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"Efeito do extrato da folha de Psidium cattleianum na expressão protéica de Streptococcus mutans, no biofilme bacteriano e na desmineralização do esmalte"

Tese apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, para a obtenção do título de Doutor em Odontopediatria.

Orientador: Prof. Dr. Alberto Carlos Botazzo Delbem

Co-orientador: Prof. Dr. Elerson Gaetti-Jardim Júnior

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"Cada um que passa em nossa vida, passa sozinho,
pois cada pessoa é única e nenhuma substitui outra.
Cada um que passa em nossa vida, passa sozinho,
mas não vai só, nem nos deixa sós;
leva um pouco de nós mesmos, deixa um pouco de si mesmo.
Há os que levam muito, mas há os que não levam nada;
há os que deixam muito, mas há os que não deixam nada.
Esta é a maior responsabilidade de nossa vida
e prova evidente de que duas almas não se encontram por acaso."
Antoine de Saint-Exupéry

Dedicatória

Àqueles que não tenho palavras para agradecer, deixo o meu pequeno obrigado e a minha gratidão eterna...

À Deus e Nossa Senhora,

Pelo amor incondicional.

"Estar debaixo da benção de Deus, não é estar livre de lutas e dificuldades, mas é ter a certeza que alguém luta por ti.
Não é ter muito, mas é ver Deus multiplicar o pouco que se tem.
É plantar com a certeza da colheita.
É chorar com a certeza do consolo.
É saber que onde entrares, Deus entra com você.
Quando estiveres aflito, Deus vem te socorrer,
Quando se levantarem contra ti, Deus vem te defender,
Quando sonhares, Deus vem cumprir.
É pedir e receber, lutar e vencer, plantar e colher."

Autor Desconhecido

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Cora Coralina

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"Quem tem um amigo, mesmo que um só,
não importa onde se encontre, jamais sofrerá de solidão;
poderá morrer de saudades, mas não estará só."
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"Tudo que é bom dura o tempo necessário para se tornar inesquecível."
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"O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso, existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis." Fernando Pessoa

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Epígrafe

"...você não pode ligar os pontos olhando para a frente; você apenas pode ligá-los olhando para trás. Então, você tem que confiar que os pontos vão, de alguma forma, se ligar no seu futuro. Você tem que confiar em alguma coisa – sua força, destino, vida, carma, qualquer coisa. Isso nunca me deixou na mão, e tem feito toda a diferença na minha vida.

(...) Você tem que achar o que ama. E isso é verdade tanto para sua vida profissional quanto para a pessoal. Seu trabalho vai preencher uma grande parte da sua vida, e a única maneira de se sentir verdadeiramente satisfeito é fazendo o que acredita ser um grande trabalho. E a única maneira de fazer um grande trabalho é amando o que você faz. (...) Como todos os assuntos do coração, você saberá quando encontrá-lo. E, como qualquer grande relacionamento, vai apenas se tornando melhor e melhor à medida que os anos passarem.

(...) Tenha a coragem de seguir seu coração e intuição. De alguma forma eles já sabem o que você verdadeiramente quer se tornar. Todo o resto é secundário."

Steve Jobs

Sumário

Resumo

Brighenti FL. “Efeito do extrato da folha de *Psidium cattleianum* na expressão protéica de *Streptococcus mutans,* no biofilme bacteriano e na desmineralização do esmalte” [Tese]. Araçatuba: Universidade Estadual Paulista, 2008.

Produtos naturais têm sido estudados com relação ao controle químico da microbiota patogênica do biofilme bucal. O objetivo desse estudo foi esclarecer o efeito do extrato da folha de *Psidium cattleianum* no metabolismo de *S. mutans*, no biofilme formado *in situ* e na capacidade em inibir a desmineralização do esmalte. O extrato foi obtido por decocção das folhas em água deionizada. O efeito do extrato foi avaliado *in vitro* com relação à viabilidade, expressão protéica e produção de ácido de biofilmes de *S. mutans*. No estudo *in situ*, blocos de esmalte bovino foram fixados em dispositivos acrílicos palatinos de dez voluntários. A fase experimental consistiu de três etapas de 14 dias cada, separadas por intervalo de sete dias. Os voluntários gotejaram solução de sacarose 20% (8x/dia) e a solução de tratamento (água, extrato ou um enxaguatório comercial; 2x/dia). O biofilme dentário foi analisado quanto à acidogenicidade, quantidade microorganismos e presença de polissacarídeos álcali-solúveis. Nos blocos de esmalte, foram realizadas as análises de microdureza superficial e em secção longitudinal. O extrato foi capaz de afetar a viabilidade, a produção de ácidos e a síntese protéica do biofilme formado *in vitro*. No biofilme formado *in situ*, o extrato foi capaz de diminuir a queda do pH, o acúmulo de microorganismos, a formação de polissacarídeos extracelulares e a desmineralização do esmalte. Conclui-se que o extrato foi capaz de diminuir a expressão de proteínas envolvidas no metabolismo bacteriano, a patogenicidade do biofilme bucal e a desmineralização do esmalte *in situ*.

Palavras-chave: Cárie dentária - prevenção e controle. Produtos biológicos. *Streptococcus mutans*.

Abstract

Brighenti FL. “Effect of *Psidium cattleianum* leaf extract on *Streptococcus mutans* protein expression, bacterial biofilm and *in situ* enamel demineralization” [Thesis]. Araçatuba: Universidade Estadual Paulista, 2008.

Natural products have been studied with relation to chemical control of pathogenical microorganisms of dental biofilm. The aim of this study was to clarify the effect of *Psidium cattleianum* leaf extract on *S. mutans* metabolism, on *in situ* biofilms and on enamel demineralization. The extract was obtained by decoction in deionized water. The extract was studied *in vitro* with relation to viability, protein expression and acid production of *S. mutans* biofilms. On the *in situ* study, enamel bovine blocks were placed in palatal appliances of ten volunteers. The experimental phase consisted of three stages with a duration of 14 days each and a washout period of seven days. The volunteers dripped 20% sucrose (8x/day) and the treatment solution (water, extract or a marketed mouthwash – 2x/day) on the enamel blocks. The dental biofilm was analyzed regarding the amount of microorganisms and presence of alkali-soluble polysaccharides. On the enamel blocks, surface and cross-sectional microhardnessess were evaluated. The extract was able to affect the viability, acid production and protein synthesis of the *in vitro* biofilm. On the *in situ* biofilm, the extract was able to decrease pH drop, microorganism accumulation, extracellular polysaccharides formation and enamel demineralization. It can be concluded that the extract was able to reduce the expression of proteins involved on bacterial metabolism, the pathogenicity of dental biofilm and *in situ* enamel demineralization.

Key-words: Dental caries – prevention and control. Biological products. *Streptococcus mutans*.

Introdução Geral

No desenvolvimento do tratamento da cárie, a odontologia evoluiu da simples extração dos dentes afetados para o tratamento e restauração da lesão. A identificação de lesões incipientes de cárie, o tratamento com métodos não-operatórios e a adoção de medidas preventivas representam um avanço posterior no tratamento odontológico (NIH Consensus Statement, 2001)[1].

A doença cárie progride quando há um desequilíbrio entre os fatores patológicos e os fatores fisiológicos (Featherstone, 2004)[2]. Desse modo, a filosofia preventiva da cárie visa o controle dos diversos fatores etiológicos, dentre as quais se destaca o controle farmacológico do biofilme bucal.

A participação do biofilme bacteriano no desenvolvimento da lesão de cárie foi comprovada nas décadas de 40 e 50, quando se demonstrou em animais que a lesão de cárie oclusal poderia ser prevenida com o uso de penicilina (McLure e Hewitt, 1946)[3] e que ratos livres de germes não desenvolviam lesões cariosas independente do alto teor de açúcar presente na dieta (Orland et al., 1954)[4]. Assim, o controle do biofilme cariogênico através do uso de antimicrobianos reduziria o crescimento e o metabolismo bacteriano e, conseqüentemente, o aparecimento de lesões cariosas.

Entre as bactérias envolvidas no processo carioso, os estreptococos do grupo mutans têm sido alvo de muitos estudos sobre a prevenção de cáries. Os *Streptococcus mutans* são considerados uma das espécies mais acidogênicas do biofilme bucal, pois são capazes de produzir ácidos a uma taxa maior e em um pH mais ácido do que qualquer outro estreptococo (de Soet et al., 2000)[5]. Além disso, essas bactérias são capazes de sobreviver e continuar produzindo ácidos a um pH

[1] NIH Consensus Statement: Diagnosis and Management of Dental Caries Throughout Life. 2001;18:1–30.
[2] Featherstone JDB: The Science And Practice Of Caries Prevention. J Am Dent Assoc 2000;131:887-889.
[3] McClure FF, Hewitt WL: The relation of penicillin to induce rat dental caries and oral Lactobacillus acidophilus. J Dent Res 1946;25:441-443.
[4] Orland FJ, Blayney JR, Harrison RW, Reyniers JA, Trexler PC, Wagner M, Gordon HA, Luckey TD: Use of the germfree animal technique in the study of experimental dental caries. I. Basic observations on rats reared free of all microorganisms. J Dent Res 1954 33:147-174.
[5] de Soet JJ, Nyvad B, Kilian M. Strain-related acid production by oral streptococci. Caries Res 2000;34:486–490.

baixo, de produzir altas quantidades de polissacarídeos intra e extracelular e de produzir polímeros para facilitar a aderência à superfície dentária (Kleinberg, 2002)[6].

Uma das estratégias que visam o controle da formação do biofilme cariogênico tem sido o uso de produtos naturais. A eficácia desses produtos é demonstrado por sua presença no mercado: cerca de 25% dos medicamentos prescritos mundialmente provêm de origem vegetal (World Health Organization, 2002)[7].

A bioprospecção consiste em localizar, avaliar e explorar a diversidade existente em determinada região. Através dela, é possível descobrir novas possibilidades de uso comercial e contribuir com as comunidades locais para melhorar suas condições de vida. Por sua grande extensão territorial, o Brasil é um dos países com maior número de espécies vegetais no mundo (Maciel et al. 2002)[8]. No entanto, 99,6% da flora nacional ainda não foram exploradas (Souza Brito e Souza Brito, 1993)[9].

Particularmente, o cerrado representa o segundo maior bioma do Brasil, abrangendo cerca de 20% do território nacional. É considerada a savana mais rica do mundo em biodiversidade, possuindo mais de 10.000 espécies de plantas, sendo 40% plantas endêmicas desse bioma.

Bianco (2004)[10] realizou uma triagem de diversas plantas do cerrado brasileiro e avaliou extratos provenientes da casca, caule ou folha. Destes, oito extratos apresentaram atividade antibacteriana, destacando-se o extrato de folha do vegetal *Psidium cattleianum*. Essa planta pertence à família Myrtaceae, umas das mais importantes em termos de número de espécies no cerrado (Ratter et al., 1997)[11]. Entretanto, seu potencial biológico continua inexplorado.

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[6] Kleinberg I: A mixed-bacteria ecological approach to understanding the role of the oral bacteria in dental caries causation: an alternative to Streptococcus mutans and the specific-plaque hypothesis. Crit Rev Oral Biol Med 2002;13:108-125.

[7] World Health Organization: WHO Traditional Medicine Strategy 2002–2005, Geneva: WHO; 2002.

[8] Maciel MAM, Pinto AC, Veiga Jr VF: Plantas medicinais: a necessidade de estudos multidisciplinares. Quím Nova 2002;25:429-438.

[9] Souza Brito ARM, Souza Brito A: A Forty years of brazilian medicinal plant research. J. Ethnopharmacol 1993;39:53-57.

[10] Bianco KG: Avaliação da atividade antimicrobiana de extratos vegetais de plantas da savana brasileira sobre bactérias cariogênicas e sua capacidade de desmineralização e a adesão à superfície de vidro. 2004. Dissertação (mestrado) – Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba.

[11] Ratter JA, Ribeiro JF, Bridgewater S: The Brazilian cerrado vegetation and threats to its biodiversity. Annals of Botany 1997;80:223-230.

Frente ao exposto, o presente estudo tem como objetivo elucidar o mecanismo de inibição do extrato da folha de *Psidium cattleianum* e avaliar seu efeito no biofilme formado *in situ* e na desmineralização do esmalte.

Essa tese está dividida em dois capítulos, conforme descrito abaixo:

- Capítulo 1: **"Effect of *Psidium cattleianum* leaf extract on *Streptococcus mutans* viability, protein expression and acid production"**, artigo aceito para publicação no periódico Caries Research*;

- Capítulo 2: **"*In situ* demonstration of the potential anticaries properties of *Psidium cattleianum*'s leaf extract"**, artigo preparado para envio ao periódico Caries Research*.

* Anexo 1

Capítulo 1: Effect of *Psidium cattleianum* leaf extract on *Streptococcus mutans* viability, protein expression and acid production

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Short title: Effect of *Psidium cattleianum* extract on *S. mutans*

Key words: antibacterial agents, plant extract, proteome, *Streptococcus mutans*

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Abstract

Plants naturally produce secondary metabolites that can be used as antimicrobials. The aim of this study was to assess the effects of *Psidium cattleianum* leaf extract on *Streptococcus mutans*. The extract (100%) was obtained by decoction of 100 g leaves in 600 ml deionized water. To assess killing, *S. mutans* biofilms were treated with water (negative control) or various extract dilutions (100, 50, 25% (v/v) in water) for 5 or 60 min. To evaluate the effect on protein expression biofilms were exposed to water or 1.6% (v/v) extract for 120 min, proteins were extracted and submitted to two-dimensional difference gel electrophoresis. Differentially expressed proteins were identified by mass spectrometry. The effect of 1.6% (v/v) extract on acid production was determined by pH measurements and compared to a water control. Viability was similar after 5 min treatment with the 100% extract or 60 min with 50% extract (about 0.03% survival). There were no differences in viability between biofilms exposed to 25 or 50% extract after 60 min treatment (about 0.02% survival). Treatment with 1.6% extract significantly changed protein expression. The abundance of 24 spots was decreased compared to water ($p < 0.05$). The extract significantly inhibited acid production ($p < 0.05$). It is concluded that *P. cattleianum* leaf extract kills *S. mutans* grown in biofilms when applied at high concentrations. At low concentrations it inhibits *S. mutans* acid production and reduces expression of proteins involved in general metabolism, glycolysis and lactic acid production.

Introduction

Dental caries is an infectious disease widespread in all regions of the world and considered by the World Health Organization (WHO) as a major public health problem [Petersen, 2003]. *Streptococcus mutans* plays an important role in dental caries due to its special characteristics. It is able to metabolize a wide variety of carbohydrates and to synthesize all of its required amino acids [Ajdic et al., 2002]. Furthermore, it is able to produce large amounts of lactic acid and to tolerate environment adversities such as extremes in sugar concentration and pH [Hamada and Slade, 1980]. These properties make it one of the most important caries causing bacteria [Ajdic et al., 2002]. Studying the effect that an antimicrobial compound has on *S. mutans* can help to predict its efficacy in preventing dental caries.

Plants naturally produce substances in response to chemical or environmental factors, such as microbial invasion or ultraviolet radiation [Grayer and Harborne, 1994; Martini et al., 2004]. The use of natural products is a successful strategy for the discovery of new medicines since 25% of the modern medicines are made from plants that were first used traditionally [World Health Organization, 2002].

The study of natural products in caries prevention involves research on promising compounds such as propolis [Koo et al., 2002], teas [Matsumoto et al., 1999] and other plant extracts [Yanagida et al., 2000; Leitão et al, 2004].

Still, there are a lot of challenges to overcome, especially because of lack of good scientific evidence concerning the efficacy of these herbal preparations. Thus, the WHO stimulates the pharmacological evaluation of complementary and alternative medicine to create stronger evidence on the safety, efficacy and quality of these products and practices [World Health Organization, 2002]. A suitable way to elucidate these features is to study the mechanism of action of herbal preparations.

