UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

GLÁUBER CAMPOS VALE

COMPOSIÇÃO MICROBIOLÓGICA E BIOQUÍMICA DO BIOFILME DENTAL FORMADO EM DIFERENTES TEMPOS E SUA RELAÇÃO COM A DESMINERALIZAÇÃO DO ESMALTE

Dissertação apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do Grau de Mestre em Odontologia, Área de Cariologia.

PIRACICABA
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CIRURGIÃO-DENTISTA

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SUMÁRIO

RESUMO

O biofilme dental exposto a sacarose *in situ* por 13 dias ou mais apresenta altas concentrações de polissacarídeos extra-celulares (PEC), altas contagens da lactobacilos e baixa concentração inorgânica. Entretanto, essas mudanças e suas conseqüências em estágios inferiores de formação de biofilme são desconhecidas. Assim, o objetivo deste estudo foi avaliar a composição microbiológica e bioquímica do biofilme dental formado na presença de sacarose ou glicose + frutose em diferentes tempos, com a finalidade de observar a dinâmica de maturação do biofilme e sua relação com a desmineralização do esmalte. Doze voluntários adultos utilizaram em 3 fases cruzadas de 14 dias, um dispositivo intra-oral palatino contendo 6 blocos de esmalte dental humano, os quais foram expostos 8 vezes ao dia aos seguintes tratamentos: água destilada e deionizada (T1), solução de glicose 10% + frutose a 10% (T2) ou solução de sacarose a 20% (T3). O biofilme foi coletado após 3, 7 e 14 dias de formação e avaliado quanto à composiçao microbiológica e bioquímica. A análise microbiológica consistiu nas contagens de microrganismos totais (MT), estreptococos totais (ET), estreptococos do grupo mutans (EM), lactobacilos (LB), %EM/MT, %EM/ET e LB/MT. As variáveis bioquímicas avaliadas foram Ca, F, P_i, polissacarídeos intra (PIC) e extracelulares (PEC) no biofilme. Nos espécimes dentais, a perda mineral do esmalte seccionado longitudinalmente foi determinada. Maior desmineralização foi encontrada nos blocos submetidos ao T3 do que nos tratados com T1 e T2 ($p < 0,05$), sendo a perda mineral considerada significante a partir de 7 dias ($p < 0,05$). As concentrações de F, Ca e P_i no biofilme dental foram menores no T2 e T3 do que no T1 ($p < 0,05$),), sendo que para F e Ca não houve diferença entre os tempos ($p > 0,05$) e para P_i, 7 e 14 dias mostraram maiores concentrações que no biofilme de 3 dias ($p < 0,05$). As concentrações de PIC foram significantemente maiores no T2 e T3 do que no T1 ($p < 0,05$), havendo um aumento com 7 e 14 dias, enquanto as de PEC foram maiores no T3 do que no T1 e T2 ($p < 0,05$), não mostrando diferença entre os tempos. Em relação à composição microbiana, os resultados mais evidentes foram em relação à contagem de LB e %LB/MT que apresentaram-se maiores no biofilme tratado com T2 e T3 do que no T1, entretanto essa diferença só foi observada a partir do 7º dia ($p < 0,05$). Os resultados sugerem que mudanças na composição do biofilme formado na presença de sacarose já são evidentes a partir do 3º dia de formação, entretanto a perda mineral só é significativa com 7 dias.

ABSTRACT

Dental biofilm exposed *in situ* to sucrose for 13 days or longer presents high concentration of extracellular polysaccharide (EPS), high lactobacilli counts and low inorganic concentration. However, these changes and their consequences at earlier stages of biofilm formation are unknown. Thus, the aim of this study was to evaluate the microbiological and biochemical composition of dental biofilm formed in the presence of sucrose or glucose + fructose at different periods, in order to observe its dynamic of maturation and its relationship with enamel demineralization. Twelve adult volunteers wore, for 3 crossover phases of 14 days, an intra-oral palatal appliance containing 6 human enamel blocks, which were exposed 8 times/day to the following treatments: distilled and deionized water (T1), 10% glucose + 10% fructose solution (T2) or 20% sucrose solution (T3). The biofilm was collected after 3, 7 and 14 days of formation and evaluated with regard to microbiological and biochemical composition. Microbiological analyses consisted in counts of total microorganisms (TM), total streptococci (TS), mutans streptococci (MS), lactobacilli (LB), %MS/TM, %MS/TS and %LB/TM. The biochemical variables evaluated were F, Ca, P_i, intra (IPS) and extracellular (EPS) polysaccharides. In dental specimens, enamel cross-sectional mineral loss was determined. Higher mineral loss was found in enamel blocks treated with T3 than those exposed to T1 and T2 ($p < 0.05$), however only with 7 days the mineral loss was considered significant ($p < 0.05$). The concentrations of F, Ca and P_i in dental biofilm were lower in T2 and T3 than in T1 ($p < 0.05$). Also, for F and Ca no difference was observed among the periods ($p > 0.05$) and for P_i, 7 and 14-day biofilm showed higher concentrations than 3-day biofilm ($p < 0.05$). IPS concentrations were significantly higher in T2 and T3 than in T1 ($p < 0.05$), showing increased concentrations with 7 and 14 days ($p < 0.05$), whereas EPS concentration did not show statistical difference among the periods ($p > 0.05$), but showed higher values in T3 than in T1 and T2 ($p < 0.05$). With respect to microbiological composition, the most evident results were related to LB counts and %LB/TM that showed higher values in T2 and T3 than in T1 ($p < 0.05$), but this difference was observed only with 7-day biofilm ($p < 0.05$). The results suggest that the changes on composition of biofilm formed under sucrose exposure are evident at 3 days of formation, however the mineral loss is only significant with 7 days of biofilm formation.

1. INTRODUÇÃO GERAL

A cárie dental é uma doença multifatorial e seu desenvolvimento está diretamente relacionado ao alto consumo de carboidratos (Bowen *et al*., 1980), os quais são fermentados por bactérias cariogênicas a ácidos orgânicos que provocam a desmineralização do substrato dental (Gibbons & van Houte, 1975). Várias pesquisas (Staat *et al.*, 1975; Newbrun *et al.*, 1982; Sheiham, 2001; Nobre dos Santos *et al.*, 2002), incluindo o estudo clássico de Vipeholm (Gustafsson *et al.*, 1954), mostram significativa relação entre o consumo de sacarose e cárie dental. Assim, dos carboidratos presentes na dieta, a sacarose é considerada o mais cariogênico, pois além de ser fermentável, promovendo queda de pH e seleção microbiana (Marsh, 1991) no biofilme, também é substrato para a síntese de polissacarídeos extracelulares (PEC) (Rolla *et al*., 1985; Bowen, 2002).

Os PEC aumentam a porosidade do biofilme dental e difusão de substrato para a superfície do esmalte (Dibdin & Shellis, 1988), aumentando a queda de pH na interface biofilme-dente (Zero *et al.*, 1986), o que resulta no aumento de espécies acidúricas e acidogênicas (Marsh, 2003). Assim, os PEC aumentam a cariogenicidade do biofilme, sendo considerados um dos principais fatores de virulência. Em acréscimo, altas freqüências de exposição à sacarose podem modificar as características bioquímicas do biofilme, apresentando altas concentrações de polissacarídeos insolúveis e baixas concentrações de cálcio, fósforo inorgânico e flúor (Cury *et al*., 1997), além de promover maior desmineralização do esmalte dental (Cury *et al.*, 1997; Paes Leme *et al.*, 2004).

