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RENZO ALBERTO CCAHUANA VÁSQUEZ

EFEITO DA FREQÜÊNCIA DE EXPOSIÇÃO À SACAROSE SOBRE A COMPOSIÇÃO DO BIOFILME DENTAL E A DESMINERALIZAÇÃO DO ESMALTE NA PRESENÇA DE FLUORETO

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

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RENZO ALBERTO CCAHUANA VÁSQUEZ
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Orientadora: Profa. Dra. Cínthia Pereira Machado Tabchoury
Co-orientador: Prof. Dr. Jaime Aparecido Cury

Banca Examinadora:

Profa. Dra. Cínthia Pereira Machado Tabchoury
Profa. Dra. Marília Afonso Rabelo Buzalaf
Prof. Dr. Pedro Luiz Rosalen

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SUMÁRIO

RESUMO

Nos últimos anos tem sido sugerido que a freqüência de consumo de açúcares poderia ser incrementada, se fluoreto (F) a partir da água ou dentifrício fosse utilizado. Mas o seu efeito na desmineralização do esmalte e principalmente na composição do biofilme dental formado sob várias freqüências de consumo de açúcares não foi totalmente explorado. Desta forma, o objetivo deste estudo foi avaliar, na presença do uso de fluoreto, o efeito da freqüência de exposição à sacarose na composição microbiológica e bioquímica do biofilme dental e sua relação com a desmineralização do esmalte dental. Dez voluntários adultos saudáveis, vivendo em uma área fluoretada, usaram dispositivos intra-orais palatinos contendo 4 blocos de esmalte dental hígido humano, durante 3 fases experimentais de 14 dias cada. Os voluntários escovaram seus dentes usando dentifrício fluoretado, contendo 1100 ppm de F na forma de NaF, 3 vezes ao dia. Foram avaliadas as seguintes freqüências de exposição à solução de sacarose 20%: 0 (controle), 2, 4, 6, 8 e 10 vezes/dia. Em cada fase experimental, foram testadas 2 freqüências (0-2, 4-6, 8-10). Ao final de cada fase experimental, o biofilme dental foi coletado, os blocos de esmalte foram removidos dos dispositivos e foram realizadas as seguintes análises: contagem de microrganismos totais (MT), estreptococos totais (ET), estreptococos do grupo mutans (EM) e lactobacilos (LB); porcentagem de estreptococos do grupo mutans em relação a microrganismos totais (%EM/MT) e estreptococos totais (%EM/ET); porcentagem de lactobacilos em relação a microrganismos totais (%LB/MT); determinação da quantidade de polissacarídeo extracelular (PEC) e intracelular (PIC); análise da concentração de íons F, cálcio (Ca) e fósforo inorgânico (P_i) no estroma e fluido do biofilme dental e avaliação de perda mineral do esmalte seccionado longitudinalmente (ΔZ). Os resultados mostraram perdas minerais (ΔZ) estatisticamente maiores que o controle ($p<0,05$) nas freqüências maiores que 6 x/dia. Entretanto, quantidade de biofilme, contagens de MT, ET, LB e concentração de PEC aumentaram, enquanto as concentrações de F, Ca e P_i no estroma diminuíram significativamente ($p<0,05$), com freqüências de exposição à sacarose menores que 6 x/dia. Em conclusão, F é capaz de reduzir a perda mineral do esmalte dental se o consumo de sacarose não for maior que 6 vezes ao dia, mas mudanças na composição bioquímica e microbiológica do biofilme dental são observadas com menores freqüências de uso.

ABSTRACT

In the last years, it has been suggested that the frequency of consumption of sugars could be increased, if fluoride from water or dentifrice is used. However, its effect on enamel demineralization and mainly on composition of dental biofilm formed under various frequencies of consumption of sugars has not been completely explored. Therefore, the objective of this study was to evaluate, in the presence of fluoride, the effect of the frequency of sucrose exposure on the microbiological and biochemical composition of dental biofilm and its relationship with demineralization of dental enamel. Ten healthy adult volunteers, living in a fluoridated area, wore intra-oral palatal appliances containing four sound human dental enamel blocks, during 3 experimental phases of 14 days each. The volunteers brushed their natural teeth with fluoridated dentifrice, containing 1100 ppm of F, in the form of NaF, 3 times/day. The following frequencies of exposure to 20% sucrose were evaluated: 0 (control), 2, 4, 6, 8 and 10 times/day. In each experimental phase, 2 frequencies were tested (0-2, 4-6, 8-10). At the end of each experimental phase, dental biofilm was collected, enamel blocks were removed from the appliance and the following analysis were conducted: counts of total microorganisms (TM), total streptococci (TS), mutans streptococci (MS) and lactobacillus (LB); percentage of mutans streptococci in relation to total microorganisms (%MS/TM) and total streptococci (%MS/TS); percentage of lactobacillus in relation to total microorganisms (%LB/TM)concentration of extracellular (EPS) and intracelular (IPS) polysaccharides; fluoride (F), calcium (Ca), inorganic phosphorus (P_i) concentration in whole biofilm and fluid; and mineral loss of longitudinally sectioned enamel (ΔZ). The results showed significantly higher ($p<0.05$) mineral losses (ΔZ) than control group for the frequencies higher than 6 x/day. However, biofilm mass, TM, TS, LB counts and EPS concentration increased, while F, Ca and Pi concentration in the whole biofilm decreased significantly ($p<0.05$) in the frequencies of sucrose exposure lower than 6 x/day. In conclusion, fluoride is able to reduce enamel demineralization if sucrose consumption is not higher than 6 x/day, but changes in the biochemical and microbiological composition of the dental biofilm are observed with lower frequencies of use.

1 INTRODUÇÃO GERAL

Cárie dental é considerada uma doença multifatorial que afeta, em países em desenvolvimento, 60 – 90% das crianças em idade escolar e uma grande maioria dos adultos (Petersen, 2003). Vários fatores estão envolvidos no processo de cárie, sendo os mais importantes: a susceptibilidade do hospedeiro, a presença de microrganismos cariogênicos e o consumo de carboidratos, principalmente a sacarose (Bowen, 1991). Entre os diversos açúcares da dieta, a sacarose é considerada o carboidrato mais cariogênico, porque é fermentada pelos principais microrganismos cariogênicos a ácidos orgânicos e também leva à formação de polissacarídeos extracelulares (PEC), que aumentam a porosidade do biofilme dental, permitindo a penetração de substratos acidogênicos para as camadas mais internas do biofilme com a conseqüente produção de ácidos próximo à estrutura dental (Newbrun, 1969; Dibdin & Shellis, 1988). Os PEC também melhoram a aderência bacteriana no esmalte (Rölla, 1989). Além dos PEC, a metabolização de sacarose leva à formação de polissacarídeos intracelulares (PIC), que podem ser utilizados como substrato na ausência de nutrientes e podem prolongar a exposição de ácidos sobre a estrutura dentária (Tanzer *et al.*, 1976).

