva elimina os excessos de nutrientes e a microbiota passa longos períodos de tempo na ausência de nutrientes. Estes fenômenos são conhecidos como episódios de "fartura-miséria" e podem causar mudanças microbiológicas, aumentando a proporção de espécies como *S. mutans* e *S. sobrinus* (Loesche, 1986; Marsh, 2006).

S. mutans é considerado um importante microrganismo no desenvolvimento da cárie dental humana (Hamada and Slade, 1980; Loesche, 1986), porque além de utilizar carboidratos fermentáveis da dieta, especialmente a sacarose, para produzir ácidos, ele sintetiza polissacarídeos extracelulares insolúveis (PECI) e solúveis (PECS) por meio de três enzimas de glucosiltransferases: GTFB, GTFC e GTFD (Colby and Russell, 1997), que são codificadas respectivamente pelos genes *gtfB, gtfC* e *gtfD* (Kuramitsu, 1993). PECI são importantes factores de virulência do *S. mutans* porque eles têm a importante papel na adesão e no acúmulo de microrganismos na superfície dental (Loesche, 1986, Hojo et al., 2009) e formam partes da matriz extracelular que é responsável pela integridade estrutural do biofilme dental (Yamashita et al., 1993; Schilling and Bowen, 1992, Xiao and Koo, 2009, Koo et al, 2009). PECS servem como reservas de energia externos para os microrganismos (Colby e Russell, 1997). Por outro lado, existe evidência que a estrutura dos PECI pode ser modificada por dextranases e/ou mutanases, remodelando e ramificando a matriz extracelular (Hayacibara et al., 2004).

Biofilmes de *S. mutans* são usados como uma forma de avaliar biofilme dental in vitro devido às dificuldades que existem para desenvolver estudos in vivo em situações cariogênicas ou que possam ser prejudiciais para os voluntários. Modelos in vitro usam simulações simples de situações in vivo para estudar a fisiologia e comportamento de biofilmes frente a diferentes condições (Sissons, 1997).

Embora muitos estudos contribuíram para o entendimento de biofilmes dentais com relação a expressão gênica (Li e Burne, 2001; Shemesh, 2007), adaptação ácida (Li et al., 2001);

e resistência antimicrobiana (Koo et al., 2006), estes estudos foram executados com protocolos de crescimento de biofilme que usam exposição constante de carboidratos como fonte de nutrientes que não simula as verdadeiras alternâncias de episódios de "fartura e miséria" que acontecem na cavidade bucal na presença de açúcares. Por outro lado, estes modelos não utilizam susbtrato dental, impossibilitando a avaliação de desmineralização dental provocada pelos biofilmes.

Outro modelo experimental para o estudo do biofilme dental são os modelos in situ que não conseguem controlar todas as condições como os estudos in vitro, mas é conduzido em situações reais. Poucos estudos sobre expressão gênica de biofilme dental formado in situ têm sido realizados, e um destes estudos (Aires et al., 2008) mostrou o aumento de níveis de PECI quando a concentração de exposição à sacarose foi aumentada o que pode indicar que a expressão gênica de *gtfB*, *gtfC, gtfD* e *dexA* pode ser modificada nessas condições.

PROPOSIÇÃO

O presente estudo teve como objetivos:

a) Desenvolver um modelo de crescimento de biofilme de *S. mutans* que simule episódios de "fartura-miséria" que acontecem no ambiente bucal através do efeito da concentração e frequência de exposições de sacarose sobre o biofilme dental e sobre a desmineralização do esmalte dental.
b) Validar este modelo através da relação dose resposta da clorexidina sobre biofilmes de *S. mutans* e sobre a desmineralização do esmalte.
c) Avaliar o efeito da sacarose na expressão gênica de *gtfB, gtfC, gtfD* e *dexA* de biofilmes dentais formados in vitro e in situ.

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CAPÍTULO 1

Development of an improved *S. mutans* biofilm model

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Short title: *S. mutans* biofilm improved model
Key Words: dental biofilm, *S. mutans*, enamel demineralization, sucrose.

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ABSTRACT

Since biofilm models should simulate oral environmental conditions, the objective of this research was to standardize a model of biofilm formation of *S. mutans,* simulating 'feast-famine' episodes of sucrose exposure that happen in the oral cavity and allowing the quantification of dental demineralization. *S. mutans* UA159 biofilms were grown during 5 days on bovine enamel slabs at 37^{o}C and 10% CO_2 in ultra-purified culture media. To standardize the model, the effect of sucrose concentration (1, 5, 10 and 20%) 8x/day and the frequency of exposure (0, 2, 4 and 8x/day) were evaluated. Bacterial viability, biofilm acidogenicity, biomass and extracellular polysaccharides were determined, and enamel demineralization was evaluated by surface hardness loss. The results showed that sucrose at 10% or 20% and the frequency of exposure 8x/day provoked similar changes on biofilms and also enamel demineralization than sucrose 1% exposed constantly used as control. The standardized model was sensitive to detect changes in the biofilm metabolism in different conditions and the use of 10% sucrose 8x/day is suggested to study *S. mutans* biofilm instead of 1% sucrose exposure constantly.

INTRODUCTION

Dental biofilm is a complex community of microorganisms found on a tooth surface embedded in a matrix of polymers of host and bacterial origin (Marsh, 2003; 2006). In oral environment, microorganisms of dental biofilm are exposed to large amounts of sugar in a short period of time and some microorganisms have the capacity to use these carbohydrates to synthesize extracellular polysaccharides, store energy and produce acid. After this rapid sugar exposition, dental biofilm passes through to long periods of sugar starvation. These phenomenons are known as feast or famine episodes and the high frequency of these episodes can cause a microbiological selection, increasing a proportion of acid-tolerant species like *S. mutans* and *S. sobrinus* (Loesche, 1986; Marsh, 2006).

S. mutans is one of the most cariogenic microorganisms of dental biofilm due to the capacity to use dietary carbohydrates to synthesize extracellular polysaccharide and has acidic and acid tolerant properties (Loesche, 1986). Many researches have focused in *S. mutans* biofilms as a way of evaluating cariogenic biofilm due to research difficulties to develop in vivo studies in cariogenic situations (Sissons, 1997).

Sucrose is considered the most cariogenic carbohydrate, because it is fermented by cariogenic microorganisms, and serves as a substrate for the synthesis of extracellular (EPS) polysaccharides in dental plaque (Paes Leme et al., 2006). EPS are important virulence factors because they promote bacterial adherence to the tooth surface (Rölla, 1989; Schilling and Bowen, 1992), contribute to the structural integrity of dental biofilms (Koo et al., 2009; Xiao and Koo, 2009), change the porosity of the biofilm (Dibdin and Shellis, 1988) and consequently increase enamel demineralization (Cury et al. 2000). Also, the presence of sucrose into dental biofilm causes higher demineralization in dental substrate than other sugars like starch (Ribeiro et al., 2005, Thunheer et al., 2008) and glucose + fructose (Cury et al., 2000).

Many studies about gene expression (Shemesh et al., 2007) and antibacterial tests (Koo et al., 2006, Coenye et al., 2007; Deng et al., 2009) of *S. mutans* biofilm used protocols of growth with constant sucrose exposure as nutrient source to microorganisms, which does not simulate the real alternations of “feast or famine” episodes that happen in oral environment when dietary carbohydrates are present in oral cavity. In addition, these protocols do not use dental substrate and it does not allow the evaluation of dental caries in different conditions.

Therefore, the aim of this study was to develop a *S. mutans* biofilm improved model that simulates "feast or famine" episodes that allow the evaluation of biofilm changes and dental demineralization.

MATERIAL AND METHODS

Experimental design

To standardize the model, the effect of biofilm exposition to sucrose concentrations from 1%, to 20%, 8x/day simulating alternations of abundance and absence of sugar in the oral cavity was studied. As control, the biofilm exposed constantly to 1% sucrose was evaluated. Based on the results of the first experiment concerning enamel demineralization, the second experiment was conducted in which sucrose concentration was fixed and the frequency of sucrose exposure from 0x to 8x/day was tested.

For the two experiments, *S mutans* UA159 biofilms were formed on saliva-coated bovine enamel slabs suspended vertically in ultrafiltered tryptone-yeast extract broth (UTYEB) containing 1% sucrose at 37 °C, 10% CO_2 during 8 h; then the biofilms were grown in UTYEB containing 0.1 mM glucose at 37°C, 10% CO_2 during 136 h. Biofilm acidogenicity was determined daily through pH measurement of culture media, which was changed daily. In the final of each experiment, biofilms were collected and viable cells, biofilm biochemical composition and enamel mineral loss were analyzed. For statistical evaluation, each biofilm was considered as an experimental block.

Enamel preparation

Bovine incisor teeth, whose roots were removed, were stored in 2% formol solution for a period of at least 30 days (White, 1987). The tooth crown was fixed in an acrylic base and, with two parallel disks spaced 4 mm, a longitudinal slice was obtained from the central part of the dental specimen. Using two parallel disks spaced 7 mm, this slice was transversally cut. The dentin of this 7x4 mm dental slab was totally worn in a grinder machine (Phoenix Beta, Buehler, Lake Bluf, IL, USA), using 400-grit aluminum oxide abrasive paper. Enamel surfaces were flattened, polished and baseline enamel surface hardness was determined on outer enamel surface by making 3 indentations, spaced 100 μm from each other, using a Knoop indenter with

a 25 g load for 5 s and a Future-Tech FM microhardness tester coupled to software FM-ARS 900. Slabs presenting hardness of 333.0 ± 10.7 kg/mm^2 were randomly divided in six groups (n=4) to sucrose experiment and four groups (n=6) to frequency experiment.

Each slab was individually placed in 1 mL of a solution containing 0.06 mM P_i and 0.08 mM Ca^{++} and sterilized by autoclaving (condition previously standardized). The sterilized slabs were anchored vertically in metallic devices and suspended in 24-well culture plate.

Biofilm growth

UTYEB was used as culture media (Koo et al., 2003) and depending on the experimental phase, the media contained 1% glucose, 1% sucrose or 0.1 mM glucose as described further. *S. mutans* UA159 colonies were transferred to UTYEB containing 1% glucose and incubated at 37°C and 10% CO_2 to reactivate the microorganisms. The slabs, on which human salivary pellicle was formed, were individually positioned in well containing 2.0 mL of the inoculum and were incubated at 37 °C, 10% CO_2 to allow bacterial adhesion on acquired pellicle. All these procedures were done according to Koo et al. (2003) but after 8 h (previously standardized) incubation the slabs were transferred to the fresh UTEYB containing 0.1 mM glucose (salivary basal concentration) and incubated for additional 16 h at 37 °C, 10% CO_2. On the next day, biofilms on enamel slabs were transferred to fresh UTYEB containing 0.1 mM glucose and they were exposed 8x/day during 1 min to 10% sucrose (containing 1.23 mM Ca, 0.74 mM P_i and 0.023 μg F/mL, previously standardized) at predetermined times (8:00, 9:30, 11:00, 12:30, 14:00, 15:30, 17:00 and 18:30 h). This procedure was repeated for the next 3 days. After each sucrose exposure, the biofilms on enamel slabs were washed 3 times in 0.9% NaCl. The pH of the culture media was determined every 24 h and the media was changed to fresh one.

Treatments

For the first experiment (n=4) the groups tested were: 1%, 5%, 10% and 20%, 8x/day and 1% sucrose constant; for the second experiment (n=6) the group tested were 0x (control group), 2x, 4x and 8x/day of sucrose exposure at 10% concentration. The concentrations and frequencies evaluated as a cariogenic challenges were based in previous in situ studies (Aires et al., 2006; Ccahuana-Vásquez et al., 2007).

Biofilm collection

After the assigned experimental time of biofilm growth, the enamel slabs containing the biofilms were washed 3 times in 0.9% NaCl and individually transferred to microcentrifuge tubes containing 1 ml of 0.9% NaCl. The tubes were sonicated at 7 W for 30 s (Branson, Sonifier 50, USA) to detach the biofilms formed on the slabs (Aires et al., 2008). The slabs were carefully removed from the suspension and stored for enamel demineralization determination. Aliquots of the suspension were used for determination of biofilm bacterial viability, biomass (dry weight and total soluble proteins) and extracellular polysaccharides.

Biomass Determination

Biofilm dry weight determination was done according to Koo et al. (2003) from 200 µl of the suspension. For total soluble protein determination (Lowry et al., 1951), 50 µl of the suspension was transferred to a microcentrifuge tube, to which the same volume of 2M NaOH was added. The tube was vortexed and placed at 100°C for 15 min, centrifuged (10000 *g* for 10 min, 4°C) and in the supernatant the concentration of soluble protein was determined (DC Protein Assay, Bio-Rad, USA) .

Bacterial viability

An aliquot of 100 µl of the suspension was diluted in 0.9% NaCl in series up to 10^{-7} and 2 drops of 20 µL of each dilution were inoculated on BHI agar (BD, Sparks, USA) to determine viable microorganism number (Herigstad et al., 2001). The plates were incubated for 48 h at 37°C, 10% CO_2. Colonies formation units (CFU) were counted and the results were expressed as CFU/mg of biofilm dry weight (Aires et al., 2008).