Psidium belongs to the Myrtaceae and is native to tropical America. Traditionally, plants from *Psidium* spp. are used to treat scurvy in Asia and Africa, to treat cough and pulmonary diseases in Bolivia and Egypt, as an anti-inflammatory and hemostatic agent in China and as an anti-diarrhea compound in Mexico [Lozoya et al., 1994; Jaiarj et al., 1999]. Recently, studies have demonstrated the antimicrobial activity of leaf extract from *Psidium* spp. against bacteria that causes diarrhea [Gnan and Demello, 1999; Tona et al., 1999; Arima and Danno, 2002; Voravuthikunchai et al., 2004]. However, activity against oral pathogens has not been studied yet.

With the help of proteomics it is possible to quantify many proteins simultaneously and to study the structure, function and the control of biological systems. It is also possible to evaluate the effect of stress conditions, such as antimicrobials, starvation, acid shock, etc. on living cells [Svensater et al., 2000; Welin et al., 2003; Len et al., 2004]. Analyzing these properties helps to clarify the therapeutic action of an antimicrobial on bacteria. Ways of increasing efficacy can also be found by optimizing the concentration, time and frequency of application or by combining this compound with other preventive measure.

The aim of this study was to evaluate the bacteriocidal effect of *P. cattleianum*'s leaf extract on *S. mutans* biofilms, to study the effect of the extract on *S. mutans*' protein expression and to assess its effect on *S. mutans'* acid production.

Materials and Methods

Extract preparation

Harvesting of *P. cattleianum* leaves was authorized by the Brazilian Environment and Natural Resources Institute (IBAMA, permit# 0129208).

Only healthy leaves were selected for the extract preparation. The leaves were dried at 37^{o}C for one week, after being washed in tap water and deionized water. The leaves were then ground to a fine powder. Aqueous extract was obtained by decoction in deionized water (100 g/600 mL) for 5 min at 100^{o}C and at 55^{o}C for an additional 1 h. The solution was then filter sterilized with 0.22 µm mixed cellulose esters membranes (Millipore; Billerica, USA). The extract was stored in dark bottles at -20^{o}C until further use.

Bacterial strain, growth conditions and culture medium

Streptococcus mutans UA159 was kept at -80^{o}C in 26% w/v glycerol. *S. mutans* was streaked on brain heart infusion (BHI) agar with 1.5% v/v bacto-agar (Becton, Dickinson and Company, Sparks, USA) and grown at 37^{o}C anaerobically (80% N_2, 10% CO_2 and 10% H_2) for 72 h to obtain separate colonies.

Antibacterial testing

Viability assays were done in duplicate in order to determine the antibacterial activity of the *P. cattleianum* extract. Water was used as control. One colony of *S. mutans* from the streak-plate was used to inoculate 25 mL of BHI and incubated

anaerobically overnight. A fresh inoculum was prepared by adding 200 µL of this culture (containing 2.4 x 10^9 ± 2.3 x 10^7 CFU/mL) to 10 mL half-strength BHI supplemented with 50 mM PIPES (1,4-piperazinediethanesulfonic acid, Sigma Chemical Co., St. Louis, USA) and 0.2% sucrose, adjusted to pH 7.0. Biofilms were grown anaerobically on pegs attached to the lids of microtiter/culture plates (Greiner Bio-One Gmbh, Frickenhausen, Germany) immersed in wells containing 200 µL/well of the fresh inoculum. After 8 h, the medium was refreshed and the biofilm was incubated anaerobically for an additional 16 h.

Next, the biofilm was washed in PBS (8.0 g NaCl, 0.2 g KCl, 1.0 g Na_2HPO_4 and 0.2 g KH_2PO_4 per liter, adjusted to pH 7.4) and treated with water or extract (undiluted (100%) and at 50% or 25% (v/v) of the undiluted extract) during 5 or 60 minutes. The biofilm was washed again in PBS and harvested by cutting off the peg, submerging it in 1 mL of cysteine peptone water (CPW; 8.5 g NaCl, 5 g Bacto yeast extract, 1 g Bacto peptone, 0.5 g cysteine HCl per liter, pH 7.3) and sonicating on ice in an ultrasonic vibra cell (VC130; Sonics & Materials Inc., Newtown, CT, USA) for 30 x 1 s; amplitude 40, 30 W.

The bacterial suspension was diluted in CPW, spiral plated on BHI agar and colonies were counted after incubation for 72 h at 37^{o}C in an anaerobic chamber. The results were expressed in percentage survival (CFU of treated sample/ average CFU of water group).

Determination of sub-lethal concentration

It was essential to estimate the concentration of the extract that was necessary to induce a detectable change in protein expression before conducting protein expression experiments. This concentration should not be too high (all cells are damaged to such an extent they can no longer produce proteins) and should not be too low (there is no effect on the cells, thus no change in protein expression). Because the killing curve of the extract was very steep (data not shown), we decided to determine the sublethal concentration of the extract and use this concentration for further experiments. To establish the sublethal concentration, which was defined as the highest concentration at which viability was not affected, viability assays were done in duplicate. Semi-defined medium (SDM) was used for this experiment and the protein expression experiment because the extract precipitated with BHI and other media. SDM consisted of an electrolyte solution (76 mM K_2HPO_4, 15 mM KH_2PO_4, 10

mM $(NH_4)_2SO_4$, 35 mM NaCl, 2 mM $MgSO_4.7H_2O$; pH 7.0), supplemented with filter-sterilized vitamins (0.04 mM nicotinic acid, 0.1 mM pyridoxine HCl, 0.01 mM pantothenic acid, 1 µM riboflavin, 0.3 µM thiamine HCl, and 0.05 µM D-biotin), amino acids (4 mM L-glutamic acid, 1 mM L-arginine HCl, 1.3 mM L-cysteine HCl, and 0.1 mM L-tryptophan), 0.3% (w/v) yeast extract, and 18 mM glucose [modified from Li et al., 2002]. Glucose was used instead of sucrose in these experiments because it is very difficult to harvest proteins from sucrose grown biofilm cells. One colony of *S. mutans* was used to inoculate 20 mL of a semi-defined medium and incubated anaerobically for 16 h. A fresh inoculum was prepared by adding 1 mL of this culture (containing $6.8.10^7 \pm 5.7.10^6$ CFU/mL) to 10 mL SDM. Biofilms were grown on the bottom of microtiter plates containing 200 µL/well of the fresh inoculum, to make them as similar as possible to the biofilms used for the protein expression experiment. The medium was refreshed after 24 h and treatment with water (control) or extract was done 30.5 h after the initial inoculation. Extract concentrations of 1.6% and 0.8% v/v (100% being the original extract obtained from the leaves) were selected on the basis of preliminary experiments. The biofilm was treated for 30, 60, 90 or 120 min by adding 10 µL of diluted extract to the medium on top of the biofilm. Next, the medium from each well was removed and the biofilm was re-suspended in 0.2 ml of CPW by pipetting vigorously and plated as described above. This experiment was repeated twice.

Biofilm formation and treatment for protein extraction

One colony of *S. mutans* was used to inoculate 20 mL of SDM and incubated anaerobically for 16 h. A fresh incoculum was prepared by adding 10 mL of this culture (containing $4.9 \times 10^7 \pm 1.5 \times 10^7$ CFU/ml) to 100 mL SDM. 90 mL inoculated medium was transferred to large polystyrene (Ø 14cm, Bibby Sterilin Ltd, Stone, Staffordshire, United Kingdom) plates to allow biofilm development and medium was refreshed after 24 h.

At 30.5 h after the initial inoculation 4.5 mL of extract (1.6% (v/v) final concentration) or water were added to the medium above the biofilm. After an additional 2 h, the biofilm cells were harvested with a 50 mL disposable pipette and centrifuged (5 min, 37°C, 3939*g*). The supernatant was poured off and the pellet was frozen with liquid nitrogen and stored at -70℃ until further analysis. This experiment was performed in triplicate.

2D-DIGE (two dimensional difference gel electrophoresis)[1]

Protein extraction and two dimensional difference gel electrophoresis were done as described before [Luppens and ten Cate, 2005]; pH range of the 18 cm-IEF-strips was 4-7 and run time was adjusted accordingly, IEF-strips were equilibrated two times and in the second equilibration step DTT was replaced by 4.5% iodoacetamide. For all samples the same amount of protein was loaded on the strips and each protein sample was run in duplicate. Gel analysis was performed using DeCyder 6.5 software (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Only proteins detected in all gels were analyzed. Spots with a volume smaller than 10^4 or bigger than 10^9 were excluded. The ratio for each spot was determined by dividing the standardized spot abundance of the extract sample by the standardized spot abundance of the corresponding water sample. The mean ratio was determined by averaging the ratios for all samples.

Mass spectrometry[1]

For mass spectrometric identification of protein spots a sample containing 150 µg of unlabelled pooled standard was subjected to 2D-electrophoresis as described above. The gel was stained for 10 min with 200 ml 90°C Coomassie R-350 solution (1 PhastGel Blue tablet per 1.6 L 10% acetic acid) and destained for 2 h with 10% acetic acid. Protein spots of interest from the stained 2D gels were cut out using a sterile scalpel and digested with trypsin by the in-gel method of Shevchenko et al. [1996].

The collected eluates of the extracted peptides from each sample were dried in vacuo and redissolved in 8 µL 50% acetonitrile in 2% formic acid. For identification MS/MS data was acquired with a QTof1 (Waters, Manchester, UK) coupled with an Ultimate nano LC system (LC Packings Dionex, Sunnyvale, CA, USA). The peptides of each sample were separated on a nano-analytical column (75 µm i.d. X 15 cm C18 PepMap, LC Packings, Dionex) with a gradient of 1-50% acetonitrile in 0.1% formic acid. The flow of 300 nl/min was directly electrosprayed in the QTof1 operating in data-dependent MS and MS/MS mode. The Masslynx software (Waters, Manchester, UK) for the data acquisition was also used for the processing of the low CID MS/MS spectra. All MSMS spectra were used for a data search in MASCOT against the total

[1] Anexos 2 e 3

MSDB database with the fixed modification of carbamidomethyl for cysteine and a variable modification of oxidized methionine. All identifications were above the calculated threshold of MASCOT which was for these settings typically around a score of 60. See for further details http://www.matrixscience.com.

Effect of the extract on acid formation

Because of spectral interference of the extract it was not possible to measure lactic acid concentrations directly. To determine the effect of the plant extract on the acid formation of *S. mutans* an overnight culture was prepared by adding *S. mutans* UA159 to 25 mL BHI and incubated overnight at 37ºC under anaerobic conditions. The overnight culture was centrifuged for 10 min at 3939g at 4ºC and the pellet was resuspended in 5 mL CPW, followed by another centrifugation step after which the pellet was resuspended in 20 mL CPW. Subsequently the suspension was divided in two 10 mL portions and pre-incubated for 5 min at 37ºC. To these suspensions either 160 µL plant extract (final concentration 1.6%) or MilliQ water was added. The pH was adjusted to approximately pH 6.9 with 0.5 M NaOH. To start the acid production, 100 µL 20% (w/v) glucose was added to both suspensions which were incubated at 37ºC. The acid production was monitored for 45 min using a pH meter. Three replicate experiments were done.

Statistical Analysis

The null hypothesis tested was that treatment with *P. cattleianum* leaf extract does not change protein expression, viability or acid production of *S. mutans*. The significance limit was set at 5%.

For antibacterial testing and determination of sub-lethal concentration, GMC software was used [Campos, 2003]. First, normality and homogenicity of the samples were tested using, respectively, the Kolmogorov-Smirnov's test and Cochran's test. Data from antibacterial testing presented non-normal and non-homogeneous distribution. Thus, a Kruskal-Wallis test was carried out at each data point. For determination of sub-lethal concentration, changes among the groups at each data point were tested with one-way ANOVA.

The effect of plant extract on acid production by *S. mutans* was tested by comparing the pH at 45 min using a t-test (SPSS 14.0 software).

Statistical analysis for protein expression was done using the tool from the DeCyder 6.5 software. Multiple comparison between gels were done with unpaired one-way-ANOVA. False discovery rate was applied. Next, each differentially expressed spot was observed graphically in the Protein Table mode of the Biological Variation Analysis Module to elucidate whether they were up- or downregulated and from which group they originated.

Results

P. cattleianum leaf extract had an antibacterial effect on *S. mutans* biofilms (Figure 1). It can be observed that a larger number of bacteria were killed after 60 min than after 5 min exposure for each concentration used. There were no differences in survival after treatment with 25 or 50% (v/v) extract. Cell survival was similar after 5 min treatment with the undiluted extract or 60 min treatment with 50% (v/v) extract.

P. cattleianum leaf extract significantly ($p = 0.032$ at 45 min) inhibited acid production by *S. mutans* at a low (1.6%) concentration (Figure 2). The acidification of the medium was less in the presence of the extract than in its absence.

Several concentrations of the extract were tested regarding their effect on the viability of *S. mutans* biofilm cells (data not shown). The results for the two highest concentrations of extract that did not reduce *S. mutans* viability after 2 h treatment are shown in Figure 3. There were no statistical differences on cell survival after these treatments (ANOVA, $p<0.05$). On the basis of these results and a trial 2D-experiment (data not shown) 1.6% (v/v) extract was chosen for the study on the effect of the extract on protein expression in *S. mutans*.

<Insert Figure 1>
<Insert Figure 2>
<Insert Figure 3>

The abundance of 24 out of 683 spots decreased after exposure to the extract and for none of the spots did it increase in comparison with water-treated bacteria. From 22 spots of these, 24 proteins could be identified by mass spectrometry (Table

1)[1]. The other two proteins could not be identified because they were not visible on the Coomassie stained gel owing to their small volume.

The downregulated proteins RpdA, Fus and GroEL were each found in two separate spots and two spots contained two proteins (marked in the table by the same symbol). One of the latter spots contained lactate dehydrogenase (ldh) and the mannose specific PTS component IIAB (manL). Judging from the gel image (which contained one very large and one small peak very close to each other), the knowledge that ldh is a highly abundant protein in *S. mutans* [Nakano et al., 2005], and the mass-spectrometry data (with 18 peptide hits for ldh and 3 for manL), we assume that the majority of the spot volume and the decrease in spot volume could be attributed to lactate dehydrogenase. The downregulated proteins are mostly involved in carbohydrate metabolism, amino acid metabolism, nucleotide metabolism, lipid metabolism and translation.

<Insert Table 1>

Discussion

In this study we show that *P. cattleianum* extract has an antibacterial effect on *S. mutans*. Thus, in addition to treatment of intestinal infections, it may be used as herbal medicine for the prevention of dental caries.

The compounds with antibacterial activity known to be present in *P. cattleianum* are all phenolic compounds. Three of them are flavonoids (kaempferol, quercetin and cyanidin) and one of them is tannin (ellagic acid) [National Genetic Resources Program, 2005]. One of the mechanisms considered responsible for phenolic toxicity to microorganisms is enzyme inhibition by the oxidized form of the phenolic compound [Mason and Wasserman, 1987]. Cranberry juice, which contains some of the same polyphenols as *P. cattleianum*, inhibits glucosyltransferase activity of *S. mutans* [Koo et al., 2006]. Oolong green teas are known to contain polyphenols, which inhibit glucosyltransferase production in *S. mutans* [Matsumoto et al., 1999]. In our experiments glucosyltransfereses could not be detected since they are extracellular enzymes.

[1] Anexo 4

Flavonoids are secondary metabolites naturally synthesized by plants as a reaction to microbial infection [Dixon et al., 1983]. Their activity is probably due to their ability to form complexes with extracellular and soluble proteins and with bacterial cell walls [Cowan, 1999]. Kaul et al. [1985] provided a summary of the activities and modes of action of several types of flavonoids and showed that quercetin is universally effective in reducing infectivity of viruses. Martini et al. [2004] studied the antibacterial activity of seven flavonoids isolated from *Combretum erythrophyllum*, including kaempferol and quercetin, against Gram positive and Gram negative bacteria. However, the results of the individual compounds were not as good as the complete extract [Martini and Eloff, 1998], suggesting that there may be a synergistic effect between these compounds.