Com relação à composição bioquímica, estudos anteriores demonstraram que o biofilme dental formado na presença de sacarose apresenta menor concentração de íons cálcio (Ca), fósforo inorgânico (Pi) e flúor (F) do que aquele formado na sua ausência (Cury *et al.*, 1997, 2000; Aires *et al.*, 2006). Em acréscimo, o biofilme dental formado *in situ*, em presença de sacarose, sofre modificações na sua composição microbiológica, apresentando um aumento nas contagens de lactobacilos quando comparado com biofilme que não foi exposto à sacarose (Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005). Além

disso, também foi verificado um aumento da concentração de PEC nos biofilmes dentais expostos à sacarose (Cury *et al.*, 1997, 2000; Pecharki *et al.*, 2005; Aires *et al.*, 2006).

Estes estudos de análise de biofilme dental foram realizados utilizando o biofilme como um todo. Entretanto, os principais eventos de trocas iônicas nos processos de desmineralização e remineralização ocorrem no fluido do biofilme dental, que é a porção aquosa entre as bactérias e a matriz do biofilme (Tatevossian & Gould, 1976), além de ser responsável pelo equilíbrio de solubilidade do tecido mineral, determinando se o esmalte-dentina será desmineralizado ou não quando ocorre uma queda de pH (Pearce, 1998). Nesse sentido, uma avaliação separada das concentrações inorgânicas do fluido e do estroma (parte sólida) do biofilme dental seria de fundamental interesse para o entendimento dos fenômenos envolvidos no processo de cárie dental.

Antes do uso difundido do fluoreto como meio preventivo em relação ao desenvolvimento de cárie dental, estudos foram realizados em humanos (Gustafsson *et al.*, 1954; Harris, 1963) e evidenciaram uma relação direta entre a freqüência de consumo de carboidratos e o desenvolvimento de cárie, o que também foi confirmado por estudos com animais (König, 1969) e *in situ* (Cury *et al.*, 1997, 2001). Entretanto, atualmente o uso do fluoreto é amplamente difundido e está presente em diferentes formas, desde água de abastecimento até dentifrício. Muitos estudos mostram claramente que o fluoreto é um efetivo agente cariostático, reduzindo a desmineralização e ativando a remineralização do esmalte dental (ten Cate & Rempt, 1986) e também, dependendo da concentração, inibindo enzimas bacterianas (Marquis, 1995).

Desta forma, um importante declínio na prevalência de cárie dental tem sido observado nas últimas décadas não somente nos países industrializados (Brathall, 1996),

mas também em países em desenvolvimento (Narvai *et al.*, 1999), o que é atribuído ao uso difundido do fluoreto. Na atualidade, os programas de prevenção de cárie incluem alguma forma de uso contínuo de fluoreto por parte das populações, questionando o papel da freqüência de consumo de carboidratos no desenvolvimento da cárie dental. Muitos países têm registrado uma diminuição da prevalência de cárie em vários grupos etários de suas populações, apesar de não existir uma mudança significativa no consumo de açúcares e de ter evidência do aumento do uso de carboidratos refinados na forma de doces e refrigerantes (Duggal *et al.*, 2001). Em acréscimo, Gibson & Williams (1999) sugerem que o uso de dentifrícios fluoretados pode ser mais importante no controle da cárie que a restrição no consumo de açúcares. Porém, estudos epidemiológicos recentes realizados, sob condições atuais de acesso a produtos fluoretados com crianças (Karjalainen *et al.*, 2001) e adolescentes (Arcella *et al.*, 2002) de países industrializados mostraram uma relação direta entre freqüência de consumo de açúcares e presença de cárie.

Não obstante, atualmente, a relação entre o consumo de açúcar e cárie dental é fraca, o que poderia ser atribuído ao uso difundido do fluoreto (Burt e Pai, 2001). De fato, dados experimentais in situ mostram que demineralização de esmalte na presença de fluoreto, na forma de água de abastecimento (Cury *et al.*, 2001) ou dentifrício (Duggal *et al.*, 2001), é detectável só quando a freqüência de exposição de sacarose é maior que 6 vezes ao dia. Entretanto, o efeito da alta freqüência da exposição à sacarose sobre a composição bioquímica e microbiológica do biofilme dental não foi totalmente explorado. Isto poderia ser relevante e poderia contribuir para explicar porque uma

associação entre consumo de açúcar e cárie dental ainda é encontrado, mesmo em países em desenvolvimento (Karjalainen *et al.*, 2001).

2 PROPOSIÇÃO

O objetivo deste estudo foi avaliar, na presença de fluoreto, a composição microbiológica e bioquímica do biofilme dental formado em função da exposição a várias freqüências de consumo de sacarose e sua relação com a desmineralização do esmalte dental.

3 CAPITULO

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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Effect of frequency of sucrose exposure on dental biofilm composition and enamel demineralization in the presence of fluoride

R.A. Ccahuana-Vásquez, G.C. Vale, C.P.M. Tabchoury, L.M.A. Tenuta, A.A. Del Bel Cury, J.A. Cury

Faculty of Dentistry of Piracicaba, UNICAMP, Piracicaba, SP, Brazil

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Address for correspondence:

Jaime A Cury
Av. Limeira 901
13414-903, Piracicaba, SP
Brazil
Phone: #55-19-34125303, Fax: #55-19-34125212
E-mail: jcury@fop.unicamp.br

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ABSTRACT

It has been shown that the frequency of sugar consumption could be increased if fluoride from water or dentifrice is used, but its effect on enamel demineralization and mainly on dental biofilm composition has not been totally explored. Ten volunteers, living in a fluoridated area, wore palatal appliances during 14 days, exposed human enamel slabs to 20% sucrose solution 0 (control), 2, 4, 6, 8 or 10 times/day and used fluoride dentifrice 3 times/day. Enamel demineralization differed significantly from control for sucrose frequencies higher than 6x/day. However, biofilm mass, total microbiota, total streptococci, lactobacilli counts and insoluble extracellular polysaccharide concentration increased, while Ca, P_i and F concentration in whole biofilm decreased significantly, with frequencies of sucrose exposure lower than 6x. The findings confirm that fluoride is able to reduce enamel demineralization if sucrose consumption is not higher than 6 x/day, but changes in the biochemical and microbiological composition of the biofilm are observed with lower frequencies of use.

