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Cecilia Claudia Costa Ribeiro

"COMPOSIÇÃO E CARIOGENICIDADE DO BIOFILME DENTAL FORMADO *IN SITU* NA PRESENÇA DE AMIDO E SACAROSE"

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

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Mestre em Odontopediatria

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que nunca limitaram meus sonhos
e me ensinaram que com perseverança tudo é possível.

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pelo amor e pela paciência na espera da minha volta.

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SUMÁRIO

RESUMO

Tendo em vista que experimentos em animais e *in vitro* sugerem que a combinação de amido com sacarose é mais cariogênica que sacarose isoladamente, o objetivo desse estudo *in situ* foi avaliar os efeitos dessa associação de carboidratos na acidogenicidade, na composição bioquímica e microbiológica do biofilme dental, bem como na desmineralização do esmalte decíduo. Este estudo foi do tipo cego e cruzado, com 2 fases experimentais de 14 dias cada. Quinze voluntários utilizaram dispositivos intra-orais palatinos contendo blocos de esmalte, obtidos a partir de incisivos humanos decíduos, os quais foram submetidos a 4 grupos de tratamentos: (T1) água ou controle negativo; (T2) 2% amido; (T3) 10% sacarose e (T4) 2% amido + 10% sacarose. Dois tratamentos diferentes (split-mouth) foram avaliados no mesmo dispositivo, sendo que para tal foram aleatorizados entre T1 / T3 ou T2 / T4 na primeira fase, invertendo os tratamentos na segunda fase. As soluções de tratamento foram gotejadas sobre os blocos dentais 8 vezes ao dia. O biofilme formado sobre os blocos foi analisado em relação à atividade da amilase, acidogenicidade, e composição bioquímica e microbiológica. A perda mineral foi avaliada pela microdureza do esmalte seccionado longitudinalmente. Não foi encontrada diferença entre perda mineral dos blocos dentais do grupo controle e do tratado com o amido ($P > 0,05$). A maior área de desmineralização foi observada para a associação amido + sacarose ($P < 0,05$) em relação aos demais grupos. Essa associação também resultou na maior contagem de lactobacilos ($P < 0,05$). Em conclusão, os resultados do presente estudo indicam que o amido usado isoladamente não é cariogênico para esmalte humano decíduo. Os dados também sugerem que o amido aumenta o potencial cariogênico da sacarose e este efeito parece estar relacionado à seleção de lactobacilos no biofilme dental formado.

ABSTRACT

Since in vitro and animal studies suggest that the combination of starch with sucrose is more cariogenic than sucrose alone, the objective of the present study was to evaluate *in situ* the effects of this association on the acidogenicity, biochemical and microbiological composition of dental biofilm, as well as on deciduous enamel demineralization. This study was blind and had a cross-over design, with two 14-day experimental phases. Fifteen volunteers wore intra-oral palatal appliances containing blocks of enamel, obtained from human deciduous teeth, which were submitted to 4 groups of treatments: (T1) water or negative control; (T2) 2% starch; (T3) 10% sucrose and (T4) 2% starch + 10% sucrose. Two different treatments (split-mouth) were evaluated in the same appliance and were randomized between T1/T3 or T2/T4 in the first phase, inverting the treatments in the second phase. The treatment solutions were dripped onto the dental blocks 8 times a day. The biofilm formed on the blocks was analyzed with regard to amylase activity, acidogenicity and biochemical and microbiological composition. Mineral loss was evaluated by cross-sectional microhardness. No significant difference was found between mineral loss of the dental blocks of control group and blocks treated with starch ($P > 0.05$). The greatest mineral loss was observed for the association starch + sucrose ($P < 0.05$). Also, this association resulted in the highest lactobacillus count in the biofilm formed ($P < 0.05$). In conclusion, the results of the present study indicate that starch alone is not cariogenic for human deciduous enamel. The data also suggest that starch may increase the cariogenic potential of sucrose and this effect seems to be related to the selection of lactobacillus in the dental biofilm formed.

INTRODUÇÃO GERAL

O papel da sacarose na cariogenicidade da dieta é bem conhecido. Tem sido aceito que o biofilme dental formado na presença de sacarose é mais cariogênico por apresentar alta concentração de polissacarídeos insolúveis, os quais alteram a matriz do biofilme, tornando-o mais poroso e por esse motivo facilitando a difusão através do mesmo (Dibdin & Shellis, 1988). Em acréscimo, o biofilme formado na presença de sacarose apresenta baixas concentrações de cálcio (Ca), fósforo inorgânico (P_i) e fluoreto (F) (Cury *et al*., 1997, 2000, 2003), os quais são relevantes para manter o equilíbrio mineral do dente com o meio ambiente.

O amido tem sido apontado como não cariogênico ou pouco cariogênico, quando usado como única fonte de carboidrato na dieta. Tal observação é suportada por experimentos de acidogenicidade do biofilme dental (Stephan, 1940; Imfeld; 1977; Lingström *et al*., 1989), por estudos experimentais com animais (König & Grenby, 1965; Hefti & Schmid, 1979), por estudos controlados em humanos (Gustaffson *et al*., 1954), por dados epidemiológicos (Marthaler & Froesch, 1967; Fisher, 1968; Newbrun *et al*., 1980) e por experimentos *in situ* (Lingström *et al*., 1994), os quais demonstraram que o amido é menos cariogênico que a sacarose.

Nas dietas primitivas o amido era consumido como fonte principal de energia e na ausência de sacarose. Entretanto, nas dietas contemporâneas ele é

consumido ao mesmo tempo ou intercalado com a sacarose, o que pode influenciar o processo da doença cárie. A elevada prevalência de cárie dental nos países industrializados tem sido atribuída ao freqüente consumo de sacarose e alimentos amiláceos contendo açúcar (Bibby, 1975).

A associação de amido à sacarose é comum na alimentação infantil, chegando a ser utilizada, segundo Mattos-Graner *et al*. (1998), por 22,5% das crianças que fazem uso de mamadeira. Nesse estudo foi observado que o grupo com maior prevalência de lesões de cárie fazia uso de mamadeira com adição de cereais e sacarose ao leite, dando indícios que adição de produtos amiláceos à dieta com sacarose pode torná-la mais cariogênica e influenciar a manifestação precoce da doença.

A avaliação da cariogenicidade da combinação amido e sacarose tem sido alvo de estudo em experimentos com animais (Green & Hartles, 1967; Firestone *et al*., 1982; Mundorff-Shrestha *et al.,* 1994).

Os resultados de estudo em ratos (Green & Hartles, 1967) apontaram para uma tendência da associação de amido à sacarose ser mais cariogênica que a sacarose e também mostraram que a sacarose adicionada ao amido cru foi mais cariogênica que sacarose associada a um amido processado. Os autores levantaram a questão de por que amido cru, a princípio não cariogênico, quando associado à sacarose se tornar mais cariogênico que a sacarose adicionada ao amido processado, este moderadamente cariogênico.

Num outro estudo em ratos, após infecção com cepas de *Streptococcus mutans* e *Actinomyces naeslundi*, foi avaliado o efeito do amido isolado ou

associado à sacarose. Os resultados mostraram uma maior incidência de lesões de cárie em dentina para o grupo no qual a sacarose foi usada em associação com amido do que para o uso da mesma quantidade de sacarose isoladamente (Firestone *et al*., 1982). Segundo os autores, esses resultados podem ter implicações na dieta humana, mas o exato mecanismo de interação entre sacarose e amido ainda não é completamente claro, se é um interação física (adesividade) ou metabólica.

Nesse mesmo contexto, em um estudo em ratos infectados com *S. sobrinus*, Mundorff-Shrestha *et al.* (1994), avaliando o potencial cariogênico de alimentos, encontraram que todos com potencial cariogênico igual ou maior que a sacarose apresentavam 1% ou mais de amido na sua composição.

Resultados de estudos *in vitro* também sugerem que o biofilme dental formado na presença dessa associação seria, a princípio, mais cariogênico que aquele formado na presença desses componentes de forma isolada (Vacca-Smith *et al*., 1996; Kopec *et al*., 1997).

Vacca-Smith *et al*. (1996) encontraram *in vitro* que os hidrolisados de amido produzidos pela alfa-amilase adsorvida à hidroxiapatita com a enzima GTF-B levaram ao aumento da síntese de glucanos pela sacarose. Esses glucanos formados na presença de amido não foram susceptíveis à enzima mutanase e aumentaram a adesão de algumas cepas de *S. mutans* e de *A. naeslundi,* indicando que os mesmos possuem propriedades físicas e bioquímicas diferentes daqueles formados na presença isolada da sacarose. Essa estrutura diferenciada dos glucanos sintetizados na presença do amido foi confirmada posteriormente por Kopec *et al*. (1997), sendo relatado que os glucanos, que normalmente

apresentam ligações glicosídicas tipo 1-3, na presença do amido, apresentaram maior quantidade de ligações do tipo 4. Essa maior síntese de glucanos na presença de amido e a maior adesão observada *in vitro* talvez possa contribuir *in vivo* para a aderência e acúmulo do biofilme na superfície dental.