Studies reviewed by Scalbert [1991], show that tannins are active against filamentous fungi, yeasts, and bacteria. Tannins have the ability to inactivate proteins by forming irreversible complexes that may lead to loss of function of microbial adhesins, enzymes, cell envelope transport proteins [Cowan, 1999].

From the data above, the combination of the tannin and flavonoid compounds that are present in *P. cattleianum* is expected to have a general inhibiting and killing effect on bacteria, and to inhibit the expression and activity of certain enzymes. The present study corroborates with these findings. At high concentrations the extract has a bactericidal effect on *S. mutans* and at low concentrations it inhibits expression of certain proteins involved in general metabolism.

Surprisingly, for all differentially expressed proteins the abundance was lower in the extract exposed cells than in the control. In cells exposed to the extract a significant reduction in the abundance of proteins essential for RNA synthesis and protein synthesis was found. The most interesting effect of the extract on *S. mutans* was the reduction in expression of seven proteins involved in carbohydrate metabolism. Ajdic et al. [2002] identified the presence of the genes coding for the complete glycolytic pathway in *S. mutans* UA159 and showed that carbohydrate metabolism is their main energy supply. Of the seven proteins mentioned above, five are enzymes that are essential for glycolysis and lactic acid production by *S. mutans*, and thus for the development of dental caries.

These proteomics results suggest that besides the bactericidal effect at high concentrations, at low concentration the extract decreases lactate production by *S. mutans*. The inhibition of *S. mutans'* acid production by the extract, which was shown

in the current study, supports this hypothesis, since lactic acid is the major acid that is produced by *S. mutans*. Kashket et al. [1985] and Leitão et al. [2004] support these findings by showing that natural products have the ability to interfere with lactic acid production of cariogenic microorganisms.

The mechanism of action disclosed by the proteome analysis and the measurement of acid production using sub-lethal concentrations of the extract should not be considered definitive. Our results give more insight in the effect of the extract on *S. mutans* at low concentration but not on the mechanism of bacterial killing that is observed at high concentrations.

In conclusion, the results of the present study show that *P. cattleianum* leaf extract can kill *S. mutans* grown in biofims at high concentrations and that at low concentrations, it has a general effect on *S. mutans* metabolism. Of particular interest is the downregulation of enzymes involved in lactic acid production and the inhibiting effect of the extract on acid production. This makes the extract an interesting candidate for use in the prevention and treatment of dental caries. The extract will be tested further in a clinical study that is under way at the moment.

Acknowledgements

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Legends

Table 1: Proteins and their corresponding genes downregulated in *S. mutans* biofilms exposed to *P. cattleianum* leaf extract (1.6% relative concentration) for 2 h.

Figure 1: Effect of different concentrations of *P. cattleianum* leaf extract on viability of *S. mutans* biofilms after 5 (circles) or 60 (squares) minutes exposure (averages for duplicates samples $\pm$ standards errors). Distinct letters show statistical difference between treatment groups (Kruskal-Wallis, $p < 0.05$). [% survival = 100(survival extract treated sample/survival water-treated sample)].

Figure 2: Effect of the presence of 1.6% *P. cattleianum* leaf extract (triangles) or water control (squares) on acidification by *S. mutans*.

Figure 3: Viability of *S. mutans* biofilm cells after exposure to water and two sub-lethal concentrations (% of undiluted extract) of the extract in time. Error bars represent the standard error.

Table 1:

Protein[1]	Gene[2]	Gene name[3]	Ratio (mean)[4]	p value[5]
Amino acid metabolism				
D-alanine-D-alanyl carrier protein ligase	SMU.1691c	*dltA*	-1.6	0.025
S-adenosylmethionine synthetase	SMU.1573c	*metK*	-1.5	0.014
Threonine dehydratase	SMU.234	*ilva*	-1.6	0.005
Carbohydrate metabolism				
6-phosphofructokinase	SMU.1191c	*pfk*	-1.5	0.020
Fructose-bisphosphate aldolase	SMU.99	*sorG*	-1.6	0.008
L-lactate dehydrogenase, fructose-1,6-diphosphate dependent‡	SMU.1115	*ldh*	-1.5	0.037
Phosphoglycerate kinase	SMU.361	*pgk*	-1.5	0.040
Putative dTDP-glucose-4,6-dehydratase	SMU.1457c	*rmlB*	-1.3	0.045
Putative enolase	SMU.1247	*eno*	-1.4	0.022
Putative PTS system, mannose-specific component IIAB‡	SMU.1877	*manL*	-1.5	0.037
Lipid metabolism				
Putative trans-2-enoyl-ACP reductase	SMU.1742c	*fabK*	-1.6	0.011
Nucleotide metabolism				
Adenylate kinase†	SMU.2005c	*adk*	-1.5	0.006
CTP synthetase (UTP-ammonia lyase)	SMU.97	*pyrG*	-1.4	0.042
DNA-directed RNA polymerase alpha subunit	SMU.2001c	*rpoA*	-1.4	0.022
Orotate phosphoribosyltransferase	SMU.1221c	*pyrE*	-1.3	0.035
Pyrimidine regulatory protein PyrR†	SMU.856	*pyrR*	-1.5	0.006
Translation				
30S ribosomal protein S1	SMU.1200c	*rpsA*	-1.6	0.006
30S ribosomal protein S1	SMU.1200c	*rpsA*	-1.6	0.013
Elongation factor EF-2	SMU.359	*fus*	-1.6	0.037
Elongation factor EF-2	SMU.359	*fus*	-1.7	0.016
Other				
chaperonin GroEL	SMU.1954c	*GroEL*	-1.7	0.011
chaperonin GroEL	SMU.1954c	*GroEL*	-1.4	0.049
Hypothetical protein SMU.1760c	SMU.1760c	-	-1.3	0.040
Hypothetical protein SMU.229	SMU.229c	*ip*	-1.5	0.039

[1]When a spot contained two proteins, both proteins are marked by the same symbol.

[2]http://www.genome.ad.jp/kegg/

[3]http://www.oralgen.lanl.gov/

[4](Standardized abundance water treated sample)/(standardized abundance extract treated sample)

[5]ANOVA

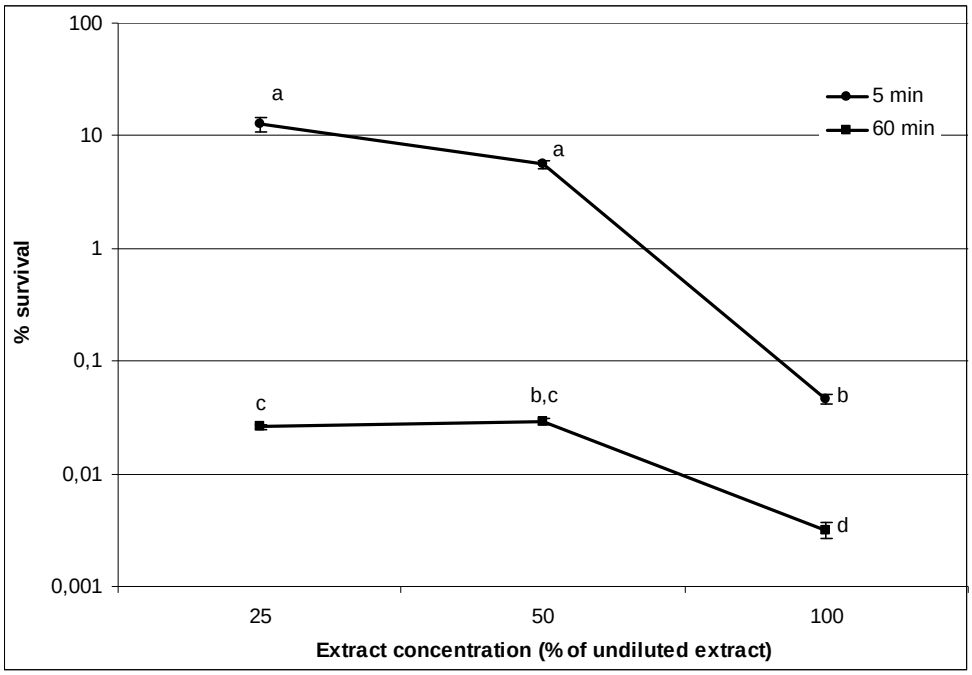

Figure 1

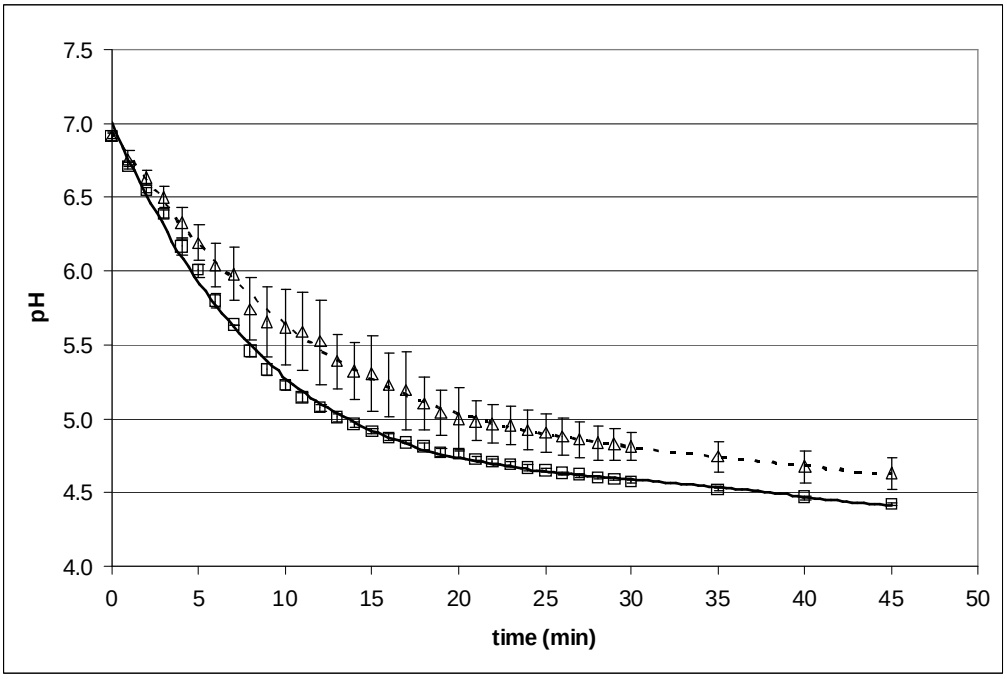

Figure 2

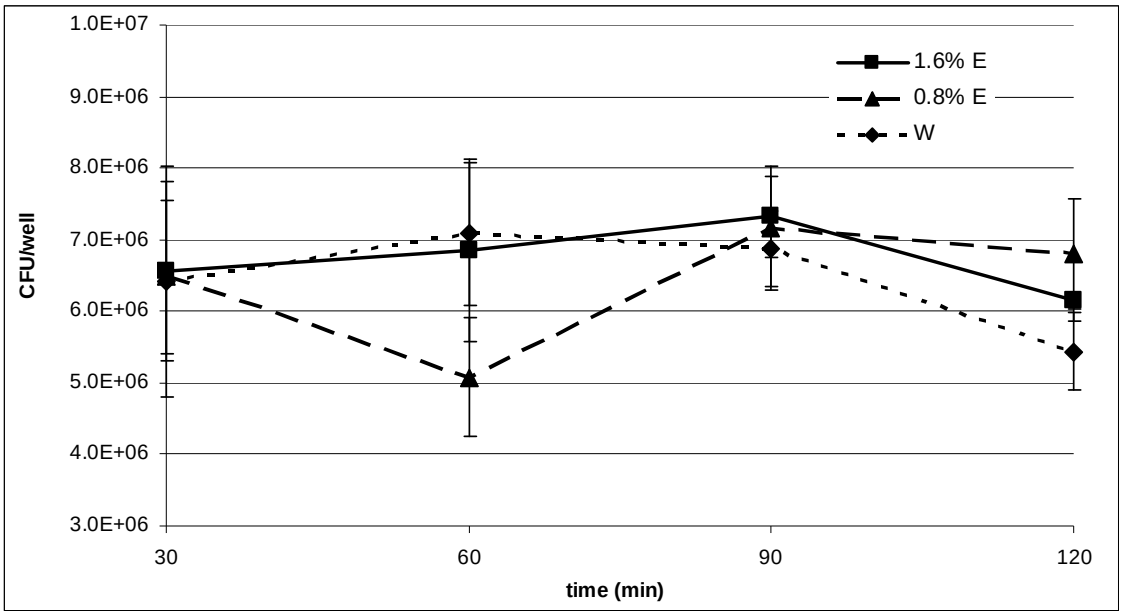
1.0E+07
9.0E+06
8.0E+06
7.0E+06
6.0E+06
5.0E+06
4.0E+06
3.0E+06
CFU/well
30
60
90
120
time (min)
1.6% E
0.8% E
W

Figure 3

Capítulo 2: *In situ* demonstration of the potential anticaries properties of *Psidium cattleianum*'s leaf extract

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Short title: Effect of *Psidium cattleianum* extract on dental biofilm

Key words: antibacterial agents, dental caries – prevention and control, plant extract

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Abstract

Natural products have been studied with relation to chemical control of dental biofilm. The aim of this *in situ* study was to evaluate the effect of *P. cattleianum*'s leaf extract on enamel demineralization and to assess its effect on biochemical and biological composition. Extract was obtained by decoction in deionized water. Enamel bovine blocks were selected on basis of their surface microhardness (SMH). The study was divided in three experimental phases according to the treatment solution: water, extract and a marketed mouthwash (positive control). A washout period of one week was allowed between each phase. Ten volunteers wore palatal appliances containing the enamel blocks during 14 days and dripped 20% sucrose (eight times/day) and the treatment solution (twice/day) outside the mouth. On the 12th and 13th days of the experiment dental plaque pH was measured with a microelectrode. On the 14th day, biofilms were harvested and analyzed with regard to total anaerobic microorganism (TM), total streptococci (TS) or mutans streptococci (MS) and extracellular polysaccharides in dental biofilm. Percentage change of SMH (%SMH) and integrated area were also analyzed. In comparison to water, the extract showed a smaller pH drop and a reduction on TM, TS and MS and on the amount of carbohydrates in dental biofilm. %SMH and integrated area showed a decrease in enamel demineralization by the extract when compared to water. In conclusion, *P. cattleianum* leaf extract reduced enamel demineralization, sucrose fermentation, microorganism accumulation and extracellular polysaccharides production of dental biofilm developed *in situ*.

Introduction

The factors associated to the development of dental caries are related to the teeth, the oral microflora and the substrate [Keys, 1969]. The infectious-contagious nature of dental caries implies on the adoption of several measures to control dental biofilm trough use of antimicrobial agents [Köhler et al., 1983]. Moreover, microbiological control associated to restorative treatment is fundamental to reestablish the oral health of the patient [Zickert et al., 1987].

Natural products have been studied with relation to chemical control of dental biofilm. Teas, propolis and other extracts [Koo et al., 2002; Smullen et al., 2007] have demonstrated efficacy against bacteria that causes dental decay.

Once therapeutic properties of medicinal plants are still based on empiric knowledge, the World Health Organization stimulates the pharmacological evaluation of phytotherapic drugs [World Health Organization, 2002]. Despite the stimuli and the current interest on medicinal use, less than 15% of medicinal plants potential from tropical area was studied [Plottikin, 1991].

Psidium cattleianum is commonly known as strawberry guava. It belongs to Myrtaceae family and is native to tropical America. Studies have shown that the leaf extract of plants from this family, such as *P. guajava* is able to reduce gingivitis and bad odour [Jaiarj et al., 2000]. Previous study from our research group has shown that *P. catteianum* leaf extract is able to inhibit the expression of proteins involved in general metabolism, especially those involved in carbohydrate metabolism of *S. mutans* biofilms [Brighenti et al., 2008]. However, the effect of these extracts on prevention of dental decay has not been evaluated.