INTRODUCTION

Caries is a sugar-dependent bacterial disease and among the dietary carbohydrates sucrose is considered the most cariogenic since in addition to being fermented in acids, it is metabolized in intra (IPS) and extracellular polysaccharide (EPS) by microorganisms from dental biofilm [Newbrun, 1967; Bowen, 2002].

The constant low pH, induced by sucrose fermentation, not only triggers a shift in the balance of resident plaque microflora to a more cariogenic one [Marsh, 1991], as well as it is responsible for the demineralization of the underneath dental mineral tissues. IPS can be involved with dental caries by prolonging the exposure of tooth surfaces to organic acids and maintaining a lower fasting pH in the matrix of the plaque [Tanzer et al., 1976].

EPS, mainly the insoluble one, changes the structure of biofilm matrix, increasing its porosity [Dibdin and Shellis, 1988] and allowing sugar diffusion into the deepest part of the biofilm, which would result in low plaque pH values due to microbial catabolism [Zero et al., 1986]. Also, insoluble EPS may enhance bacterial adherence to enamel-dentine surface [Rölla, 1989; Schilling and Bowen, 1992], increasing plaque formation and accumulation. Furthermore, low concentrations of calcium (Ca), inorganic phosphate (P_i) and fluoride (F) have been found in whole biofilm formed in the presence of sucrose [Cury et al., 1997, 2000]. These ions in biofilm fluid are responsible by solubility equilibrium of mineral tissue and their concentrations determine if enamel-dentine will be demineralized or not when the pH drops [Pearce, 1998]. Additionally, the changes provoked by sucrose in dental biofilm are dependent of its frequency of exposure and concentration [Cury et al., 1997; Paes Leme et al., 2004; Aires et al., 2006].

However, at present, the relationship between sugar consumption and caries is weak, which could be attributed to the widespread use of fluoride [Burt and Pai, 2001]. In fact, experimental in situ data have shown that enamel demineralization in the presence of fluoride, either from water supply [Cury et al., 2001a] or dentifrice [Duggal et al., 2001], is detectable only when the frequency of sucrose exposure is higher than six times/day. However, the effect of this high frequency of sugar consumption on the biochemical and microbiological composition of the biofilm formed has not been totally explored. This may be relevant and could contribute to explain why an association

between sugar consumption and caries is still found, even in developing countries [Karjalainen et al., 2001].

Therefore, this study aimed to evaluate, in the presence of fluoride use, the effect of the frequency of sucrose exposure on biochemical and microbiological composition of dental biofilm formed and its relationship with enamel demineralization.

MATERIALS AND METHODS

Experimental Design

This study was approved by the Research and Ethics Committee of FOP-UNICAMP (Protocol N° 100/2004). It was carried out in three phases of 14 days each, during which ten volunteers, 19-31 years old, wore acrylic palatal appliances, containing two sets of two slabs (duplicate) of human dental enamel located on the right and left sides of the appliance. In each experimental phase, each set of two slabs was submitted outside the mouth to one of the following frequencies of exposure to 20% sucrose solution: 0 (control) or 2, 4 or 6 and 8 or 10 times/day. Thus, in the first phase, the volunteers were randomly assigned to subject the slabs to the frequencies 0 and 2, or 4 and 6 or 8 and 10 x/day. The volunteers who did the treatments 0 and 2 in the first phase, in the following did 4 and 6, and 8 and 10; those that did 4 and 6, did 8 and 10, and 0 and 2, and those that did 8 and 10 in the first phase, in the following did 0 and 2, and 6 and 8, so that after the three phases all the volunteers were subjected to all the treatments. The use of two treatments in the same intra-oral appliance (split-mouth design) was supported by the absence of a cross-effect in previous studies [Cury et al., 2001b; Hara et al., 2003; Paes Leme et al., 2004; Pecharki et al., 2005; Ribeiro et al., 2005]. After each phase, the effect of the treatments was analyzed in the biochemical and microbiological composition of the biofilm formed, as well as in mineral change in enamel. For the statistical analysis, the volunteer was considered as an experimental block. This study was blind only with respect to the examiner, since the volunteers were instructed to follow the frequencies of sucrose exposure.

Enamel Slabs and Palatal Appliance Preparation

One hundred and twenty dental enamel slabs (4 X 4 X 2 mm) were obtained from the middle third of the buccal and lingual face of sound human impacted third molars and

were randomly distributed to the different treatments. An acrylic resin intra-oral palatal appliance, containing two lateral cavities measuring 9 X 5 X 3 mm, in which two slabs of enamel were placed on each side, was made for each volunteer, for each phase of the study. Plastic meshes were fixed over the cavities to protect the enamel slab surfaces from mechanical attrition, leaving a 1-mm space for accumulation of dental biofilm. Colourless and red acrylic resin was used to fix the meshes, indicating where each treatment should be made [Cury et al., 2001b; Paes Leme et al., 2004; Pecharki et al., 2005; Ribeiro et al., 2005]. Further details of appliance preparation are given in previous publications [Cury et al., 1997; 2000; Hara et al., 2003].

Treatments

Solution of 20% sucrose, prepared every 48 h, was used as a cariogenic challenge based on results of biofilm formation and enamel mineral loss found in previous studies [Cury et al., 1997; 2000; Aires et al. 2006] but the length of the study was shortened for 14 days due to more recent findings [Paes Leme et al., 2004; Pecharki et al 2005; Ribeiro et al., 2005; Aires et al., 2006].

The volunteers were instructed to remove the appliances from the oral cavity and drip 2 drops of the solution on each set of enamel slabs at the following time, according to the frequency of sucrose exposure: 2x (11:00 and 21:00 h); 4x (8:00, 11:00, 15:30 and 21:00 h); 6x (8:00, 11:00, 14:00, 15:30, 19:00 and 21:00 h); 8x (8:00, 9:30, 11:00, 14:00, 15:30, 17:00, 19:00 and 21:00 h) and 10x (8:00, 9:30, 11:00, 12:30, 14:00, 15:30, 17:00, 19:00, 20:00 and 21:00 h). The excess of fluid was removed with gauze and five minutes later the device was re-inserted in the mouth. A wash-out interval of one week was established between the experimental phases. The volunteers were also instructed to wear the appliances all the time, removing them only during the meals [Cury et al., 1997, 2000].