Tendo em vista que a associação de produtos amiláceos à sacarose parece ter implicações na composição e estrutura do biofilme dental, o que influenciaria na manifestação e progressão da doença cárie, estudos em humanos mais próximos das condições *in vivo* se fazem necessários para avaliar esta hipótese.

PROPOSIÇÃO

O objetivo deste estudo experimental foi avaliar *in situ* a acidogenicidade, a composição bioquímica e microbiológica do biofilme dental formado pela associação amido mais sacarose, e seu efeito na desminerelização do esmalte decíduo.

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

CAPÍTULO

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COMPOSITION AND CARIOGENICITY OF DENTAL BIOFILM FORMED IN SITU IN THE PRESENCE OF STARCH AND SUCROSE.

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ABSTRACT

Since in vitro and animal studies suggest that the combination of starch with sucrose may be more cariogenic than sucrose alone, this study assessed *in situ* the effects of this association on the acidogenicity, biochemical and microbiological composition of dental biofilm, as well as on enamel demineralization. During two phases of 14 days each, 15 volunteers wore palatal appliances containing blocks of human deciduous enamel, which were submitted to 4 groups of treatments: (T1) water (negative control); (T2) 2% starch; (T3) 10% sucrose and (T4) 2% starch + 10% sucrose. The solutions were dripped onto the blocks 8 times a day. The biofilm formed on the blocks was analyzed with regard to amylase activity, acidogenicity, and biochemical and microbiological composition. Demineralization was determined on enamel by cross-sectional microhardness. The greatest mineral loss was observed for the association starch + sucrose ($P < 0.05$). Also, this association resulted in the highest lactobacillus count in the biofilm formed ($P < 0.05$). In conclusion, the findings suggest that starch may increase the cariogenic potential of sucrose and this effect seems to be related to the selection of lactobacillus in the dental biofilm formed.

INTRODUCTION

Among dietary carbohydrates, starch has been pointed out as non-cariogenic or slightly cariogenic, when used as the sole source of carbohydrate in the diet. This observation has been supported by experiments of dental biofilm acidogenicity (Stephan, 1940; Imfeld, 1977; Lingström *et al*. 1989), experimental studies with animals (König & Grenby, 1965; Green & Hartles, 1967; Hefti & Schmid, 1979), controlled studies in humans (Gustaffson *et al.* 1954), epidemiological data (Marthaler & Froesch, 1967; Fisher, 1968; Newbrun *et al*. 1980) and *in situ* experiments (Lingström *et al*. 1994), which demonstrated that starch is less cariogenic than sucrose.

However, while in primitive diets starch was consumed as the main energy source, in contemporary ones it is consumed simultaneously or interspersed with sucrose. This association, which can be used by 22.5% of bottle–fed children (Mattos-Graner *et al.* 1998), may influence dental biofilm composition and consequently dental caries. Thus, a greater prevalence of caries lesions was found in children that consume milk supplemented with a combination of cereal and sucrose (Mattos-Graner *et al.* 1998). Such observation in humans is supported by the results of experimental caries studies in animals (Firestone *et al.* 1982; Mundorff-Shrestha *et al*. 1994), suggesting that starch would enhance the cariogenic potential of sucrose.

The explanation for the greater cariogenicity of the association of dietary starch with sucrose may reside in the dental biofilm formed. Therefore, it has been

accepted that dental biofilm formed in the presence of sucrose is more cariogenic due to its high concentration of extracellular insoluble polysaccharides, which alter the matrix of the biofilm, making it more porous (Dibdin & Shellis, 1988). Furthermore, the biofilm formed from sucrose presents low concentrations of calcium, inorganic phosphorous and fluoride (Cury *et al*. 1997; 2000; 2003), which are relevant to maintain mineral equilibrium between teeth and the oral environment (Pearce, 1998).

These polysaccharides are produced from sucrose by bacterial enzymes named glucosyltransferases (GTF). Thus, it has been shown *in vitro* that in the presence of starch, not only the synthesis of insoluble glucans by GTF-B increases, but these glucans present a biochemical (Vacca-Smith *et al.* 1996) and physical structure (Kopec *et al.* 1997) different from those formed in the presence of sucrose alone. In addition, in the presence of these 'new polysaccharides', an increase in the adherence of strains of *Actinomyces naeslundi* and mutans streptococci to hydroxyapatite was also observed (Vacca-Smith *et al.* 1996). This greater adhesion observed *in vitro* may possibly contribute to the *in vivo* adherence and accumulation of biofilm on dental surface.

Considering that the effect of the dietary combination of starch and sucrose seems to be involved in the formation of dental biofilm structure, which would influence caries development, studies closer to *in vivo* conditions in humans are necessary in order to assess this hypothesis. Therefore, this experimental research aimed to study *in situ* the acidogenicity, the biochemical and microbiological composition of the dental biofilm formed in the presence of the combination of

starch and sucrose, and its relationship with demineralization of deciduous dental enamel.

MATERIALS AND METHODS

Experimental Design

This study was approved by the Research and Ethics Committee of Faculty of Dentistry of Piracicaba (Protocol No. 132/2002). It was carried out in two phases of 14 days each, during which 15 volunteers, 18-33 years old, wore acrylic palatal appliances, containing two sets of 4 blocks of human deciduous dental enamel. In each phase, each set of 4 blocks was submitted to one of the following treatments: T1- distilled and deionized water (negative control), T2- 2% starch solution (Sigma, soluble starch - 85H0799), T3- 10% sucrose solution and T4- 2% starch + 10% sucrose solution. The volunteers were randomly assigned to the different treatments and those that dripped T1 and T3 in the first phase, dripped T2 and T4 in the second phase, and vice versa (Fig. 1). The use of 2 treatments (split-mouth) in the same intra-oral palatal appliance (Fig. 2A) was supported by the absence of across-effect in previous studies conducted (Cury *et al*. 2001; Hara *et al*. 2003; Paes Leme *et al*. 2004; Pecharki *et al.* 2004). On the 13th day of the experiment, the biofilm formed on the most posterior blocks of each group (Fig. 2B) was collected for determination of amylase activity; on this day the acidogenicity of the dental biofilm formed was also determined (Fig. 2C). On the 14th day of each phase, the biofilms were collected for biochemical and microbiological analyses (Fig. 2D); in the enamel the variation of mineral content was determined (Fig. 2E). For statistical analysis, the volunteer was considered as an experimental block.

This study was blind only with respect to the examiner, since the volunteers were able to identify the treatments by the flavor and consistency of the solutions.

Enamel blocks and palatal appliance preparation

Two hundred and forty dental enamel blocks (3 x 3 x 2 mm) were obtained from the middle third of the buccal face of sound human deciduous incisors, which were randomly distributed to the different treatments. Acrylic resin intra-oral palatal appliances, containing two lateral cavities measuring 13 x 4 x 3 mm, in which four blocks of enamel were placed on each side, were made for each volunteer. Plastic meshes were fixed over the cavities to protect the enamel block surfaces from mechanical attrition, leaving a 1-mm space for accumulation of dental biofilm. Further details of appliance preparation are described in previous publications (Cury *et al.* 1997; 2000; Hara *et al*. 2003).

The concentration of 10% sucrose used in the present experiment is close to that found in some infant formulas that contain sucrose in their composition or even simulates the addition of 1 soupspoon of sugar in a baby's bottle with 150 mL. The 2% starch concentration was adopted in the experiment because it is the maximum solubility of the starch used (Sigma, soluble starch - 85H0799) and also because it is the concentration frequently found in the infant formulas available in the market.

Treatments

Throughout the entire experiment, the volunteers used a dentifrice containing 1100 μg F/g (NaF) and silica as abrasive, consumed water optimally fluoridated (0.67 mg F/L) and received instructions as previously described (Cury

et al. 2000). During the 14 days of each experimental phase, eight times per day (8.00, 9.30, 11.00, 14.00, 15.30, 17.00, 19.00, 21.00 h), the volunteers removed the appliances from the oral cavity and dripped the treatment solutions on the dental blocks. The excess of fluid was removed with gauze in an attempt to avoid carry-across effect of the treatments and after 5 min the appliance was replaced in the mouth. A washout interval of 14 days was established between the experimental phases. Considering the crossover design of this study, no restriction was made with regard to the volunteers' diet, but they were instructed to remove the appliances during their meals (Cury *et al*. 1997; 2000).