A presença de estreptococos do grupo mutans é um fator importante para a cariogenicidade do biofilme, já que esses microrganismos, além de acidúricos e acidogênicos, produzem PEC a partir da sacarose, dos quais os glucanos insolúveis estão diretamente relacionados com cárie dental (Zero *et al.*, 1886; van Houte., 1994; Cury *et al.*, 2001). No entanto, pesquisas recentes sugerem que a capacidade dos *S. mutans* sintetizarem glucanos insolúveis seja mais importante que seus níveis no biofilme (Mattos-Graner *et al.*, 2000; Nobre dos Santos *et al.*, 2002). Já a relação direta entre consumo de sacarose e lactobacilos (Staat *et al.*, 1975; Fejerskov, 1997), pode ser explicada pela alta tolerância ácida que esses microrganismos apresentam, sobrevivendo

em ambientes de baixo pH, como os resultantes da metabolização da sacarose por microrganismos orais (Bradshaw *et al*., 1989).

Entre as mudanças microbiológicas que ocorrem no biofilme na presença de sacarose, alguns estudos (Pecharki *et al*., 2005; Ribeiro *et al.*, 2005) mostram uma maior contagem de estreptococos do grupo mutans e lactobacilos no biofilme formado na presença de sacarose em comparação ao grupo controle negativo. Essa tendência também foi observada no trabalho de Ccahuana-Vásquez *et al*. (2006), que avaliaram várias freqüências de exposição à sacarose. Tais pesquisas foram realizadas *in situ* com o tempo de formação de biofilme de 14 dias.

Com relação às alterações bioquímicas do biofilme, a concentração de íons é um fator relevante na determinação da saturação do biofilme e, conseqüentemente, no desenvolvimento de cárie (Ashley & Wilson, 1977; Margolis & Moreno, 1992; Pearce, 1998). As concentrações de cálcio e fósforo são relevantes em termos de desenvolvimento de cárie, já que há uma relação inversa entre as concentrações desses íons no biofilme dental (Ashley & Wilson, 1977; Grobler *et al.*, 1982; Shaw *et al*., 1993) e no fluido do biofilme (Margolis *et al*., 1993) e experiência de cárie. Avaliando a composição bioquímica do biofilme formado após 28 dias *in situ* na presença de sacarose e de seus monossacarídeos constituintes, glicose + frutose, que são fermentáveis, mas não atuam como substrato para a síntese de PEC, Cury *et al.* (2000) observaram em ambos os tratamentos menores concentrações de flúor, cálcio e fósforo e um aumento significativo na concentração de polissacarídeos insolúveis no grupo sacarose. Recentemente, foi demonstrado que o aumento da concentração de sacarose também promove maior concentração de PEC, menor concentração inorgânica e maior perda mineral do esmalte (Aires *et al.*, 2006). Essas evidências sugerem que a maior cariogenicidade do biofilme, além dos PEC, também está relacionada com a menor concentração inorgânica.

Entretanto, a maioria das pesquisas *in situ* que apontam essa tendência de maior cariogenicidade da sacarose foram realizadas com períodos de formação de biofilme de 14 (Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005; Aires *et al.*, 2006) até 28 dias (Cury *et al.*, 1997, 2000). Sabe-se que o desenvolvimento do biofilme dental pode ser dividido em vários estágios arbitrários que compreendem: a formação da película; adesão de células bacterianas simples (0-4 h); crescimento de bactérias aderidas,

levando à formação de microcolônias distintas (4-24 h); sucessão e co-agregação microbiana que levam a uma alta diversidade de espécies (1-14 dias) e comunidade clímax ou biofilme maduro (14 dias ou mais) (Marsh & Nyvad, 2005). Por outro lado, deve-se observar que a formação do biofilme é um processo altamente dinâmico e que a adesão, o crescimento, a remoção e a readesão de microrganismos podem ocorrer ao mesmo tempo (Marsh & Bradshaw, 1995).

Dessa forma, torna-se importante avaliar as mudanças na composição do biofilme dental formado na presença de sacarose em tempos inferiores com a finalidade de observar a dinâmica de maturação do mesmo.

2. PROPOSIÇÃO

O objetivo desse estudo foi avaliar a composição microbiológica e bioquímica do biofilme dental formado *in situ* na presença de sacarose ou glicose + frutose por 3, 7 e 14 dias e sua relação com a desmineralização do esmalte dental.

3. CAPÍTULO

Este trabalho foi realizado no formato alternativo, conforme deliberação número 001/98 da Comissão Central de Pós-Graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP).

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3.1 Timing Effect on Dental Biofilm Exposed to Sucrose *In Situ**

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ABSTRACT

Dental biofilm exposed *in situ* to sucrose for 13 days or longer presents high concentration of extracellular polysaccharide (EPS), high lactobacilli counts and low inorganic concentration. Since these changes and their consequences at earlier stages of biofilm formation are unknown, 12 volunteers subjected enamel slabs to the treatments: water, 10% glucose + 10% fructose and 20% sucrose solution. The biofilms formed during 3, 7 and 14 days were analyzed biochemically and microbiologically, and mineral loss (ΔZ) was evaluated on enamel. Statistically higher ΔZ value was found for sucrose treatment after 7 days. On the 3rd day, sucrose significantly increased lactobacilli, EPS, intracellular polysaccharide and decreased the inorganic concentration in the biofilm; however, the only significant difference compared to glucose-fructose treatment was a higher insoluble EPS concentration. The data suggest that although sucrose induces significant enamel demineralization only after 7 days of biofilm accumulation, changes in the biofilm composition are observed earlier.

INTRODUCTION

Sucrose is a unique cariogenic carbohydrate, since besides being fermentable to acids it is substrate for synthesis of extracellular polysaccharides (EPS) in dental biofilm.

Thus, low pH induced by sucrose fermentation triggers a shift in the balance of resident plaque microflora to a more cariogenic one, according to the ecological plaque hypothesis (Marsh, 2003), which has been supported by long term sugar consumption-diet (Staat *et al*., 1975) and *in situ* experimental studies (Minah *et al*., 1981; Pecharki *et al.,* 2005; Ribeiro *et al*., 2005). Furthermore, this low pH is also responsible for enamel-dentine demineralization, whose detection is time-dependent.

EPS (mainly insoluble glucans) enhance bacterial adherence to the tooth surface (Schilling and Bowen, 1992) and contribute to the structural integrity of dental biofilms. They also increase the porosity of biofilm formed, allowing sugar diffusion into its deepest parts (Dibdin and Shellis, 1988), which would result in low plaque pH values due to microbial catabolism (Zero *et al*., 1986). The relationship between EPS and caries has been supported by *in situ* and clinical studies (Zero *et al*., 1986; Cury *et al*., 1997; 2000; Mattos-Graner *et al*., 2000; Nobre dos Santos *et al*., 2002; Tenuta *et al.*, 2003; Paes Leme *et al*., 2004; Pecharki *et al*., 2005; Ribeiro *et al*., 2005; Aires *et al*., 2006).