Throughout the entire experiment, volunteers brushed their natural teeth and the appliance, except the area of enamel slabs, with a dentifrice containing 1100 μg F/g (NaF) and silica as abrasive. Brushing was carried out three times a day after the main mealtimes. The volunteers lived in an optimally fluoridated city (0.7 mg F/ L, for the region), drank and consumed foods prepared with this water. Considering the crossover design of this study, no restriction was made with regard to the volunteers' diet. They

also received oral and written information to refrain from using any antibacterial substance.

Biofilm Analysis

Microbiological Analysis

On the 14th day of each experimental phase, approximately ten hours after the last exposure to sucrose solution and with the volunteers at fasting and without brushing their teeth, the dental biofilm formed on the enamel slabs was collected; a homogeneous aliquot was used for the microbiological analyses and the rest of the biofilm was used for the biochemical analysis.

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 (Sonics and Materials, Danbury, CT, USA) at 40 W, 5% amplitude, six pulses of 9.9 s each [Bowen et al., 1986]. The suspensions were diluted in 0.9% NaCl in series up to 10^{-5} and the dilutions were inoculated using the drop plate method [Herigstad et al., 2001]. The plates were divided in 3 equal areas for each dilution, which were inoculated in duplicate. In each area, 3 drops of 20 µL of the same dilution were placed. The suspensions were inoculated on blood agar to determine total microorganisms (TM), Rogosa SL agar (Difco 248020) to determine lactobacillus (LB), mitis salivarius agar to determine total streptococci (TS) and mitis salivarius agar plus 0.2 units bacitracin/ml (MSB) to determine mutans streptococci (MS) populations [Gold et al., 1973]. The plates were incubated for 48 h at 37°C in 10% CO_2. The blood agar plates were additionally incubated for 24 h at 37°C in aerobiosis. The colony-forming units (CFU) were counted and the results were expressed as CFU/mg of wet plaque, percentage of mutans streptococci group (%MS/TM) and lactobacilli (%LB/TM) in relation to total microorganisms and percentage of mutans streptococci group (%MS/TS) in relation to total streptococci.

Biochemical Analysis

On the 14th day of each experimental phase, after aliquot removal for microbiological analysis, the rest of the biofilm was collected using a plastic spatula and immediately placed inside an oil-filled centrifuge tube [Vogel et al., 1997]. After determination of the sample weight (± 0.01 mg), the tube was centrifuged for 10 min

(21,000 *g*) at 4°C to separate the fluid from the biofilm solids. The fluid was recovered with oil-filled capillary micropipettes and deposited under mineral oil on the bottom of a plastic petri dish until the analyses.

The tip of the centrifuge tube was cut [Vogel et al., 1997], and the remaining biofilm was centrifuged into a tube containing 0.5 M HCl (0.5 mL/10 mg of biofilm wet weight) for extraction of acid-soluble whole biofilm Ca, P_i and F [Cury et al., 1997, 2000]. The samples were agitated 3 h at room temperature, neutralized with 2 M NaOH containing TISAB III (Orion 940911) 50% (0.125 mL/10 mg biofilm wet weight), centrifuged, and the supernatant was immediately analyzed for F. The remaining supernatant was kept frozen until Ca and P_i analyses.

To the precipitate, 1 M NaOH (0.5 mL/10 mg biofilm wet weight) was added for extraction of insoluble EPS [Cury et al., 2000]. After 3 h at room temperature under constant agitation, supernatant was collected for analysis. To the precipitate, 1 M NaOH (0.5 mL/10 mg biofilm wet weight) was added 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 analyzed for total carbohydrate according to Dubois et al. [1956].

For analyses of Ca and P_i in the biofilm fluid, quartz nanoliter volume pipettes [Vogel et al., 1990] were used to transfer standardized volumes of the samples or standards into Ca or P_i sensitive colorimetric reagents [Vogel et al., 1983]. The absorbance of the mixtures, after mixing, was then read using a micro-cuvette (Hellma, 105.202, Müllheim, Germany) in a Beckman DU-70 spectrophotometer. For the analyses of Ca and P_i in the whole biofilm, the standards also contained TISAB III. For F analysis in the fluid, samples were diluted with TISAB III (1:9) under microscope on the surface of an oil-covered inverted F electrode [Vogel et al., 1997]. A micro-reference electrode was used to close the circuit, and the signal was read using a high-impedance electrometer (WPI, FD223, Sarasota, FL) and graphically observed using the program Plot 1 (Paffenbarger Research Center, ADA Foundation, Gaithersburg, MD). The acid extract of whole biofilm was previously diluted with TISAB III and analyzed for F as described above.

Enamel Analysis

At the end of each experimental phase, the enamel slabs were removed from the appliances, longitudinally sectioned 1 mm from one of the sides, embedded in acrylic resin and the cut surfaces were exposed and polished. Cross-sectional microhardness (CSMH) was measured according to Cury et al. [2000], but the indentations were made at 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 integrated mineral loss (ΔZ) for each treatment was calculated [White and Featherstone, 1987].

Statistical Analysis

The assumptions of equality of variances and normal distribution of errors were checked for all the response variables tested and those that did not satisfy were transformed [Box et al., 1978]. The data of biofilm biomass, counts of TM and TS, Ca in fluid, Ca and P_i in whole biofilm were transformed into $\log_{10}$; counts of MS into $\log_{10}(X+1)$; counts of LB into $(X+1)^{0.1}$; F in fluid into $1/\sqrt{x}$ and %LB/TM into $1/\sqrt{(x+1)}$. These transformed variables and the original data of EPS, IPS, P_i, F in whole biofilm and mineral loss were submitted to analysis of variance (ANOVA) followed by the Tukey test, with the exception of the variable ΔZ, for which was applied the Duncan test. The variables %MS/TM and %MS/TS were analyzed by the Friedman test. The software SPSS for Windows 11.0 was used and the significance level was fixed at 5%.

RESULTS

The effect of the frequency of sucrose exposure evaluated by ANOVA was statistically significant for most of the analyses made ($p < 0.05$) except for MS counts ($p = 0.308$), %MS/TM ($p = 0.086$), %MS/TS ($p = 0.146$), and F ($p = 0.128$) and P_i ($p = 0.962$) in biofilm fluid.