Polysaccharide analyses

From 100 µl of the suspension, insoluble and soluble extracellular polysaccharides were extracted according to Aires et al. (2008) and analyzed for total carbohydrate according to Dubois et al. (1956). The results were normalized by biofilm dry weight.

Enamel demineralization assessment

At the end of each experimental phase, enamel slabs surface hardness (SH) 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 % SHL was calculated (baseline SH – SH after in vitro test) x100/baseline SH). Surface hardness loss was used as indicator of enamel demineralization (Cury et al., 2000).

Statistical analysis

The assumptions of equality of variances and normal distribution of errors were checked for all the response variables tested and those that did not satisfy were transformed (Box et al., 1978). The relationship between sucrose concentrations and frequencies of sucrose exposure with variables evaluated was estimated by regression analysis. Variables of viable cells, polysaccharides and %SHL of concentration experiment; viable cells, EPS and total proteins of frequency experiment were transformed to log10. All the variables were submitted to analysis of variance (ANOVA), with the exception of pH of the culture media that was analyzed by repeated measured ANOVA followed by Tukey. The software SPSS for Windows 15.0 was used and the significance level was fixed at 5%.

RESULTS

Regarding to sucrose concentration, statistically significant linear effect among sucrose concentration and biofilm dry weight, total soluble proteins, viable bacteria, SEPS and enamel demineralization was found (Table 1). No effect on IEPS was observed. Culture media pH of 5% sucrose 8x/day was lower than 1% sucrose 8x/day and higher than the other groups at 48, 72, 96 and 120 h of biofilm growth (Fig. 1A). Dry weight (Fig. 1B) and %SHL (Fig 1D) of 10% and 20% sucrose groups were higher than 1% and 5% sucrose concentration and similar than control group ($p<0.05$). Total proteins of 1% sucrose were similar to 5% sucrose and lower than the other groups (Fig. 1B). Bacterial viability in 1% sucrose was higher than the other groups (Fig. 1C). IEPS in control group was higher than those in experimental groups ($p<0.05$) and no differences were observed between experimental groups ($p>0.05$); SEPS of 10% and 20%

sucrose were higher than the other groups ($p>0.05$), which did not differ from each other ($p<0.05$).

In relation to the frequency of sucrose exposure following the results of the first experiment, 10% sucrose was used as cariogenic challenge. Statistically significant linear effect among frequency of sucrose exposure and biofilm dry weight, total soluble proteins, viable bacteria, IEPS and enamel demineralization was found (Table 3). No effect on SEPS was observed. Culture media pH decreased according to the increase of frequency of sucrose exposure at 48, 72, 96 and 120 h of biofilm growth (Fig. 2A). Biomass (Fig. 2B) and IEPS (Table 4) increased according to the increase of frequency exposure and all the groups were different ($p<0.05$). Bacterial viability (Fig.2C) values at frequency of 8x/day was lower than the other groups ($p<0.05$). %SHL at frequency of 8x/day was higher than the other groups, which did not differ from each other (Fig. 2D). No differences were found among the frequencies to SEPS ($p>0.05$).

DISCUSSION

The development of in vitro models has contributed to the understanding of different aspects of biofilms, such as, matrix frameworks (Sutherland, 2001), channels of nutrient transport (Costerton, 1999), quorum sensing (Suntharalingam and Cvitkovitch, 2005), gene expression (Shemesh, 2007) and higher resistant to antibacterial compounds compared to planktonic cells (Costerton, 1999; Mah and O'Toole, 2001). In spite of these advances in biofilm research, improvements can be performed to simulate as close as possible the real conditions that happen in vivo biofilm.

Dose-response effect was observed in both experiments to biomass, viable bacteria and enamel demineralization, independent of the way to increase the carbohydrate supply. SEPS in concentration experiment and IEPS in frequency experiment showed dose-response effect, suggesting that the production of extracellular polysaccharide can be regulated in different ways when amount of frequency of sucrose exposure is modified.

In the presence of sucrose in the experimental concentration 1% sucrose and frequencies 0x and 2x groups, the microorganisms produced acid low levels above the critical enamel pH. Biomass data showed higher values of dry weight and total proteins according to the increase in

sucrose exposure in both experiments (Fig. 1B and 2B). Dietary carbohydrates are rapidly fermented by *S. mutans* (Colby and Russell, 1997) and the presence of large amounts of sucrose stimulated the increase of metabolism activity and consequently the biomass increased. Viable bacteria counts diminished according to the increase of sucrose concentration (Fig 1C) and frequency of sucrose exposure (Fig. 2C). These acidic conditions provoked that *S. mutans* alters its physiology to survive activating acid tolerance response to enhance the survival at low pH, but these changes did not avoid the decrease of viable number in agreement to Welin-Neislands and Svensäter (2007).

Levels of enamel mineral loss were higher according to the increase of sucrose concentration (Fig. 1D) in agreement to Tehrani et al. (1983) and Aires et al. (2006). However, only 10% and 20% sucrose groups showed similar demineralization than control group. The lower acid production of 1% and 5% sucrose concentration groups (Fig. 1A) can be responsible of lower mineral loss than the other groups. The same profile was observed when frequency of sucrose exposure was increased (Fig. 2D). The frequency 8x/day provoked higher demineralization than the other groups in agreement with in situ study of Ccahuana-Vásquez et al. (2007).

The production of EPS is considered the main virulence factor of cariogenic biofilm in the development of dental caries (Loesche, 1986), and concentration and frequency experiments showed different profiles of EPS production: concentration experiment did not show differences in IEPS between the experimental groups and EPS of 10% and 20% groups were higher than the other groups. In the other hand, frequency experiment exhibited IEPS high levels according to the increase of the frequencies and no differences in the groups were observed to EPS. The way of carbohydrate supply to dental biofilm may influence and modified the gene expression of glucosyltranferases (Shemesh, 2007) and dextranases (Colby and Rusell, 1997) enzymes responsible for the production and degradation of EPS.

In conclusion, this in vitro model demonstrated to be sensible to detect differences of *S. mutans* biofilms metabolism in the presence of sucrose in more physiological conditions. The use of the concentration of 10% sucrose, and the frequency 8x/day is suggested to study *S. mutans* biofilm instead of culture media containing 1% sucrose constantly.

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Table 1. Correlation (r) and significance (p) between sucrose concentration (1 – 20%) and the response variables.

Variable response	r	p
Biofilm dry weight	0.85	<0.001
Soluble total proteins	0.732	<0.001
Viable bacteria	0.73	<0.001
% Surface hardness loss	0.881	0.001
IEPS	0.31	0.243
SEPS	0.911	<0.001

*IEPS: Insoluble Extracellular Polysaccharides
SEPS: Soluble Extracellular Polysaccharides

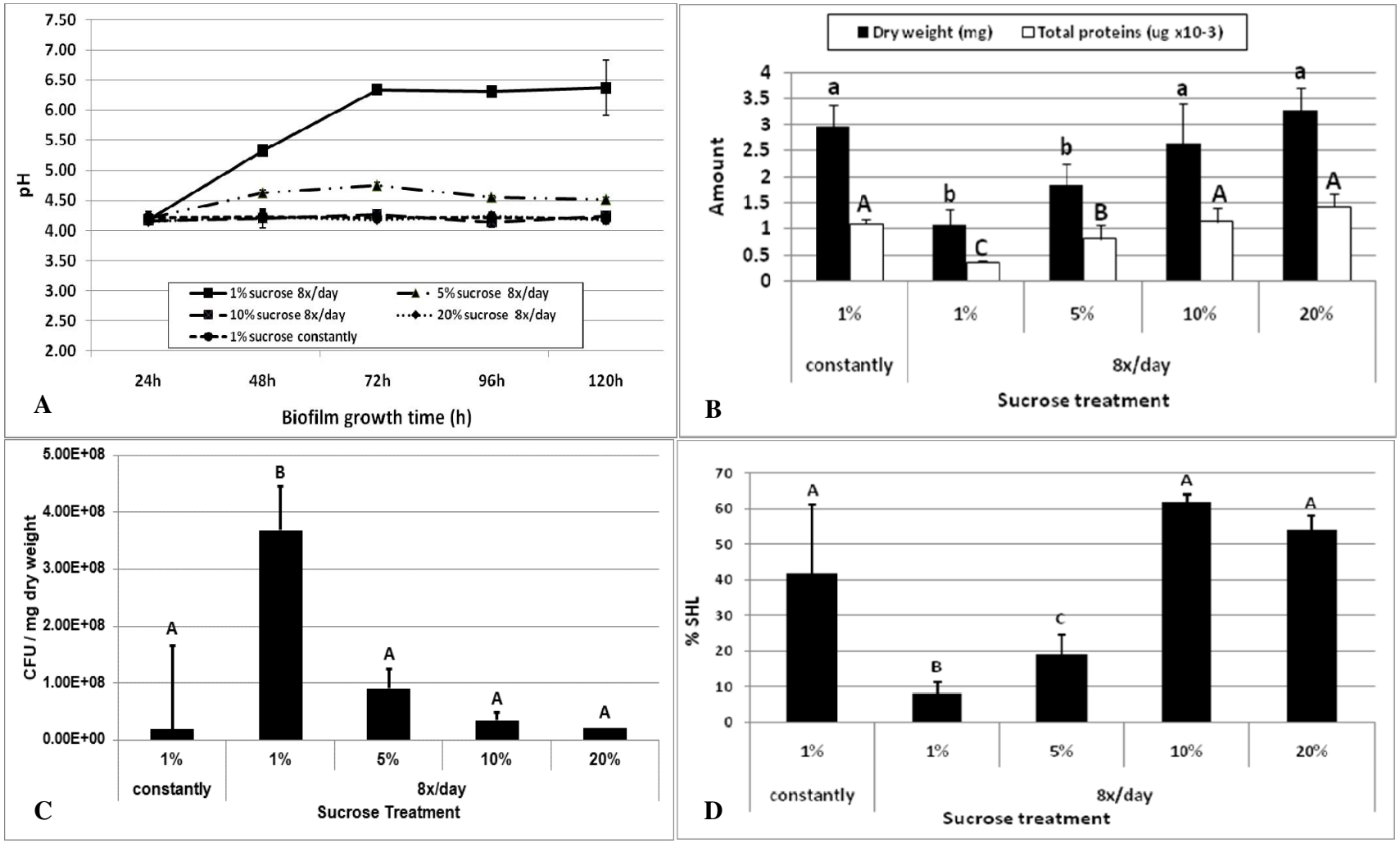

Fig. 1. Sucrose concentration effect on *S. mutans* biofilms. (A) pH of the culture media (24, 48, 72, 96 and 120 h data). The statistical significance among the treatments is described in Results section. (B) Means of biofilm dry weight (mg) and amount of soluble proteins (μg x 10^{-3}). (C) Means of viable bacteria (CFU/mg dry weight). (D) Means of enamel demineralization (%SHL). Results are according to sucrose treatments. Vertical bars denote standard deviations and significant differences among the groups are indicated by different letters ($p < 0.05$).

Table 2. Means (n=4) of insoluble (IEPS) and soluble (SEPS) extracellular polysaccharides in *S. mutans* biofilms according to the sucrose concentrations. Significant differences among the treatments, for each polysaccharide, are indicated by different letters ($p < 0.05$).

Sucrose treatment	IEPS (ug/mg)	SEPS (ug/mg)
1% constantly	199.04 ± 15.77a	13.75 ± 1.72a
1% 8x/day	110.41 ± 47.92b	15.97 ± 3.66a
5% 8x/day	121.92 ± 11.45b	19.87 ± 4.32a
10% 8x/day	113.79 ± 16.9b	30.49 ± 3.38b
20% 8x/day	136.75 ± 14.88ab	40.72 ± 5.96b

Table 3. Correlation (r) and significance (p) between frequency of sucrose exposure (0 – 8x/day) and the response variables.