Therefore, EPS are considered critical virulence factors in the dental biofilm formed in the presence of sucrose (Bowen, 2002), but simultaneously it has been found that sucrose reduces calcium (Ca), inorganic phosphorus (P_i) and fluoride (F) concentrations in the dental biofilm (Cury *et al*., 1997; 2000; 2003; Paes Leme *et al*., 2004; Pecharki *et al*., 2005; Ribeiro *et al*., 2005; Aires *et al*., 2006), and these ions are relevant in maintaining the mineral equilibrium between the tooth and the oral environment (Margolis *et al*., 1988).

On the other hand, these changes induced by sucrose on biofilm composition have been experimentally observed in long term studies conducted during 14 up to 28 days of biofilm accumulation since the main objective was to evaluate enamel demineralization (Cury *et al*., 1997, 2000; Tenuta *et al*., 2003; Paes Leme *et al*.,

2004; Pecharki *et al*., 2005; Ribeiro *et a*l., 2005; Aires *et al*., 2006). However, it would be relevant to evaluate these changes in earlier stages of biofilm formation and accumulation for addressing a more effective caries control.

This study aimed to evaluate the changes in the biochemical and microbiological composition of dental biofilm formed *in situ* in the presence of sucrose as a function of time. To reach the aim, two controls were idealized: a negative (water) and an active (glucose-fructose, mixture of monosaccharides components of sucrose that are only fermentable).

MATERIAL AND METHODS

Experimental design

This study, approved by the Research and Ethics Committee of FOP-UNICAMP (Protocol no. 053/2004), had a crossover blind design and was performed in three phases, during which 12 healthy adult volunteers, 17-27 years old, wore acrylic intra-oral palatal appliances containing six enamel slabs, which were extraorally subjected to the treatments: distilled and deionized water (negative control); mixture of 10% glucose and 10% fructose solution (active control) or 20% sucrose solution, 8 times a day. The volunteers were randomly allocated to the treatments and after 3, 7 and 14 days the biofilms formed were collected for microbiological and biochemical analyses as well as enamel demineralization was evaluated. For statistical analysis, the volunteer was considered a block.

Enamel Slabs and Appliance Preparation

Two hundred and sixteen human enamel slabs (4 x 4 x 2 mm), prepared as previously described (Cury *et al*., 2000), were randomized to the treatments and periods. The volunteers wore palatal appliances containing six enamel slabs placed separately, three of them located on right and left posterior sides of the appliance (Hara *et al.*, 2003 for details).

Treatments

Eight times a day, the volunteers removed the appliances and dripped one drop of the solutions onto each enamel slab according to the treatment protocol, and after 5 min the appliance was replaced in the mouth. During a 7-day pre-experimental period, washout periods of 7 days and the experimental phases, the volunteers brushed their natural teeth with nonfluoridated toothpaste, but drank fluoridated water (0.70 mg F/L). Other details are described elsewhere (Cury *et al*., 2000).

Dental Biofilm Analysis

Dental biofilm was collected from 2 enamel slabs at random, but one from each side of the appliance, after 3, 7 and 14 days of biofilm formation and ten hours after the last exposure to treatments. A homogeneous aliquot was used for the microbiological analyses and the rest of the biofilm was dehydrated for the biochemical analyses.

The microbiological analyses were made as described in Pecharki *et al*. (2005) and Ribeiro *et al*. (2005). The colony-forming units (CFU) of total microbiota (TM), total streptococci (TS), mutans streptococci (MS) and lactobacillus (LB) were counted and expressed as CFU/mg dental biofilm (wet weight); the percentage of mutans streptococci and lactobacilli in relation to total microbiota (%MS/TM and %LB/TM) and mutans streptococci in relation to total streptococci (%MS/TS) were calculated.

Ca, P_i, F and insoluble EPS were extracted from dehydrated biofilms, as previously described (Pecharki *et al*., 2005). After extraction of insoluble EPS, the precipitate was treated with 1 mol/LM NaOH (0.2 mL/mg biofilm dry weight) at 100°C for 1 h for extraction of intracellular polysaccharide (IPS), which was done at 100°C for 1 h. Supernatants containing the polysaccharides were precipitated with 75% ethanol and analysed for total carbohydrate (Dubois *et al*.,1956). F was determined as previously described (Cury *et al*., 2000), and Ca and P_i were determined colorimetrically with Arsenazo III and malachite green/molibdate, respectively (Vogel *et al*., 1983).

Dental Enamel Analysis

Enamel mineral loss (ΔZ) was evaluated by cross-sectional microhardness as described elsewhere (Paes Leme *et al*., 2004).

Statistical Analysis

The variables were analyzed using split-plot analysis of variance (ANOVA), considering the treatments as plots and the time of biofilm formation as subplots, with the volunteers considered as statistical blocks. The assumptions of equality of variances and normal distribution of errors were checked for all the response variables, and those that did not satisfy were transformed (Box *et al*., 1978). The values of ΔZ were transformed by square root; LB, F, P_i and IPS by $\log_{10}$, Ca by the power of -0.3 and insoluble EPS and MS were transformed, respectively, by the power of 0.2 and 0.1, allowing a parametric statistical analysis of the data. The data of %LB/TM, %MS/TM and %MS/TS were not transformed. The data were assessed by analyses of variance (ANOVA) and Tukey test, except for %LB/TM, %MS/TM and %MS/TS, when a non-parametric analysis was performed. For all statistical analyses, SAS software system (version 8.02. SAS Institute Inc., Cary, NC, USA) was used, and the significance level was set at 5%.

RESULTS

A significant effect ($p<0.05$) for both factors treatment and time of biofilm formation and their interaction was observed in the variables LB, %LB/TM, %MS/TS, Ca concentration and ΔZ, while for concentrations of P_i and IPS, a statistically significant effect ($p<0.05$) was found only for the isolated factors (Table 1). With regard to a significant effect only for treatment ($p<0.05$), it was observed in EPS concentration. %MS/TM and F concentration showed a statistical effect ($p<0.05$) for treatment and the interaction treatment *vs.* time. For MS counts in dental biofilm, none of the factors showed a significant effect ($p>0.05$).

With respect to the effect of time of biofilm formation, within each treatment/group, there was a statistically significant increase of LB counts, %LB/TM, and IPS concentration ion biofilm formed and ΔZ on enamel in sucrose treatment (Table 2). However, ΔZ was the only variable that showed different time profile compared with

the controls, either negative or active. Thus, a increase of IPS concentration over the time was also found for the negative control group, and LB counts, %LB/TM and IPS concentration as well increased due to the glucose-fructose treatment. Furthermore, Ca and F concentrations did not change as a function of time of biofilm accumulation in sucrose and glucose-fructose groups, while significantly increased in the negative control group (Table 2).