Dental Biofilm Analysis

Determination of amylase activity in dental biofilm

On the 13th day of each experimental phase, approximately 10 h after the last exposure to treatments, with the volunteers in fasting condition, and without having brushed their teeth, the biofilm formed on the most posterior enamel block on each side of the appliance (Fig. 2B) was collected. Homogenization was done with the aid of a tissue micro-grinder, using 20 µL of 1.5% NaCl solution, containing 2.0 mg F/mL, for each 1 mg of biofilm (Dodds & Edgar, 1986). This suspension was incubated in 1% starch solution (Merck, soluble starch - F1000552.139) buffered with 0.02 M phosphate pH 6.9 for 15 min. Amylase activity was determined by means of the Bernfeld (1955) colorimetric method, using maltose as standard and the absorbance was determined in a spectrophotometer (Beckman DU-70®) at 540 nm. The results were expressed in U/g/min.

Dental Biofilm Acidogenicity Assessment.

On the same 13th day of the experiment, after biofilm collection for amylase activity analysis, with the intra-oral appliances positioned in the volunteers' oral cavities (Fig. 2C), the biofilm pH was determined after overnight fasting and 5 min after treatment with the respective solutions. A contact micro-electrode (Beetrode® MEPH-3L, WPI, USA) connected to a pH meter (Orion- 720-A, USA) in combination with a reference electrode (Orion- 9002, USA) was used. For this analysis, the plastic mesh that covered the third dental block on each side of the appliance was dislocated to facilitate positioning of the electrode in the biofilm. A salt bridge was created in a 3 M KCl solution between the reference electrode and the volunteer's finger (Lingström *et al*. 1994). pH measurement at time 0 (baseline) was done and the intra-oral appliance was removed from the oral cavity and the respective treatment solutions dripped onto the blocks. After 1 min, the appliance was replaced in the oral cavity and after 4 min had elapsed, the pH was determined again ($pH_{5\ min}$). After these procedures, the plastic mesh was replaced and the volunteers continued carrying out the treatments until the following day. By means of a computer program (Larsen & Pearce, 1997), the pH data were converted into hydrogenionic concentration (cH^+) and the cH^+ area between the times of 0 and 5 min was calculated.

The determination at 5 min, as a parameter of plaque acidogenicity rather than time-curve pH, was chosen after a pilot study conducted with 3 volunteers, which showed that it was suitable to compare the difference of fermentation between starch and sucrose. Furthermore, this procedure allowed the analysis of acidogenicity in up to 8 volunteers in the same morning period.

Dental Biofilm Composition Analysis

On the 14th day of each experimental phase, under the same conditions as described for the previous day, the dental biofilm from the three remaining blocks was collected (Fig. 2D); a homogeneous aliquot was used for microbiological analyses and the rest of the biofilm was dehydrated for the biochemical analyses.

For the microbiological analyses, the dental biofilm was weighed (± 0.01 mg) in sterile microcentrifuge tubes, suspended in 0.9% NaCl solution (1 mL / mg wet weight) and sonicated using Sonicador Vibra Cell at 40 W, amplitude 5%, 6 pulses of 9.9 s each (Bowen *et al.* 1986). The suspensions were diluted in 0.9% NaCl in series up to 1:10^5 and automatically inoculated in duplicate (Spiral Plater®) in the following culture mediums: blood agar, for total microbiota; mitis salivarius agar plus 0.2 units of bacitracin/mL (MSB), for mutans streptococci group (Gold *et al.* 1973); Rogosa SL agar (Difco 248020), for lactobacillus and CFAT medium (Zylber & Jordan, 1982), for *Actinomyces naeslundi.* The plates were incubated in 10% CO_2 at 37^{o}C for 48 h (blood agar, MSB, Rogosa) or for 72 h (CFAT). The blood agar plates were additionally incubated for 24 h at 37ºC in aerobiosis. The colony forming units (CFU) were counted and the results expressed in CFU/mg of dental biofilm wet weight and in percentage of mutans streptococci group (%SM), lactobacillus (%LB) and *Actinomyces naeslundi* (%AC) in relation to total microorganisms.

After removing the aliquot for microbiological analysis, the rest of the collected biofilm was dried over P_2O_5 for 24 h and treated as previously described (Cury *et al.* 1997; 2003), but with a modification in the proportion of the solutions in

the extraction of the inorganic components (100 µL de HCl/mg dry biofilm weight) and in the extraction of the insoluble polysaccharides (200 µL de NaOH/mg dry biofilm weight). Fluoride (F), calcium (Ca), inorganic phosphorus (P_i) and insoluble polysaccharide (IP) analyses were done as previously described (Cury *et al.* 1997; 2000; 2003).

Dental Enamel Analysis

Two blocks from each treatment, which were placed in the most anterior position (Fig. 2E), were longitudinally sectioned through the center for enamel cross-sectional microhardness (CSMH) determination. The CSMH was determined according to Cury *et al*. (2000), and the impressions were made at the distances of 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160, 180, and 200 µm from the outer enamel surface. CSMH values were converted to mineral content (vol %) according to Featherstone *et al*. (1983), and the area of mineral loss (∆Z) for each treatment was calculated (White & Featherstone, 1987). The microhardness tester Future-Tech FM, coupled to software FM-ARS, was used with a 25-g load for 5 s.

Statistical Analyses

For all the analyses, the experimental unit considered was the volunteer. The assumptions of equality of variances and normal distribution of errors were checked, and the data that violated these statistical principles were transformed (Box *et al*. 1978). The data of biofilm biomass, cH^+ area, counts of total microorganism, lactobacillus and *Actinomyces naeslundi*, Ca and ΔZ were transformed into $\log_{10}$. The values of %LB, %AC and IP were transformed by power of -0.5; amylase activity by power of 2.5; F by power of 0,1; initial pH by

power of 3, pH_{5min} by power -1; P_i by power of -0.3 and counts of mutans streptococci group by power of -0.1. The analysis of variance (ANOVA) followed by Tukey test was used for all the variables, with the exception of the variable %SM, for which Friedman test followed by t-test was used. The software SAS (version 8.02, SAS Institute Incorporation, Cary: NC, 1999) was used and the significance level fixed at 5%.

RESULTS

The analysis of variance showed a significant effect of treatments for the majority of the variables studied (P<0.001), except for the amylase activity in the biofilm (P=0.231).

With regard to the baseline pH of the biofilm (Table 1), no statistical difference was observed (P>0.05) between treatments sucrose and starch + sucrose, but both presented significantly lower values (P<0.05) than treatments negative control and starch, which did not differ from each other (P>0.05). For pH_{5min} and cH^+ area (Table 1), also no difference was found (P>0.05) between the treatments sucrose and starch + sucrose, and they presented lower pH_{5min} values and higher cH^+ area values (P<0.05) than the treatment with starch; the negative control differed significantly (P<0.05) from all the other treatments.

The total microorganism count (Table 2) in the biofilm of the negative control group was statistically higher (P<0.05) than that of the sucrose and starch + sucrose groups, which did not differ from each other (P>0.05). The starch treatment did not differ from the other treatment groups (P>0.05).

In the populations of mutans streptococci group (Table 2), the highest

values were found for the treatments sucrose and starch + sucrose, which differed from the other groups ($P<0.05$), but did not differ from each other ($P>0.05$). No significant difference was observed between the control group and the treatment with starch ($P>0.05$). The same observations were found for %SM in relation to total microorganisms.

In relation to the lactobacillus count (Table 2), the treatment starch + sucrose presented the greatest values, significantly differing ($P<0.05$) from all the other treatments. The sucrose and starch treatments did not differ from each other ($P>0.05$), but presented higher counts ($P<0.05$) than those of the control group. With respect to %LB in relation to total microorganisms, the treatment starch + sucrose presented the highest values ($P<0.05$), which did not differ significantly only from the sucrose treatment (Table 2). The starch treatment did not differ ($P>0.05$) from the control or the sucrose treatments; however, a statistical difference ($P<0.05$) was found between the latter treatments.

For the *Actinomyces naeslundi* count (Table 2), the highest values were presented by the treatments sucrose and starch + sucrose, which did not show difference from each other ($P>0.05$), but differed from the other groups ($P<0.05$). No significant difference was found between the control group and the treatment with starch ($P>0.05$) either. The same pattern was observed for %AC in relation to the total microorganisms.

The biomass formed on the dental blocks in the presence of treatments sucrose and starch + sucrose (Table 3) was significantly greater than that of the other groups ($P<0.05$), and did not differ from each other ($P>0.05$). No significant

difference was found between the negative control and the treatment with starch (*P*>0.05) either.

For the F concentration in the dental biofilm (Table 3), the lowest values were found in the treatments sucrose and starch + sucrose, which did not differ from each other (*P*>0.05), but differed significantly from the other groups (*P*<0.05); whereas the highest values were observed in the negative control, which differed statistically (*P*<0.05) from all the other groups. The same pattern was observed for the variables Ca and P_i concentration in the biofilm (Table 3).