Variable response	r	p
Biofilm dry weight	0.963	<0.001
Soluble total proteins	0.94	<0.001
Viable bacteria	0.469	0.024
% Surface hardness loss	0.9	<0.001
IEPS	0.85	<0.001
SEPS	0.081	0.72

*IEPS: Insoluble Extracellular Polysaccharides
SEPS: Soluble Extracellular Polysaccharides

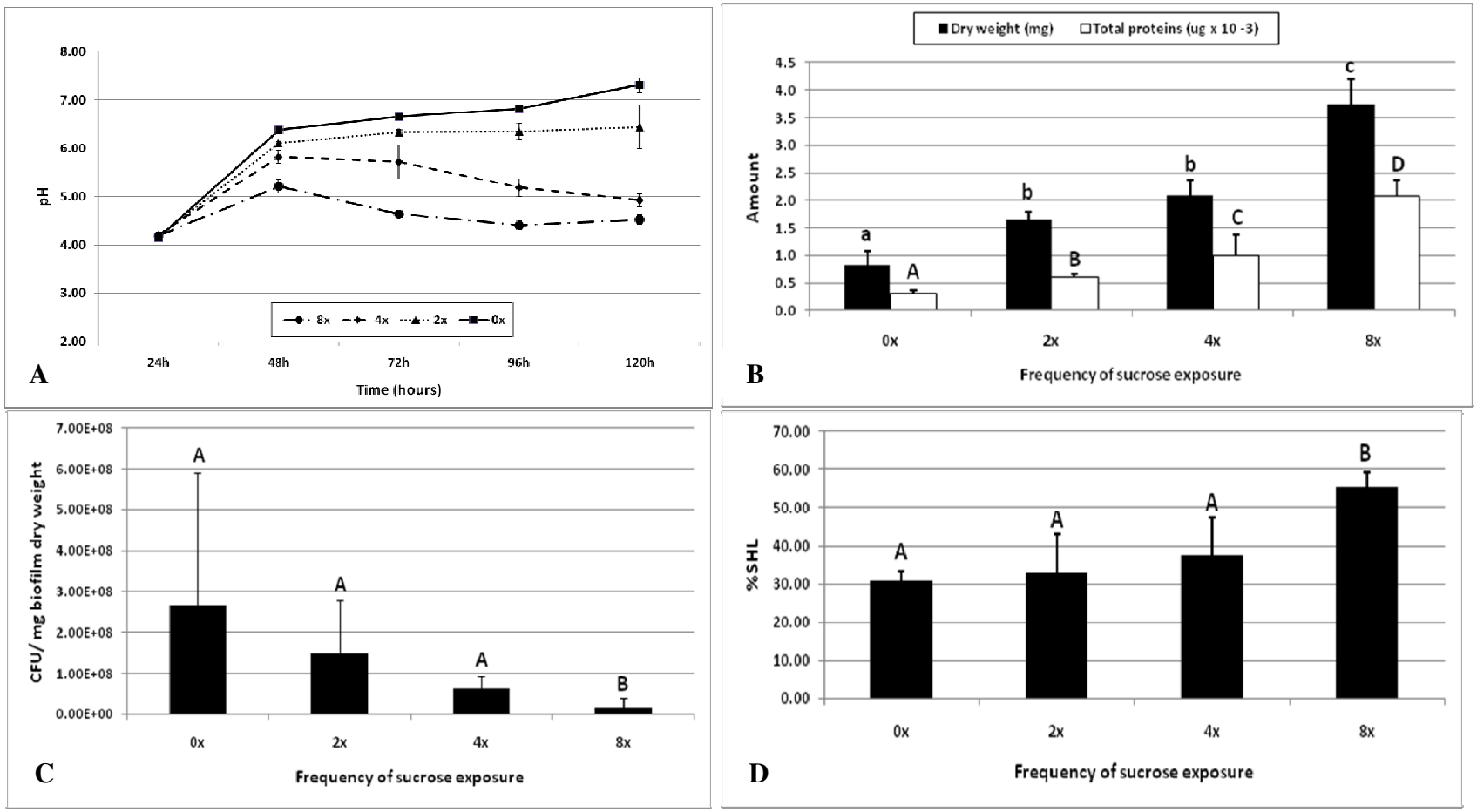

Fig. 2. Frequency of sucrose exposure effect on *S. mutans* biofilms. (A) pH of the culture media (24, 48, 72, 96 and 120 h data). The statistical significance among the treatments is described in Results section. (B) Means of biofilm dry weight (mg) and amount of soluble proteins (μg x 10^{-3}). (C) Means of viable bacteria (CFU/mg dry weight). (D) Means of enamel demineralization (%SHL). Results are according to the frequencies. Vertical bars denote standard deviations and significant differences among the groups are indicated by different letters ($p < 0.05$).

Table 4. Means (n=6) of insoluble (IEPS) soluble (SEPS) extracellular polysaccharides in *S. mutans* biofilms according to the frequency of sucrose exposure. Significant differences among the frequencies, for each polysaccharide, are indicated by different letters ($p < 0.05$).

Frequency/day	Extracellular Polysaccharides	
	IEPS (ug/mg of biofilm dry weight)	SEPS (ug/mg of biofilm dry weight)
0x	24.00 ± 10.15c	25.57 ± 14.99a
2x	76.50 ± 7.18b	20.31 ± 2.29a
4x	104.61 ± 11.01a	18.12 ± 4.13a
8x	111.86 ± 11.62a	26.52 ± 4.31a

CAPÍTULO 2

Improvement and validation of a *S. mutans* biofilm model

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ABSTRACT

Background

Since biofilm models should be validated, this research aimed to validate a model of biofilm formation of *S. mutans,* which simulating 'feast-famine' episodes of sucrose exposure that happen in the oral cavity, showed dose-response susceptibility to antimicrobials and allowed the evaluation of substances with anticaries potential.

Results

S. mutans UA159 biofilms were grown during 5 days on bovine enamel slabs at 37 Celsius degree, 10% carbon dioxide. To validate the model, the biofilms were treated 2x/day with chlorhexidine digluconate at 0.012, 0.024 and 0.12% (concentration with recognized anti-plaque effect) and 0.05% NaF (concentration with recognized anti-caries effect). Chlorhexidine digluconate showed dose-response effect, decreasing biomass, bacterial viability and enamel demineralization ($p<0.05$). Moreover, 0.05% NaF did not show antimicrobial effect, but had similar effect than 0.12% chlorhexidine digluconate, decreasing enamel demineralization ($p<0.05$).

Conclusion

The model developed has potential to evaluate the effect of substances on biofilm growth and enamel demineralization.

BACKGROUND

Dental biofilm is an organized microbiologic community enclosed in a matrix of extracellular material and attached to dental surfaces [1]. In some conditions, such as high carbohydrate consumption, the presence of high amount of sugars can change the biochemical and microbiological composition of biofilm, leading to an increase in the proportion of pathogenic species, transforming a healthy biofilm in to a cariogenic one and can develop dental caries disease [2].

In the oral cavity, microorganisms of dental biofilm are exposed to large amounts of sugar during short periods of time and some microorganisms have the capacity to use these carbohydrates to produce acid, synthesize extracellular polysaccharides and store energy. After this rapid sugar exposure, dental biofilm passes for long periods of sugar starvation. These physiological conditions of bacteria growth are known as 'feast or famine' episodes [3] and can cause microbiological selection, increasing a proportion of acid-tolerant species, like *S. mutans* in biofilm [4, 5].

S. mutans is considered the most cariogenic microorganisms of dental biofilm due to its capacity to use dietary carbohydrates as sucrose, to synthesize extracellular polysaccharides (EPS) and to its acidogenic and aciduric properties [4]. EPS are important virulence factors of *S. mutans,* because they promote bacterial adherence to the tooth surface [6, 7], contribute to the structural integrity of dental biofilms [8, 9], change the porosity of the biofilm [10] and consequently increase enamel demineralization [11]. Therefore, *S. mutans* biofilms have been used to evaluate its cariogenic properties due to difficulties to develop in vivo studies in controlled cariogenic situations [12].

However, most *S. mutans* biofilm model protocols to evaluate antimicrobial substances use sucrose exposure constantly as nutrient source to biofilm growth [13, 14, 15], which subjects the biofilm to a constant acid stress, not simulating the real alternations of "feast or famine" episodes that occur in the oral cavity. Also, these protocols do not use dental substrates to evaluate the effect of antimicrobial substances on dental demineralization provoked by the attached biofilm. Furthermore, an important requirement of biofilm models is that they should show a dose-response effect against antimicrobial substances. Concerning to oral biofilm models, chlorhexidine has been used as 'gold standard' because is considered the most efficient

topical substance to reduce dental plaque, a kind of biofilm [16]. Also, it is recognized that although fluoride is the most important anticaries substance [17], its antibacterial effect is limited [18] and a model should simulate the main mechanism of fluoride on dental caries.

Therefore, this study aimed to validate a *S. mutans* biofilm model that simulating sucrose exposure that occurs in the oral environment allows the evaluation of antimicrobial substances on biofilm formation and on enamel demineralization.

METHODS

Experimental design

This *S. mutans* biofilm model was modified from Koo et al. [13] and the main modifications were the use of dental substrate and the simulation of 'feast or famine' episodes of sucrose exposure. Previously, the sucrose concentration and the frequency of exposure were standardized (data not shown). The findings showed dose-response effect of sucrose concentration and frequency, and 10% sucrose at frequency of 8x/day was chosen to validate the model concerning antibacterial dose-response effect on biofilm and enamel demineralization.

Therefore, *S. mutans* UA159 biofilms were formed on saliva-coated bovine enamel slabs suspended vertically in ultrafiltered tryptone-yeast extract broth (UTYEB) at 37°C, 10% CO_2 during 5 days [13] but exposed 1 min, 8x/day to 10% sucrose. After 48 h, the growth of some biofilms (n=4) was stopped (baseline) and the others (n=4) were grown for more 3 days and treated 2x/day during 1 min with one of the following solutions: 1) 0.9% NaCl (Control), 2) 0.012% CHX, 3) 0.024% CHX, 4) 0.12% CHX and 5) 0.05% NaF. In all biofilms; biomass, viable bacteria and biochemical composition were determined, and on enamel slabs mineral loss was assessed. The pH of the culture media was determined at 24 h as an indicator of biofilm acidogenicity. For statistical evaluation each biofilm was considered as an experimental block.

Enamel preparation

Bovine incisor teeth, whose roots were removed, were stored in 2% formol solution for a period of at least 30 days [19], The tooth crown was fixed in an acrylic base and; with two parallel disks spaced 4 mm, a longitudinal slice was obtained from the central part of the dental specimen. Using two parallel disks spaced 7 mm, this slice was transversally cut. The dentin of

this 7x4 mm dental slab was totally worn in a grinder machine (Phoenix Beta, Buehler, Lake Bluf, IL, USA), using 400-grit aluminum oxide abrasive paper. Enamel surfaces were flattened, polished and baseline enamel surface hardness was determined on outer enamel surface by making 3 indentations, spaced 100 µm from each other, using a Knoop indenter with a 25 g load for 5 s and a Future-Tech FM microhardness tester coupled to software FM-ARS 900. Slabs presenting hardness of 331.7 ± 13.8 kg/mm^2 were randomly divided in six groups (n=4).

Each slab was individually placed in 1 mL of a solution containing 0.06 mM P_i and 0.08 mM Ca^{++} and sterilized by autoclaving (condition previously standardized).

Biofilm growth

UTYEB was used as culture media [13] and, depending on the experimental phase, the media contained 1% glucose, 1% sucrose or 0.1 mM glucose as described further. *S. mutans* UA159 colonies were transferred to UTYEB containing 1% glucose and incubated at 37 °C and 10% CO_2 to reactivate the microorganisms. The sterilized slabs were anchored vertically in metallic devices, treated with clarified human saliva to form pellicle acquired, individually positioned in each well with 2.0 mL of UTYEB containing 1% sucrose and inoculum and were incubated at 37 °C, 10% CO_2 to allow bacterial adhesion on acquired pellicle. All these procedures were done according to Koo et al. [13]. After 8 h of incubation (previously standardized) the slabs were transferred to the fresh UTEYB containing 0.1mM glucose (salivary basal concentration) and incubated for additional 16 h at 37 °C, 10% CO_2. On the next day, biofilms on enamel slabs were transferred to fresh UTYEB containing 0.1 mM glucose and were exposed 8x/day during 1 min to 10% sucrose (containing 1.23 mM Ca, 0.74 mM P_i and 0.023 µg F/mL, previously standardized) at predetermined times (8:00, 9:30, 11:00, 12:30, 14:00, 15:30, 17:00 and 18:30 h). This procedure was repeated for the next 3 days. After each sucrose exposure, the biofilms on enamel slabs were washed 3 times in 0.9% NaCl. The pH of the culture media was determined every 24 h and the media was changed to fresh one.

Treatments

The CHX solutions were prepared from 20% chlorhexidine digluconate (Sigma, Steinheim, Germany) using sterilized distilled water. From 0.12% CHX, solutions 5 (0.024%)

and 10 (0.012%) times less concentrated were prepared to evaluate dose-response effect. The solution of 0.05% NaF was prepared and sterilized by autoclaving. The treatments were made 2x/day, after the first and the last sucrose exposure of the day. After each treatment, the biofilms on enamel slabs were washed 3 times in 0.9% NaCl.

Biofilm collection

After the assigned experimental time of biofilm growth, the enamel slabs containing the biofilms were washed 3 times in 0.9% NaCl and individually transferred to microcentrifuge tubes containing 1 ml of 0.9% NaCl. The tubes were sonicated at 7W for 30 s (Branson, Sonifier 50, USA) to detach the biofilms formed on the slabs [20]. The slabs were carefully removed from the suspension and stored for enamel demineralization determination. Aliquots of the suspension were used for determination of biofilm bacterial viability, biomass (dry weight and total soluble proteins) and polysaccharides.

Biomass Determination

Biofilm dry weight determination was done according to Koo et al. [13] from 200 µl of the suspension. For total soluble protein determination [21], 50 µl of the suspension was transferred to a microcentrifuge tube, to which the same volume of 2 M NaOH was added. The tube was vortexed and placed at 100°C at 15 min, centrifuged (10000 g for 10 min, 4°C) and in the supernatant the concentration of soluble protein was determined (DC Protein Assay, Bio-Rad, USA).