Table 1 shows that, according to the increase of the frequency of sucrose exposure, compared with the control: Ca, P_i and F concentrations in whole biofilm were statistically lower from sucrose exposure 2x/day on; LB counts and %LB/TM were higher from 4x/day on; biomass and EPS concentration were higher, while TM and TS

counts were lower from 6x/day on; concentrations of IPS and Ca in biofilm fluid and enamel mineral loss were higher from the frequency of sucrose 8x/day on.

DISCUSSION

The results of mineral loss (Table 1) show that 20% sucrose solution used at a frequency higher than 6 times/day causes significant demineralization on enamel in comparison with the control. These results are in agreement with in situ studies conducted by Cury et al. [2001a] and Duggal et al. [2001], when these authors evaluated the effect of sucrose frequency in volunteers using only one source of F, water or dentifrice, respectively. The present findings were observed when these sources were combined, suggesting that there was no additive effect, increasing enamel resistance to demineralization at higher frequency of sucrose exposure. This can be explained by the fact that the isolated effect of fluoride ion present in saliva from water or dentifrice was evaluated, since the biofilm was not mechanically disrupted by brushing; also the topical effect of the dentifrice forming products in enamel did not occur. Nevertheless, the findings show the relevance of fluoride use and give support to epidemiological studies in children [Karjalainen et al., 2001; Marshall et al., 2003] and adolescents [Arcella et al., 2002], showing that, in this modern age of extensive fluoride exposure, dental caries is only associated with high frequency of sugar consumption. On the other hand, although enamel mineral losses observed at frequencies of sucrose exposure lower than 8 x/day were not statistically different from the control, the values observed for the frequencies 4 and 6x/day were consistently higher than the control, suggesting that they may not be considered as a safe sugar consumption just because fluoride is used. Therefore, controlling the consumption of sugar remains a justifiable part of caries prevention [Burt and Pai, 2001], mainly root caries, since dentine is less resistant than enamel.

Thus, the frequency of sucrose exposure lower than 8x/day was enough to induce biochemical and microbiological changes in the biofilm formed (Table 1). Lactobacilli were selected in the biofilm formed with frequency of sucrose exposure of 4 x/day. This is in accordance to Bradshaw et al. [1989] and Bradshaw and Marsh [1998], who observed a predominance of LB in *in vitro* multi-species communities according to the decreased pH of the media. The selection of LB has also been found in situ when the biofilm was formed in presence of sucrose [Pecharki et al., 2005] or starch [Ribeiro et al.,

2005] in comparison with the control group. Although LB are not implicated with caries initiation, these findings may have consequences for caries progression either in enamel or dentine since they are found in high counts in caries lesions [Loesche and Syed, 1973]. Furthermore, the present results have implication for the cariogenicity of other dietary carbohydrate than sucrose, such as starch [Ribeiro et al., 2005].

Although statistically significant effect of sucrose frequency on MS counts or percentage were not found (Table 1), the changes provoked by these microorganisms on dental biofilm may be considered more relevant than their counting. Thus, insoluble EPS increased significantly from sucrose frequency of 6 x/day on. The relationship between sucrose exposure, EPS and caries development has been demonstrated in several studies and it is the unique property that differentiate sucrose from the other cariogenic carbohydrates [Cury et al., 2000]. Thus, mutant strains of *S. mutans* defective in the *gtf* genes, especially *gtfB* and *gtfC*, are significantly less cariogenic in animals than the parent strains [Yamashita et al., 1993]. In situ studies have shown that high concentration and frequency of sucrose exposure increased EPS concentration in the biofilm matrix, lowered fasting pH values and enhanced enamel demineralization when compared to biofilms formed in the absence of sucrose [Cury et al., 1997; Cury et al., 2000; Ribeiro et al., 2005; Aires et al., 2006]. Furthermore, clinical studies have also suggested that synthesis of EPS is related to caries-activity in children [Mattos-Graner et al., 2000; Nobre dos Santos et al., 2002]. Therefore, the findings should be considered when diet counseling is discussed in the presence or not of fluoride use. Additionally, the increase of EPS concentration in biofilm according to the frequency of sucrose exposure would explain why biomass increased while TM or TS counts decreased (Table 1), considering that EPS occupy a large volume of biofilm matrix.

When all variables of this study are considered, only the concentration of IPS in whole biofilm and that of Ca in the fluid showed the same significance as enamel mineral loss since these three variables differed when comparing control with sucrose exposure 8 x/day. The high IPS concentration found in biofilm exposure to sucrose higher than 8 times a day explains the low fasting pH found in biofilm formed in situ [Pecharki et al., 2005; Ribeiro et al., 2005] and also would explain the highest concentration of Ca in biofilm fluid observed in the present study. Thus, this reserve polysaccharide is

breakdown during the night, low pH is found, enamel is dissolved and Ca is released to the biofilm fluid. It has been suggested that IPS can promote the formation of dental caries by prolonging the exposure of tooth surfaces to organic acids and maintaining a lower fasting pH in the matrix of the plaque [Tanzer et al., 1976] and the present study supports this assumption.

However, the most sensitive biofilm variables to frequency of sucrose exposure were Ca, P_i and F concentrations in the whole biofilm. Biofilm exposed to sucrose 2 x/day showed lower concentrations of these ions compared with the control (Table 1). These findings are in agreement with the in situ results of Cury et al. [1997] and Paes Leme et al. [2004], and the lower Ca concentration corroborates with in vitro data found by Pearce et al. [2002]. However, the means of this low concentration of Ca, P_i and F with regard to caries is not known yet and has also been found when other carbohydrates were evaluated [Cury et al., 2000; 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 would prevent the precipitation of minerals in biofilm matrix [Tenuta et al., 2005]. Furthermore, this low concentration has not been found in the fluid of biofilm, which would be more relevant regarding enamel demineralization.

In conclusion, although enamel demineralization in the presence of fluoride is found only when sucrose is used more than 6 x/day, biochemical and microbiological changes in biofilm formed are observed with lower frequencies of exposure.