As an intermediate stage between *in vitro* and *in vivo* studies, *in situ* experiments allow the exploration of laboratorial findings in a situation that mimics the development of natural caries and yet use sensitive detection methods [Zero, 1995]. The aim of this study was to evaluate the effect of *P. cattleianum*'s leaf extract on enamel demineralization and to assess its effect on biochemical and biological composition using an *in situ* dental caries model.

Material and Methods

Extract preparation

Harvesting of *Psidium cattleianum* leaves was authorized by the Brazilian Environment and Natural Resources Institute (IBAMA, permit# 0129208).

Only healthy leaves were selected for the extract preparation. The leaves were dried at 37℃ during one week after being washed in tap water and deionized water. The leaves were then grinded until a thin powder was achieved. Aqueous extract was obtained by decoction in deionized water (100g / 600mL) for 5 min at 100℃ and at 55℃ for an additional 1 h. The solution was then filter sterilized with 0.22 µm mixed cellulose esters membranes (Millipore™, Billerica, USA). The extract was stored in dark bottles at -20℃ until further use [Brighenti et al., 2008].

Enamel blocks preparation and analysis[1]

Enamel blocks measuring 4 x 4 mm were obtained from bovine incisor teeth previously stored in 2% formaldehyde solution (pH 7.0) for one month [White and Featherstone, 1987], and had their surface serially polished. Surface (SMH) and cross-sectional enamel microhardnessess measurements were made using a Shimadzu HMV-2000 microhardness tester (Shimadzu Corp., Kyoto, Japan). For baseline SMH (SMH_1), five indentations spaced 100 µm from each other were made (25 g load, 10 s) in the center of the enamel block, as described by Vieira et al. [2005]. The blocks used in the present study had their SMH_1 between 360 and 398 KHN.

After the experimental phase, SMH was again measured (SMH_2). Five indentations spaced 100 µm from each other and from the baseline were made. The percentage change of SMH (%SMH) was calculated [$\%SMH = 100(SMH_2 - SMH_1)/SMH_1$].

To perform cross-sectional microhardness tests, the blocks were longitudinally sectioned through the center. One of the halves was embedded in acrylic resin with the cut face exposed and gradually polished.

Three rows of nine indentations spaced 100 µm from each other were made at different distances (10, 30, 50, 70, 90, 110, 170, 220 and 330 µm) from the outer enamel surface under a 25g load for 10s. Lesion depth was measured from enamel surface up to the distance where hardness represented 95% of the value for sound enamel. The mean values from all three measuring points at each distance from the surface were then averaged and integrated area (KHN x µm) was calculated until a depth of 170 µm [Ögaard et al., 1988; Arends and Bosch, 1992; Sullivan et al., 1995].

[1] Anexos 5, 6 e 7

In situ experiment[1]

This study was previously approved by the Human Ethical Committee (protocol # 2005-02188). Ten healthy volunteers aged 23-34 years were selected. One week before the experiment began and during the whole experiment, the panels used non fluoridated dentifrice.

The crossover *in situ* experiment comprised of three phases of 14 days each. The volunteers wore palatal appliances containing four enamel bovine blocks covered by a plastic mesh to enable biofilm accumulation and to protect it from disturbance [Cury et al., 2000]. 1 mm of space was left between the enamel and the plastic mesh.

Eight times a day, the volunteers removed the palatal appliance from the mouth and dripped 20% sucrose in the enamel blocks for 5 minutes. Twice a day, deionized water (negative control), extract or Original Listerine Antiseptic™ (Johnson & Johnson, New Brunswick NJ, USA - positive control) were dripped 1 min after sucrose application and the appliance was replaced in the mouth 4 min later. A washout period of seven days was allowed between each phase. The volunteers received oral and written instruction to wear the appliance all the time and to remove it during meals or oral hygiene. They were not allowed to use any antimicrobial or fluoride product during the experiment.

Assessment of biofilm acidogenicity[1]

pH measurements were done according to Pecharki et al. [2005] using a palladium microelectrode (Beetrode™ NMPH3, World Precision Instruments Inc., Sarasota, FL, USA) with biofilms at overnight fasting to assure that no bacterial carbohydrate was stored [Denepitiya and Kleinberg, 1984]. 3 mol L^{-1} KCl was used to create a salt bridge between the reference electrode and the volunteer's finger [Lingström and Birkhed, 1993].

After baseline measurement (pH_1), treatment with sucrose was done as previously described and new pH measurements were done after 5 min (pH_2). Next, pH variation was calculated ($\Delta pH = pH_1 - pH_2$). To detect whether the pH variation was due to a structural change in the biofilm or due to the presence of the treatment solutions, pH measurements were done in two different days: on the 12th day, only

[1] Anexos 8 a 11

sucrose was applied and on the 13th day, sucrose and the treatment solutions were applied.

Analysis of dental biofilm composition[1]

At the end of the experiment, the volunteers returned the palatal appliances. The plastic mesh was removed and biofilms were harvested and weighed. Around 5 mg of the biofilms were re-suspended in phosphate saline buffer ("PBS": 8.0 g NaCl, 0.2 g KCl, 1.0 g Na_2HPO_4 and 0.2 g KH_2PO_4 per liter, adjusted to pH 7.4; 1mL / mg of biofilm) and sonicated on ice in an ultrasonic cell disruptor (XL; Misonix Inc., Farmingdale, NY, USA) for 6 x 9.9 s; amplitude 40 W [Bowen et al. 1986]. The suspensions were diluted in PBS and plated in duplicate in Brain Heart Infusion agar, Mitis Salivarius agar or Mitis Salivarius Sucrose Bacitracin agar to analyze, respectively: total anaerobic microorganism (TM), total streptococci (TS) or mutans streptococci (MS) [Gold et al., 1973]. The presence of TS and MS was confirmed by Gram staining. Colony forming units (CFU) were counted after incubation for 72 h at 37^{o}C in an anaerobic chamber. The results were expressed in CFU/mg wet weight.

The remaining biofilm was dried with phosphorus pentoxide (Vetec Química Fina Ltda., Duque de Caxias, RJ, Brazil) for 12 h at room temperature. Insoluble extracellular polysaccharides (EPS) were extracted by adding 1.0 mol L^{-1} NaOH (10μL/ mg dry weight) to the biofilm. The samples were vortexed for 1 min and after 3 h under agitation at room temperature, they were centrifuged (1 min, 11,000*g* at room temperature) [Nobre dos Santos et al., 2002]. Supernatants were precipitated with 75% cooled ethanol overnight, centrifuged and re-suspended in 1.0 mol L^{-1} NaOH [Ccahuana-Vásquez et. al, 2007]. Carbohydrate analysis was done by the phenol-sulfuric acid procedure [Dubois, 1956]. The results were expressed as μg/mg dry weight.

Statistical analysis

The null hypothesis tested was that treatment with *P. cattleianum* leaf extract does not change the parameters analyzed. Statistical analysis was carried out using GraphPad Prism Version 3.02 (GraphPad Software Inc., San Diego, CA, USA). Data from %SMH, integrated area, EPS and ΔpH showed equality of variances (Bartlett's

[1] Anexos 12 e 13

test) and normal distribution (Kolmogorov-Smirnov's test) and were submitted to analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test. Data from microorganism counts (TM, TS and MS) and differences of ΔpH between the 12th and 13th day were analyzed with two-tailed unpaired *t* test. The significance limit was set at 5%.

Results

P. cattleianum leaf extract had an antibacterial effect on microorganisms counts (Figure 1). It can be observed that a larger number of bacteria were killed after treatment with the extract than after treatment with the positive control. There were no differences on mutans streptococci survival after treatment with water or the positive control ($p<0.05$).

<insert Figure 1>

P. cattleianum's leaf extract and the positive control reduced surface demineralization (%SMH) and lesion depth (integrated area) when compared to the water group. In addition, the extract showed a better performance than the positive control ($p<0.05$) (Figure 2).

<insert Figure 2>

When compared to water, ΔpH was lower in the extract and positive control on both measurements (12th and 13th days). However, there were no differences on pH drop in the two situations investigated (sucrose only or sucrose + treatment) ($p<0.05$) (Figure 3). The use of the extract also decreased the amount of extracellular polysaccharides when compared to the positive or negative controls. No differences in EPS were observed after treatment with water or the positive control ($p<0.05$).

<insert Figure 3>

Discussion

The studies of natural products against oral pathogens available to date attempt mostly to verify *in vitro* their antibacterial properties and mechanism of action [Yanagida et al., 2000; Duarte et al., 2006; Percival et al., 2006; Smullen et al., 2007]. Plants from *Psidium* spp. are traditionally used to treat several diseases worldwide [Jaiarj et al., 1999] and the antibacterial effect of these plants have been tested against bacteria that causes diarrhea [Gnan and Demello, 1999; Voravuthikunchai et al., 2004]. The evaluation of dental products using *in situ* caries models helps to presume clinical outcomes and allows the evaluation of many features from dental biofilm and hard tissues. Still, there are no data of the effect of natural products using the *in situ* caries model employed in this study.

The pH measurement carried out in the present study can be considered an indirect measure of acid production. The results observed in this *in situ* experiment corroborate with previous *in vitro* observations. Brighenti et al. [2008] showed a decrease on *S. mutans* proteins from carbohydrate metabolism and a reduction on pH drop after the exposure to the extract.

On the other hand, the decrease in enamel demineralization observed in the extract group can be attributed not only to the inhibition of these proteins but also to membrane-associated proteins, such as glycosyltransferases (GTF) [Duarte at al., 2006]. Thus, the decrease of extracellular polysaccharides formation observed after sucrose application is possibly related to inhibition of GTF activity in dental biofilm. A positive correlation was found between the amount of EPS formed and the presence of TM and TS but not to MS (r values 0.9016, 0.9967 and 0.4386 respectively). Besides the reduction of bacterial accumulation, the control of biofilm matrix formation found after the use of the extract may control the pathogenicity of dental biofilm by also changing its thickness and porosity [Dibdin and Shellis, 1988; Cury et al., 2000]. Together with the control of microorganisms, the control of polysaccharides accumulation is and important approach for the prevention of biofilm-associated diseases.

The data of this study demonstrate that the extract is also able to interfere on biofilm ecology. The number of total anaerobic microorganism, total streptococci and mutans streptococci were statistically reduced after the use of the extract. However, a weak correlation was found between MS and the parameters analyzed (%SMH, integrated area, EPS and ΔpH on 12^{th} and 13^{th} days - r values -0.2609, -0.1058,

0.4386, 0.1395 and 0.1712, respectively). These findings are in agreement to those in the literature [van Ruyven et al., 2000; Tenuta et al., 2003].

Independently of the role of specific microorganisms in dental caries development, it is clear that the extract affects dental biofilm properties, which is reflected in the decrease on enamel surface demineralization and lesion depth. The protective effect of the extract should not be attribute to only one factor but to the association of all features evaluated, leading to a shift in dental biofilm pathogenicity. It is important to state that the extract studied does not have considerably amounts of fluoride, calcium or phosphate on its composition (data not shown).

The positive control is composed by a combination of purified essential oils and has the American Dental Association seal of approval [Council on Dental Therapeutics, 1988]. It also contains cleaning agents, surfactants and preservatives that collaborate with inhibition of microorganism growth of the active compounds. Our findings corroborate with those in the literature only partially [Minah et al., 1989; Pan et al., 2000]. In the present study, the commercial mouthwash showed either poorer results in comparison to the extract or similar outcomes in comparison to the negative control. The fact that the extract used in the present study is a crude concentrate obtained by decoction of the leaves makes these findings even stronger. This may be due to synergistic effect between different compounds found in the plant extract, as previously described [Martini and Eloff 1998; Koo et al. 2003].

In conclusion, the results of the present study show that *P. cattleianum* leaf extract reduces enamel demineralization, acidogenic potential, microorganism accumulation and extracellular polysaccharides production of *in situ* dental biofilm. Having shown clearly the potential activity of *P. cattleianum*'s leaf extract to interfere in the biofilm pathogenicity, the aggregation of this plant extract into dental products may be an important approach to control biofilm-associated dental diseases and should be evaluated in the future.

Acknowledgements

The authors would like to express their gratitude to the volunteers for their valuable participation. We also thank Mario Luis da Silva and Maria dos Santos Fernandes for the laboratorial assistance in this study. This study was supported by, CAPES (DS and PDEE -4446/05), CNPq (471634/2007-7) and FAPESP (06/00726-8).

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Legends:

Figure 1: Viability of total anaerobic microorganism, total streptococci and mutans streptococci after exposure to different treatments (mean ± se, n=10). Distinct letters show statistical difference between treatment groups (unpaired t-test, $p < 0.05$).

Figure 2: Effect of different treatment groups on percentage change of surface microhardness (%SMH) and integrated area (KHN x µm) (mean ± se, n=10). Distinct letters show statistical difference between groups (ANOVA, $p < 0.05$).

Figure 3: Effect of different treatment solutions on alkali-soluble carbohydrates (µg /mg dry weight) and pH variation (ΔpH) (mean ± se, n=10). Distinct letters show statistical difference between treatment groups (ANOVA, $p < 0.05$) and between differences on the pH measurements of the 12^{th} and 13^{th} days (unpaired t-test, $p < 0.05$).

Figures:

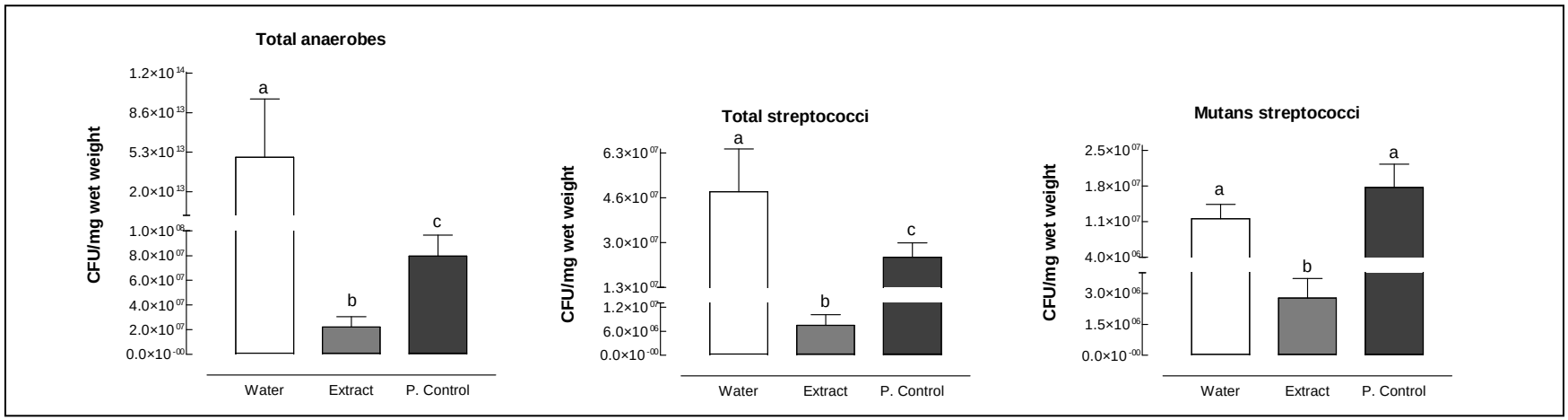

Figure 1

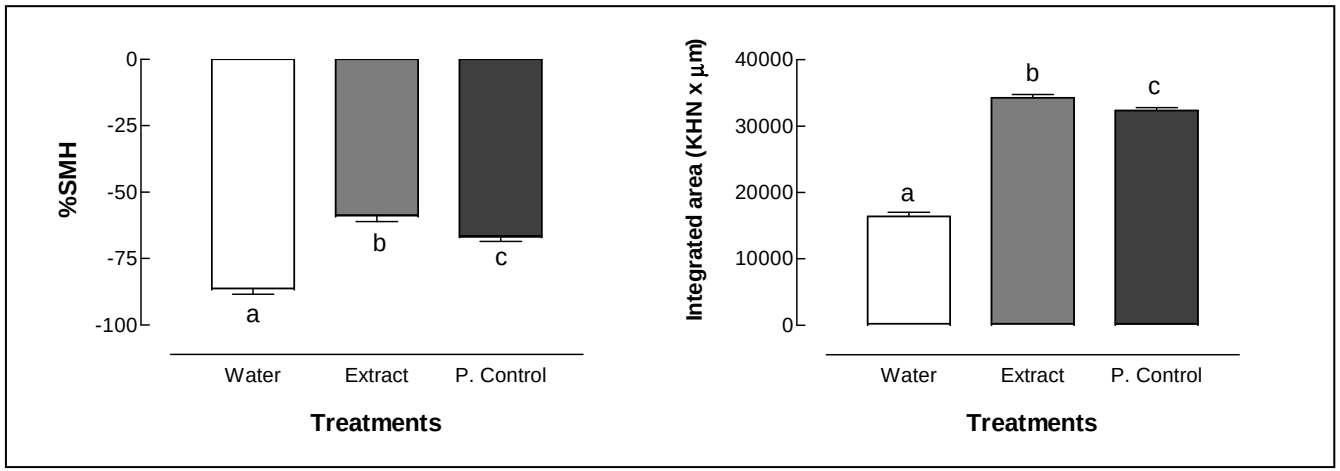

Figure 2

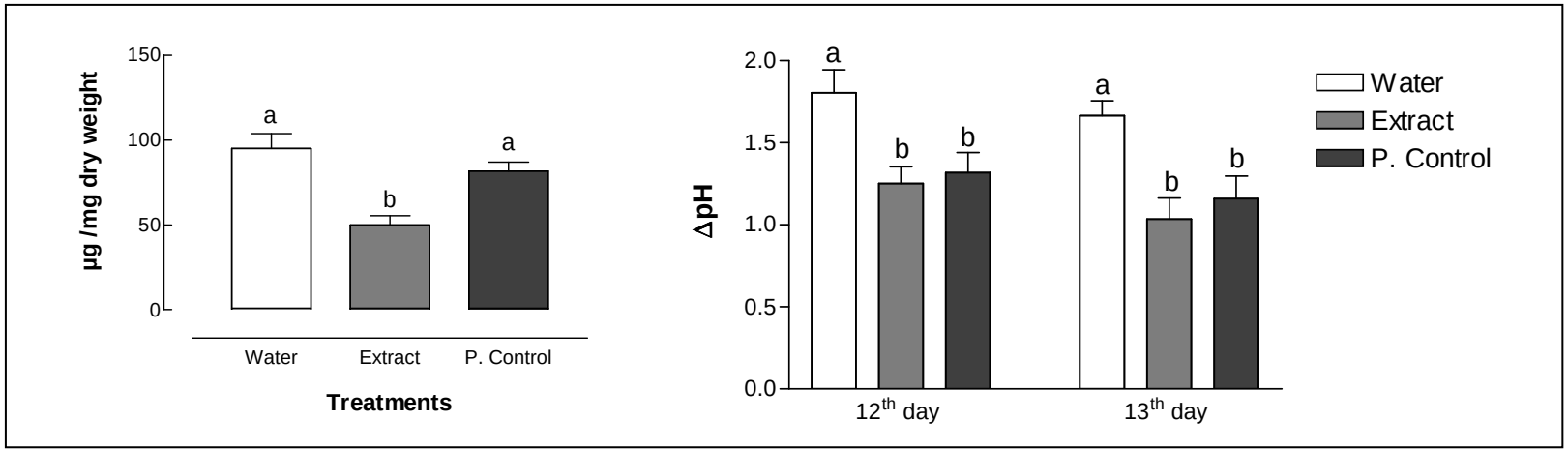
µg /mg dry weight
150
100
50
0
a
b
a
Water
Extract
P. Control
Treatments
ΔpH
2.0
1.5
1.0
0.5
0.0
a
b
b
a
b
b
12th day
13th day
Water
Extract
P. Control

Figure 3

Anexos

ANEXO 1: ORIENTAÇÃO AOS AUTORES – REVISTA CARIES RESEARCH

Aims and Scope

'Caries Research' is an international journal, the aim of which is to promote research in dental caries and related fields through publication of original research and critical evaluation of research findings. The journal will publish papers on the aetiology, pathogenesis, prevention and clinical control or management of dental caries. Papers on health outcomes related to dental caries are also of interest, as are papers on other disorders of dental hard tissues, such as dental erosion. Aspects of caries beyond the stage where the pulp ceases to be vital are outside the scope of the journal. The journal reviews papers dealing with natural products and other bacterial inhibitors against specific criteria, details of which are available from the Editor.

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Copies of any ‘in press’ papers cited in the manuscript must accompany the submission. Manuscripts reporting on clinical trials must be accompanied by the CONSORT checklist (see below).

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Types of Papers

Original papers or Short Communications are reports of original work (including systematic reviews and meta-analyses). Both have the structure outlined below but for Short Communications the abstract should be less than 100 words and the manuscript should not exceed 3 printed pages, equivalent to about 9 manuscript pages (including tables, illustrations and references). *Reviews* can have a freer format but should nevertheless commence with a Title page, an Abstract and an Introduction defining the scope.

Current topics are concise articles that present critical discussion of a topic of current interest, or a fresh look at a problem, and should aim to stimulate discussion. *Letters to the Editor*, commenting on recent papers in the journal, are published occasionally, together with a response from the authors of the paper concerned.

Preparation of Manuscripts

Text should be one-and-a-half-spaced, with wide margins. All pages should be numbered, starting from the title page. A conventional font, such as Times New Roman or Arial, should be used, with a font size of 11 or 12. Avoid using italics except for Linnaean names of organisms and names of genes.

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(b) Papers published only with DOI numbers: Theoharides TC, Boucher W, Spear K: Serum interleukin-6 reflects disease severity and osteoporosis in mastocytosis patients. Int Arch Allergy Immunol DOI: 10.1159/000063858.
(c) Monographs: Matthews DE, Farewell VT: Using and Understanding Medical Statistics. Basel, Karger, 1985.
(d) Edited books: DuBois RN: Cyclooxygenase-2 and colorectal cancer; in Dannenberg AJ, Dubois RN (eds): COX-2. Prog Exp Tum Res. Basel, Karger, 2003, vol 37, pp 124-137.
(e) Patents: Diggens AA, Ross JW: Determining ionic species electrochemically. UK Patent Application GB 2 064 131 A, 1980.
(f) World Wide Web: Chaplin M: Water structure and behavior. www.lsbu.ac.uk/water, 2004.

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ANEXO 2: PROTOCOLO PARA ELETROFORESE BIDIMENSIONAL[1]

PROTEIN EXTRACTION

1. Materials
   a) Sufficient amount of cells (> 50 µg wet weight)
   b) Gloves
   c) Blue and yellow pipet tips that have only been touched with gloves, they do not need to be sterile
   d) Eppendorf tubes that have only been touched with gloves, they do not need to be sterile
   e) Protease inhibitor
   f) Ice
   g) Cooled (4℃) centrifuge for eppendorf tubes;
   h) Scales
   i) -80℃ freezer
   j) Pre-cooled Eppendorf tube holder
   k) Beaker with water and eppendorf floater
   l) Sonicator
   m) Universal tubes with sterile demineralised water and 70% alcohol

2. Solutions that can be prepared in advance
   a) Lysis buffer

	Amount/40 ml
30 mM Tris (pH 8.0)	0.145 g
7 M Urea	16.800 g
2 M Thio-urea	6.080 g
4% (w/v) CHAPS	1.600 g
Add 2 µl concentrated HCl/ml to get about pH 8.0. Store at -20℃.	

3. General remarks and precautions:
   a) The sample should be prepared to completely solubilize, disaggregate, denaturate and reduce the proteins in the sample. This may be different for every sample.

[1] De acordo com o protocolo preconizado no Centro Acadêmico de Odontologia em Amsterdam (ACTA) e baseado em Ettan Dige: Users' Manual - Amersham Biosciences, 2003

b) Avoid repeated thawing of samples.
c) Concentration of salts and other charged small molecules in the sample should be maximum 10 mM for rehydration loading.
d) Pharmalyte and IPG buffer do not have to be counted as salts. They are similar substances.
e) SDS final concentration should be maximally 0.25%. The ratio of nonionic detergents (such as CHAPS, Triton X-100 or NP-40) to SDS should be at least 8:1
f) EPS can interfere with 1D, remove it by precipitation. Use Clean-up kit and respuspend in lysis buffer.
g) Nucleic acids can interfere with 1D, break them by sonication or remove them by using DNAse/RNAse or precipitation.
h) Maximum concentration of Pharmalyte allowed for our system is 0.5 %.

4. DIGE remarks and precautions:
   a) In the lysis buffer do not use amides or Pharmalytes. pH should be between 8 and 9. Use CHAPS instead of Triton. The latter is made in bulk and thus not very pure.
   b) Primary amino-acids in the medium interfere with fluorescent labelling, so first precipitate extracellular proteins before you label them.

5. Cell harvesting:
   a) Pre-weigh the tube that the sample will be in after harvesting
   b) Make the harvesting step as short as possible
   c) Take a bacterial sample. E.g. pour off the biofilm medium
   d) Centrifuge as short as possible (5 min 37°C 45000 rpm)
   e) Completely pour off the supernatant
   f) Freeze the pellet with liquid nitrogen

6. Protein extraction (perform maximally one week after cell harvesting):
   a) Defrost lysis buffer and protease inhibitor at room temperature. Put lysis buffer on ice after it is completely defrosted
   b) Take the tube with cell pellet from the -80°C freezer

c) Weigh the tube and calculate the wet weight of the cell pellet. Always keep the tubes on ice
d) Add about 5 µl of lysis buffer per 1 mg of wet weight
e) Resuspend the pellet by pipetting
f) Use 300-500 µl of sample for the next steps. Freeze the remaining sample (for possible later use) or discard into chemical waste.
g) Add 20 µl of protease inhibitor per ml lysis buffer added. Vortex briefly
h) Freeze for 30-40 min in at -20°C, defrost for 5 min in water at room temperature, vortex. Repeat 2 more times, defrost and put on ice
i) In fume hood: sonicate on ice water at 55-60% for 20 sec, stop for 1 min. Repeat 11 more times
j) Leave on ice for at least 15 min
k) Centrifuge for 10 min at 4 °C, 13000 r.p.m.
l) Distribute supernatant over several (4-5) Eppendorf tubes. One should contain about 20 µl, to be used for subsequent quantification (preferably the same or the next day).
m) Store at –80 °C for up to 3 months.

7. Protein determination using 2-D Quant Kit
   a) Prepare working color reagent by mixing 100 parts of color reagent A with one part of color reagent B. Each individual assay requires 1 ml working color reagent.
   b) Prepare standard curve (0–50 µg) using the 2 mg/ml BSA standard solution. Prepare microcentrifuge tubes (in duplicate) containing 1–50 µl of the sample to be assayed. The useful range of the assay is 0.5–50 µg.
   c) Add 500 µl precipitant to each microcentrifuge tube. Vortex and incubate 2–3 min at room temperature.
   d) Add 500 µl co-precipitant. Mix briefly.
   e) Centrifuge (at least 10 000 × g) for 5 min.
   f) Remove supernatant. Centrifuge briefly to bring remaining supernatant to bottom of tube. Remove remaining supernatant with micropipette. Proceed rapidly to avoid resuspension or dispersion of pellet. There should be no visible liquid remaining.

g) Add 100 µl copper solution and 400 µl distilled or deionized water to each tube. Vortex to dissolve the precipitated protein. Ensure that the pellet is completely resuspended by vortexing thoroughly.
h) Add 1 ml working color reagent to each tube. Ensure instantaneous mixing by introducing the reagent as rapidly as possible.
i) Incubate at room temperature for 15–20 min.
j) Read the absorbance at 480 nm.
k) Generate standard curve by plotting the absorbance of the standards against the quantity of protein and estimate protein concentration of samples by comparison to the standard curve. The recommended concentration of the protein sample is 5-10 mg/ml.

PROTEIN LABELLING

1. Materials
   a) Gloves
   b) Pipet tips that have only been touched with gloves, they do not need to be sterile
   c) Eppendorf tubes that have only been touched with gloves, they do not need to be sterile
   d) Ice
   e) Cy2, Cy3, Cy5
   f) DMF

2. Solutions that can be prepared in advance
   a) 10 mM L-Lysine (DIGE)

	Amount/10 ml
10 mM L-Lysine	0.0183 g
Make up to 10 ml with distilled water. Store at 4℃.	

3. Requirements for a cell lysis buffer

It is essential that the pH of the protein solution used with a CyDye DIGE Fluor minimal dye is between pH 8.0–9.0 by the careful addition of dilute sodium hydroxide 50 mM. Ensure that the pH remains between pH 8.0–9.0, by including a buffer such as Tris, HEPES or Bicarbonate in the protein solution. The buffer should be at a concentration of approximately 30 mM. Higher buffer concentrations may affect

isoelectric focusing. Failure to include a suitable buffer will mean that the pH of the solution may fall below pH 8.0 resulting in little or no protein labelling. The standard cell lysis buffer is required to work at +4℃ so the pH sh ould be checked when the solution is chilled.

Note: The protein solution should not contain any added primary amine compounds before labelling as these will compete with the protein for dye.

4. Calculating the amount of CyDye DIGE Fluor minimal dye required to label a protein lysate
   a) Reconstitute the stock CyDye DIGE Fluor minimal dye in dimethylformamide (DMF) to create a 1 nmol/µl dye stock solution
   b) For labelling, an aliquot of the CyDye DIGE Fluor minimal dye stock solution should be diluted to a concentration of 400 ρmol/µl.
   c) It is recommended that 400 ρmol of dye is used to label 50 µg of protein. If the ratio of dye:protein is too low, sensitivity may be decreased. If the ratio of dye:protein is too high, there is a possibility of multiple dye molecules per protein and this could lead to multiple spots per protein on the gel

5. Protein sample labelling
   a) Prepare of an internal standard by pooling the same amount of protein from all of the samples. Sufficient internal standard must be prepared to allow enough to be included on every gel in the experiment.
   b) Add a volume of protein sample equivalent to 50 µg to a microfuge tube.
   c) Add 1 µl of working dye solution to the microfuge tube containing the protein sample (i.e. 50 µg of protein is labeled with 400 ρmol of dye). Mix thoroughly by vortexing.
   d) Centrifuge briefly in a microcentrifuge to collect the solution at the bottom of the tube. Leave on ice for 30 min in the dark.
   e) Add 1 µl of 10 mM lysine to stop the reaction. Mix and spin briefly in a microcentrifuge. Leave for 10 min on ice, in the dark.

FIRST DIMENSION (ISOELECTRIC FOCUSING)

1. Materials
   a) IEF-aparatus and accessories (rehydration strip holder, strip holder, electrodes)
   b) Gloves
   c) Pipet tips that have only been touched with gloves, they do not need to be sterile
   d) Eppendorf tubes that have only been touched with gloves, they do not need to be sterile
   e) Ice
   f) Dry strip cover fluid

2. Solutions that can be prepared in advance
   a) Bromophenol blue stock solution

	Amount/10 ml
1% Bromophenol blue	100 mg
50 mM Tris-base	60 mg
Double distilled water	to 10 ml

   b) Rehydation stock solution

	Amount/25 ml (125 µl/7 cm strip)
8 M Urea	12 g
2% (w/v) CHAPS	0.5 g
0.002% Bromophenol blue	50 µl of stock
1% Pharmalyte	250 µl
Double distilled water	to 25 ml
Just before use add 0.2% DTT	5 mg per 2.5 ml

   c) 2x Sample buffer

	Amount/25 ml (sample volume)
2 M Thio-urea	3.8 g
7 M Urea	10.5 g
2% pH 3-10 Pharmalyte for IEF	0.5 ml
2% DTT	0.5 g
4% CHAPS	1.0 g

3. Overview
   a) Use damp electrode pads.
   b) Ensure the Immobiline™ DryStrip does not dry out.
   c) Ensure correct orientation of the strips and the strip holder on the Ettan IPGphor™ IEF unit.
   d) Use running conditions appropriate to the protein sample type/load and strip.
   e) Do not programme the Ettan IPGphor IEF unit to deliver more than 50 µA per Immobiline DryStrip.
   f) Protein samples, labeled with the different dyes, are mixed together so that they are focused on the same strip. This ensures that the protein samples, labeled with different dyes, are subject to exactly the same electrophoretic running conditions.