The IP concentration (Table 3) found in the biofilms treated with sucrose and starch + sucrose did not differ from each other (*P*>0.05), but were statistically greater (*P*<0.05) than those for the negative control and starch groups, which did not differ from each other (*P*>0.05).

In the ΔZ analysis, the negative control and the starch treatment did not differ from each other (Table 4), but presented statistically lower values (*P*<0.05) than the other groups. The greatest mineral loss was found for the treatment starch + sucrose (*P*<0.05).

DISCUSSION

The results of mineral loss (Table 4) show that starch at 2%, used at the frequency of 8 times a day, did not cause demineralization on deciduous human dental enamel, confirming results observed with permanent teeth. The findings also confirm studies conducted with animals (König & Grenby, 1965; Green & Hartles, 1967; Hefti & Schmid, 1979) and epidemiological studies (Fisher, 1968; Newbrun

et al. 1980), which also found little or no caries in enamel when starch is used as the only carbohydrate source in the diet.

The absence of demineralization in the dental blocks exposed to the treatment with starch (T2) cannot be attributed to a cross-effect during the study, since sucrose (T3) caused significant more enamel demineralization than T1, which was dripped on the opposite side of the same oral appliance (Table 4). The non-cariogenicity of starch may be explained by the biofilm acidogenicity parameters (Table 1), as the baseline pH was similar to that of the negative control (T1). Thus, although starch presented higher cH^+ area values than control and caused a significant pH drop after 5 min. (Table 1), these values were still above the critical pH for enamel dissolution, which is around 5.5. In addition, the insoluble polysaccharide (IP) concentration in the matrix of the biofilm formed in the presence of starch (T2) did not differ from that of the negative control (Table 3). Considering the role of these IP in dental biofilm cariogenicity (Rölla *et al.* 1985; Dibdin & Shellis, 1988; Cury *et al*. 2000; Mattos-Granner *et al*. 2000; Nobre dos Santos *et al*. 2002), this observation would give greater support to the absence of demineralization observed in the presence of starch (Table 4).

The mineral content data of the dental blocks treated with the starch + sucrose association (T4) presented the greatest value of mineral loss. This *in situ* data is consistent with the results found in observational studies in humans (Mattos-Graner *et al.* 1998) and in experiments with animals (Firestone *et al.* 1982; Mundorff-Sherstha *et al.* 1994), in which the association starch + sucrose was more cariogenic than sucrose alone.

With regard to the inorganic composition of the dental biofilm, it does not appear to contribute to the greater cariogenicity of the combination of starch + sucrose, as the concentrations of F, Ca and P_i found for this association did not differ statistically from T3 (Table 3).

With regard to the insoluble polysaccharide (IP) in the biofilm formed, our *in situ* findings (Table 3) did not confirm the *in vitro* data showing higher IP concentration formed in presence of starch + sucrose than sucrose alone (Vacca-Smith *et al.* 1996). However, in the present study the total amount of alkali-soluble polysaccharides (Cury *et al.* 1997; 2000) was determined, not differentiating the insoluble polysaccharides synthesized by the different GTFs. Also, the methodology used did not allow confirmation whether the IP formed *in situ* by treatment T4 are structurally different from those formed in the exclusive presence of T3, as shown *in vitro* (Kopec *et al.* 1997). Further studies should be conducted to evaluate *in situ* these possibilities.

Thus, the most evident effect of the combination starch + sucrose in the biofilm formed seems to be related to bacterial ecology, since in the presence of this treatment there was a greater ($P < 0.05$) absolute lactobacillus count than in presence of sucrose alone (Table 2). Our results showed that, although starch (T2) did not result in enamel demineralization (Table 4), it caused an increase in the populations of lactobacillus in the biofilm formed when compared to the negative control (Table 2). Thus, although the pH drop during starch fermentation (Table 1) did not reach a critical pH for enamel solubility, this was sufficient for bacterial selection (Marsh, 1994). Such effect on the dental biofilm - selecting more aciduric bacteria both by starch and by sucrose - may help explain the question posed by

Green & Hartles (1967) whether the consumption of a mixture of highly cariogenic sucrose with a virtually non-cariogenic starch would lead to more dental caries in rats than when sucrose is mixed with moderately cariogenic roll-dried starch.

With regard to the *Actinomyces naeslundi* populations in the biofilm formed, the results of this *in situ* study (Table 2) did not confirm those obtained *in vitro*, which showed greater adherence of these microorganisms in the presence of glucans synthesized from starch and sucrose (Vacca-Smith *et al.* 1996), since the difference between T4 and T3 was not statistically significant ($P>0.05$).

In conclusion, the results of this *in situ* study indicate that starch used alone is not cariogenic to deciduous human enamel, confirming the existing evidence for permanent teeth. They also give support to the hypothesis that association with this carbohydrate may increase the cariogenic potential of sucrose. This effect seems to be associated with the ecological change in the dental biofilm, selecting lactobacillus.

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Table 1: Analysis of dental biofilm pH (mean± SD; n) according to the treatments, which differ statistically ($P < 0.05$) when followed by distinct lower case superscript letters.

ANALYSIS	*TREATMENTS			
	T1	T2	T3	T4
baseline pH	7.5 ± 0.4 [a] (n=14)	7.4 ± 0.5 [a] (n=15)	6.6 ± 0.6 [b] (n=14)	6.7 ± 0.8 [b] (n=15)
$pH_{5\ min}$	7.2 ± 0.4 [a] (n=14)	6.4± 0.7 [b] (n=13)	5.3 ± 0.3 [c] (n=14)	5.2 ± 0.3 [c] (n=14)
cH^+ area (µmol/L x min)	0.1 ± 0.2 [a] (n=14)	2.0± 2.0 [b] (n=13)	18.3 ± 11.7 [c] (n=14)	22.7 ± 13.3 [c] (n=14)

*T1 (H_2O); T2 (2% starch); T3 (10% sucrose) and T4 (2% starch + 10% sucrose)

Table 2: Microbiological analysis (mean ± SD; n) of dental biofilm according to the treatments, which differ statistically ($P < 0.05$) when followed by distinct lower case superscript letters.

ANALYSES	*TREATMENTS			
	T1	**T2**	**T3**	**T4**
Total Microorganims (CFU/mg x 10^7)	3.2 ± 3.1 ^{a} n=14	1.9 ± 1.2 ^{ab} n=15	0.9 ± 0.6 ^{b} n=14	1.1 ± 0.8 ^{b} n=15
Mutans streptococci (CFU/mg x 10^3)	0.02 ± 0.04 ^{a} n=14	0.07 ± 0.12 ^{a} n=15	8.51 ± 16.37 ^{b} n=14	7.98 ± 18.36 ^{b} n=15
% Mutans streptococci	0.0001 ± 0.0000 ^{a} n=14	0.0012 ± 0.002 ^{a} n=15	0.22 ± 0.46 ^{b} n=14	0.17 ± 0.40 ^{b} n=15
Lactobacilli (CFU/mg x 10^5)	0.004 ± 0.006 ^{a} n=14	1.1 ± 3 ^{b} n=15	8.2 ± 18.1 ^{b} n=14	18.1 ± 3 ^{c} n=15
% Lactobacilli	0.017 ± 0.00 ^{a} n=14	1.8 ± 5.6 ^{ab} n=15	12.6 ± 22.9 ^{bc} n=14	23.1 ± 29.6 ^{c} n=15
A. naeslundi (CFU/mg x 10^5)	2.3 ± 7.7 ^{a} n=14	3.1 ± 8.1 ^{a} n=15	7.0 ± 10.1 ^{b} n=14	8.4 ± 10.8 ^{b} n=15
% *A. naeslundi*	2.8 ± 7.2 ^{a} n=14	2.8 ± 7.6 ^{a} n=15	10.4 ± 15.7 ^{b} n=14	7.3 ± 7.0 ^{b} n=15

*T1 (H_2O); T2 (2% starch); T3 (10% sucrose) and T4 (2% starch + 10% sucrose)

Table 3: Biochemical analysis (mean ± SD; n) of dental biofilm according to the treatments, which differ statistically ($P < 0.05$) when followed by distinct lower case superscript letters.