Bacterial viability

An aliquot of 100 µl of the suspension was diluted in 0.9% NaCl in series up to 10^{-7} and 2 drops of 20 µL of each dilution were inoculated on BHI agar (BD, Sparks, USA) to determine viable microorganism number [22]. The plates were incubated for 48 h at 37°C, 10% CO_2. CFU were counted and the results were expressed as CFU/mg of biofilm dry weight [20].

Polysaccharide analyses

From 100 µl of the suspension, insoluble and soluble extracellular polysaccharides were extracted according to Aires et al. [20] and analyzed for total carbohydrate according to Dubois et al. [23]. The results were normalized by biofilm dry weight.

Enamel Demineralization assessment

At the end of each experimental phase, enamel slabs surface hardness (SH) 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 hardness loss (% SHL) was calculated (baseline SH – SH after in vitro test) x100/baseline SH). Surface hardness loss was used as indicator of enamel demineralization [11].

Statistical analysis

The assumptions of equality of variances and normal distribution of errors were checked for all the response variables tested and those that did not satisfy were transformed [24]. The relationship between CHX concentrations and the variable evaluated was estimated by regression analysis. When significant correlation was found, the data were submitted to ANOVA followed by Tukey test, with the exception of acidogenicity that was analyzed by repeated measured. Original data were used with the exception of viable cells that were transformed to $\log_{10}$. The software SPSS for Windows 15.0 was used and the significance level was fixed at 5%.

RESULTS

Statistically significant linear effect between CHX concentration and biofilm dry weight, total soluble proteins and viable bacteria, and enamel demineralization was found (Table 1), showing dose-response effect for these variables. No effect on polysaccharides was found.

Concerning the pH of the culture media (Fig. 1A), the groups did not differ statistically after 48 h of the biofilm growth. At 72 h of biofilm growth, the pH of media for the 0.12% CHX treatment was similar than NaF 0.05% group but higher than the other groups. At the 72 h and

120 h of biofilm growth, the pH of the NaF 0.05% was similar to control group, but lower than the other groups ($p<0.05$).

The values of dry weight and proteins (Fig. 1B) showed that biomass of the biofilm treated with 0.12% CHX was lower than the control ($p<0.05$) and did not differ statistically from the baseline value ($p>0.05$). However, the biomass of biofilm treated with 0.05% NaF did not differ from the control ($p>0.05$) and was higher than the baseline values ($p<0.05$) (Fig. 1B).

The counts of viable bacteria in the biofilm (Fig. 1C), normalized by biofilm dry weight, was significantly lower ($p<0.05$) for the treatment with 0.12% CHX compared with the baseline and the control group. However, the counts of viable bacteria of biofilm treated with 0.05% NaF did not differ from the control and the baseline values ($p>0.05$).

Concerning enamel demineralization (Fig. 1D), 0.12% CHX and 0.05% NaF significantly reduced %SHL compared with the control ($p<0.05$), but these treatments did not differ between them ($p>0.05$).

DISCUSSION

Biofilm models are important tools to evaluate the biochemical and microbiological composition of biofilm formed under different conditions or the changes provoked on the substratum surface where the biofilm is attached. Therefore, the conditions of biofilm formation and the substratum used must be closer of the real life.

The improved model of *S. mutans* biofilm growth was validated and dose-response effect of CHX on *S. mutans* biofilm was showed for most variables (Table 1). Therefore, the model is sensible to show biofilm and enamel demineralization changes in the presence of antimicrobial substances. The treatment 2x/day with 0.12% CHX showed bactericidal effect, killing a great proportion of viable bacteria of the biofilm, decreasing the biofilm capacity of producing acids, avoiding the increase of biofilm mass (dry weight and total proteins) and consequently enamel demineralization process was stopped (Fig. 1). This effect may be attributed to CHX molecule ability to bind to the negatively charged bacterial cell surface, altering and disrupting the integrity of cell membrane, causing bacterial death [25, 26]. The concentration of 0.012% CHX had a bacteriostatic effect, without interfering on viable bacteria counts but affected acid production level that was lower than control group but it was not able to avoid enamel

demineralization. At this sublethal stage, CHX effects are reversible; removal of excess of CHX by neutralizers allows the bacterial cell to recover [26]. This implies that the structural damages caused by 0.012% CHX was minor than 0.024% and 0.12% CHX. The results found with this model are supported by a clinical trial showing that 0.12% CHX is more effective to reduce *S. mutans* CFU than lower concentrations [27].

Opposite to CHX, fluoride did not show effect on biofilm formation based on biomass (Fig. 1B) and viable bacteria counts (Fig. 1C). It also did not inhibit sucrose fermentation, since the pH of media was not different compared with the control (Fig. 1A), however it reduced enamel demineralization (Fig. 1D). The findings are supported by the knowledge that it is necessary at least 10 ppm of fluoride constantly in the media to inhibit sugar fermentation [28]. In this model, simulating the clinical use of mouthrinse, although the biofilm has been treated with 225 ppm of fluoride the time was during only 1 min. The absence of antimicrobial effect of 0.05% NaF but its effect reducing enamel demineralization suggest that the main effect of F on caries control is physicochemical [29, 30].

Also, the findings suggest that CHX did not have direct effect on the synthesis of extracellular polysaccharides (Table 1). This result apparently disagrees of Koo et al. [13] but it reflects the way as the results were expressed. Koo et al. [13] expressed the results in amount of polysaccharides found in the biofilm and in the present study the results were normalized by biofilm dry weight. Since the biofilm weight increased (Fig 1B) but the viable bacteria decreased (Fig. 1C), the reduction of EPS amount should be attributed to bacterial death and not to a specific effect of CHX inhibiting the synthesis of extracellular polysaccharides.

In conclusion, the results suggest that this *S. mutans* improved model can be used to test the effect of antimicrobials agents on biofilm growth and on enamel demineralization.

AUTHOR'S CONTRIBUTIONS

RACV participated in the design of the study, carried out the experiment, analyzed and interpreted the data, performed statistical analysis and wrote the manuscript. JAC was the intellectual author of the study, designed it, helped in the interpretation of the data and revised the manuscript. The authors read and approved the final manuscript.

ABREVIATIONS

EPS: extracellular polysaccharides, **CHX**: chlorhexidine digluconate, **UTYEB**: ultrafiltered tryptone-yeast extract broth, **CFU**: colony forming units, **IEPS**: Insoluble extracellular polysaccharide, **SEPS**: Soluble extracellular polysaccharide, **IPS**: intracellular polysaccharide, **%SHL**: percentage of enamel surface hardness loss.

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Effect of sucrose and starch on dental biofilm formation and on dentin demineralization. *Caries Res* 2008; 42:380-386.

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FIGURES LEGENDS

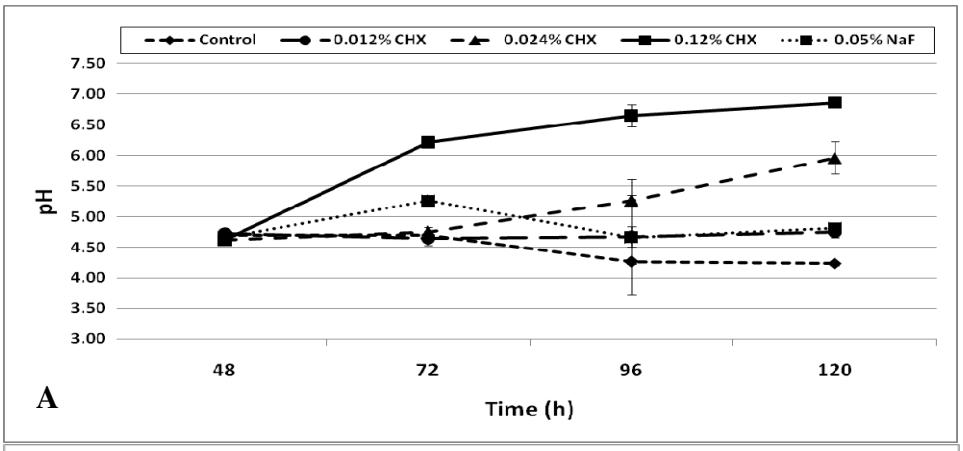
Fig. 1. Baseline group and the effect of the treatments on *S. mutans* biofilm and on enamel demineralization (n=4). (A) pH of the culture media after 48 of biofilm growth in the absence of treatments and at each 24 h after beginning the treatments (72, 96 and 120 h data). The statistical significance among the treatments is described in Results section. (B) Means of biofilm dry weight (mg) and amount of soluble proteins (μg x 10^{-3}) for the baseline and

according to the treatments. (C) Means of viable bacteria (CFU/mg dry weight) in the biofilms grown 48 h in absence of the treatments (baseline) and after 3 days of treatments described. (D) Means of enamel demineralization (%SHL) after 48 h of biofilm growth in absence of the treatments (baseline) and after 3 days of the treatments described. Vertical bars denote standard deviations and significant differences among the groups are indicated by different letters ($p < 0.05$).

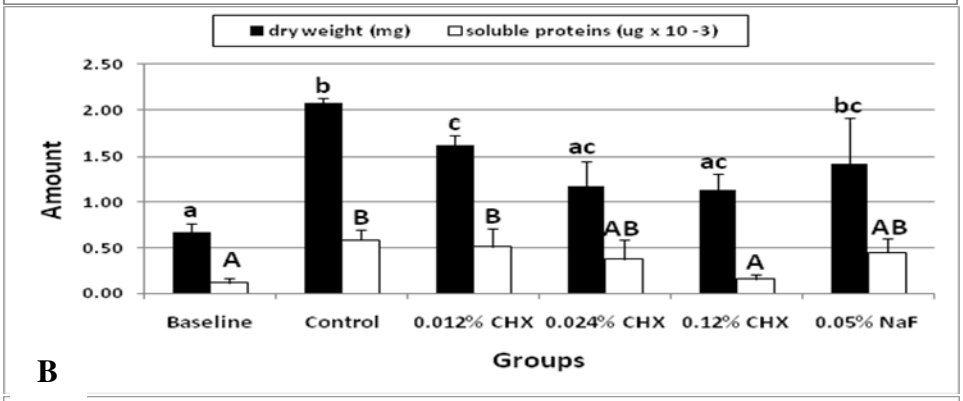
TABLES

Table 1. Correlation (r) and significance (p) between chlorhexidine concentration (0 – 0.12%) and the response variables.

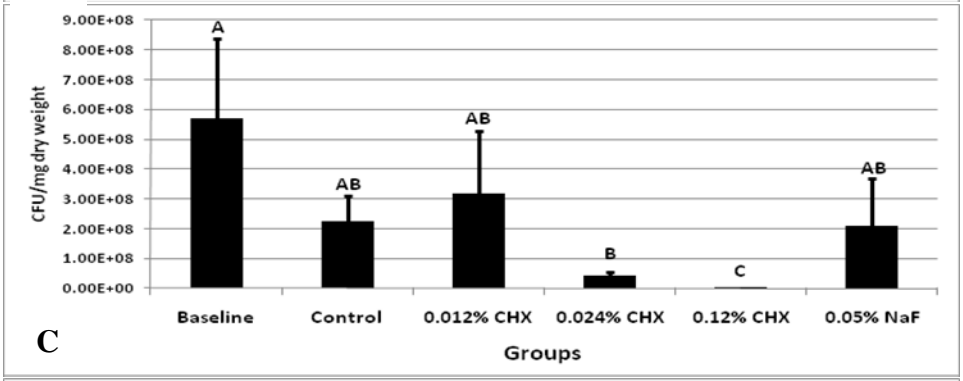
Variables	r	p
Dry weight	0.808	<0.001
Proteins	0.767	0.001
Viable bacteria	0.689	0.006
%SHL	0.628	0.009
IEPS	0.148	0.615
SEPS	0.249	0.412


Control
0.012% CHX
0.024% CHX
0.12% CHX
0.05% NaF
pH
7.50
7.00
6.50
6.00
5.50
5.00
4.50
4.00
3.50
3.00
48
72
96
120
Time (h)

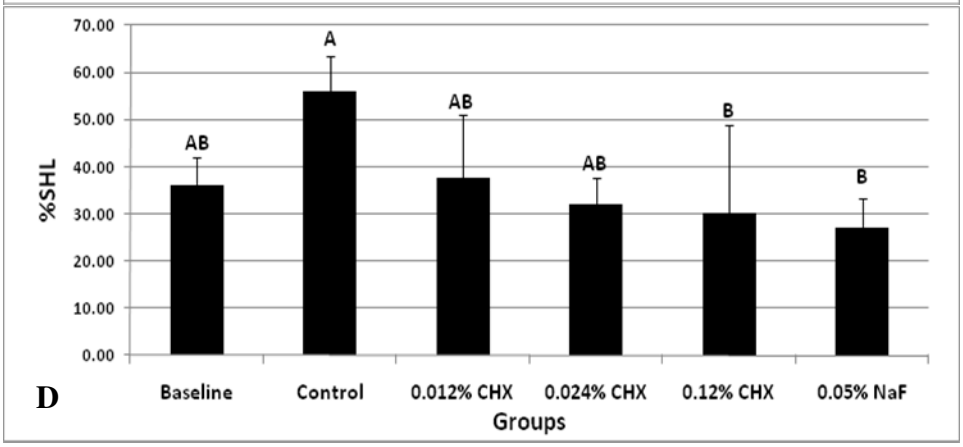
A


dry weight (mg)
soluble proteins (ug x 10 -3)
Amount
2.50
2.00
1.50
1.00
0.50
0.00
a
A
b
B
c
B
ac
AB
ac
A
bc
AB
Baseline
Control
0.012% CHX
0.024% CHX
0.12% CHX
0.05% NaF
Groups

B


CFU/mg dry weight
9.00E+08
8.00E+08
7.00E+08
6.00E+08
5.00E+08
4.00E+08
3.00E+08
2.00E+08
1.00E+08
0.00E+00
A
AB
AB
B
C
AB
Baseline
Control
0.012% CHX
0.024% CHX
0.12% CHX
0.05% NaF
Groups

C


%SHL
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
AB
A
AB
AB
B
B
Baseline
Control
0.012% CHX
0.024% CHX
0.12% CHX
0.05% NaF
Groups

D

CAPÌTULO 3

Sucrose effect on gene expression of *gtfBCD* and *dexA* of dental biofilms formed in vitro and in situ

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Short title: Gene expression in dental biofilms

Key Words: dental biofilm, *S. mutans*, gene expression, sucrose.