Considering the effect of treatments at each time evaluated (Table 3), sucrose exposure for 7 and 14 days significantly increased LB counts, %LB/TM, %MS/TM, %MS/TS, EPS and IPS concentrations and ΔZ, and significantly decreased Ca, P_i and F concentrations compared to the negative control. The changes in LB counts, %LB/TM, Ca, P_i, F, EPS and IPS concentrations were already observed on the 3rd day. However, the only variables that significantly differed from the glucose-fructose treatment/group were insoluble EPS at all times and ΔZ on the 7th and 14th day of biofilm accumulation.

DISCUSSION

The findings showed that undisturbed dental bioflm exposed 8x/day to sucrose or glucose-fructose significantly provoked an increase on enamel mineral loss (ΔZ) as a function of time compared with the negative control treatment group (Table 2). These data agree with *in vivo* and *in situ* studies (Holmen *et al*., 1988; Tenuta *et al*., 2003, respectively), showing that completely undisturbed dental plaque for more than one week results in visible enamel demineralization. The data also showed (Table 3) that biofilm treated with sucrose induced higher enamel loss than that exposed to glucose-fructose solution. This result already observed on the 7th day of biofilm accumulation agrees with the 28-day study conducted by Cury *et al*. (2000). However, enamel demineralization on the 3rd day of sucrose treatment is numerically higher than that found for glucose-fructose and the negative control group (Table 3), although not statistically different. Thus, the data suggest that the changes caused by sucrose on dental biofilm and its consequences for enamel occurs earlier.

Therefore, sucrose was able to significantly increase mutans streptococci and lactobacilli in dental biofilm compared with the negative control treatment group

(Table 3), but the difference with regard to glucose-fructose group did not reach statistically different significance. These *in situ* findings are in agreement with *in vivo* observation (Staat *et al*., 1975) as well as they confirm *in vitro* data (Bradshaw *et al*., 1989) about the aciduric property of these microorganisms, surviving and predominating in acidic medium generated by carbohydrate fermentation. The data also showed that lactobacilli increased faster and in higher amounts than mutans streptococci (Tables 2 and 3), which is in agreement with *in vitro* (Bradshaw *et al*., 1989) and *in situ* studies (Pecharki *et al*., 2005; Ribeiro *et al*., 2005). The predominance of these microorganisms can be explained, since the experimental model used, where the biofilm is undisturbed for 13 days, may resembles proximal plaque or the ecological niche of a carious lesion, where LB are known to be present at high counts (Loesche and Syed, 1973). On the other hand, the lower increase of mutans streptococci, in agreement with *in vivo* study (Boyar *et al*., 1989), suggests that the change provoked by this microorganism in dental biofilm may be more relevant than its levels (Cury *et al.,* 2001).

In fact, regarding the biochemical changes induced by sucrose in the biofilm, the concentration of insoluble EPS was the only differential in comparison with the negative control (water) and active control (glucose-fructose) treatment effects. Insoluble EPS concentration was statistically higher in biofilm exposed to sucrose than both controls soon on the 3rd day of biofilm formation (Table 3) and this concentration stayed high during the 14 days of biofilm accumulation (Table 2). The findings are in agreement with the role of this kind of polysaccharide on caries development, enhancing bacterial adherence to enamel (Bowen, 2002) and changing the properties of biofilm matrix (Dibdin and Shellis, 1988), which would explain the greatest cariogenic potential of sucrose compared with other carbohydrates (Cury *et al*., 2000; Ribeiro *et al*., 2005). Furthermore, the findings about insoluble EPS change in the beginning of biofilm formation reinforce that one of the best strategies to caries control would be to interfere with the enzymes responsible for the synthesis of these polysaccharides (Hayacibara *et al*., 2004; Xavier *et al*., 2005).

Thus, although IPS can promote the formation of dental caries (Tanzer *et al*., 1976), its intracellular storage is not a unique attribute of sucrose. Indeed, statistically higher IPS concentrations were found in biofilm exposed either to sucrose

or glucose-fructose than that in the negative control group (Table 3), but the differences between the carbohydrates groups were not statistically different at any time of biofilm accumulation. On the other hand, this high IPS concentration was found 10 h after the last carbohydrate exposure (see M&M), suggesting that this source of energy is not totally depleted during the night, so that the cariogenic effect can be prolonged during the day. This experimental observation is relevant in terms of dietary counseling, mainly considering root caries, since dentine is more susceptible to demineralization than enamel and critical pH for dentine has been found in fasting biofilm exposed to sucrose (Pecharki *et al*., 2005; Ribeiro *et al*., 2005).

With regard to the inorganic composition of the biofilm (Table 3), Ca, Pi and F concentrations were lower in biofilms exposed to sucrose and glucose-fructose compared with the negative control group, but the difference between the carbohydrate treatments was not statistically different, which is in accordance with the 28-day study conducted by Cury *et al*. (2000). This low concentration due to sucrose treatment has also been found in biofilm accumulated for 14 days (Tenuta *et al*., 2003; Paes Leme *et al*., 2004; Pecharki *et al*., 2005; Ribeiro *et al*., 2005; Aires *et al*., 2006) and the present findings show that it occurs earlier. However, the means of this low concentration of Ca, P_i and F with regard to caries is not known yet and it has also been found when other carbohydrates were evaluated, such as starch (Ribeiro *et al*., 2005). Additionally, there is no explanation for this low concentration of ions found in the presence of sucrose (Cury *et al*., 2000) and the present data give support for the alternative hypothesis that constant low pH maintained in the biofilm by sugar fermentation would prevent the precipitation of minerals in biofilm matrix (Tenuta *et al.,* 2005). Furthermore, the statistically significant increase of Ca and F over the time of biofilm accumulation observed for the negative control group (Table 2) is another evidence in this direction.

In summary, insoluble EPS seems to be the only differential change induced by sucrose on dental biofilm composition compared with its monosaccharide components, and although significant enamel demineralization occurs only after 7 days of biofilm accumulation, changes in the biofilm composition are observed earlier.

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Table 1. Results of ANOVA (p-values)

Factors and interactions	LB[a]	%LB/TM[b]	MS[c]	%MS/TM	%MS/TS[d]	Ca[e]	P_i[f]	F[g]	EPS[h]	IPS[i]	ΔZ[j]
Treatment	<.001	<.001	ns[k]	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
Time	<.001	<.001	Ns	ns	.042	.006	<.001	ns	ns	<.001	<.001
Treatment*Time	.012	.005	Ns	0.025	.007	.007	Ns	<.001	ns	ns	<.001

[a] LB = lactobacilli counts

[b] TM = total microorganisms

[c] MS = mutans streptococci

[d] TS = total streptococci

[e] Ca = calcium

[f] P_i = inorganic phosphorus

[g] F = fluoride

[h] EPS = extracellular polysaccharide

[i] IPS = intracellular polysaccharide

[j] ΔZ = Area of enamel mineral loss

[k] ns = non-statistically significant

Table 2. Effect of the time (days) of biofilm formation and accumulation within each treatment/group (mean ± SD, n)[a].