ANALYSES	*TREATMENTS			
	T1	**T2**	**T3**	**T4**
Amylase activity, U/g/min	24.2 ± 3.5 [a] n=14	22.4 ± 4.5 [a] n=15	25.3 ± 3.1 [a] n=14	24.1 ± 4.1 [a] n=15
Biomass, mg dry weight	2.2 ± 1.2 [a] n=14	2.6 ± 1.6 [a] n=15	5.0 ± 2.0 [b] n=14	5.2 ± 3.8 [b] n=15
F, µg/g	468.4 ± 401.8 [a] n=10	239.8 ± 251.3 [b] n=15	119.9 ± 179.3 [c] n=14	55.7 ± 144.3 [c] n=15
Ca, µg/mg	45.9 ± 50.9 [a] n=10	17.4 ± 25.4 [b] n=15	5.1 ± 7.9 [c] n=14	4.9 ± 10.5 [c] n=15
P_i, µg/mg	27.1 ± 29.5 [a] n=10	11.6 ± 14.6 [b] n=15	4.1 ± 4.6 [c] n=14	3.6 ± 5.0 [c] n=15
Insoluble polysaccharide, µg/mg	47.5 ± 22.8 [a] n=10	49.8 ± 13.5 [a] n=15	181.6 ± 115.8 [b] n=14	201.6 ± 137.6 [b] n=15

*T1 (H_2O); T2 (2% starch); T3 (10% sucrose) and T4 (2% starch + 10% sucrose)

Table 4: Analysis of dental enamel (mean ± SD; n) according to the treatments, which differ statistically ($P < 0.05$) when followed by distinct lower case superscript letters.

ANALYSIS	*TREATMENTS			
	T1	**T2**	**T3**	**T4**
Mineral loss, ΔZ	447.9 ± 169.0 [a] n=14	420.0 ± 160.1 [a] n=15	955.6 ± 543.6 [b] n=14	1421.8 ± 653.8 [c] n=14

*T1 (H_2O); T2 (2% starch); T3 (10% sucrose) and T4 (2% starch + 10% sucrose)

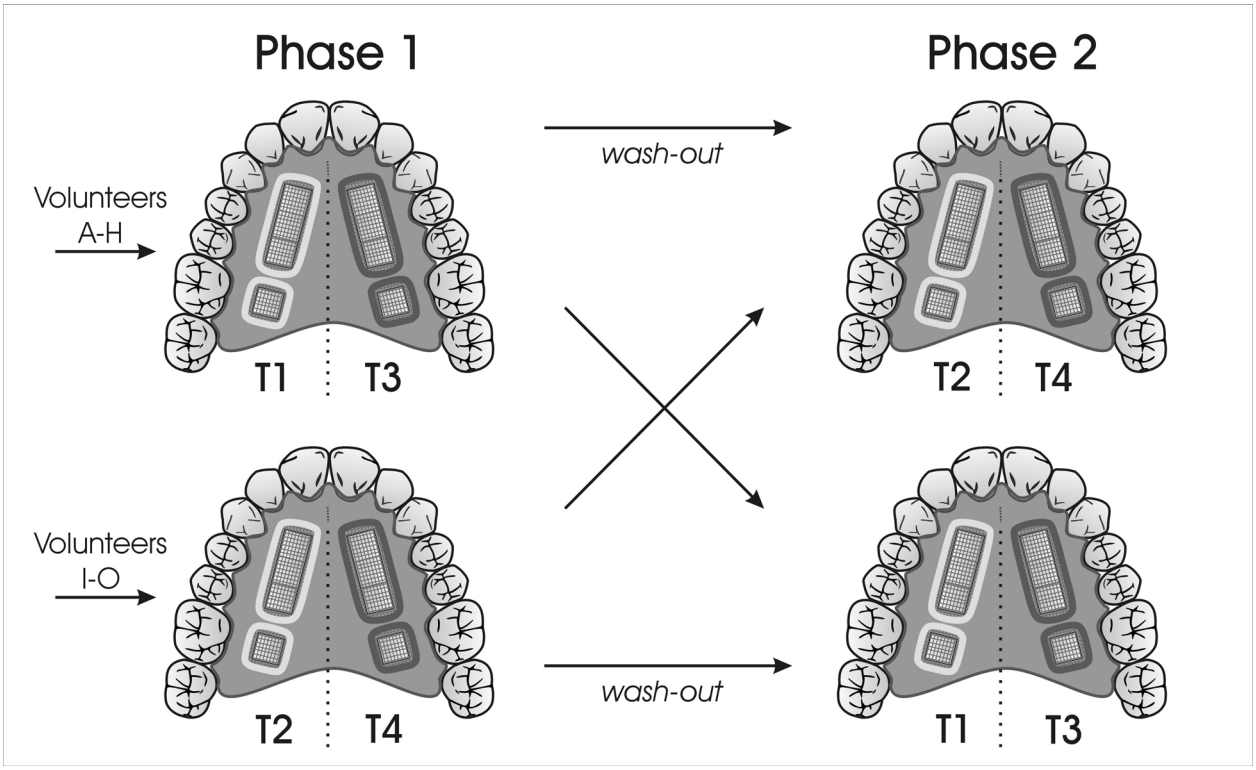
Phase 1
Phase 2
Volunteers
A-H
wash-out
T1
T3
T2
T4
Volunteers
I-O
wash-out
T2
T4
T1
T3

Fig. 1

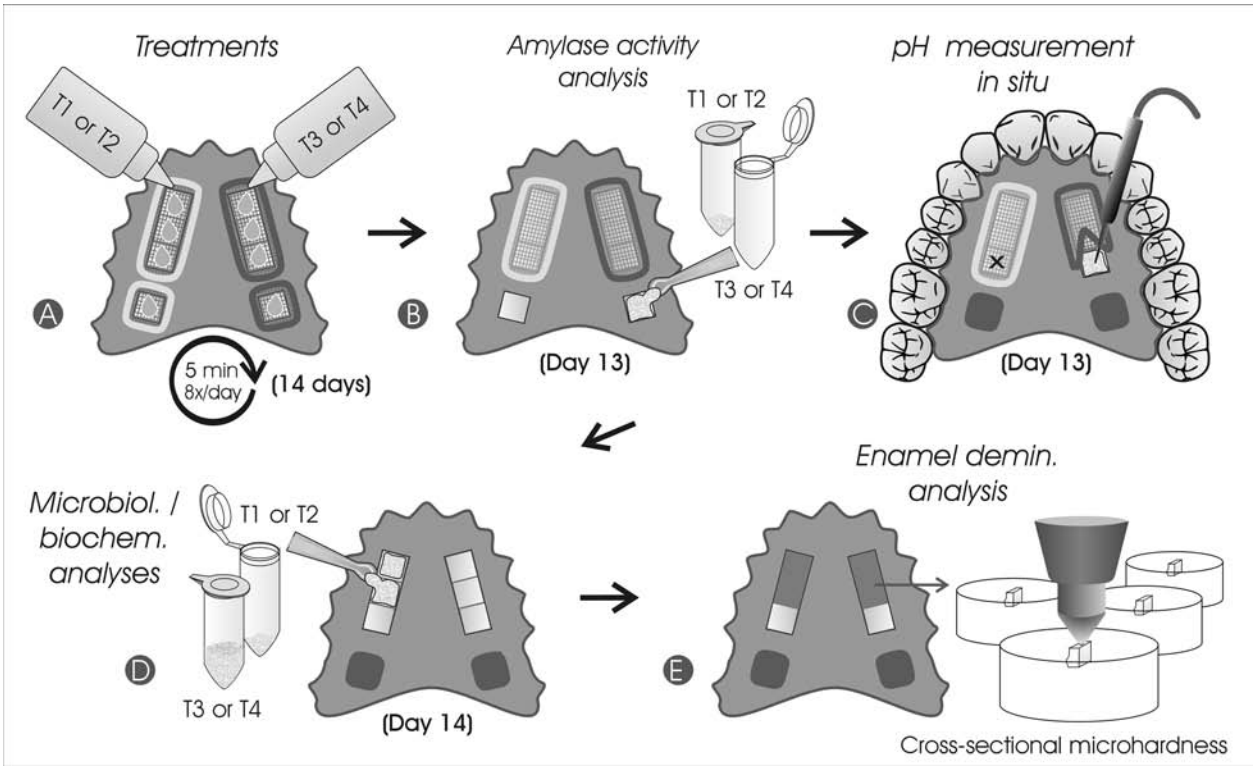
Treatments
T1 or T2
T3 or T4
A
5 min
8x/day
(14 days)
Amylase activity
analysis
T1 or T2
T3 or T4
B
(Day 13)
pH measurement
in situ
C
(Day 13)
Enamel demin.
analysis
E
Cross-sectional microhardness
Microbiol. /
biochem.
analyses
T1 or T2
D
T3 or T4
(Day 14)

Fig. 2

Figure captions

Fig.1- Diagram of the experimental design conducted.

Fig.2 - Diagram of the treatments and the analyses made.

CONCLUSÃO GERAL

Os resultados do presente estudo *in situ* indicam que o amido usado isoladamente não é cariogênico para esmalte humano decíduo, confirmando as evidências que existiam para dentes permanentes. Eles também dão suporte para a hipótese de que a associação daquele carboidrato com a sacarose aumenta o potencial cariogênico da mesma. Este efeito parece estar associado a alterações ecológicas no biofilme dental, selecionando bactérias mais acidúricas, como por exemplo, lactobacilos.