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ABSTRACT

Extracellular polysaccharides are considered virulence factors of cariogenic biofilm and their production and degradation are regulated by glucosyltransferases and dextranases that are decoded by *gtfB*, *gtfC, gtfD* and *dexA* genes. Since there is no consensus about the expression regulation of these genes, the objective of this study was to evaluate in vitro the effect of sucrose exposure constantly or intermittently on *gtfBCD* and *dexA* gene expressions of *S. mutans* biofilm and to evaluate in situ the effect of sucrose exposure on expression of these genes in dental biofilm. In the in vitro experiment, *S. mutans* UA159 biofilms were formed on saliva-coated bovine enamel slabs in batch culture and they were grown in ultrafiltered tryptone-yeast extract broth at 37^{o}C, 10% CO_2 during 5 days. They were exposed to 1% sucrose constantly (control group) or 10% sucrose 8x/day (intermittent group) and after 48, 72 and 120 h of growth; the biofilms were collected for analysis. For in situ experiment, a crossover, blind study was conducted in two phases of 7 days each and nine adult volunteers wore intraoral palatal appliances containing root dentin slabs, which were extraorally submitted 8 times/day to sucrose solutions at 1%, 5%, 10% and 20% . On the 7th day, dental biofilm were collected for microbiological, insoluble (IEPS) and soluble (SEPS) extracellular polysaccharides and *gtfB*, *gtfC, gtfD* and *dexA* expression analysis. In vitro results of intermittent group showed in relation to control group that *gtfB, gtfC, gtfD* and *dexA* expression of intermittent group could be constant along the time evaluated different than constant sucrose exposure group in which gene expression increased along the time. In situ results showed Lactobacilli (LB), % LB/Total microorganisms, IEPS and SEPS increased and RNA crude and purified decreased according to the increase of sucrose concentration. However, there were no statistically significant differences in *gtfB* gene expression among the groups and it was not possible to detect levels of *gtfC*, *gtfD* and *dexA* gene expression. In conclusion, it was possible to quantify in vitro the expression of *gtfB, gtfC, gtfD* and *dexA* of *S. mutans* biofilm but in dental biofilm formed in situ only *gtfB* expression can be determined which does not to regulated by sucrose concentration.

INTRODUCTION

Dental caries is a biofilm sugar dependent disease (Fejerskov, 2004) and its development is associated with the presence of cariogenic microorganisms, such as mutans streptococci and lactobacilli (van Houte et al., 1994) and high consumption of carbohydrates, like sucrose (Paes Leme et al 2006).

S. mutans is an important microorganism in the development of human dental caries (Hamada and Slade, 1980; Loesche, 1986) and use carbohydrates, especially sucrose, as substrate to ferment acids and to synthesize extracellular polysaccharides (EPS) through three glucosyltransferases: GTFB, GTFC and GTFD (Colby and Russell, 1997), that are encoded respectively by glucosyltransferases genes *gtfB, gtfC* and *gtfD* (Kuramitsu, 1993). Among EPS, insoluble polysaccharides (IEPS) have an important role in the microorganism adhesion and accumulation in dental surface (Loesche, 1986; Cross et al., 2007) and establish an extracellular matrix that is responsible to the structural integrity of dental biofilm (Yamashita et al., 1993; Schilling and Bowen, 1992; Li e Burne, 2001). Soluble polysaccharides (SEPS) serve as external energy source to bacteria (Colby and Rusell, 1997). In other hand, there is evidence showing that the structure of EPS could be modified by dextranase and/or mutanase during EPS synthesis by GTFs causing linkage remodelling and branching, which influences the bacterial binding sites on these glucans (Hayacibara *et al.*, 2004). The presence of glucanohydrolases may have an impact on the development, physical properties, and bacterial binding sites of the polysaccharide matrix in dental biofilms (Colby and Rusell, 1997, Paes Leme et al., 2006).

S. mutans biofilms are used as a way of evaluating dental biofilm in vitro due to research difficulties to develop in vivo studies in cariogenic situation (Sissons, 1997). In vitro models use simplified simulations of the in vivo situation in order to be able to study biofilm physiology in the laboratory. The advantage of these models is that the growth conditions of the cells can be defined and controlled much better than in vivo. The disadvantage is that there is not one general model that can be used to study all aspects of biofilms (Luppens and ten Cate, 2005). Although studies about *gtfs* gene expression of *S. mutans* contributed to the knowledge of changes in cariogenic microorganisms, theses studies were performed with planktonic bacteria (Fujiwara et al., 2002) or with biofilms that used protocols of growth that use sucrose exposure constantly as nutrient source (Li e Burne, 2001; Yoshida and Kuramitsu, 2002; Koo et al., 2006), which do not

simulate the real alternations of "feast or famine" episodes that happens in oral environment in the presence of sugars.

Other experimental model to study dental biofilm is in situ model that do not have all conditions controlled as in vitro biofilm model but is conducted in real situations (Zero et al., 1995). The effect of sucrose on the biochemical composition of dental formed in situ has been extensively studied (Cury et al., 2000, Ccahuana- Vásquez et al., 2007) concerning EPS but there are few data about the expression of the genes (Aires et al., 2008) that decoded the enzymes responsible for the synthesis and degradation of these polysaccharides.

Therefore, the objectives of this study were to evaluate the effect of sucrose concentration on *gtfB*, *gtfC, gtfD* and *dexA* gene expression in vitro using *S. mutans* biofilm growth model and in more real situation on dental biofilm formed in situ.

MATERIALS AND METHODS

In vitro study

Experimental Design

An in vitro study was performed, in which *S. mutans* UA159 biofilms were subjected to 1% sucrose constantly (control) or 10% sucrose intermittently (1 min, 8x/day) simulating "feast and famine" episodes that happen in the oral environment. These biofilms were formed on saliva-coated bovine enamel slabs suspended vertically in ultrafiltered tryptone-yeast extract (UTYE) broth at 37°C, 10% CO_2 and were collected after 48 h, 72 h and 120 h of growth to gene expression analysis.

Enamel preparation

Bovine incisor teeth, whose roots were removed, were stored in 2% formol solution for a period of at least 30 days (White, 1987). The tooth crown was fixed in an acrylic base and with two parallel disks spaced 4 mm a longitudinal slice was obtained from the central part of the dental specimen. Using two parallel disks spaced 7 mm, this slice was transversally cut. The dentin of this 7x4 mm dental slab was totally worn in grinder machine (Phoenix Beta, Buehler, Lake Bluf, IL, USA), using 400-grit aluminum oxide abrasive paper. Enamel surfaces were flattened and polished. Each slab was individually placed in 1 mL of a solution containing 0.06

mM P_i and 0.08 mM Ca^{++} and sterilized by autoclaving (condition previously standardized). The sterilized slabs were anchored vertically in metallic devices and two were slabs suspended in each well of 24-culture plate.

Biofilm growth and collection

UTYEB was used as culture media (Koo et al., 2003) and depending on the experimental phase, the media contained 1% glucose, 1% sucrose or 0.1 mM glucose as described further. *S. mutans* UA159 colonies were transferred to UTYEB containing 1% glucose and incubated at 37°C, 10% CO_2 to reactivate the microorganisms. The slabs, on which human salivary pellicle was formed, were individually positioned in well containing the inoculum and were incubated at 37 °C, 10% CO_2 to allow bacterial adhesion on acquired pellicle. All these procedures were done according to Koo et al. (2003) but 8 h (previously standardized) after incubation the slabs were transferred to the fresh UTEYB containing 0.1 mM glucose (salivary basal concentration) and incubated for additional 16 h at 37°C, 10% CO_2. On the next day, biofilms on enamel slabs were transferred to fresh UTYEB containing 0.1 mM glucose and they were exposed 8x/day during 1 min to 10% sucrose (containing 1.23 mM Ca, 0.74 mM P_i and 0.023 μg F/mL, previously standardized) at predetermined times (8:00, 9:30, 11:00, 12:30, 14:00, 15:30, 17:00 and 18:30 h). Biofilms of both groups grew during 48 h, 72 h and 120 h. The culture media were changed daily. The concentration of 10% sucrose and a frequency 8x/day of experimental group were used as a cariogenic challenge based in previous in vitro study (data not shown). After each sucrose exposure, biofilms on slabs were washed 3 times in 0.9% NaCl to remove sugar excess. In the final of each phase, biofilms were washed 3 times in 0.9% NaCl and transferred 4 biofilms on slabs to 1 ml RNA later solution and stored until analysis (n=4 to each time of growth).

In situ study

Experimental Design

This study was approved by the Research and Ethics Committee of FOP-UNICAMP (Protocol N° 042/2006). During two phases of 7 days each, 9 volunteers wore acrylic palatal appliances, containing twelve bovine dentine slabs (six on each side for each treatment). The volunteers were randomly assigned in two groups and were instructed to extraorally treat the

slabs with 2 concentrations in the same appliance: 1% and 10% or 5% and 20% sucrose solution. The use of two treatments in the same intra-oral appliance (split-mouth design) was supported by the absence of a cross-effect in previous in situ studies (Pecharki et al., 2005; Ccahuana-Vásquez et al., 2007). In the final of each phase, *gtfB, gtfC, gtfD* and *dexA* gene expression of the biofilms were analyzed. After a washout period, new dentin slabs were placed in the appliance for the next experimental phase. All volunteers did all the treatments assigned in the final of two phases. For the statistical analysis, the volunteer was considered as an experimental block. This study was double blind with respect to the examiner and the volunteers; examiner analyzed samples previously coded to ensure blindness, since the volunteers were instructed to follow the concentrations of sucrose exposure.

Dentin Slabs and Palatal Appliance Preparation

Two hundred and sixteen dentin slabs (5 X 5 X 2 mm) were obtained from the upper third of bovine incisor root. The outer and inner surfaces of dentin were polished or flattened, and the slabs were randomly divided according to the concentrations of sucrose use. An acrylic resin intra-oral palatal appliance, containing two lateral cavities measuring 16 X 11 X 3 mm, in which six dentin slabs were placed on each side, was made for each volunteer. Plastic meshes were fixed over the cavities to protect the dentin slab surfaces from mechanical attrition, leaving a 1-mm space for accumulation of dental biofilm. Colourless or red acrylic resin was used to fix the meshes, indicating the different treatments used at the different sides of the appliances (Pecharki et al., 2005; Ccahuana-Vásquez et al, 2007).

Volunteers

Nine volunteers (24 – 35 years old), who fulfilled inclusion (mean stimulated saliva flow rate $\geq$ 0.7 ml/min and ability to comply with the experimental protocol) and exclusion criteria (antibiotic use for the last 2 months before starting the study, use of any form of medication that modifies salivary secretion, use of fixed or removable orthodontic appliance, periodontal disease, general/systemic illness, participated in this study. They were informed about the procedures and written consent was obtained prior to the commencement of the study.

Treatments

Solutions of 1%, 5%, 10% and 20% sucrose and frequency of 8x/day were used as cariogenic challenge according to previous studies (Aires et al., 2006; Ccahuana- Vásquez et al. 2007). The volunteers were instructed to remove the appliances from the oral cavity and drip one drop of the solution on each dentin slab at the following time: 8.00, 9.30, 11.00, 12.00, 13.30, 14.30, 16.00 and 17.30 h. The excess of fluid was removed with gauze and five minutes later the device was re-inserted in the mouth. A wash-out interval of one week was established between the experimental phases. The volunteers were also instructed to wear the appliances all the time, removing them only during the meals (Cury et al, 2000). Throughout the entire experiment, volunteers brushed their natural teeth and the appliance, except the area of enamel slabs, with a dentifrice containing 1450 µg F/g. Brushing was carried out three times a day after the main mealtimes**.** The volunteers lived in an optimally fluoridated city (0.7 mg F/ L, for the region), drank and consumed foods prepared with this water. Considering the crossover design of this study, no restriction was made with regard to the volunteers diet. They also received oral and written information to refrain from using any antibacterial substance.