VARIABLES	TREATMENTS and TIME								
	CONTROL			GLUCOSE-FRUCTOSE			SUCROSE		
	3 days	7 days	14 days	3 days	7 days	14 days	3 days	7 days	14 days
[b]LB, UFC/mg x 10^5	0.003 ± 0.007 A n = 11	0.028 ± 0.068 A n = 11	0.06 ± 0.11 A n = 12	2.5 ± 8.4 A n = 12	9.0 ± 11.5 B n = 12	48.3 ± 70.9 B n = 11	0.7 ± 2.3 A n = 12	11.9 ± 25.2 B n = 12	47.6 ± 44.5 B n = 11
%LB/TM[c]	0.001 ± 0.002 A n = 11	0.009 ± 0.022 A n = 11	0.015 ± 0.03 A n = 12	1.0 ± 3.1A n = 12	4.1 ± 5.4 B n = 12	22.7 ± 27.4 B n = 12	2.7 ± 8.0 A n = 12	6.2 ± 9.7B n = 12	22.7 ± 23.3 B n = 12
[d]MS, UFC/mg x 10^3	3.6 ± 9.5 A n = 11	1.5 ± 3.2 A n = 10	1.7 ± 5.2 A n = 12	2.8 ± 7.7 A n = 12	2.8 ± 4.8 A n = 12	6.0 ± 8.7 A n = 11	4.2 ± 10.4 A n = 12	6.9 ± 11.5 A n = 12	6.5 ± 9.4 A n = 11
%MS/TM	0.013 ± 0.035 A n = 11	0.004 ± 0.007 A n = 11	0.003 ± 0.008 A n = 12	0.009 ± 0.024 A n = 12	0.006 ± 0.011 AB n = 12	0.243 ± 0.78 B n = 12	0.010 ± 0.02 A n = 12	0.02 ± 0.03 A n = 12	0.036 ± 0.06 A n = 12
%MS/TS[e]	0.024 ± 0.07 A n = 11	0.005 ± 0.01 A n = 11	0.003 ± 0.009 A n = 12	0.013 ± 0.035 A n = 12	0.026 ± 0.074 A n = 12	0.14 ± 0.30 B n = 12	0.014 ± 0.025 A n = 12	0.06 ± 0.09 A n = 12	0.062 ± 0.098 A n = 12
[f]Ca, µg/mg	12.4 ± 6.1 A n = 8	23.0 ± 11.8 AB n = 9	34.4 ± 17.1 B n = 9	4.1 ± 1.2 A n = 9	7.1 ± 5.7 A n = 9	3.6 ± 0.9 A n = 9	3.2 ± 1.0 A n = 9	3.7 ± 2.0 A n = 9	4.8 ± 5.4 A n = 9
[g]P_i, µg/mg	14.1 ± 8.9 A n = 8	28.8 ± 13.9 B n = 9	42.5 ± 24.7 B n = 9	5.9 ± 2.4 A n = 10	11.2 ± 10.9 B n = 11	7.9 ± 4.8 B n = 12	6.8 ± 3.8 A n = 10	8.9 ± 8.6 B n = 12	6.9 ± 1.6 B n = 12
[h]F, µg/g	30.0 ± 41.8 A n = 8	44.9 ± 31.6 AB n = 8	75.7 ± 65.9 B n = 8	13.5 ± 12.5 A n = 8	9.9 ± 12.0 A n = 7	8.0 ± 9.9 A n = 7	11.8 ± 19.6 A n = 8	7.6 ± 12.5 A n = 8	12.7 ± 28.2 A n = 8
Insoluble [i]EPS, µg/mg	39.5 ± 28.7 A n = 8	44.9 ± 20.6 A n = 9	45.5 ± 17.4 A n = 9	40.6 ± 26.0 A n = 10	34.9 ± 15.3 A n = 9	55.6 ± 33.4 A n = 12	165.4 ± 96.3 A n = 9	219.0 ± 170.2 A n = 12	237.0 ± 214.5 A n = 12
[j]IPS, µg/mg	11.8 ± 8.8 A n = 8	20.6 ± 22.0 B n = 9	14.1 ± 4.9 B n = 9	14.7 ± 6.9 A n = 10	28.8 ± 14.9 B n = 11	24.6 ± 8.9 B n = 12	27.1 ± 13.4 A n = 10	36 ± 18.8 B n = 12	34.9 ± 11.5 B n = 12
ΔZ[k]	400.8 ± 151.5 A n = 12	362.4 ± 129.9 A n = 12	451.4 ± 194.9 A n = 12	359.8 ± 226.4 A n = 12	409.4 ± 194.8 AB n = 12	672.2 ± 323.9 B n = 12	519.2 ± 175.3 A n = 12	819.6 ± 302.3 A n = 11	1359 ± 394.0 B n = 12

[a] Time, within each treatment, whose means are followed by distinct capital letters statistically differ from each other ($p<0.05$).

[b] LB = lactobacilli; [c] TM = total microorganisms, [d] MS = mutans streptococci; [e] TS = total streptococci; [f] Ca = calcium; [g] P_i = inorganic phosphorus; [h] F = fluoride; [i]EPS = extracellular polysaccharide, [j] IPS = intracellular polysaccharide; [k]ΔZ = Area of enamel mineral loss

Table 3. Effect of the treatments within each time of biofilm formation and accumulation (mean ± SD, n)[a].