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* De acordo com a norma UNICAMP/FOP, baseada no modelo Vancouver. Abreviatura dos periódicos em conformidade com o Medline

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ANEXO 1

British Journal of Nutrition

British Journal of Nutrition

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Manuscript #	BJN-2004-009830
Current Revision #	0
Submission Date	2nd Jul 04
Current Stage	Under Consideration
Title	Composition and cariogenicity of dental biofilm formed in situ in the presence of starch and sucrose
Running Title	Starch and sucrose effect on dental caries
Manuscript Type	Research Article
Manuscript Comment	Hard copy sent to office - authors had trouble uploading files
Corresponding Author	Jaime Cury (University of Campinas)
Contributing Authors	Cinthia P M Tabchoury, Altair Cury, Livia Tenuta, Pedro Rosalen, Cecilia Ribeiro
Abstract	Since in vitro and animal studies suggest that the combination of starch with sucrose may be more cariogenic than sucrose alone, this study assessed in situ the effects of this association on the acidogenicity, biochemical and microbiological composition of dental biofilm, as well as on enamel demineralization. During two phases of 14 days each, 15 volunteers wore palatal appliances containing blocks of human deciduous enamel, which were submitted to 4 groups of treatments: (T1) water (negative control); (T2) 2% starch; (T3) 10% sucrose and (T4) 2% starch + 10% sucrose. The solutions were dripped onto the blocks 8 times a day. The biofilm formed on the blocks was analyzed with regard to amylase activity, acidogenicity, and biochemical and microbiological composition. Demineralization was determined on enamel by cross-sectional microhardness. The greatest mineral loss was observed for the association starch + sucrose ($P < 0.05$). Also, this association resulted in the highest lactobacillus count in the biofilm formed ($P < 0.05$). In conclusion, the findings suggest that starch may increase the cariogenic potential of sucrose and this effect seems to be related to the selection of lactobacillus in the dental biofilm formed.
First Editor	Not Assigned
Key Words	starch, sucrose, dental biofilm, demineralization, enamel

Manuscript Items

1. Author Cover Letter. File # PDF (160Kb)
2. Article File # PDF (381Kb)

UNICAMP

COMITÊ DE ÉTICA EM PESQUISA

UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

CERTIFICADO

Certificamos que o Projeto de pesquisa intitulado "Composição e cariogenicidade do biofilme dental formado *in situ* na presença de amido e sacarose", sob o protocolo nº **132/2002**, da Pesquisadora ***Cecilia Claudia Costa Ribeiro***, sob a responsabilidade da Profa. Dra. **Cinthia Pereira Machado Tabchoury**, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa – FOP.

Piracicaba, 06 de novembro de 2002

We certify that the research project with title "Composition and cariogenicity of the dental biofilm formed *in situ* in presence of starch and sucrose", protocol nº **132/2002**, by Researcher ***Cecilia Claudia Costa Ribeiro***, responsibility by Prof. Dr. **Cinthia Pereira Machado Tabchoury**, is in agreement with the Resolution 196/96 from National Committee of Health/Health Department (BR) and was approved by the Ethical Committee in Research at the Piracicaba Dentistry School/UNICAMP (State University of Campinas).

Piracicaba, SP, Brazil, November 06 2002

Prof. Dr. Pedro Luiz Rosalen
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Antonio Bento Alves de Moraes
Coordenador
CEP/FOP/UNICAMP

ANEXO 3

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Título da Pesquisa

Composição e cariogenicidade do biofilme dental formado *in situ* na presença de amido e sacarose.

Objetivo da Pesquisa

Avaliar se o biofilme dental formado *in situ* na presença de amido mais sacarose é mais cariogênico que aquele formado pelo uso desses carbohidratos de forma isolada.

Justificativa

A associação de produtos amiláceos à sacarose, o que ocorre freqüentemente na alimentação infantil, pode ter implicações na formação do biofilme dental e, assim, influenciar na manifestação precoce da doença cárie. Experimentos em animais e estudos *in vitro* dão sustentação a esta hipótese, relatando que esta associação na dieta pode ser mais cariogênica que o uso isolado da sacarose. Entretanto, nenhum trabalho avaliou a composição do biofilme formado *in situ* na presença de amido mais sacarose e se, nessas condições, ele é mais cariogênico que o formado na presença desses carboidratos isoladamente.

Procedimentos

Será realizado um estudo do tipo cruzado que compreenderá 4 tratamentos, sendo duas fases de 14 dias cada, durante as quais vocês utilizarão dispositivos intra-orais palatinos contendo 8 blocos de esmalte decíduo humano (4 em cada lado). Os tratamentos serão:

Tratamento 1: água destilada e deionizada (água d. d.);

Tratamento 2: solução de amido a 2%;

Tratamento 3: solução de sacarose a 10%;

Tratamento 4: solução de amido a 2% e de sacarose a 10% .

O estudo compreenderá 4 tratamentos, porém serão feitos somente 2 cruzamentos. Assim, enquanto em uma etapa da pesquisa metade dos voluntários submeterão os blocos dentais aos tratamentos 1 e 3 (por exemplo), os demais voluntários farão os tratamentos 2 e 4. Numa segunda etapa será feita o cruzamento entre voluntários e tratamentos. O fato de que o estudo será dividido em apenas duas fases de 14 dias cada lhes proporcionará maior comodidade.

Desconfortos e Riscos

1. Vocês poderão apresentar discreta halitose durante o período experimental, o que poderá ser resolvido com adequada higiene da parte interna do dispositivo intra-oral.
2. O uso das soluções será apenas como gotas sobre os blocos de esmalte presentes nos dispositivos intra-orais, não implicando em qualquer aumento de risco de cárie dental nos voluntários.
3. O dispositivo intra-oral pode causar um leve desconforto, que é, entretanto, semelhante ao desconforto causado por um aparelho ortodôntico móvel. Durante todo o período da pesquisa, acompanhamentos semanais serão realizados, para verificar as condições do aparelho e da sua saúde bucal

O benefício que vocês terão será um auxílio indireto, contribuindo para a realização deste projeto e o conhecimento que vocês adquirirão sobre o potencial cariogênico da associação amido e sacarose. Este conhecimento poderá ser utilizado futuramente em prol da população, com particular interesse para conhecimentos em relação à cárie de mamadeira.

Forma de acompanhamento e assistência

Os pesquisadores envolvidos na pesquisa estarão à disposição de vocês para ajuste no aparelho intra-oral a fim de minimizar qualquer desconforto.

Garantia de esclarecimento

Você tem garantia de que receberá resposta ou esclarecimento de qualquer dúvida quanto aos procedimentos, riscos, benefícios e outros assuntos relacionados à pesquisa. Também os pesquisadores supracitados assumem o compromisso de proporcionar informação atualizada obtida durante o estudo, ainda que esta possa afetar a vontade do indivíduo em continuar participando. Qualquer dúvida ou problema com o dispositivo intra-oral, por favor, comunicar-nos com a maior brevidade possível.

Tel: 3412-5303 (Laboratório de Bioquímica)

3412-5393 (Sala dos alunos da pós-graduação – Cariologia)

3434-4869 (Profa. Cínthia – residência)

3434-5378 (Cecilia - residência)

Formas de ressarcimento

Vocês serão ressarcidos de eventuais despesas com o transporte-alimentação para a retirada das amostras contidas nos dispositivos.

Formas de indenização

Não há danos previsíveis decorrentes desta pesquisa.

Garantia de sigilo

Os pesquisadores asseguram a sua privacidade quanto aos dados confidenciais envolvidos na pesquisa.

Liberdade para se recusar em participar da pesquisa

A decisão de fazer parte desta pesquisa é voluntária. Você pode escolher se quer ou não participar, assim como poderá desistir de participar a qualquer momento.

SUA ASSINATURA INDICA QUE VOCÊ DECIDIU PARTICIPAR DA PESQUISA COMO VOLUNTÁRIO E QUE LEU E ENTENDEU TODAS AS INFORMAÇÕES ACIMA EXPLICADAS.