Biofilm collection

On day 7 of each experimental phase, after 20 minutes of the last sucrose exposure of the day, the biofilm formed on the slabs were collected. Then, 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 W for 60 s using a Digital Sonifier Unit, model S-150D (Branson Ultrasonics Corp., Danbury, Conn., USA) to microbiological and extracellular polysaccharides analyses. To gene expression analysis, the rest of biofilm was collected, weighed and kept in RNA *Later* solution (Ambion, Austin, Tex., USA) to preserve the integrity of the RNA.

Microbiological analysis

An aliquot of 100 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 (TM); mitis salivarius agar to determine total

streptococci (TS) mitis salivarius agar plus 0.2 units bacitracin per ml and 15% sucrose (MSB), for mutans streptococci group (MS) (Gold et al., 1973); Rogosa SL agar, for lactobacillus (LB), and CFAT medium (Zylber and Jordan, 1982), for *Actinomyces* (AC). The plates were incubated in 10% CO_2 at 37°C for 48 h (blood agar, MSB, Rogosa) or 72 h (CFAT). The colony-forming units (CFU) were counted and the results expressed as CFU/mg dental biofilm wet weight and as proportions of total streptococci (%TS/TM), mutans streptococci (%MS/TM), lactobacilli (%LB/TM), and *Actinomyces* (%AC/TM) in relation to total microorganisms.

Polysaccharide analyses

An aliquot of biofilm suspension (200 µl) was centrifuged at 10000 *g* for 10 min at 4°C, then the supernatant was collected to analysis of soluble extracellular polysaccharide (SEPS) (Aires et al., 2008). To the precipitate, 200 µl of 1 M NaOH was added for extraction of insoluble EPS (Cury et al., 2000). After 3 h at room temperature under constant agitation, supernatant was collected for analysis (Aires et al., 2008). Supernatants containing the polysaccharides were precipitated with 75% ethanol at -20°C, 30 min and analyzed for total carbohydrate according to Dubois et al. (1956).

Laboratorial Analyses

RNA extraction and purification

The RNA extraction and purification analyses were performed according to Cury and Koo (2007), and modifications of Aires et al., (2008) regarding to washing/sonication steps were made using 1.0 ml phosphate-buffered saline (PBS) and homogenization in NAES was made by sonication using one 10-second pulse at 6 W. The slabs were removed after the first washing/sonication in phosphate-buffered saline and biofilm wet weight determined after the first centrifuged (Analytical Plus AP 250D, Ohaus Corp., Florham Park, N.J., USA). The quantification of RNA extracted and its purification were made essentially as described by Cury and Koo (2007). Extracted RNA samples were joined to purification procedure forming pools.

Real Time quantitative PCR

Real-time quantitative RT-PCR was performed to evaluate gene expression of *gtfB, gtfC, gtfD* and *dexA* 1.0 μg of RNA and specific primers (Fujiwara et al., 2002) were used to generate cDNA (iScript cDNA synthesis kit, Bio-Rad Laboratories, Ca, USA). 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 MiniOpticon real-time PCR detection system with iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Calif., USA) and specific primers for *gtf B , gtf C, gtf D* and *dexA* as described by Koo et al . (2006). Standard curves for each gene were prepared as described by Yin et al. (2001) and Koo et al. (2006). Total cDNA abundance was normalized using *S. mutans* specific16S rRNA gene as a housekeeping control (Koo et al., 2006).

Statistical analysis

The assumptions of equality of variances and normal distribution of errors were checked for the response variables tested and those that did not satisfy were transformed (Box et al., 1978). To in vitro study, biofilm wet weight data were analyzed by unpaired t test ($p<0.05$). RNA extraction, purification and gene expression data were presented by descriptive statistic because biofilms were mixed to form pools.

From in situ study, the data of wet weight and purified RNA and *gtfB* were transformed into $\log_{10}$ and the original data of crude RNA and %DNA removed were submitted to analysis of variance (ANOVA) followed by the Tukey test. Also, the relationship between biofilm wet weight, concentration of sucrose and crude RNA, purified RNA and gene expression were estimated by regression analysis. The software SPSS for Windows 15.0 was used and the significance level was fixed at 5%.

RESULTS

With regard to in vitro study, biofilm wet weight of constant group was higher than intermittent group in all times evaluated (Fig. 1). The quantity of crude and purified RNA of control group (1% sucrose constant) showed lower values than experimental group at the three times (Table 1). Regarding to gene expression, *gtfB* expression of constant group increased

according to the time and was higher than the intermittent group in all times evaluated. *gtfC* and *gtfD* expression showed similar profiles: constant and intermittent groups were similar at 48 h, constant group was higher to 72 h and lower to 120 h than intermittent group. *dexA* expression of the constant group increased according to the time and was lower than intermittent group in all the times (Fig. 2).

For in situ study, statistically significant linear effect among sucrose concentration and biofilm wet weight, LB, % LB/TM, SEPS, IEPS, crude RNA and purified RNA were found (Table 2). No effects in the other variables were observed. TS, LB, LB/TM (Table 3), wet weight, IEPS and SEPS (Table 4), showed the values of 20% sucrose concentration were higher than 1% sucrose concentration ($p<0.05$). The other microbiological variables did not show differences between the groups (Table 3). Also, there were not differences among the groups to crude RNA, %DNA removed and purified RNA ($p>0.05$) (Table 5). *gtfB* gene expression (Fig. 3) increased according to the increase of sucrose concentration, but was not statistically significant ($p>0.05$). None *gtfC*, *gtfD* and *dexA* gene expression levels were detectable.

DISCUSSION

Dental in vivo studies have been difficult to perform in extreme situations as high cariogenic challenge due to risk of exposing volunteers or patients to conditions that could damage their health. In vitro and in situ models have received increasing recognition as tools for the study of both fundamental and applied aspects of dental caries (Zero, 1995; Sissons, 1997). However, in vitro and in situ models of dental biofilm formation have limitations about biofilm amount and these circumstances are challenges to molecular biology methodologies.

Regarding to the first experiment, biofilms formed in four slabs (total growth area of 224 mm^2) were joined to obtain enough amount of RNA to perform extraction process. Biofilm weight of intermittent sucrose exposure was lower than control ($p<0.05$) at all times (Fig.1). The constant supply of sucrose activates the biofilm metabolic activity increasing biofilm mass in comparison to intermittent sucrose exposure. The formation of pools, after RNA extraction, was necessary to perform PCR real time analysis. The descriptive results about crude and purified RNA showed that the constant exposure of carbohydrate on *S. mutans* biofilm provoked lower RNA concentration in comparison to intermittent exposure (Table 2). The presence of higher

values of IEPS in constant sucrose exposure than intermittent can influence the extraction and purification of RNA from dental biofilms decreasing RNA concentration values (Cury and Koo, 2007; Cury et al., 2008). The expression of genes evaluated showed different profiles among the groups due to *S. mutans* biofilm gene expression is altered to environment and nutritional factors as culture media, amount of sucrose (Shemesh et al., 2007) and type of carbohydrate used in sugar metabolism (Duarte et al., 2008). The expression of *gtfB* in both groups evaluated was lower than the other genes probably due to high levels of *gtfB* expression are related with the initial stages of biofilm formation, in the presence of carbohydrate, because IEPS are important factor in microbial adhesion (Loesche, 1986). *dexA* expression showed high levels than the other genes in the three times evaluated and *dexA* expression of intermittent group was higher than the constant group in all times suggesting that in "feast or famine" episodes exists a permanent activity in the remodelling of polysaccharides of biofilm matrix. The genes evaluated showed constant expression in all times evaluated indicated continous production of EPS and constant remodellation.

Regarding to in situ study, low levels of MS and %MS/TS were shown in all groups (Table 3). MS are encountered less frequently at the advancing front of dentin caries where lactobacilli, prevotellae and *Bifidobacterium* are more prevalent (Aas et al., 2008; Takahashi and Nyvad, 2008). However, the changes provoked by these microorganisms into the dental biofilm can be considered more relevant than their abundance (Ccahuana-Vásquez et al., 2007). Also, it is suggested that other acidogenic and aciduric bacteria than MS, including 'low-pH' non-MS and *Actinomyces* (van Houte et al., 1994; Sansone et al., 1993, Marsh 2006) can be responsible for the initiation of caries.

LB and %LB/TM increased according to the increase of sucrose concentration (Table 3). The presence of carbohydrates in high frequency provoke, in the biofilm internal environment, constants drop of pH that can be favourable to the predominance of microorganisms aciduric different to MS as LB, non-MS, Bifidobacterium or *Actinomyces* (van Houte et al., 1996). Also, LB are not implicated in caries initiation and these findings can be more important for caries progression since these microorganisms are found in high counts in caries lesions (Loesche and Syed, 1973, Preza et al, 2008, Preza et al, 2009).

The increase of sucrose concentrations did not influence on AC variables (Table 3), this result is in agreement with Mikx et al. (1975) in which no effect of the sucrose concentration in the diet on AC counts was observed in animal study. The %AC/TM in all groups (means between 14.46 -26.82%) indicate that these microorganisms are among the most predominant bacteria in dental biofilm associated with root caries according to Hoshino (1985) and Preza et al. (2008). However, a recent research that used molecular microarray analysis concluded that AC are not prevalent in root caries (Preza et al., 2009).

Biomass and extracellular polysaccharides increased according to the increase the sucrose concentration is similar than dental biofilm formed on enamel dental (Aires et al., 2006).. The increase of amount of sucrose caused a higher metabolic activity and consequently more production of biofilm mass and polysaccharides. The decrease of RNA extracted and purified values and the increase of IEPS and EPS values according to the increase of sucrose concentration (Table 5) confirms the influence of matrix EPS on extraction and purification of RNA from biofilms (Cury and Koo, 2007; Cury et al., 2008). No differences were observed among the groups to *gtfB* expression (Fig. 3) similar to Shemesh et al. (2007) did not observe differences in *gtfB* expression of *S. mutans* biofilms formed in vitro in Triptone-Yeast culture media when compared the growth in 1% and 4% sucrose concentration. However, IEPS and EPS values increased according to the increase of sucrose concentration (Table 4). Extracellular polysaccharides can be produced by other microorganisms different of *S. mutans* (Banas and Vickerman, 2003; Vacca-Smith et al., 2000). The absence of *gtf C, gtfD* and *dexA* values can be due to the low levels of *S. mutans* in relation to the other microorganisms of dental biofilm formed in situ under high cariogenic challenge and high levels of sugars (Ccahuana-Vásquez et al., 2007) and consequently small quantity of RNA was produced referent of this gene and RT PCR analysis was not able to detect (Aires et al., 2008).

In conclusion, it was possible to quantify in vitro the expression of *gtfB, gtfC, gtfD* and *dexA* of *S. mutans* biofilm in physiological conditions of sucrose exposure but in dental biofilm formed in situ only gtfB expression can be determined which seems not be regulated by sucrose concentration.