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Table 1. Microbiological and Biochemical (mean ± DS) analyses of dental biofilm according with the frequency of sucrose exposure

Analysis	*Treatments (sucrose frequency/day)					
	0 x (control)	2 x	4 x	6 x	8 x	10 x
Biomass, mg wet weight	6.7 ± 5.3^{a} n = 9	$14.0 \pm 13.2^{a,b}$ n = 9	$14.6 \pm 8.6^{a,b}$ n = 10	$19.1 \pm 11.1^{b,c}$ n = 10	$21.1 \pm 8.5^{b,c}$ n = 9	27.8 ± 10.5^{c} n = 10
TM, CFU/mg x 10^7	12.5 ± 7.6^{a} n = 9	$3.7 \pm 2.1^{a,b}$ n = 9	$4.5 \pm 6.3^{a,b}$ n = 10	2.9 ± 1.3^{b} n = 10	2.4 ± 1.2^{b} n = 9	2.4 ± 1.6^{b} n = 10
TS, CFU/mg x 10^6	73.2 ± 54.1^{a} n = 9	$21.7 \pm 20.3^{a,b}$ n = 9	$25.0 \pm 41.9^{a,b}$ n = 10	10.5 ± 7.3^{b} n = 10	14.1 ± 9.8^{b} n = 9	11.7 ± 6.8^{b} n = 10
LB, CFU/mg x 10^5	0.0066 ± 0.0^{a} n = 9	$1.6 \pm 3.1^{a,b}$ n = 9	$30.2 \pm 68.8^{b,c}$ n = 10	$35.3 \pm 54.5^{c,d}$ n = 10	$92.2 \pm 113.0^{d,e}$ n = 9	94.7 ± 122.0^{e} n = 10
MS, CFU/mg x 10^3	0.2 ± 0.3^{a} n = 9	4.2 ± 11.6^{a} n = 9	2.6 ± 6.2^{a} n = 10	0.3 ± 0.3^{a} n = 10	0.6 ± 0.1^{a} n = 9	2.6 ± 7.6^{a} n = 10
% LB/ TM	0.0007 ± 0.00^{a} n = 9	$0.7 \pm 1.3^{a,b}$ n = 9	$12.5 \pm 20.9^{b,c}$ n = 10	$18.6 \pm 27.0^{c,d}$ n = 10	33.9 ± 27.5^{d} n = 9	34.5 ± 27.3^{d} n = 10
% MS/ TM	0.0003 ± 0.0^{a} n = 9	0.0005 ± 0.3^{a} n = 9	0.01 ± 0.03^{a} n = 10	0.0013 ± 0.00^{a} n = 10	0.0032 ± 0.01^{a} n = 9	0.01 ± 0.03^{a} n = 10
% MS/ TS	0.0007 ± 0.00^{a} n = 9	0.09 ± 0.28^{a} n = 9	0.04 ± 0.09^{a} n = 10	0.01 ± 0.01^{a} n = 10	0.01 ± 0.01^{a} n = 9	0.02 ± 0.09^{a} n = 10
F in fluid, µM	5.9 ± 9.7^{a} n = 9	3.2 ± 2.0^{a} n = 9	6.2 ± 5.2^{a} n = 9	4.6 ± 2.5^{a} n = 9	10.1 ± 12.4^{a} n = 9	14.6 ± 18.1^{a} n = 9
Ca in fluid, mM	1.0 ± 0.7^{a} n = 7	$1.5 \pm 0.8^{a,b}$ n = 7	$1.4 \pm 0.7^{a,b}$ n = 9	$1.8 \pm 0.8^{a,b}$ n = 9	2.9 ± 1.7^{b} n = 9	3.3 ± 1.9^{b} n = 10
P_i in fluid, mM	10.4 ± 6.1^{a} n = 9	11.1 ± 6.5^{a} n = 9	11.8 ± 3.3^{a} n = 9	10.9 ± 2.3^{a} n = 9	12.0 ± 4.9^{a} n = 9	11.5 ± 4.5^{a} n = 10
F in whole, µmol/g	3.9 ± 1.7^{a} n = 9	2.1 ± 1.8^{b} n = 9	$0.8 \pm 0.8^{b,c}$ n = 10	0.3 ± 0.4^{c} n = 10	0.1 ± 0.0^{c} n = 9	0.1 ± 0.1^{c} n = 10
Ca in whole, µmol/g	129.7 ± 87.3^{a} n = 9	43.8 ± 36.1^{b} n = 9	$28.1 \pm 27.7^{b,c}$ n = 10	18.5 ± 16.7^{c} n = 10	10.9 ± 4.6^{c} n = 9	10.8 ± 4.6^{c} n = 10
P_i in whole, µmol/g	157.5 ± 72.4^{a} n = 9	59.6 ± 41.3^{b} n = 9	$27.9 \pm 16.9^{b,c}$ n = 10	$21.5 \pm 7.6^{c,d}$ n = 10	$19.0 \pm 11.7^{c,d}$ n = 9	15.1 ± 4.5^{d} n = 10
EPS, µg/mg	3.5 ± 1.1^{a} n = 9	6.2 ± 3.1^{a} n = 9	$15.5 \pm 10.5^{a,b}$ n = 10	$25.8 \pm 17.1^{b,c}$ n = 10	$42.5 \pm 23.1^{c,d}$ n = 9	55.5 ± 13.7^{d} n = 10
IPS, µg/mg	2.2 ± 1.4^{a} n = 9	3.7 ± 2.3^{a} n = 9	$3.9 \pm 1.4^{a,b}$ n = 10	$4.3 \pm 2.5^{a,b}$ n = 10	5.3 ± 1.8^{b} n = 9	5.5 ± 2.2^{b} n = 10
Mineral loss, ΔZ	497.6 ± 191.3^{a} n = 10	473.6 ± 216.1^{a} n = 10	$631.1 \pm 376.2^{a,b}$ n = 10	$645.0 \pm 281.3^{a,b}$ n = 10	789.6 ± 435.5^{b} n = 10	812.5 ± 289.5^{b} n = 10

*Treatments whose means are followed by distinct lower case letters differ statistically ($P < 0.05$).

4 CONCLUSÃO GERAL

Os resultados do presente estudo sugerem que embora a desmineralização do esmalte dental na presença do fluoreto tenha sido observada somente quando sacarose foi utilizada mais que 6 vezes ao dia, mudanças na composição bioquímica e microbiológica no biofilme dental formado foram observadas com freqüências menores de exposição a este carboidrato.

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

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FACULDADE DE ODONTOLOGIA DE PIRACICABA

CERTIFICADO

Certificamos que o projeto de pesquisa "Efeito do dentifrício fluoretado na desmineralização do esmalte em função da freqüência de exposição à sacarose - estudo *in situ*", protocolo cep nº ***100/2004***, dos Pesquisadores ***Renzo Alberto Ccahuana Vasquez*** e ***Cínthia Pereira Machado Tabchoury***, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde - MS e foi aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Odontologia - UNICAMP.