_____ _____

Nome do voluntário Assinatura do voluntário

_____ _____

Nome do Representante Legal Assinatura do Representante Legal

Documento: _____

ATENÇÃO: A SUA PARTICIPAÇÃO EM QUALQUER TIPO DE PESQUISA É VOLUNTÁRIA. EM CASO DE DÚVIDA QUANTO AOS SEUS DIREITOS ESCREVA PARA O **COMITÊ DE ÉTICA EM PESQUISA DA FOP-UNICAMP.**

Endereço: Av Limeira, 901 CEP – FOP, CEP 13.414-903 Piracicaba, SP

ANEXO 4

ANEXO AO TERMO DE CONSENTIMENTO

INSTRUÇÕES AOS VOLUNTÁRIOS

Antes do início de cada fase, cada voluntário receberá um dentifrício fluoretado, frasco conta-gotas contendo a solução correspondente ao seu tratamento, estojo de aparelho ortodôntico (acomodação do dispositivo) e um dispositivo intra-oral. As instruções fornecidas a cada voluntário serão as seguintes:

INSTRUÇÕES GERAIS

- Trocar as duas soluções às segundas,quartas e sextas no Laboratório de Bioquímica Oral. As trocas de soluções são feitas para que não se acumulem fungos ou outros organismos indesejáveis
- Antes de gotejar: secar delicadamente o aparelho com gaze
- Atenção: é uma solução diferente para cada lado
- 4 blocos do lado direito do aparelho recebem solução do conta-gotas vermelho enquanto os outros 4 blocos do lado esquerdo recebem solução do conta-gotas branco
- Despejar 1 gota sobre cada bloco 8x ao dia (ver horários abaixo). É essencial para o bom andamento do experimento que se mantenha essa freqüência
- Gotejar a solução de um lado, secar o meio do aparelho; gotejar do outro lado secar novamente. Aguardar 5 min e recolocar o aparelho na boca. O uso da gaze é necessário para que não haja interferência de uma solução sobre a outra. Por favor, use!
- Utilizar o dispositivo diariamente, inclusive para dormir

- Remover o aparelho durante as refeições (qualquer tipo de refeição) mantendo-o envolvido em gaze úmida. Isso é muito importante para não ressecar os blocos e manter a placa dental (biofilme) formada sobre esses blocos em condições adequadas.

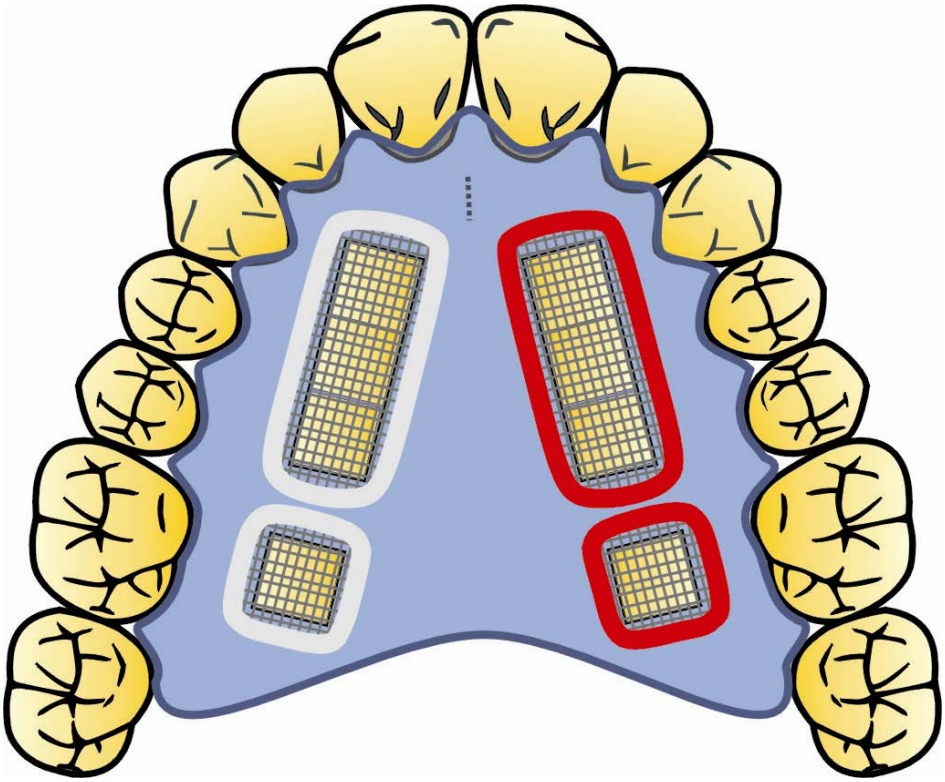
- Utilizar 3x/dia o dentifrício fluoretado fornecido (Sorriso Fresh) para escovação dental (retirar o aparelho durante a escovação). Não fazer bochechos com antissépticos durante o experimento.
- Nos dias de término de experimento, vocês deverão comparecer nos horários determinados em jejum e sem ter higienizado os dentes ou aparelho. Um delicioso café da manhã os aguardará.

Agradeço pelo apoio e colaboração!!!
Sem a sua participação não seria possível a realização deste trabalho.

QUALQUER DÚVIDA ENTRE EM CONTATO
(19) 3412-5303 (LABORATÓRIO DE BIOQUÍMICA)
(19) 3412-5393 (SALA DOS ALUNOS DA PÓS-GRADUAÇÃO)
(19) 3434-5378 (Residência)
Cecília

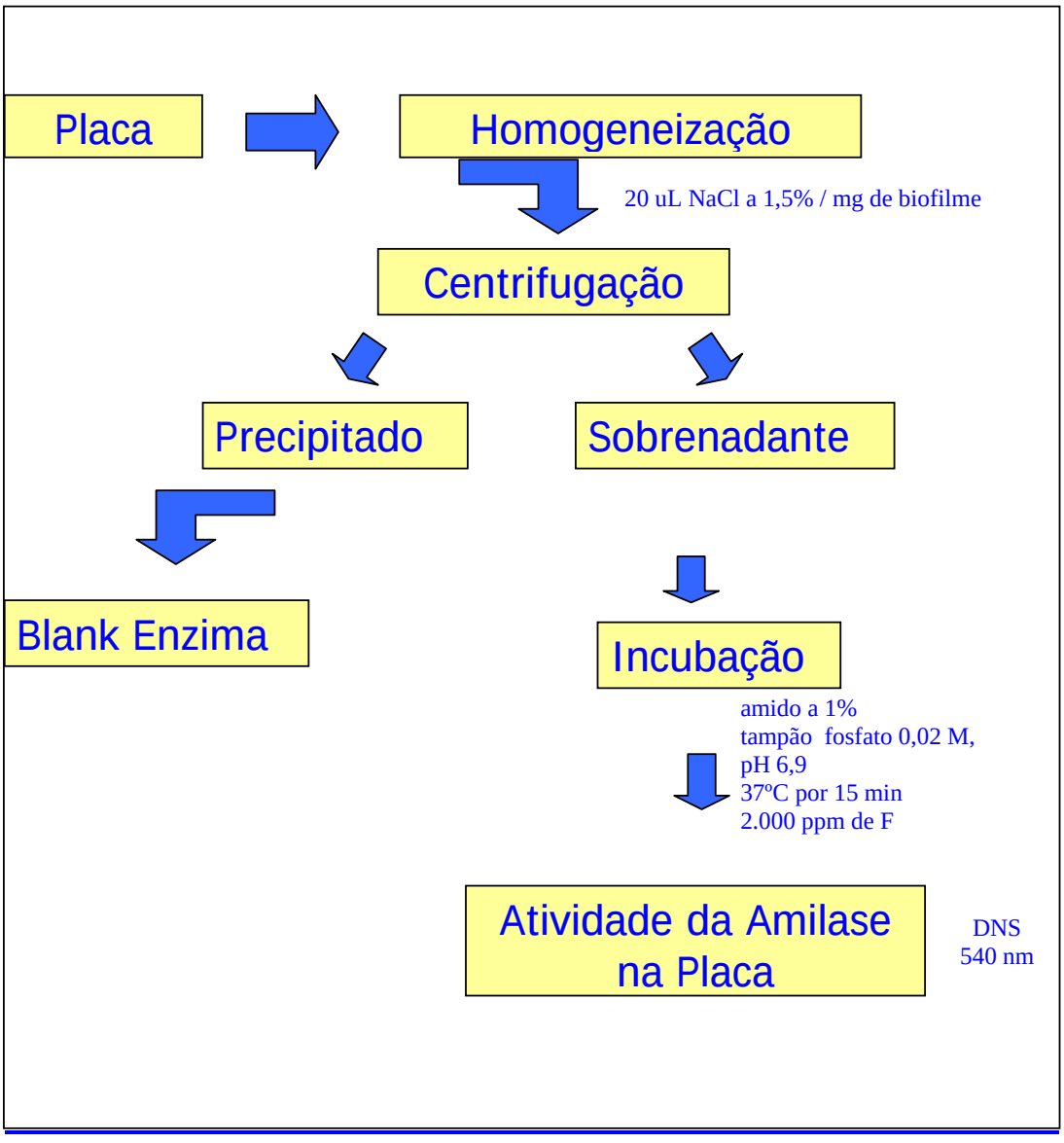
ANEXO 5

DISPOSITIVO INTRA-ORAL PALATINO



ANEXO 6

ATIVIDADE DA AMILASE

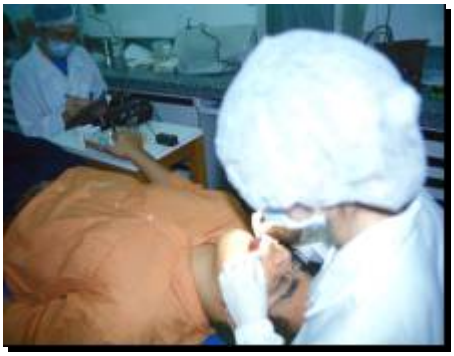


ANEXO 7

DETERMINAÇÃO DA ACIDOGENICIDADE DO BIOFILME DENTAL

A- pHmetro, microeletrodo Beetrode® e eletrodo de referência.