VARIABLES	TIME and TREATMENTS								
	3 days			7 days			14 days		
	Negative Control	Glucose-Fructose	Sucrose	Negative Control	Glucose-Fructose	Sucrose	Negative Control	Glucose-Fructose	Sucrose
[b]LB, UFC/mg x 10^5	0.003 ± 0.007 A n = 11	2.5 ± 8.4 AB n = 12	0.7 ± 2.3 B n = 12	0.028 ± 0.068 A n = 11	9.0 ± 11.5 B n = 12	11.9 ± 25.2 B n = 12	0.06 ± 0.11 A (n = 12	48.3 ± 70.9 B n = 11	47.6 ± 44.5 B n = 11
%LB/TM[c]	0.001 ± 0.002 A n = 11	1.0 ± 3.1B n = 12	2.7 ± 8.0 B n = 12	0.009 ± 0.022 A n = 11	4.1 ± 5.4 B n = 12	6.2 ± 9.7B n = 12	0.015 ± 0.03 A n = 12	22.7 ± 27.4 B n = 12	22.7 ± 23.3 B n = 12
[d]MS, UFC/mg x 10^3	3.6 ± 9.5 A n = 11	2.8 ± 7.7 A n = 12	4.2 ± 10.4 A n = 12	1.5 ± 3.2 A n = 10	2.8 ± 4.8 A n = 12	6.9 ± 11.5 A n = 12	1.7 ± 5.2 A n = 12	6.0 ± 8.7 A n = 11	6.5 ± 9.4 A n = 11
%MS/TM	0.013 ± 0.035A n = 11	0.009 ± 0.024 A n = 12	0.010 ± 0.02 A n = 12	0.004 ± 0.007A n = 11	0.006 ± 0.011 AB n = 12	0.02 ± 0.03 B n = 12	0.003 ± 0.008 A n = 12	0.243 ± 0.78 B n = 12	0.036 ± 0.06 B n = 12
%MS/TS[e]	0.024 ± 0.07 A n = 11	0.013 ± 0.035 A n = 12	0.014 ± 0.025 A n = 12	0.005 ± 0.01A n = 11	0.026 ± 0.074 AB n = 12	0.06 ± 0.09 B n = 12	0.003 ± 0.009 A n = 12	0.14 ± 0.30 B n = 12	0.062 ± 0.098 B n = 12
[f]Ca, µg/mg	12.4 ± 6.1 A n = 8	4.1 ± 1.2 B n = 9	3.2 ± 1.0 B n = 9	23.0 ± 11.8 A n = 9	7.1 ± 5.7 B n = 9	3.7 ± 2.0 B n = 9	34.4 ± 17.1 A n = 9	3.6 ± 0.9 B n = 9	4.8 ± 5.4 B n = 9
[g]P_i, µg/mg	14.1 ± 8.9 A n = 8	5.9 ± 2.4 B n = 10	6.8 ± 3.8 B n = 10	28.8 ± 13.9 A n = 9	11.2 ± 10.9 B n = 11	8.9 ± 8.6 B n = 12	42.5 ± 24.7 A n = 9	7.9 ± 4.8 B n = 12	6.9 ± 1.6 B n = 12
[h]F, µg/g	30.0 ± 41.8 A n = 8	13.5 ± 12.5 B n = 8	11.8 ± 19.6 B n = 8	44.9 ± 31.6 A n = 8	9.9 ± 12.0 B n = 7	7.6 ± 12.5 B n = 8	75.7 ± 65.9 A n = 8	8.0 ± 9.9 B n = 7	12.7 ± 28.2 B n = 8
Insoluble [i]EPS, µg/mg	39.5 ± 28.7 A n = 8	40.6 ± 26.0 A n = 10	165.4 ± 96.3 B n = 9	44.9 ± 20.6 A n = 9	34.9 ± 15.3 A n = 9	219.0 ± 170.2 B n = 12	45.5 ± 17.4 A n = 9	55.6 ± 33.4 A n = 12	237.0 ± 214.5 B n = 12
[j]IPS, µg/mg	11.8 ± 8.8 A n = 8	14.7 ± 6.9 B n = 10	27.1 ± 13.4 B n = 10	20.6 ± 22.0 A n = 9	28.8 ± 14.9 B n = 11	36 ± 18.8 B n = 12	14.1 ± 4.9 A n = 9	24.6 ± 8.9 B n = 12	34.9 ± 11.5 B n = 12
ΔZ[k]	400.8 ± 151.5 A n = 12	359.8 ± 226.4 A n = 12	519.2 ± 175.3 A n = 12	362.4 ± 129.9 A n = 12	409.4 ± 194.8 A n = 12	819.6 ± 302.3 B n = 11	451.4 ± 194.9 A n = 12	672.2 ± 323.9A n = 12	1359 ± 394.0 B n = 12

[a] Treatments, within each time, whose means are followed by distinct capital letters statistically differ from each other ($p<0.05$).

[b] LB = lactobacilli; [c] TM = total microorganisms, [d] MS = mutans streptococci; [e] TS = total streptococci; [f] Ca = calcium; [g] P_i = inorganic phosphorus; [h] F = fluoride; [i]EPS = extracellular polysaccharide, [j] IPS = intracellular polysaccharide; [k]ΔZ = Area of enamel mineral loss

4. CONCLUSÃO GERAL

As análises microbiológicas e bioquímicas do biofilme dental formado nas condições estudadas indicam que a cariogenicidade da sacarose depende de fatores ecológicos e da composição orgânica e inorgânica do biofilme dental. Os resultados do presente estudo sugerem que o biofilme formado por três dias na presença de sacarose já apresenta mudanças na sua composição, enquanto a perda mineral se mostrou significativa com sete dias.

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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 "**Influência da sacarose na diversidade genotípica de estreptococos do grupo mutans e na composição do biofilme dental formado in situ em diferentes tempos e in vivo**", protocolo nº **053/2004**, dos pesquisadores **CÍNTHIA PEREIRA MACHADO TABCHOURY, ADRIANA FRANCO PAES LEME, ALTAIR ANTONINHA DEL BEL CURY, GLÁUBER CAMPOS VALE, JAIME APARECIDO CURY, RENATA DE OLIVEIRA MATTOS GRANER e RODRIGO ALEX ARTHUR**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 07/07/2004.

Piracicaba, 27 de outubro de 2005.

The Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas, certify that project "**Influence of sucrose on genotypic diversity of mutans streptococci and on biofilm composition formed in situ at different periods and in vivo**", register number **053/2004**, of **CÍNTHIA PEREIRA MACHADO TABCHOURY, ADRIANA FRANCO PAES LEME, ALTAIR ANTONINHA DEL BEL CURY, GLÁUBER CAMPOS VALE, JAIME APARECIDO CURY, RENATA DE OLIVEIRA MATTOS GRANER and RODRIGO ALEX ARTHUR**, 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 07/07/2004.

Piracicaba – SP, Brazil, October 27 2005.

Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Prof. Dr. Thales Rocha de Mattos Filho
Diretor
FOP/UNICAMP

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

ANEXO 1

ANEXO 2

Deliberação CCPG – 001/98

Dispõe a respeito do formato das teses de Mestrado
e de Doutorado aprovadas pela UNICAMP

Tendo em vista a possibilidade, segundo parecer PG N° 1985/96, das teses de Mestrado e Doutorado terem um formato alternativo àquele já bem estabelecido, a CCPG resolve:

Artigo 1° - Todas as teses de mestrado e de doutorado da UNICAMP terão o seguinte formato padrão:

I) Capa com formato único, dando visibilidade ao nível (mestrado e doutorado) e à Universidade.
II) Primeira folha interna dando visibilidade ao nível (mestrado e doutorado), à Universidade, à Unidade em que foi defendida e à banca examinadora, ressaltando o nome do orientador e co-orientadores. No seu verso deve constar a ficha catalográfica.
III) Segunda folha interna onde conste o resumo em português e o abstract em inglês.
IV) Introdução geral.
V) Capítulo.
VI) Conclusão geral
VII) Referências bibliográficas.
VIII) Apêndices (se necessários).

Artigo 2° - A critério do orientador, 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.

Parágrafo único – Os veículos de divulgação deverão ser expressamente indicados.

Artigo 3° - A PRPG providenciará o projeto gráfico das capas bem como a impressão de um número de exemplares, da versão final da tese a ser homologada.