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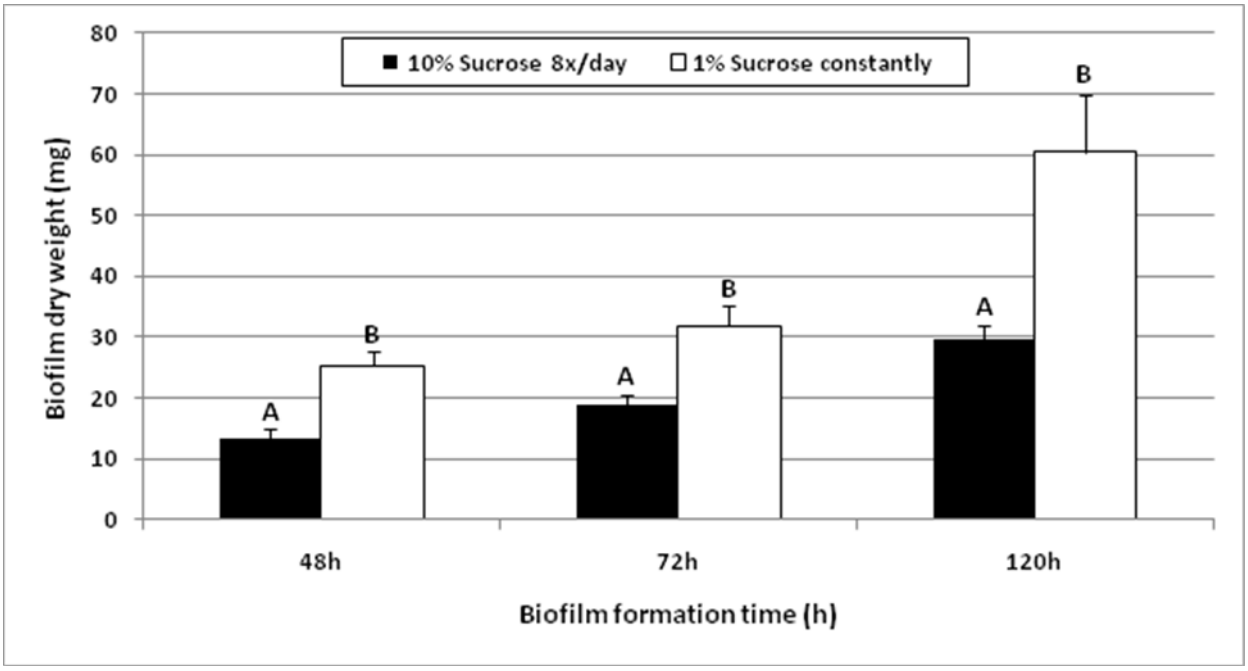

Fig 1. ***S. mutans*** **biofilm (n=4) wet weight (mg) according to the time of biofilm formation and the sucrose treatment. Different letter indicate statistical differences among the treatments in each time ($p < 0.05$).**

Table 1. Extraction and purification of RNA from *S. mutans* biofilms according to treatments and the times (mean ± SD)

Treatment	Frequency	Time (h)	*crude RNA(µg/mg biofilm wet weight)	DNA removed (%)	**purified RNA (µg/mg biofilm wet weight)
10% Sucrose	8x/day	48	2.72 ± 0.32	75.63 ± 6.47	0.65 ± 0.10
		72	3.26 ± 0.40	72.90 ± 3.75	0.89 ± 0.23
		120	2.47 ± 0.94	86.68 ± 3.61	0.33 ± 0.16
1% Sucrose	constantly	48	2.66 ± 0.38	96.20 ± 1.20	0.10 ± 0.02
		72	1.18 ± 0.03	89.62 ± 3.00	0.12 ± 0.04
		120	0.83 ± 0.04	81.15 ± 0.60	0.16 ± 0.01

*n=4; **n=2 or 3 (pools were formed)

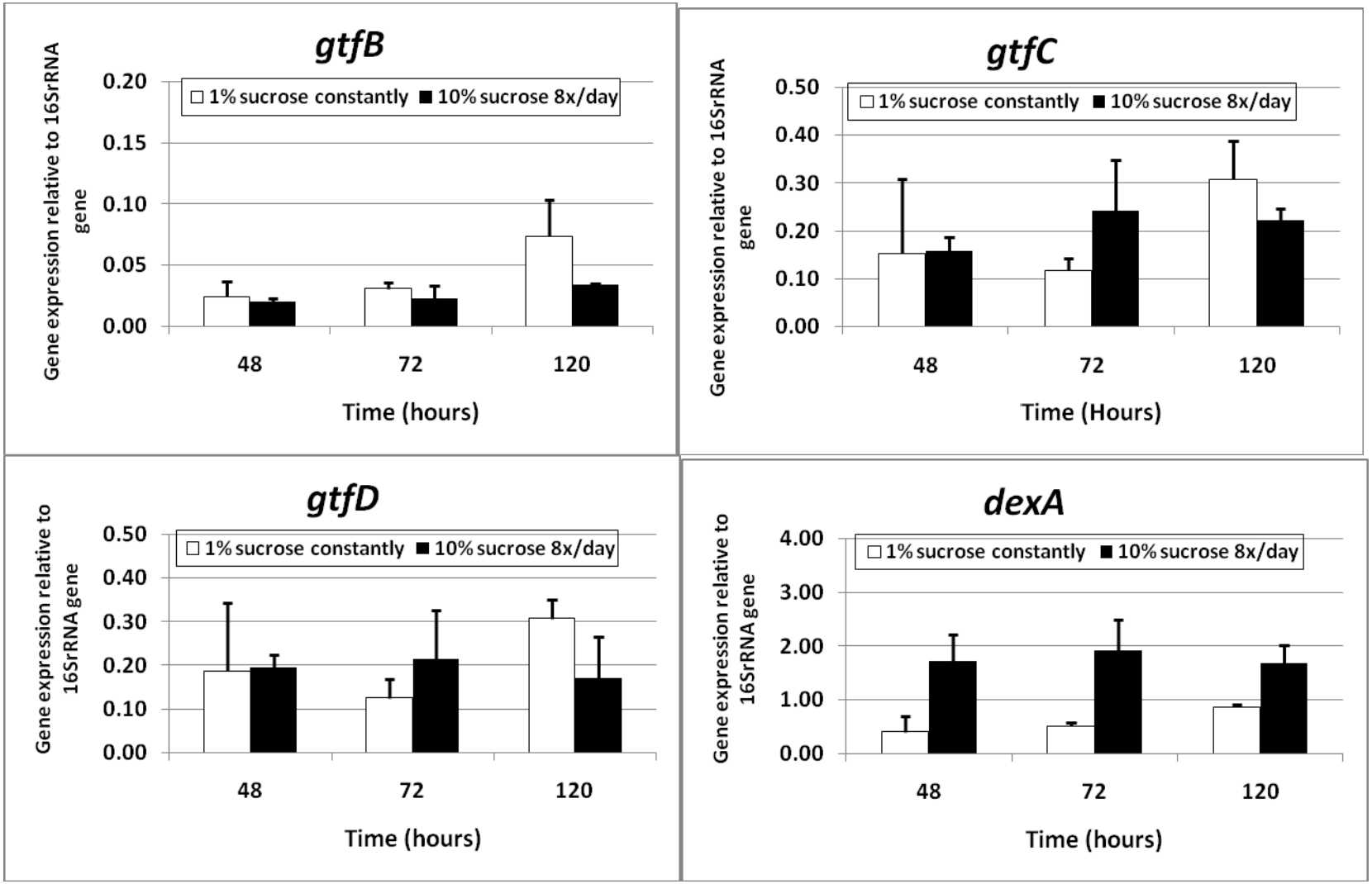

Fig 2. Gene expression of *S. mutans* biofilm after 48, 72 and 120 h of biofilm growth according to the sucrose exposure. Vertical bars denote standard deviations.

Table 2. Correlation (r) and significance (p) between sucrose concentration (1 – 20%) and the response variables.

Variable	r	p
Biofilm wet weight	0.343	0.041
Total microorganisms (TM)	0.015	0.932
Total streptococci (TS)	0.019	0.425
Mutans streptococci (MS)	0.213	0.213
MS/ TS (%)	0.116	0.505
Lactobacillus (LB)	0.35	0.046
LB/TM (%)	0.359	0.046
Actinomyces	0.029	0.870
AT/TM (%)	0.009	0.959
Insoluble extracellular polysaccharides	0.629	<0.001
Soluble extracellular polysaccharides	0.483	0.003
RNA crude	0.322	0.040
RNA purified	0.378	0.020
gtfB	0.217	0.246

Table 3. Colony forming-unit (CFU) and percentage of microorganisms on dental biofilm according to the sucrose concentrations

Variables	Sucrose concentration			
	1%	**5%**	**10%**	**20%**
TM (x $10^{5)}$	308.13 ± 179.59 a	330.57 ± 130.10 a	352. 46 ± 160.68 a	308.76 ± 258.55 a
TS (x 10^{5})	188.39 ± 103.09a	154.07 ± 111.81ab	144.93 ± 8.89ab	80.56 ± 45.58b
MS(x 10^{2})	24.78 ± 55.07a	31.31 ± 59.74a	34.42 ± 51.42a	113.98 ± 27.54a
MS/ TS (%)	0.03 ± 0.06a	0.03 ± 0.05a	0.04 ± 0.07a	0.11 ± 0.2a
LB (x 10^{4})	3.20 ± 7.94a	14.18 ± 28.91ab	73.34 ± 173.83ab	121.77 ± 191.75b
LB/TM (%)	0.06 ± 0.13a	0.51 ± 1.11ab	2.58 ± 6.05ab	3.95 ± 5.70b
AT (x 10^{5})	41.28 ± 33.66a	79.44 ± 57.49a	90.21 ± 101.97a	33.58 ± 26.30a
AT/TM (%)	14.46 ±9.11a	26.82 ± 17.29a	26.15 ± 20.50a	14.49 ± 11.66a

*n=9; TM: Total microorganisms, TS: Total Streptococci, MS: Mutans Streptococci, LB: Lactobacillus, AT: Actinomyces; sucrose concentrations whose means are followed by distinct letters differ statistically ($p < 0.05$).

Table 4. Wet weight (mg), insoluble extracellular polysaccharide [IEPS] (µg/mg biofilm wet weight) and soluble extracellular polysaccharides [SEPS] (µg/mg biofilm wet weight) concentration according to sucrose concentration for in situ study (n=9).

Variable	Sucrose concentration			
	1%	**5%**	**10%**	**20%**
Wet weight	46.06 ± 41.77a	56.77 ± 41.98a	65.97 ± 41.98ab	76.80 ± 38.62b
IEPS	6.20 ± 5.78 a	7.83 ± 5.20 a	10.22 ± 7.27 a	25.28 ± 11.59 b
SEPS	3.84 ± 1.24 a	4.05 ± 1.86a	5.70 ± 2.60ab	10.17 ± 7.87b

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

Table 5. Extraction and purification of RNA from dental biofilm according to sucrose concentration (mean ± SD; n = 7)

Sucrose concentration	RNA crude (µg/mg wet weight)	% DNA removed	RNA purified (µg/mg wet weight)
1%	1.79 ± 0.46	72.86 ± 12.13	0.51 ± 0.29
5%	1.51 ± 0.78	68.13 ± 9.28	0.48 ± 0.26
10%	1.47 ± 0.47	73.45 ± 14.00	0.40 ± 0.26
20%	1.24 ± 0.53	72.98 ± 13.69	0.28 ± 0.08

*No differences statistically significant were found ($p > 0.05$).

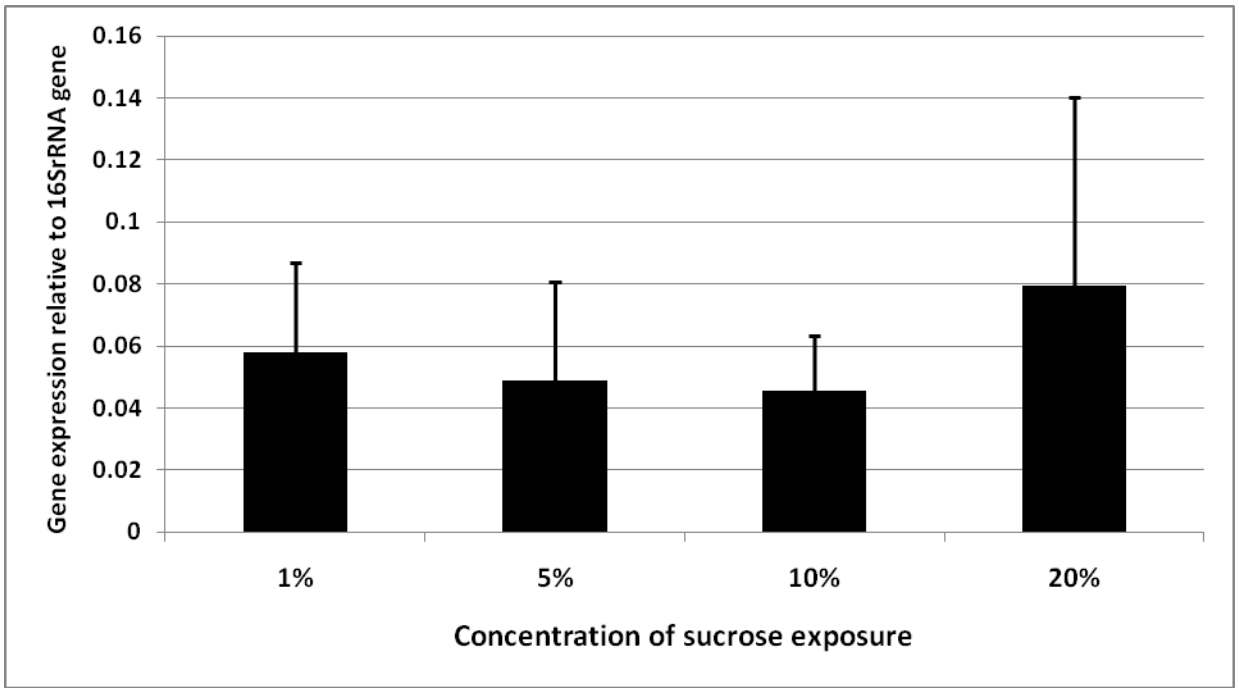

Fig. 3. Means (n=7) of *gtf B* gene expression of dental biofilm according to the concentrations of sucrose exposure. Vertical bars denote standard deviations. Significant differences were not observed among the groups ($p > 0.05$).