4. Immobiline DryStrip rehydration in the presence of protein sample

Immobiline DryStrips must be rehydrated prior to isoelectric focusing. For dilute protein samples, the in-gel rehydration method is more appropriate.

   a) Combine three differentially labeled samples into a single microfuge tube and mix. One of these samples should be the pooled internal standard.
   b) Add an equal volume of 2× sample buffer to the labeled protein samples and leave on ice for 15 minutes.
   c) For 18 cm strips, 350 µl of rehydration buffer is required. The total volume of labeled protein needs to be made up to the volume required for each strip using the rehydration buffer
   d) Add protease inhibitor to the samples (7µl/strip)
   e) Deliver the labeled protein solution slowly to the centre of the slot in the Immobiline DryStrip Reswelling Tray. Remove any large bubbles.
   f) Remove the protective cover from the strip.
   g) Position the strip with the gel side down and the pointed (acidic) end of the strip against the end of the slot closest to the spirit level and lower it onto the solution. Be sure to coat the entire strip and not to trap bubbles.
   h) Overlay each strip with 3ml PlusOne™ DryStrip Cover Fluid to prevent evaporation and urea crystallization.
   i) Slide the lid onto the re-swelling tray and allow the strips to rehydrate at room temperature overnight. Keep the samples in the dark.

5. Transferring the strip to the IPGphor Strip Holder
   a) Remove the strips from the re-swelling tray. Allow excess of cover fluid to run off.
   b) Place the strip gel side up with the basic end (flat end) flush with the flat end of the IPGphor Strip Holder.
   c) Place a damp electrode pad onto the acidic and basic ends of the gel.
   d) Clip down the electrodes firmly onto the electrode pads. Ensure that there is good contact with the strip and the metal on the outside of the strip holder.
   e) Apply at least 4 ml of cover fluid to the strip holder allowing the oil to spread so it completely covers the strip.
   f) Put the clear plastic strip cover onto the strip holder. The strip holder is now ready to load on the Ettan IPGphor IEF unit.
   g) Place the strip holders in the correct position on the IEF unit platform. The anodic (acidic) and cathodic (basic) plate areas have designated marks on them for the correct positioning of the strip holders with respect to the strip length.
   h) Cover apparatus to exclude light taking care not to cover the air vents
   i) For 18 cm strips, pH range 4-7, the following protocol should be used:

Step	Voltage (V)	Amperage	Time (h:min)	Volt-h (KVh)
1. step and hold	500	50 µA	1h	0.5
2. gradient	1000	50 µA	1h	0.8
3. gradient	8000	50 µA	3h	13.5
4. step and hold	8000	50 µA	Until it reaches 30KVh	

   j) Change electrode papers after 2h and after 3.5h.
   k) After the proteins have been focused, the strips can be stored at –70℃ in a sealed and rigid container.

SECOND DIMENSION

1. Materials for gel casting
   a) Gel caster and accessories (foam gasket, funnel, tubes, dummies)
   b) Gloves
   c) Pipet tips that have only been touched with gloves, they do not need to be sterile

d) Large graduated pipets

e) TEMED

2. Solutions that can be prepared in advance for gel casting

a) Bromophenol blue stock solution

	Amount/10 ml
1% Bromophenol blue	100 g
50 mM Tris-base	60 g
Double distilled water	to 10 ml

b) 10% SDS

	Amount/50 ml
10% SDS	5.0 g
Double distilled water	to 50 ml
Filter trough 0.45 µm filter, store at room temperature	

c) 4x Resolving gel buffer

	Amount/500 ml (25 ml/gel)
1.5 M Tris	90.85 g
Double distilled water	375 ml
HCl	adjust to pH 8.8
Double distilled water	to 500 ml
Filter trough 0.45 µm filter, store at 4°C	

3. Solutions that have to be prepared on the day itself

a) Water saturated iso-propanol/2-propanol

	Amount/1 ml (2 ml/gel)
2-propanol	0.3 ml
demi-water	0.7 ml

b) 10% Ammonium persulfate

	Amount/1 ml (5*1 ml for 7 gels)
10% Ammonium persulfate	0.1 g
Double distilled water	to 1 ml
Prepare just prior to use, make sure it makes a ‘crackling’ sound when you add the water	

c) Displacing solution

	Amount/100 ml
4× resolving buffer	25 ml
50% Glycerol 87%	57.5 ml
0.002% Bromophenol blue	200 µl
Double distilled water	17.3 ml

d) Gel recipe (12.5%) without monomer stock

	Amount/100 ml
Acrylamide	31.26 ml
N,N'-methylenebisacrylamide	16.67 ml
4× resolving gel buffer	25 ml
10% SDS solution	1 ml
MilliQ water	25.56 ml
Stirrer bar	
Dearate for 15 min at least	
Right before casting, add:	
10% ammonium persulfate	1 ml
TEMED	138 µl of a 10% fresh stock
Do not pour too much into the gel caster (pour the excess first in a graded cylinder)	

4. Materials for strip equilibration and gel electrophoresis
   a) Cast gels
   b) Gel electrophoresis apparatus and accessories (clean dummies)
   c) Gloves
   d) Blue and yellow pipet tips that have only been touched with gloves, they do not need to be sterile
   e) Large disposable graduated pipets
   f) Water bath
   g) Molecular weight markers

5. Solutions that can be prepared in advance for strip equilibration and gel electrophoresis
   a) Bromophenol blue stock solution

	Amount/10 ml
1% Bromophenol blue	100 g
50 mM Tris-base	60 g
Double distilled water	to 10 ml

b) 10x SDS electrophoresis buffer

	Amount/10 ml
25 mM Tris-base	30.3 g
192 mM Glycine	144 g
0.1% SDS	20 g
Double distilled water	to 1 liter
Use 1× for anode (lower solution)	0.7 liter needed (makes 7 l)
Use 2× for cathode (upper solution)	0.5 liter needed (makes 2.5 l)
Store at room temperature for up to 3 months	

c) Buffer for molecular weight markers

	Amount/10 ml
0.0625 M Tris-HCl	0.0757 g
2% SDS	200 mg
0.01% Bromophenol blue	100 µl
Adjust to pH 6.8 with diluted HCl	
Add 15 mg (0.1 M) DTT to 1 ml just before use	

d) Agarose sealing solution

	Amount/100 ml (2 ml/strip)
SDS Electrophoresis buffer	10 of 10× or 100 ml of 1×
0.5% Agarose (NA)	0.5 g
0.002% Bromophenol blue	200 µl
Boil, dispense 2 ml aliquots in screw cap bottles and store at room temperature	

e) 4x Resolving gel buffer

	Amount/500 ml (25 ml/gel)
1.5 M Tris	90.85 g
Double distilled water	375 ml
HCl	adjust to pH 8.8
Double distilled water	to 500 ml
Filter trough 0.45 µm filter, store at at 4°C	

f) SDS equilibration buffer

	Amount/200 ml (2*10 ml/strip)
50 mM 4× Resolving gel buffer	6.7 ml
6 M Urea	72.07 g
30% Glycerol (87% stock)	69 ml
2% SDS	4.0 g
0.002% Bromophenol blue	400 µl of stock
Double distilled water	to 200 ml
Store at –20°C in 10 ml aliquots	
Add DTT or iodoacetamide prior to use (amount/10 ml)	
Equilibration 1: DTT	100 mg
Equilibration 2: iodoacetamide (necessary with mass-spectrometry)	400 mg

6. Overview
   a) Use low fluorescence glass plates to ensure the lowest background pixel values of scanned images.
   b) Ensure that the entire casting system is clean, dry and free of any polymerized acrylamide.
   c) Equilibrate focused strips immediately before positioning at the top of the SDS-PAGE gel.
   d) Ensure that you use the recommended gel running buffer which contains 0.2% (w/v) SDS.

7. Casting homogeneous Ettan DALT gels
   a) Prepare a sufficient volume of water-saturated butanol (1-2 ml for each cassette).
   b) Make up 100 ml of displacing solution.
   c) For a full 14-gel set, make up 900 ml of acrylamide gel stock solution without adding the 10% (w/v) ammonium persulphate (APS) or 10% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED).
   d) Assemble the gel caster
   e) Connect the feed tube to a funnel held in a ring-stand above the top of the gel caster (about 30 cm). Insert the other end of the feed tube into the grommet in the bottom of the balance chamber.
   f) Fill the balance chamber with 100 ml of the displacing solution.

g) Add the appropriate volumes of APS and TEMED only when ready to pour the gels, and mix thoroughly. Once these two components are added, polymerization begins and the gel solution should be completely poured within 10 min.
h) Pour the gel solution into the funnel, taking care to avoid introducing any air bubbles into the feed tube.
i) Introduce the gel solution into the caster until it is about 1-2 cm below the final desired gel height. Stop the flow of acrylamide and remove the feed tube from the balance chamber grommet. Once the feed tube is removed, the dense displacing solution flows down the connecting tube, filling the V-well and sloped trough at the bottom of the caster. The remaining acrylamide solution is forced into the cassettes to the final gel height. The amount of gel solution required will be 800 to 850 ml for 14 gels.
j) Immediately pipette 1-2 ml of water-saturated butanol onto each gel.
k) Allow the homogeneous gels to polymerize for at least 3 h before disassembling the caster.

8. Equilibration of focused Immobiline DryStrips
   a) Prepare SDS equilibration buffer. Prior to use, prepare equilibration solutions 1 and 2. Allow 10 ml per strip for each equilibration solution.
   b) Allow the strips to defrost completely beforehand.
   c) Place the strips in individual equilibration tubes with the support film toward the wall.
   d) Add 10 ml of the DTT-containing equilibration solution 1 to each tube.
   e) Incubate the strips for 10 min with gentle agitation. Do not over-equilibrate, as proteins can diffuse out of the strip during this step.
   f) Pour off the equilibration solution 1 and add 10 ml of equilibration solution 2. Repeat step “e”. Pour off the solution and drain thoroughly.

9. Loading of focused Immobiline DryStrips
   a) During equilibration, the gel cassettes are prepared for loading by rinsing the top of the gels with deionized water, then with SDS electrophoresis running buffer for Ettan DALT, and draining all buffer from the top of the gel. Before

loading the Immobiline DryStrips, make sure that the gel surface and plates are dry

b) Place the gels in the Ettan DALT cassette rack.
c) After draining the equilibration solution, briefly rinse the strips by submerging them in a measuring cylinder containing SDS electrophoresis running buffer for Ettan DALT.
d) For each strip melt an aliquot of agarose overlay solution in a heating block or boiling water bath. Allow the agarose to cool slightly and slowly pipette the molten agarose solution, along the upper surface of the gel, up to the top of the glass plate. Take care not to introduce bubbles. Do not allow the agarose to cool or solidify.
e) Carefully place the strip in-between the two glass plates of the gel. Using a thin plastic spacer, push against the plastic backing of the strip and slide the strip between the two glass plates until it comes into contact with the surface of the gel.
f) By convention, the acidic, or pointed, end of the Immobiline DryStrip is on the left when the shorter of the two plates is facing the user. The gel face of the strip should not touch the opposite glass.

10. Inserting gels into the Ettan DALT electrophoresis buffer tank
    a) Fill the lower buffer tank with SDS electrophoresis running buffer. Turn on the control unit, switch on the pump and set the temperature to 25ºC.
    b) When the running buffer has reached the desired temperature, insert the loaded gel cassettes. Push blank cassette inserts into any unoccupied slots.
    c) Pour SDS electrophoresis running buffer for Ettan DALT into the top of the buffer tank to the fill line.
    d) Program the control unit to run for 16 h at 25 ℃, 2 W per gel. Close the lid of the buffer tank, and press start/stop to begin electrophoresis.

VISUALIZATION AND ANALYSIS

1. Materials
    a) Gloves
    b) Waterbath (in fume hood)
    c) Staining tray(s)

d) Rotary shaker

e) 2D-gels without glass plates

2. Solutions needed for hot Coomassie staining

a) Destaining solution for Coomassie (10% acetic acid)

	Amount/ 1 L
Glacial acetic acid	100 ml
Demineralised water	Add to 1 liter

b) Staining solution for Coomassie:

	Amount/1.6 L
1 PhastGel Blue tablet	
10% acetic acid	1.6 L

3. Sacanning overview

a) After the gels have run they should be scanned immediately.

b) Warp up the Typhoon Variable Mode Imager for 30 min before scanning.

c) Place the gel on the platen using the gel alignment guides;

d) Clean the scanner before use to remove dust;

e) Ensure that the gel glass plates are clean, dry and free from lint.

4. Scanning pre-settings:

a) Fluorescence Acquisition Mode:

- Select “fluorescence”;
- Choose emission filter and laser for the dyes used;
- Set PMT between 450 and 550; do a pre-scanning to check
- Sensitivity: normal;
- Select “sensitivity” in auto link mode;
- Select Tray setting and scan orientation
- Select “press sample”
- Choose Pixel size: 1000 µm for pre-scans and 100 µm for quantitative analytical scans.
- Focal Plane: 3 mm;
- Select DIGE File Naming Format to generate unique filenames

b) Press SCAN to start

c) Prior to image analysis the image files should be cropped to exclude nonessential information from the image files.

5. DeCyder 2D software analysis

   DeCyder 2D version 6.5 software comprises five modules:

   a) Image Loader: Import of scanned gel images into a project within the DeCyder database;
   b) DIA (Differential In-gel Analysis): Protein spot detection and quantitation on a set of images, from the same gel. Images must be processed in the DIA interface prior to data analysis in BVA.
      - Set estimated number of spots to 3000 or 2500;
      - Define area of interest;
      - Protein filter: volume between 10^4 and 10^5 to background subtraction, and gel artifact removal
   c) BVA (Biological Variation Analysis): Matching of multiple images from different gels to provide statistical data on differential protein abundance levels between multiple groups.
   d) XML Toolbox: Extraction of user specific data from XML files generated in Batch, DIA or BVA modules.

6. Staining:

   a) Heat the staining solution to 90 ℃
   b) Remove the gel from the glass plates. Be careful when you remove the gel, it is very fragile
   c) Pour the solution over the gel (around 200 ml per gel) which is in a glass tray.
   d) Place the tray on a laboratory shaker for 10 min (60 rev./min).

7. Destaining:

   a) In a tray on a rocking table in 10% acetic acid for at least 2 h at room temperature (don't leave overnight, you can restain if the staining becomes too faint);

b) Change solution several times (about 3 times), recycle it by pouring it through a filter filled with activated charcoal (you need about 600 ml destaining solution per gel in total).
c) Place a paper towel into the destaining solution to absorb the Coomassie dye. You can keep the gel in water to store.
d) Scan the gel on the scanner and
e) Store between plastic sheets in the refrigerator

MASS ESPECTOMETRY

1. Overview:
   a) As a positive control for in-gel digestion and extraction include 5 pmol (0.3 µg) BSA on a separate lane on a 1-D or 2-D SDS-polyacrylamide gel (is already present in molecular weight marker);
   b) Avoid keratin contamination. Keratin particles are floating in the air and are deposited on all kinds of surfaces. Given the often extremely small amounts of proteins in the samples to be analyzed, contamination of all solutions, glassware, surfaces that come into contact with gels should be prevented.