Artigo 4° - Fica revogada a resolução CCPG 17/97

ANEXO 3

JDR JOURNAL OF DENTAL RESEARCH®
Featuring Critical Reviews in Oral Biology & Medicine

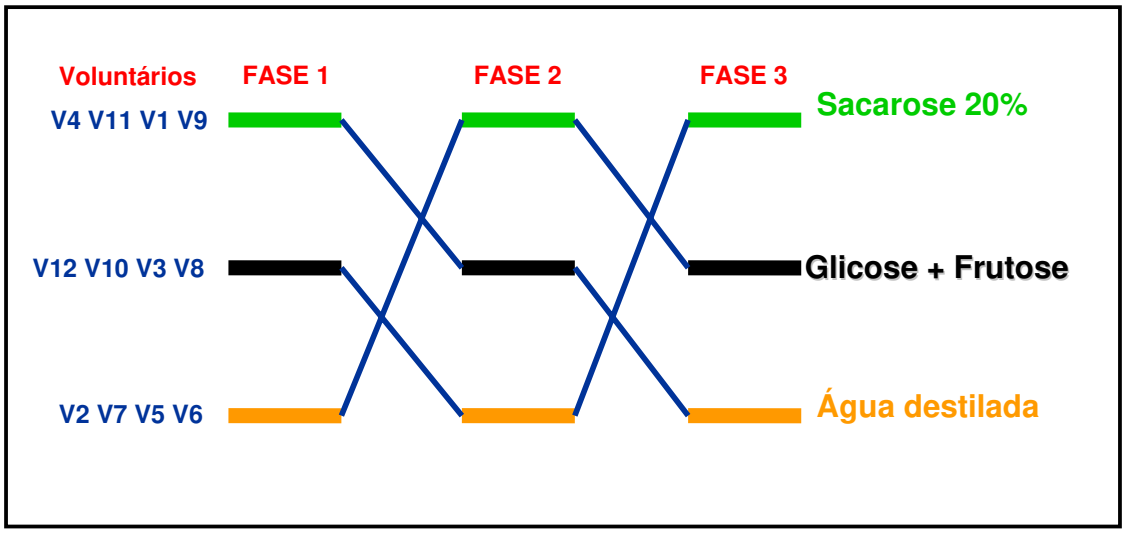
Detailed Status Information

Manuscript #	06-0042
Current Revision #	0
Submission Date	2006-01-24
Current Stage	Under Consideration
Title	Timing Effect on Composition of Dental Biofilm Exposed to Sucrose In Situ
Running Title	Sucrose, time and biofilm composition
Manuscript Type	Research Report
Special Section	N/A
Category	Biological
Manuscript Comment	Words in the abstract: 151 Words in the abstract + text: 2534 Table: 03 Cited references: 31
Corresponding Author	Jaime Cury (UNICAMP)
Contributing Authors	Cinthia Tabchoury , Glauber Vale , Rodrigo Arthur , Adriana Paes Leme , Altair Del Bel Cury
Abstract	Dental biofilm exposed in situ to sucrose for 13 days or longer presents high concentration of extracellular polysaccharide (EPS), high lactobacilli counts and low inorganic concentration. Since these changes and their consequences at earlier stages of biofilm formation are unknown, 12 volunteers subjected enamel slabs to the treatments: water, 10% glucose + 10% fructose and 20% sucrose solution. The biofilms formed during 3, 7 and 14 days were analyzed biochemically and microbiologically, and mineral loss (ΔZ) was evaluated on enamel. Statistically higher ΔZ value was found for sucrose treatment after 7 days. On the 3rd day, sucrose significantly increased lactobacilli, EPS, intracellular polysaccharide and decreased the inorganic concentration in the biofilm; however, the only significant difference compared to glucose-fructose treatment was a higher insoluble EPS concentration. The data suggest that although sucrose induces significant enamel demineralization only after 7 days of biofilm accumulation, changes in the biofilm composition are observed earlier
Associate Editor	Not Assigned
Key Words	sucrose, biofilm, polysaccharide, enamel, demineralization
Author Disclosure	• Acknowledgement Section properly discloses sponsor remuneration - no.

Stage	Start Date
Under Consideration	2006-01-24
	2006-01-24
Submission	2006-01-24