CONSIDERAÇÕES FINAIS

Modelos in vitro e in situ têm contribuído muito para o conhecimento da fisiologia e o comportamento do biofilme dental frente a diferentes tipos de estresses (Sissons, 1997; Zero, 1995). Vários modelos in vitro de crescimento microbianos foram utilizados para estudar a fisiologia (Bradshaw e Marsh, 1998), quorum sensing (Li et al., 2002), expressão de genes (Li e Burne, 2001; Shemesh et al., 2007) e resposta a antimicrobianos (Shapiro et al., 2002, Koo et al., 2003) de biofilmes de *S. mutans*. No capítulo 1 e 2, foram apresentados os processos de desenvolvimento e validação de um modelo de biofilme de *S. mutans* de fácil manipulação que simula satisfatoriamente episódios de "fartura e miséria" que acontecem quando os carboidratos da dieta estão presentes no ambiente bucal e no qual pode ser avaliada a desmineralização do substrato dental provocado pelo biofilme.

Os resultados do primeiro capítulo indicam que o modelo de biofilme proposto teve sensibilidade para mostrar diferenças de variáveis bioquímicas e pode ser utilizada para quantificar desmineralização do esmalte, característica que outros modelos não apresentam. Valores de acidogenicidade do biofilme, biomassa e desmineralização aumentaram quando a concentração de sacarose incrementou. A presença de maior quantidade de sacarose no biofilme provoca um aumento do metabolismo bacteriano que estimula a produção de ácidos e consequentemente causa maior desmineralização da superfície de esmalte. As concentrações de sacarose 10% e 20% expostas 8x/dia em condições fisiológicas mostraram os mesmos níveis de desmineralização que o grupo controle 1% fornecido constantemente. No segundo capítulo, para a validação do modelo foram utilizadas concentrações de digluconato de clorexidina (CHX) de 0,012% a 0,12%. e também foi testado o efeito do NaF 0,05%. O modelo mostrou efeito dose resposta para CHX, diminuindo os valores de biomassa, acidogenicidade, viabilidade bacteriana e desmineralização do esmalte. O F não mostrou efeito antimicrobiano, mas diminuiu a desmineralização em níveis iguais a CHX 0,12%.

O efeito da sacarose na expressão de *gtfB, gtfC, gtfD* e *dexA* no modelo in vitro desenvolvido no capitulo 1 e no biofilme formado in situ foi abordado no capitulo 3. No estudo in vitro foi comparado a expressão gênica de biofilme de *S. mutans* formados na presença de sacarose constante (controle) e intermitente (episódios "fartura e miséria"). Os resultados mostraram valores maiores de RNA extraído e purificado do grupo exposto à sacarose

intermitente que o controle e que a expressão de *gtfB, gtfC*, *gtfD* e *dexA* foi constante ao longo do tempo. Com relação ao estudo in situ, os resultados do estudo in situ mostraram valores de Lactobacilos (LB), % LB/Microrganismos totales, PECI e PECS aumentaram e RNA bruto e purificado diminuiram de acordo com aumento da concentração de sacarose confirmando que o aumento de níveis de PECI da matriz diminui a concentração de RNA (Cury e Koo, 2008). Os níveis de expressão do *gtfB* de biofilme dental mostrou que não é regulada pelo concentração de sacarose e não se conseguiu quantificar os genes *gtfC, gtfD* e *dexA*. Os baixos níveis de *S. mutans* presentes em biofilmes formados in situ sob alto desafio cariogênico (Cury et al., 2001, Ccahuana-Vásquez et al., 2007, Vale et al., 2007) podem ser responsáveis de valores baixos de RNA referente aos genes avaliados o que impossibilitou a análise gênica.

CONCLUSÃO

O modelo padronizado de crescimento de biofilme de *S. mutans* é capaz de avaliar não só mudanças bioquímicas, microbiológicas e de expressão gênica no biofilme de *S. mutans* quando crescido sob diversas condições como também avaliar a consequente desmineralização do substrato dental. Para tal, sacarose 10% 8x/dia deve ser usado ao invés de 1% sacarose constante como fonte de energia. O modelo validado tem potencial para avaliar não só susbtâncias com efeito antimicrobiano no biofilme como na desmineralização do substrato dental onde biofilme estiver aderido.

Quanto à expressão gênica foi possível quantificar in vitro a expressão de *gtfB, gtfC, gtfD* e *dexA,* sugerindo expressão constante destes genes em condições fisiológicas de exposição a sacarose. Entretanto, no estudo in situ somente a expressão de *gtfB* foi quantificável e parece não ser regulada pela concentração de sacarose.

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

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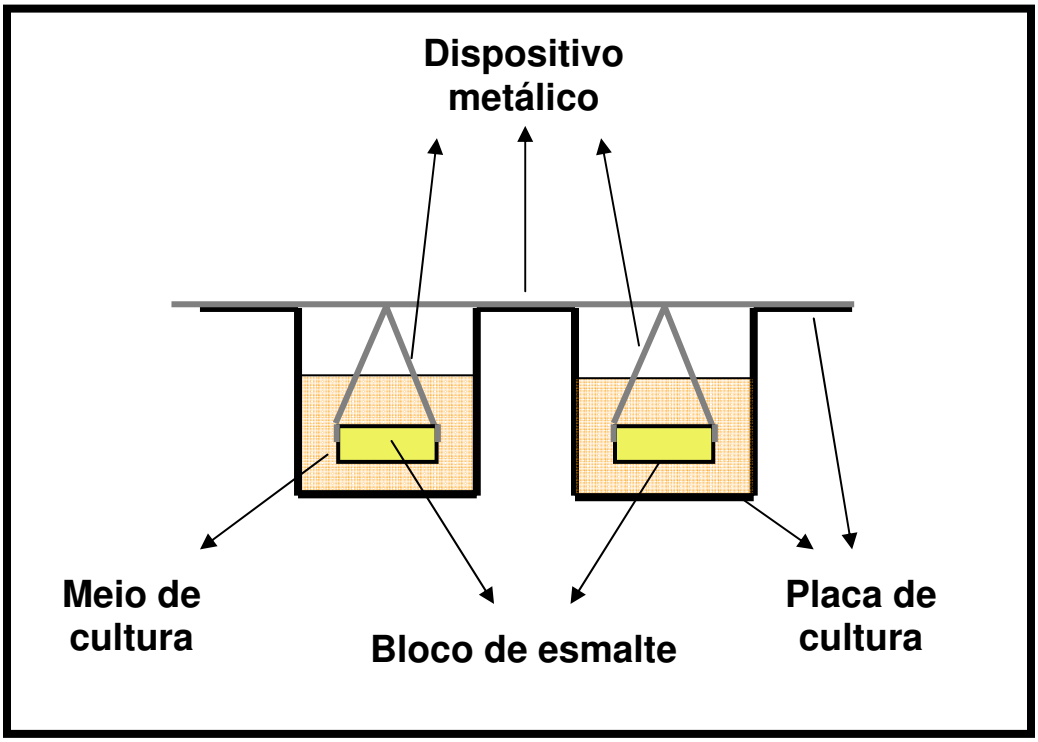
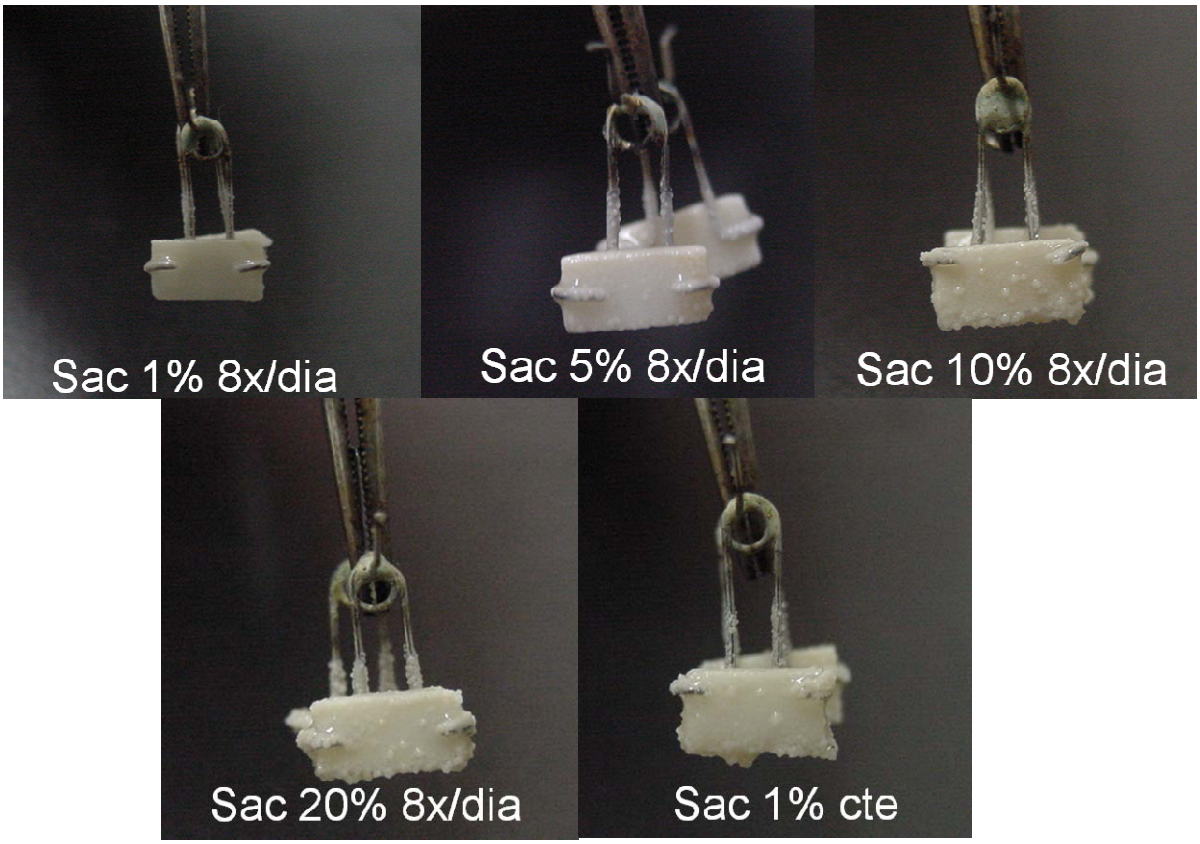
APÊNDICE 1 – Disposição dos blocos no modelo de crescimento de biofilme de *S. mutans*



APÊNDICE 2 – Fotografías do crescimento de biofilmes de *S. mutans* de acordo com os tratamentos com sacarose



ANEXO 1 – Deliberação da defesa em formato alternativo

INFORMAÇÃO CCPG/OO2/06[6]

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG n° 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "impar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2° desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

§ único - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

Artigo 3º - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em formato alternativo, desde que observados os incisos I, II, III IV, V e VII do artigo 1º.

Artigo 4º - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Um original da dissertação ou tese, em versão definitiva, impresso em folha tamanho carta, em uma só face, deve ser encaminhado à gráfica da Unicamp acompanhado do formulário "Requisição de Serviços Gráficos", onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão encaminhados à Unidade em, no máximo, cinco dias úteis.

§ 4º - No formulário "Requisição de Serviços Gráficos" deverão estar indicadas as páginas cuja reprodução deva ser feita no padrão "cores" ou "foto", ficando entendido que as demais páginas devam ser reproduzidas no padrão preto/branco comum.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

§ 6º - As páginas fornecidas para inserção deverão ser impressas em sua forma definitiva, ou seja, apenas frente ou frente/verso.

§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 13 de setembro de 2006

Profa. Dra. Teresa Dib Zambon Atvars
Presidente
Comissão Central de Pós-Graduação

ANEXO 2 – Certificado de aprovação no Comitê de Ética

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 "**Efeito da concentração e freqüência de exposição à sacarose na expressão de genes de glicosiltransferases e glucanases do biofilme dental**", protocolo nº **042/2006**, dos pesquisadores **JAIME APARECIDO CURY e RENZO ALBERTO CCAHUANA VÁSQUEZ**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 12/04/2006.

The Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas, certify that project "**Effect of concentration and frequency of sucrose exposure on glucosyltransferase and glucanase gene expression in dental biofilm**", register number **042/2006**, of **JAIME APARECIDO CURY and RENZO ALBERTO CCAHUANA VÁSQUEZ**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for researching in human subjects and was approved by this committee at 12/04/2006.

Profa. Cecília Gatti Guirado
Secretária
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

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

ANEXO 3 – Comprovante de submissão de artigo

YAHOO! EN ESPAÑOL CORREO Clásico

7409637103540607 Improvement and validation of a S. mutans biofilm model

lunes, 8 de febrero de 2010, 01:47 pm

De: "BioMed Central Editorial" <editorial@biomedcentral.com>
A: "Prof Jaime A Cury" <JCury@fop.unicamp.br>
Cc: "Prof Jaime A Cury" <JCury@fop.unicamp.br>, " Renzo A Ccahuana-Vásquez " <renzetor@yahoo.com>, "Jaime A Cury" <jcury@fop.unicamp.br>

Article title: Improvement and validation of a S. mutans biofilm model
MS ID : 7409637103540607
Authors : Renzo A Ccahuana-Vásquez and Jaime A Cury
Journal : BMC Microbiology

Dear Prof Cury

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