   The following precautions should be taken:
   - always wear gloves
   - all media used to prepare your sample, starting from any biological source, should be made free from dust by centrifugation or filtration;
   - exclusively use solutions that have been carefully prepared and used for mass spectrometric purposes;
   - bottles with solutions and vials with preparations should be closed as soon as possible after opening;
   - gels should be stained in keratin free containers;
   - special care is required for any kind of surface that contacts the gel.

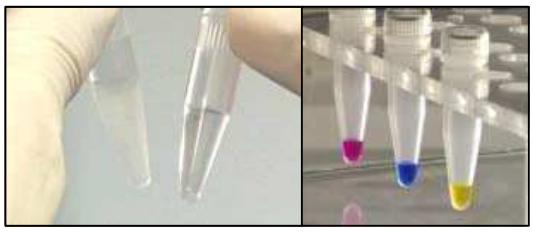
2. In gel digestion
   a) Solutions
   - Bicarbonate buffer: 100 mM NH_4HCO_3 (store at 4° C for up to 24 h);
   - Acetonitrile;
   - 1 mM HCl;

- Trypsin stock solution: dissolve contents of 1 ampoule trypsin (25 µg, Boehringer, sequencing grade) in 0.25 ml 1 mM HCl. Left over solution can be stored at -80°C.
- Digestion buffer: 12.5 µg/ml, prepare just before use and keep on ice. Add together: 1 part 100 µg/ml trypsin in 1 mM HCl, 4 parts 100 mM NH_4HCO_3 and 3 parts water;
- 20 mM NH_4HCO_3 (20 ml;)
- 5% formic acid in 50% acetonitrile (10 ml).

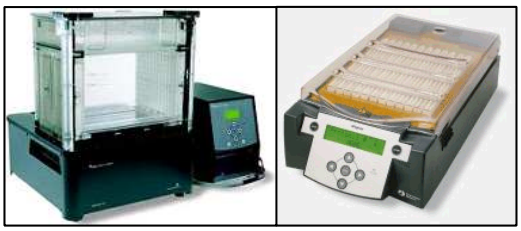
b) Wash gel with water before storing.
c) Cut out protein bands with scalpel and tweezers and transfer to 0.5 ml Eppendorf vials
d) Roughly estimate the volume of excised bands
e) Wash the gel pieces for 10 min with 100 µl 100 mM NH_4HCO_3
f) Dehydrate for 10 min in 50% acetonitrile (add 100µl acetonitrile)
g) Remove the liquid phase and swell again by rehydration in 100 µl 100 mM NH_4HCO_3
h) Dehydrate for 10 min in 50% acetonitrile (add 100 µl acetonitrile)
i) Remove the liquid phase and dry the gel pieces in the vacuum centrifuge
j) Swell the gel pieces in 2-3 times the volume of the gel pieces with 12.5 ng/µl trypsin on ice for 45 min. Check after 30 min whether you added enough digestion buffer
k) Remove all the liquid and replace by 7 µl of the same buffer (50 mM NH_4HCO_3) without trypsin, to keep the gel pieces wet; incubate overnight at 37° C in an oven or closed water bath.
l) The following morning, take off the solution above the gel pieces and transfer to an Eppendorf vial (first eluate)
m) Add 20-50 µl 20 mM NH_4HCO_3, vortex, incubate for 45 min at room temperature and transfer the eluted peptides in the vial with the first eluate
n) Repeat this step once
o) Dry the combined eluate overnight in a vacuum centrifuge
p) Extract the gel pieces three times with 50 µl 5% formic acid in 50% acetonitrile at room temperature for 20 min each time.
q) Dry the extract in a vacuum centrifuge

ANEXO 3: RESUMO DOS PROCEDIMENTOS PARA ANÁLISE DO PROTEOMA

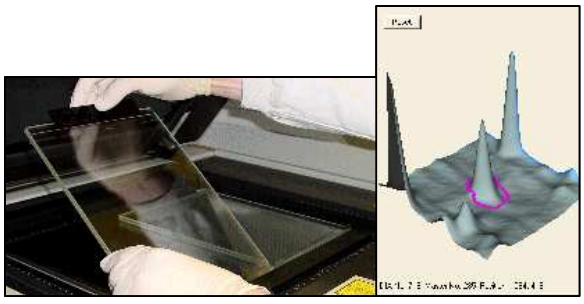
Experimento



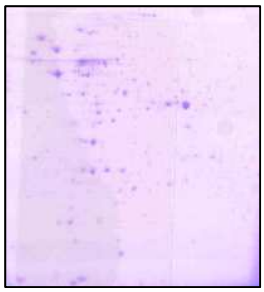
Preparo das amostras



Eletroforese bidimensional

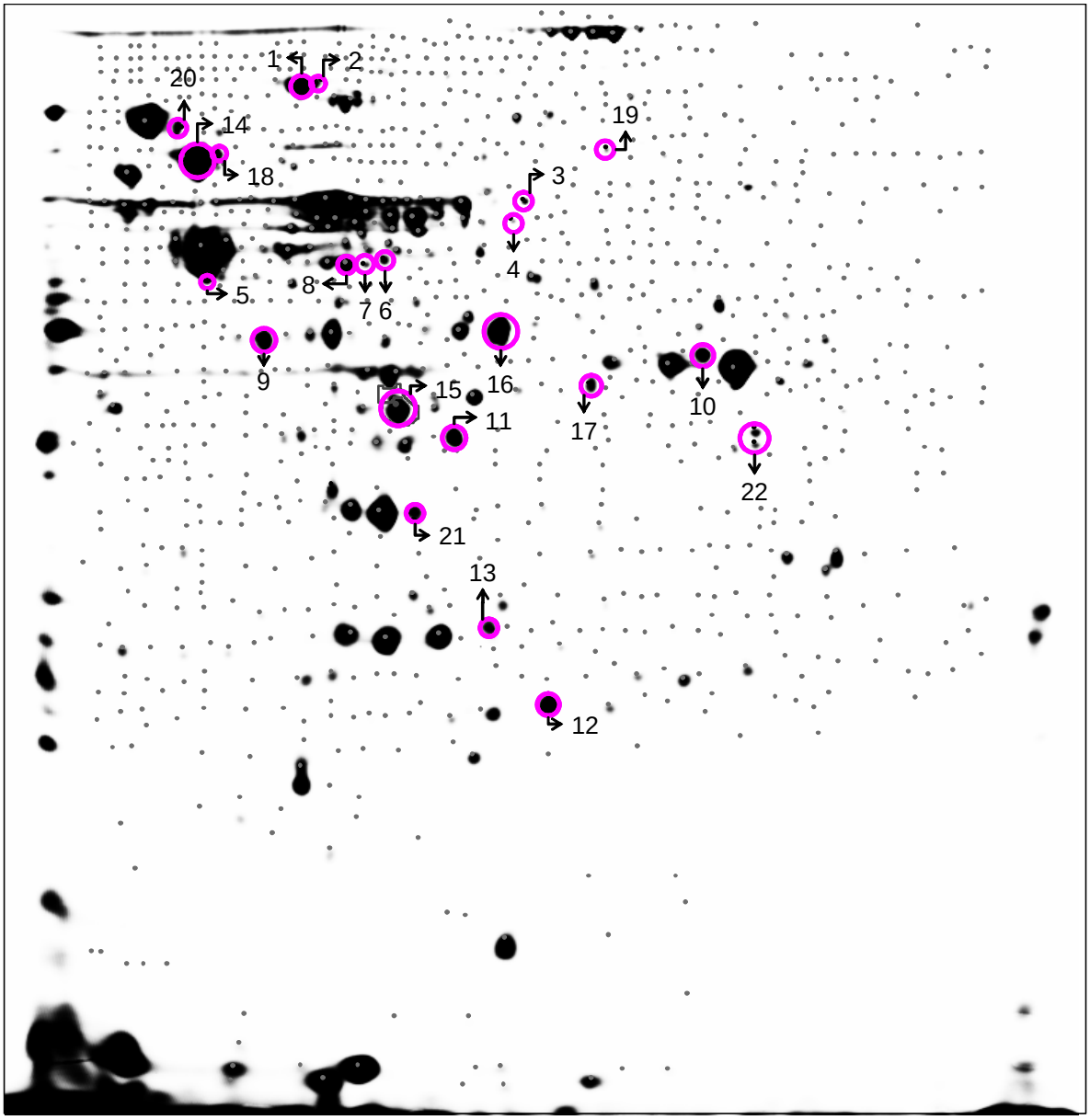


Digitalização e análise



Excisão e identificação das proteínas

ANEXO 4: MAPA DE PROTEÍNAS EXCISADAS E IDENTIFICADAS

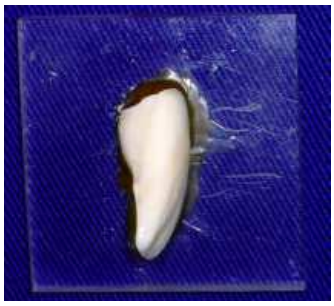


1. elongation factor EF-2	13. pyrimidine regulatory protein PyrR
2. elongation factor EF-2	adenylate kinase
3. D-alanine--D-alanyl carrier protein ligase	14. chaperonin GroEL
4. S-adenosylmethionine synthetase	15. L-lactate dehydrogenase, fructose-1,6 diphosphate dependent
5. putative enolase	Putative PTS system, mannose-specific component IIAB
6. Threonine dehydratase	16. phosphoglycerate kinase
7. 30S ribosomal protein S1	17. 6-phosphofructokinase
8. 30S ribosomal protein S1	18. Chaperonin GroEL
9. DNA-directed RNA polymerase alpha subunit	19. CTP synthetase (UTP-ammonia lyase)
10. putative dTDP-glucose-4,6-dehydratase	20. hypothetical protein SMU.229
11. hypothetical protein SMU.1760c	21. Fructose-bisphosphate aldolase.
12. orotate phosphoribosyltransferase	22. putative trans-2-enoyl-ACP reductase

ANEXO 5: PREPARO E SELEÇÃO DOS BLOCOS DE ESMALTE

- confecção dos blocos de esmalte bovino (4 x 4 mm)

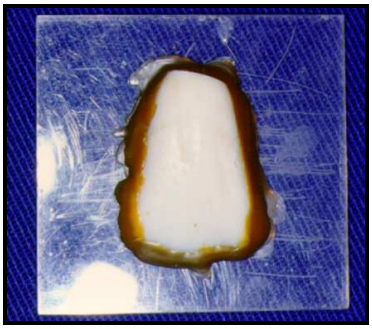
1. Coroa do dente bovino incisivo central inferior, separada da raiz através de disco diamantado de duas faces (D91, KG Sorensen, São Paulo-SP, Brasil), montado em motor de bancada (Nevoni, São Paulo-SP, Brasil), mantido sob refrigeração (água destilada/deionizada).



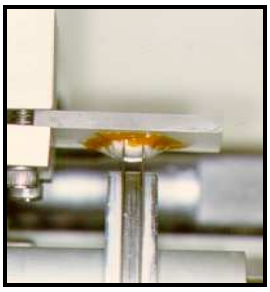
2. Coroa dentária fixada em placa de acrílico (4x4 cm e 4 mm de espessura) com cera pegajosa pela sua face proximal.

3. Placa montada em cortadeira (Isomet Low Speed Saw – Buehler, Lake Bluff, IL, EUA) sob refrigeração com água destilada/deionizada.

4. Secção da coroa utilizando disco diamantado (série 15 HC Diamond– nº 11-4244 Buehler, Lake Bluff, IL, EUA) separando a superfície vestibular da lingual.

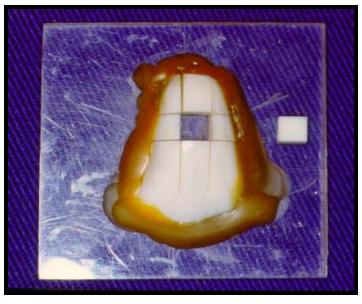


5. Face vestibular fixada na placa de acrílico.



6. Secção da face vestibular no sentido longitudinal, na porção mais plana, utilizando-se 2 discos diamantados (série 15 HC Diamond –nº 11-4243 Buehler, Lake Bluff, IL, EUA), montados em cortadeira sob refrigeração com água destilada/deionizada e separados por um disco espaçador de alumínio com 4 mm de espessura.

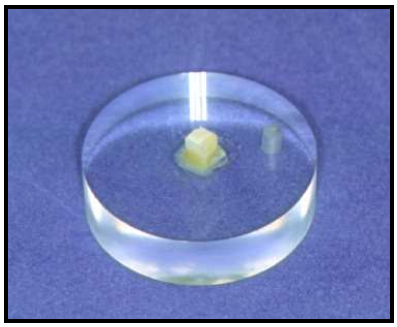
7. Secção da face vestibular no sentido transversal, na porção mais plana, utilizando-se dois discos diamantados, montados em cortadeira sob refrigeração com água destilada/deionizada e separados por um disco espaçador de alumínio com 4 mm de espessura.



8. Face vestibular do dente bovino, fixado sobre placa de resina. Ao lado, bloco de esmalte dentário.

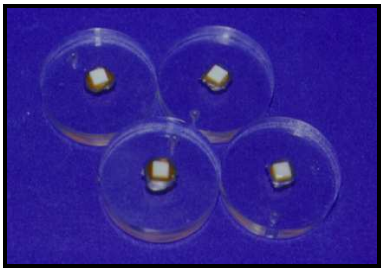
ANEXO 6: PREPARO E SELEÇÃO DOS BLOCOS DE ESMALTE

- *planificação da dentina e polimento do esmalte*



1. Bloco de esmalte fixado em disco de resina acrílica pré-fabricada (± 3 cm de diâmetro por ± 8 mm de espessura), com auxílio de cera pegajosa, com a superfície dentinária voltada para cima.

2. Politriz (APL-4 AROTEC, São Paulo-SP, Brasil utilizada para polimentos dos blocos de esmalte.



3. Blocos fixados com a superfície do esmalte voltada para cima, a qual será polida.

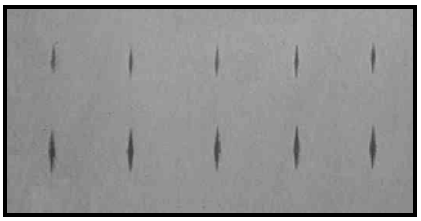
Seqüência do polimento de esmalte:

1. Ajuste da dentina para obtenção de superfícies paralelas entre esmalte e dentina, utilizando lixas de granulação 320 (CARBIMET Paper Discs, 30-5108-320, Buehler) - 2 pesos, 20 s e refrigeração a água;
2. Limpeza da superfície do esmalte utilizando contra-ângulo em baixa-rotação, taça de borracha, pedra-pomes e água deionizada;
3. Polimento seqüencial com lixas de granulação 600, 800 e 1200 (2 pesos, 30 s) e refrigeração a água. Limpeza em lavadora ultrassônica e água destilada/deionizada por 2 minutos, entre cada lixa;
4. Acabamento final com disco de papel feltro Texmet 1000 (Buehler Polishing Cloth) (1 minuto – 2 pesos) e suspensão de diamante 1 μ (Buehler);
5. Limpeza em lavadora ultrassônica utilizando solução detergente (Ultramet Sonic Cleaning Solution - Buehler) diluída 20:1 em água destilada/deionizada (3 minutos);
6. Lavagem durante 30 segundos com jato de água destilada/deionizada.

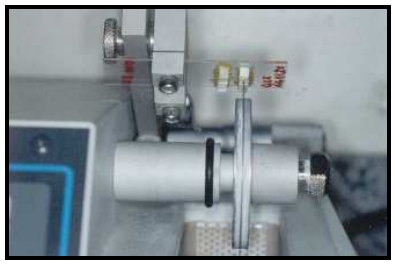
ANEXO 7: AVALIAÇÃO DA MICRODUREZA SUPERFICIAL E EM SECÇÃO LONGITUDINAL

1. Microdurômetro Shimadzu Micro Hardness Tester HMV-2.000 (Shimadzu Corporation, Kyoto, Japão), com penetrador tipo Knoop, acoplado ao Software para análise de imagem CAMS-WIN (NewAge Industries, USA).