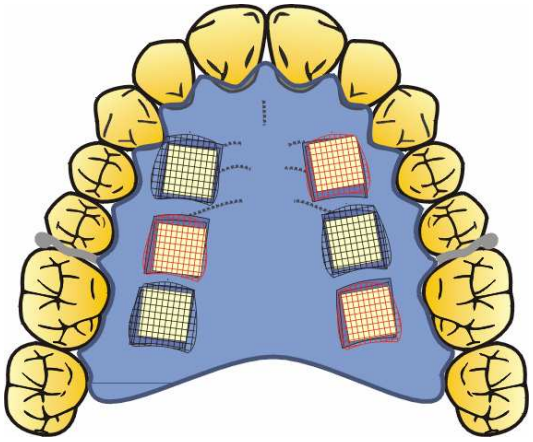
APÊNDICE 1

Fluxograma do experimento

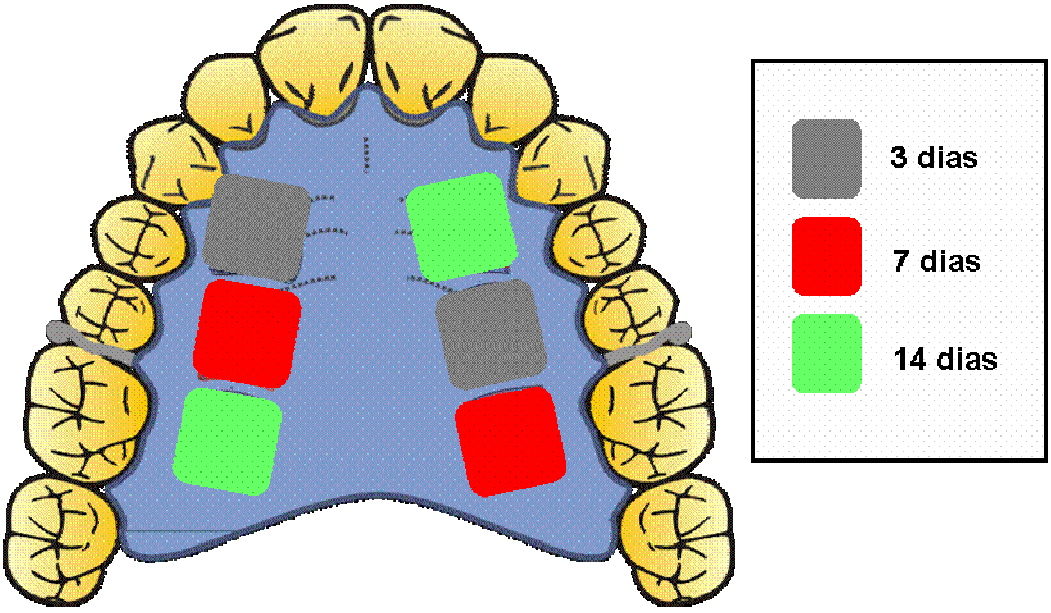


APÊNDICE 2

Esquema do dispositivo intra-oral palatino

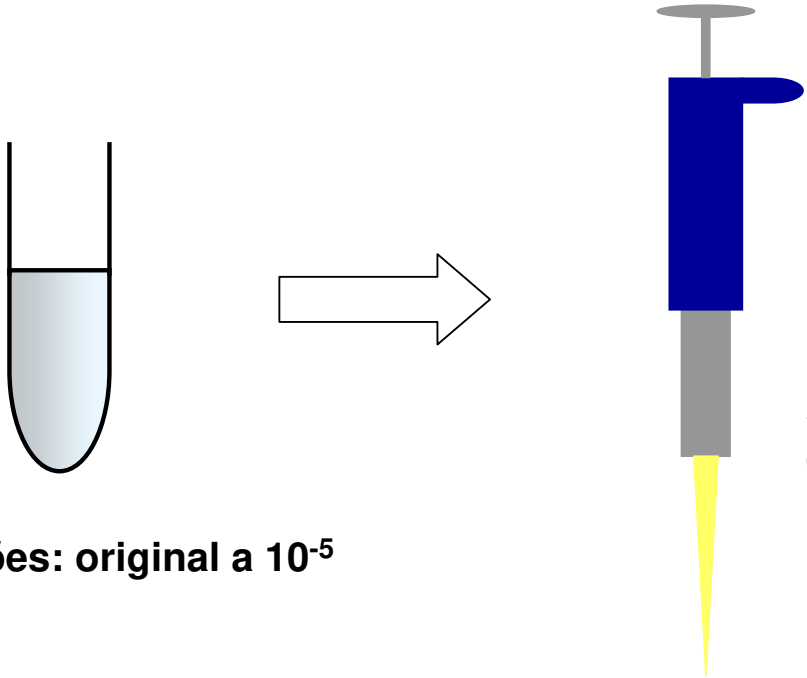


Esquema das coletas



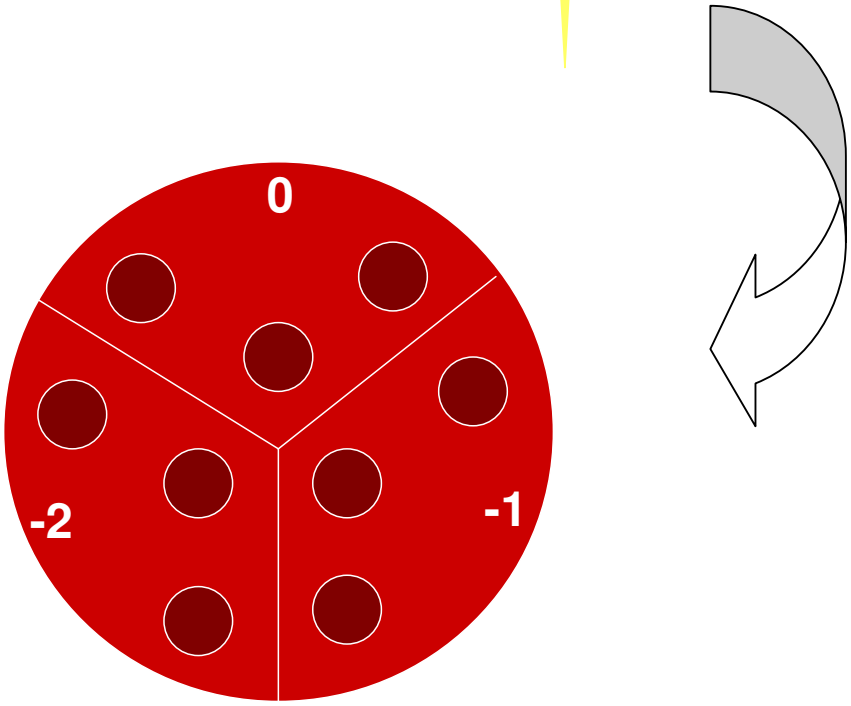
APÊNDICE 3

Esquema da análise microbiológica do biofilme dental



Alíquotas de 20 µL

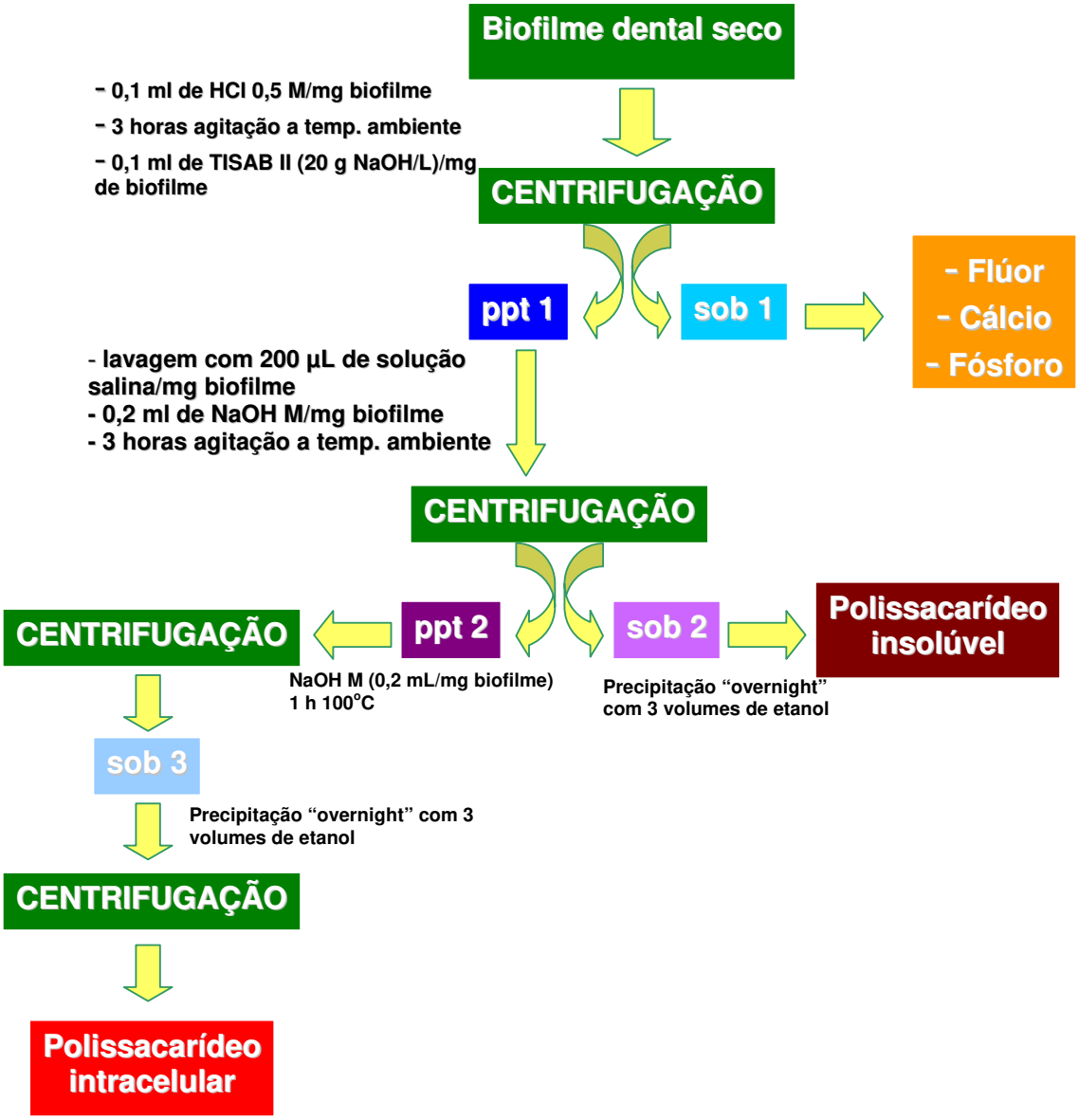
Diluições: original a 10^{-5}



- **Placas divididas em 3**
- **3 gotas em cada parte**

APÊNDICE 4

Fluxograma da análise bioquímica do biofilme dental



APÊNDICE 5

Esquema da determinação da microdureza do esmalte