We certify that the research project "Effect of fluoridated dentifrice on enamel demineralization according to the sucrose exposure frequency - *in situ* study", register number ***100/2004***, of ***Renzo Alberto Ccahuana Vasquez*** and ***Cínthia Pereira Machado Tabchoury***, is in agreement with the recommendations of 196/96 Resolution of the National Health Committee - Brazilian Health Department and was approved by the Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas - UNICAMP.

Piracicaba - SP, Brazil, September 15 2004

Cínthia P. Machado Tabchoury

Profa. Dra. Cinthia Pereira Machado Tabchoury

Secretaria
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Júnior

Coordenador
CEP/FOP/UNICAMP

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 Nº 1985/96, das teses de Mestrado e Doutorado terem um formato alternativo àquele já bem estabelecido, a CCPG resolve:

Artigo 1º - 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 2º - 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 3º - 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 4º - Fica revogada a resolução CCPG 17/97

ANEXO 3

Correio eletrônico de confirmação de que o artigo foi submetido à "Caries Research"

----- Forwarded message from r.p.shellis@bristol.ac.uk -----
> Date: Mon, 16 Jan 2006 14:59:48 +0100 (CET)
> From: r.p.shellis@bristol.ac.uk
> Reply-To: r.p.shellis@bristol.ac.uk
> Subject: Ms. No. 200601009, Caries Research
> To: jcury@fop.unicamp.br

MS: 200601009
Dear Jaime,

Thank you for submitting your manuscript entitled "Effect of frequency of sucrose exposure on dental biofilm composition and enamel demineralization in the presence of fluoride" to "Caries Research". It will now be forwarded to our reviewers and we shall inform you as soon as possible of the decision reached by the editorial board. The manuscript reference number is 200601009. Please use this number on all correspondence about the manuscript, which should be sent to the "Caries Research" editorial office at the address listed below.
For information regarding the status of your manuscript and for future submissions you can access this system by logging into the journal's online peer review system as follows:
http://www.karger.com/cre
Logon Name: jcury
Password:

With kind regards,

R P Shellis
(Editor-in-Chief, Caries Research)
Division of Restorative Dentistry
Bristol University Dental School, Bristol BS1 2LY, U.K.
Fax. +44 117-928-4778
> Tel. +44 117-928-4328
> r.p.shellis@bristol.ac.uk

ANEXO 4

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

1. Título da Pesquisa

"Efeito do dentifrício fluoretado na desmineralização do esmalte em função da freqüência de exposição à sacarose - estudo *in situ*".

2. Objetivo da Pesquisa

Avaliar a freqüência máxima de exposição à sacarose por dia na qual o dentifrício fluoretado seria capaz de controlar a desmineralização do esmalte dental humano, por meio de análises bioquímica, microbiológica, de microdureza.

3. Justificativa

Nos últimos anos tem sido sugerido que o consumo de açúcares poderia ser incrementado sem o prejuízo de formação de cárie dental, quando os sujeitos usam dentifrícios fluoretados. Entretanto, o efeito do dentifrício fluoretado na desmineralização do esmalte sob várias freqüências de consumo de açúcares não está completamente claro.

4. Procedimentos

Os voluntários usarão dispositivos intrabucais palatinos de resina acrílica contendo 4 blocos de esmalte humano. Eles escovarão seus dentes usando o dentifrício designado 3 vezes ao dia. Serão avaliadas as seguintes freqüências de exposição à solução de sacarose **20%**: 0, 2 vezes, 4 vezes, 6 vezes, 8 vezes ou 10 vezes/dia. Em cada fase experimental com duração de 14 dias, serão testadas 2 freqüências e a seqüência de grupos de freqüência de uso de sacarose (0-2, 4-6 ou 8-10) será sorteada. Ao final de cada fase experimental, a placa dental será coletada, os blocos de esmalte serão removidos dos dispositivos e as seguintes análises serão realizadas: 1) Análise bioquímica do biofilme dental; 2) Análise microbiológica 3) Análise de microdureza interna.

5. Desconfortos e Riscos

Os voluntários poderão apresentar discreta halitose durante o período experimental, o que poderá ser resolvido com adequada higiene dental. O uso da solução de sacarose será apenas como gotas sobre os blocos de esmalte presentes nos dispositivos intra-orais, não implicando em qualquer

aumento de cárie dental nos voluntários. O dispositivo intra-oral pode causar um leve desconforto, que é, entretanto, semelhante ao desconforto causado por um aparelho ortodôntico móvel. Este desconforto será minimizado por ajustes feitos previamente e durante seu uso, se necessário. Durante todo o período da pesquisa, acompanhamentos semanais serão realizados, para verificar as condições do aparelho e da sua saúde bucal. O benefício que os voluntários terão será um auxílio indireto, contribuindo para a realização deste projeto e o para a ciência.

6 Forma de acompanhamento e assistência

Os pesquisadores envolvidos na pesquisa estarão à disposição dos voluntários para ajuste no aparelho intra-oral a fim de minimizar qualquer desconforto ou para conversar sobre qualquer dúvida que possa surgir.

7 Garantia de esclarecimento

O voluntário tem garantia de que receberá resposta ou esclarecimento de qualquer dúvida quanto aos procedimentos, riscos, benefícios e outros assuntos relacionados à pesquisa ainda que isso 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: 3412-5393 (Sala dos alunos – Cariologia), 3412-5303 (Laboratório de Bioquímica)
3434-4869 (Profa. Cínthia – residência), 3413-3371 (Renzo – residência)

8 Formas de ressarcimento

Os voluntários serão ressarcidos de eventuais despesas com o transporte-alimentação para a coleta das amostras contidas nos dispositivos.

9 Formas de indenização

Não há danos previsíveis decorrentes desta pesquisa.

10 Garantia de sigilo

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

11 Liberdade para se recusar em participar da pesquisa

A decisão de fazer parte desta pesquisa é voluntária. O voluntário 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 INFOMAÇÕES ACIMA EXPLICADAS.