B- Microeletrodo Beetrode®



C- Análise da acidogenicidade do biofilme dental *in situ*

ANEXO 8

ANÁLISE DA COMPOSIÇÃO MICROBIOLÓGICA DO BIOFILME DENTAL

1 mg de biofilme dental/ mg de solução salina

diluição seriada

sonicação

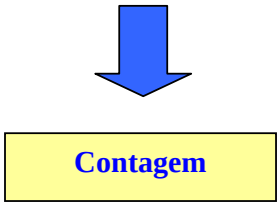
- **Agar sangue**
- **MSB**
- **CFAT**
- **Rogosa**

semeadura

Spiral Plater

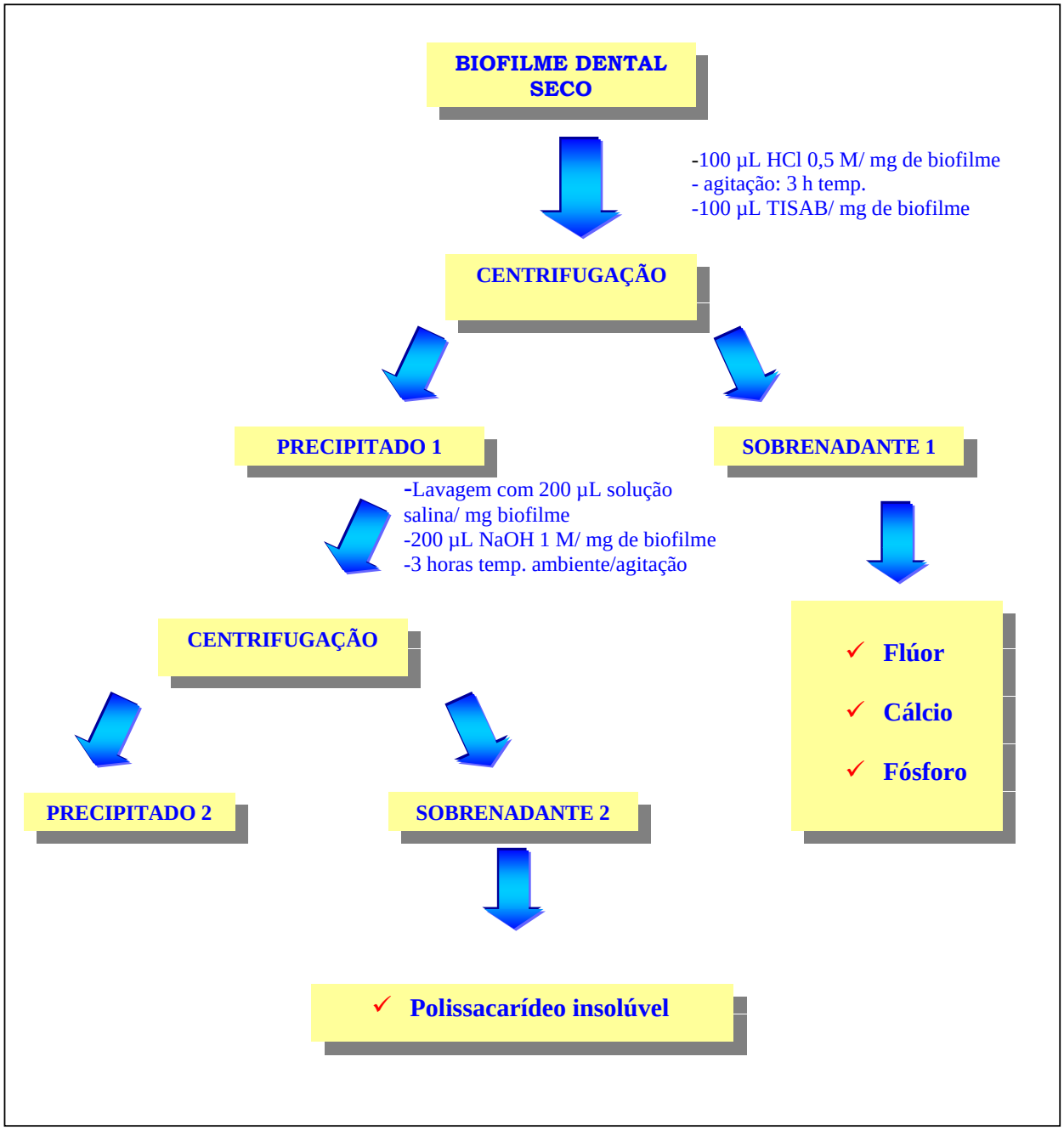
Identificação

- **Fenotipagem**
- **Gram**
- **Bioqímica**


Contagem

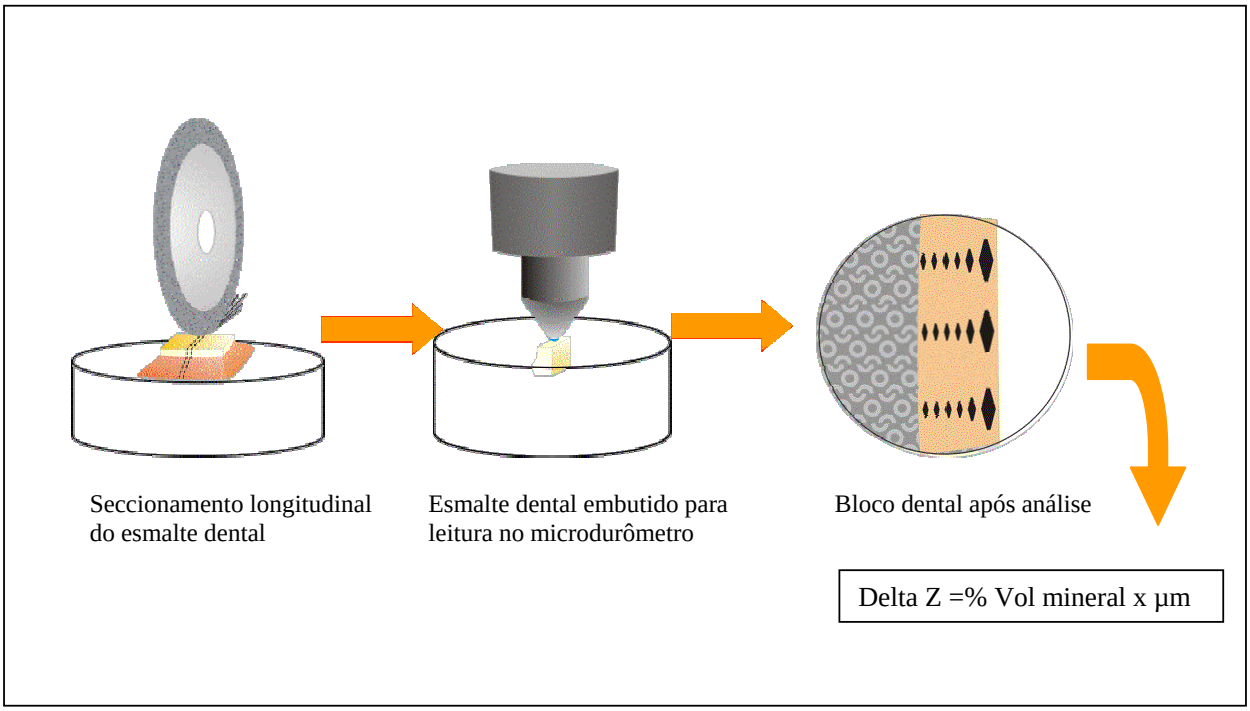
ANEXO 9

ANÁLISE DA COMPOSIÇÃO BIOQUÍMICA DO BIOFILME DENTAL



ANEXO 10

MICRODUREZA DO ESMALTE SECCIONADO LONGITUDINALMENTE



ANEXO 11

DELTA Z

CÁLCULO DAS ÁREAS INTEGRADAS

[% de volume mineral x distância da superfície (μm)]

- **ÁREA DO ESMALTE HÍGIDO** (projeção feita a partir dos dados do esmalte tratado que atingiram um platô)
- **ÁREA DE ESMALTE TRATADO**

CÁLCULO DO DELTA Z

(área de perda mineral na lesão de cárie)

ΔZ = área de esmalte hígido – área de esmalte tratado

Gráfico Delta Z