_____ _____

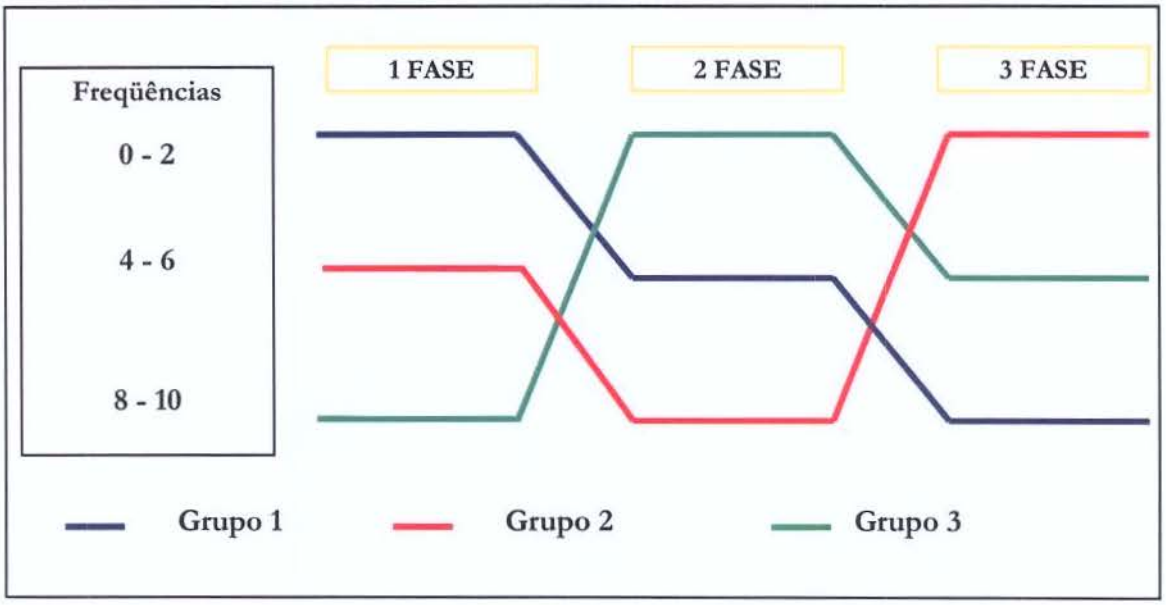
Nome do voluntário Assinatura do voluntário

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.

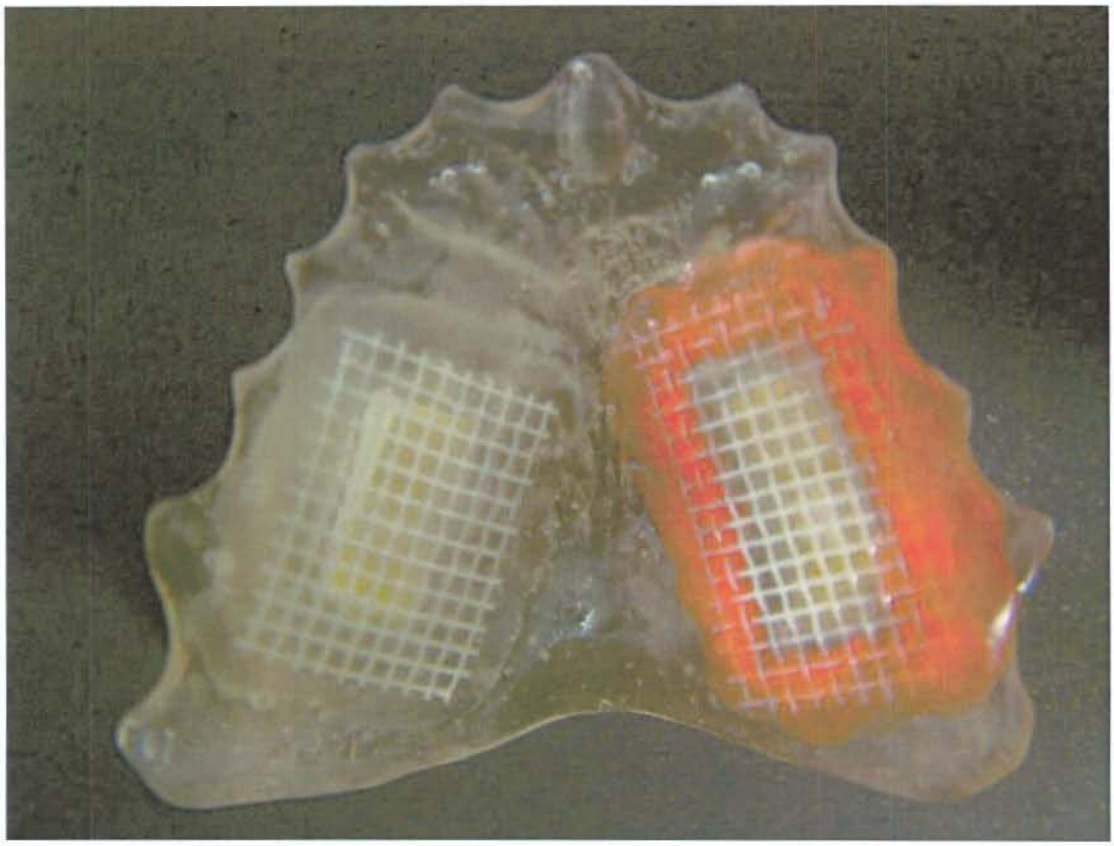
ANEXO 5

Fluxograma da fase experimental



ANEXO 6

Dispositivo intraoral palatino



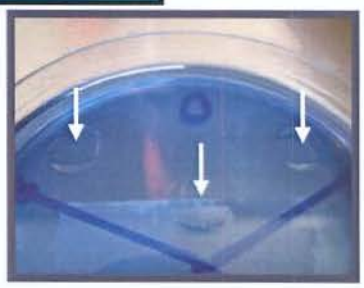
ANEXO 7

Análise Microbiológica

Coleta de biofilme dental

- Pesagem e adição de solução salina (1 mL/mg de biofilme)
- Homogeneização
- Diluição ($0 - 10^{-5}$)

Técnica da gota



Microrganismos totais

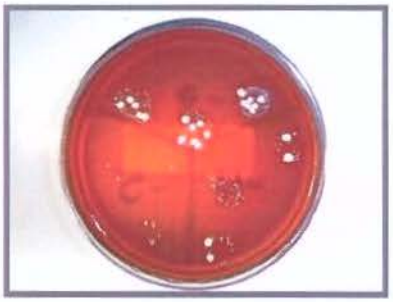
Lactobacilos

Estreptococos totais

E. do grupo mutans

Anaerobiose 48 h, 37°C

Aerobiose 24 h, 37°C (só microrganismos totais)



Contagem de UFC com Lupa Estereoscópica: 6 – 60 UFC e cálculo de porcentagem

ANEXO 8

Fluxograma do tratamento do biofilme após a retirada do fluido