2. Bloco de esmalte sendo submetido à leitura no microdurômetro.

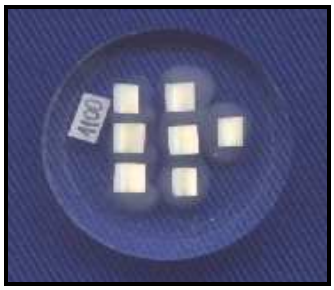


3. Fotomicrografia das impressões para análise de microdureza de superfície inicial e final do esmalte (Aumento 100x).



4. Blocos de esmalte fixados em lamínula de vidro com cera pegajosa para secção no sentido longitudinal no centro da área exposta, utilizando disco diamantado (série 15 HC Diamond - n. 11-4243 Buehler) montado em cortadeira sob refrigeração com água deionizada.

5. Inclusão dos blocos de esmalte em embutidora metalográfica (AROTEC PRE 30S) - 5 gramas de resina acrílica, pressão de 150 Kgf/cm^2, 7 min de aquecimento de e mais 7 min de resfriamento. Os blocos foram fixados em posição com cola adesiva (Super Bonder – Loctite).



6. Corpo de prova – plano longitudinal voltado para a superfície da resina acrílica.

7. Fotomicrografia das indentações da análise de microdureza em secção longitudinal do esmalte. (Aumento 100x)

Seqüência do polimento do corpo de prova:

1. Lixa de granulação 320 (1 minuto - 2 pesos), 600, 800 e 1200 (2 minutos – 2 pesos) e refrigeração a água. Limpeza em lavadora ultrassônica e água destilada/deionizada por dois minutos, entre cada lixa;
2. Acabamento final com disco de papel feltro Microcloth Supreme PSA (Buehler Polishing Cloth) e suspensão de diamante ¼ µ base-água (Buehler) por 2 minutos;
3. Limpeza em lavadora ultrassônica utilizando solução detergente (Ultramet Sonic Cleaning Buehler) diluída 20:1 em água destilada/deionizada (3 minutos);
4. Lavagem durante 30 segundos com jato de água destilada/deionizada.

ANEXO 8: APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

F. 45
PROC. 2005-02188
RUB.

COMITÊ DE ÉTICA EM PESQUISA –CEP-

OF. 014/2006
CEP
SFCD/bri

Araçatuba, aos 16 de fevereiro de 2006

Referência Processo FOA 2005-02188

O Coordenador do Comitê de Ética em Pesquisa desta Unidade, tendo em vista o parecer favorável do relator que analisou o projeto **"EFEITO DO EXTRATO DA FOLHA DE PSIDIUM CATTLEIANUM NO BIOFILME BACTERIANO E NA DESMINERALIZAÇÃO DO ESMALTE"** expede o seguinte parecer:

Aprovado:

Informamos a Vossa Senhoria que de acordo com as normas contidas na resolução CNS 215, **deverá ser enviado relatórios parciais em 16/02/2007; 16/02/2008; 16/02/2009 e o relatório final em 16/02/2010.**

Prof. Dr. Stefan Fiúza de Carvalho Dekon
Coordenador do CEP

Ciente/De acordo.
20/04/2006
Dr.Alberto Carlos Botazzo Delbem

Ilmo. Senhor
Dr.Alberto Carlos Botazzo Delbem
Araçatuba-SP-

Faculdade de Odontologia e Curso de Medicina Veterinária –
Rua José Bonifácio, 1193 CEP 16015-050 Araçatuba – SP
Tel (18) 620-3203 E-mail: diretor@foa.unesp.br

ANEXO 9: TERMO DE CONSENTIMENTO ESCLARECIDO

unesp **UNIVERSIDADE ESTADUAL PAULISTA**
"JÚLIO DE MESQUITA FILHO"
CÂMPUS DE ARAÇATUBA - FACULDADE DE ODONTOLOGIA

COMITÊ DE ÉTICA EM PESQUISA – CEP
(Resolução nº 01 de 13/06/98 – CNS)

TERMO DE CONSENTIMENTO ESCLARECIDO

I – DADOS DE IDENTIFICAÇÃO DO PACIENTE OU RESPONSÁVEL LEGAL

1. Nome do Paciente:			
Documento de Identidade nº	**Sexo:**	**Data de Nascimento:**	
Endereço:		**Cidade:**	**U.F.**
Telefone:	**CEP:**		

1. Responsável Legal:			
Documento de Identidade nº	**Sexo:**	**Data de Nascimento:**	
Endereço:		**Cidade:**	**U.F.**
Natureza (grau de parentesco, tutor, curador, etc.):			

II – DADOS SOBRE A PESQUISA CIENTÍFICA

Título do protocolo de pesquisa: Efeito do extrato da folha de *Psidium cattleianum* no biofilme bacteriano e na desmineralização do esmalte.		
1. Pesquisador responsável: Alberto Carlos Botazzo Delbem		
Cargo/função: Prof. Adjunto	**Inscr.Cons.Regional:** 39675	**Unidade ou Departamento do Solicitante:** Departamento de Odontologia Infantil e Social
3. Avaliação do risco da pesquisa: (probabilidade de que o indivíduo sofra algum dano como conseqüência imediata ou tardia do estudo). **SEM RISCO** **RISCO MÍNIMO** **RISCO MÉDIO** **RISCO MAIOR**		
4. Justificativa e os objetivos da pesquisa (explicitar): Em busca de alternativas que ofereçam a máxima eficácia com o mínimo de efeitos colaterais, produtos naturais têm sido estudados para controlar o desenvolvimento da placa bacteriana. O objetivo desse estudo será avaliar o efeito do extrato da folha de araçá (*Psidium cattleianum*), analisando a quantidade de bactérias presentes e a formação de lesões de cárie.		

5. Procedimentos que serão utilizados e propósitos, incluindo a identificação dos procedimentos que são experimentais: (explicitar)

Aparelhos intrabucais serão confeccionados após moldagem da boca de cada voluntário. Nesse aparelho, serão colocados quatro blocos de esmalte bovino protegidos por uma tela plástica. Esses aparelhos serão utilizados durante três etapas de 14 dias cada, separadas por intervalo de sete dias. Uma semana antes, os voluntários deverão iniciar o uso de pasta de dentes sem flúor. Os voluntários gotejarão uma solução de sacarose 20% e a solução de tratamento (água, clorexidina ou extrato vegetal). Após os 14 dias, será verificada a quantidade de bactérias causadoras da cárie presentes na placa bacteriana. Nos blocos de esmalte, será avaliado o tamanho da lesão de cárie formada.

6. Desconfortos e riscos esperados: (explicitar)

Os voluntários utilizarão pasta de dentes sem flúor e aparelhos intrabucais durante um total de 42 dias. Nesse período, deverão gotejar uma solução de sacarose oito vezes ao dia e a solução de tratamento duas vezes ao dia. O risco de desenvolver lesões de cárie é pequeno, uma vez que a solução de sacarose será gotejada nos blocos de esmalte fora da boca e os voluntários deverão aguardar 5 minutos antes de recolocar o aparelho na boca. Poderá ocorrer desconforto, irritação do céu da boca ou dificuldade para falar até que o voluntário se acostume com o aparelho.

7. Benefícios que poderão ser obtidos: (explicitar)

Será possível observar se o extrato vegetal utilizado possui um efeito contra as bactérias causadoras da cárie e sua possível utilização em produtos de uso odontológico, como pasta de dentes ou enxaguatórios.

8. Procedimentos alternativos que possam ser vantajosos para o indivíduo: (explicitar)

Não há.

Duração da pesquisa: 4 anos

10. Aprovação do Protocolo de pesquisa pelo comitê de ética para análise de projetos de pesquisa em / /

III - EXPLICAÇÕES DO PESQUISADOR AO PACIENTE OU SEU REPRESENTANTE LEGAL

1. Recebi esclarecimentos sobre a garantia de resposta a qualquer pergunta, a qualquer dúvida acerca dos procedimentos, riscos, benefícios e outros assuntos relacionados com a pesquisa e o tratamento do indivíduo.
2. Recebi esclarecimentos sobre a liberdade de retirar meu consentimento a qualquer momento e deixar de participar no estudo, sem que isto traga prejuízo à continuação de meu tratamento.
3. Recebi esclarecimento sobre compromisso de que minha identificação se manterá confidencial tanto quanto a informação relacionada com a minha privacidade.
4. Recebi esclarecimento sobre a disposição e o compromisso de receber informações obtidas durante o estudo, quando solicitada, ainda que possa afetar minha vontade em continuar participando da pesquisa.
5. Recebi esclarecimento sobre a disponibilidade de assistência no caso de complicações e danos decorrentes da pesquisa.
Observações complementares.

IV – CONSENTIMENTO PÓS-ESCLARECIDO

Declaro que, após ter sido convenientemente esclarecido (a) pelo pesquisador, conforme registro nos ítens 1 a 6 do inciso III, consinto em participar, na qualidade de paciente, do Projeto de Pesquisa referido no inciso II.

_____ Local, / / .

Assinatura

_____ _____

Testemunha Testemunha

Nome .....: Nome .....:
Endereço.: Endereço.:
Telefone .: Telefone .:
R.G. .....: R.G. .....:

ANEXO 10: INSTRUÇÕES AOS VOLUNTÁRIOS

Instruções gerais

1. O dispositivo intrabucal deve ser utilizado durante todo o dia e à noite, inclusive para dormir. Deve ser removido da boca apenas durante as refeições ou quando você for ingerir alguma coisa, **inclusive água ou balas, chicletes**.

2. Procure evitar que o dispositivo fique fora da boca por um período prolongado, restringindo-se ao tempo necessário para a alimentação (**máximo de 1 hora**).

2. Quando estiver fora da boca, **em nenhum momento o dispositivo deve ser deixado à seco**. Guarde-o no porta-aparelho, com uma gaze umedecida em **água deionizada**. Isso é muito importante para não ressecar os blocos e manter a placa dental (biofilme) formada sobre esses blocos em condições adequadas.

4. Utilize apenas o dentifrício e a escova fornecida.

5. Realize a higiene bucal normalmente (veja abaixo – Escovação).

6. A aplicação de sacarose ou o tratamento deve ser realizada no mínimo 30 minutos após a higienização.

7. **Não** utilize produtos para bochecho ou outros agentes tópicos de qualquer natureza na cavidade bucal durante a fase experimental. **Não** utilizar fio dental com flúor.

8. **Não** utilize vitaminas ou suplementos sistêmicos que contenham flúor durante a fase experimental.

9. **Não** utilize alimentos que possam ser fonte de flúor, como chá preto e chá verde.

10. Quando o dentifrício ou a gaze estiverem acabando, entre em contato com a pesquisadora responsável para que sejam repostos.

Procedimentos a serem realizados

1. Uma gota da solução tratamento deverá ser aplicada sobre cada bloco de esmalte, nos seguintes horários (8 vezes ao dia). É essencial para o bom andamento do experimento que se mantenha essa freqüência:

08:00h*	**15:30h***
09:30h	**17:30h**
11:30h	**19:00h**
13:30h	**21:00h**

2. Para gotejar as soluções, remova o dispositivo da boca, seque com gaze a região da telinha (com cuidado) e goteje uma gota da solução de sacarose indicada sobre cada bloco de esmalte, sem tocar a ponta do conta-gotas no dispositivo para evitar a contaminação da solução. Aguarde 5 minutos, para que a solução se difunda pela placa dental e retorne à cavidade bucal.

3. Duas vezes ao dia (nos horários marcados com *), 1 minuto após a aplicação de sacarose deve ser realizada a aplicação da solução de tratamento. Aguardar 5 minutos para recolocar o aparelho na boca.

4. Se o primeiro gotejamento do dia não puder ser realizado às 8 horas, atrase todos os outros gotejamentos de acordo com o horário do primeiro, com um intervalo de 1h 30min entre eles, até totalizar 8 por dia.

4. Quando o horário de gotejamento coincidir com um período em que o dispositivo estiver fora da boca, realize o gotejamento 5 minutos antes de retornar o dispositivo para a boca. Os gotejamentos seguintes devem ser realizados no horário pré-determinado. **Não** goteje a solução e deixe por mais de 5 minutos sem colocar o dispositivo na boca.

5. As soluções deverão ser trocadas toda semana. Solicitamos que você venha ao laboratório buscar a nova solução toda **segunda e sexta**, **das 8:00 às 9:00h**. As trocas de soluções são feitas para que não se acumulem fungos ou outros organismos indesejáveis. Se notar que houve formação de fungos antes do prazo, me procure.

6. O acúmulo de placa dental sob a tela plástica nesta fase é desejável; **não** tente removê-la.

ESCOVAÇÃO:

- Utilize somente o dentifrício fornecido.
- Realize a escovação dos dentes 3 vezes ao dia. Retomar o aparelho na boca imediatamente após a escovação.
- Sempre que escovar os dentes, escove também o dispositivo intrabucal. Você pode escovar todo o aparelho, exceto a área da telinha. Enxágüe o dispositivo com **água deionizada**. Tome cuidado para **não perturbar a placa.**
- Ao enxaguar o dispositivo, tome cuidado para que jatos de água não atinjam diretamente a tela plástica, causando a perda da placa dental acumulada.

INSTRUÇÕES PARA O TÉRMINO DE CADA FASE:

Você deverá comparecer nos horários determinados **em jejum e sem ter higienizado os dentes** ou aparelho no 13º e 14º dia após o início do experimento.

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

QUALQUER DÚVIDA ENTRE EM CONTATO!

Fernanda

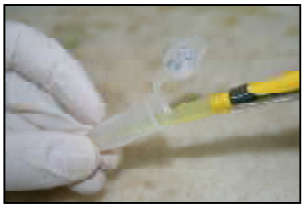
ANEXO 11: PREPARO DO DISPOSITIVO PALATINO E MEDIDA DA ACIDOGENICIDADE DO BIOFILME

	1. Dispositivo palatino confeccionado em resina acrílica (Jet – Artigos Odontológico Clássico, São Paulo-SP, Brasil), com espaço para a inserção dos blocos de esmalte de dente bovino.
	2. Blocos fixados com cera pegajosa e cobertos com tela plástica, deixando um espaço (cerca de 1 mm) entre o bloco e a tela para permitir o acúmulo do biofilme e protegê-lo da perturbação mecânica.
	1. Medição do pH com microeletrodo de contato e eletrodo de referência conectados a um analisador de íons (290A, Orion Research Inc., Boston-MA, EUA). Uma ponte salina foi criada em uma solução de KCl 3 mol L^{-1} entre o eletrodo de referência e o dedo do voluntário.
	2. Leitura do pH com o voluntário em jejum e sem realizar a higiene oral. As medições foram realizadas em dois pontos dos blocos (anterior e posterior).
	3. Após a leitura inicial, o dispositivo foi removido da boca para aplicação da sacarose (12º dia) ou da sacarose + tratamento (13º dia). Após 5 min uma nova leitura foi realizada.

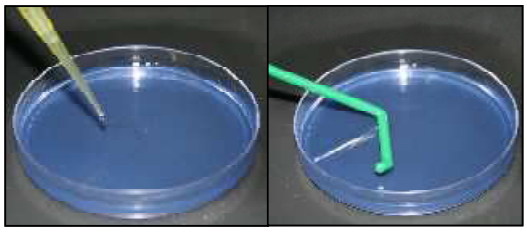
ANEXO 12: ANÁLISE DA COMPOSIÇÃO MICROBIOLÓGICA DO BIOFILME DENTAL

1 mg de biofilme dental / mL de PBS

Disruptor de células ultrassônico
(6 pulsos de 9,9 s; amplitude 40 W)



Diluição seriada

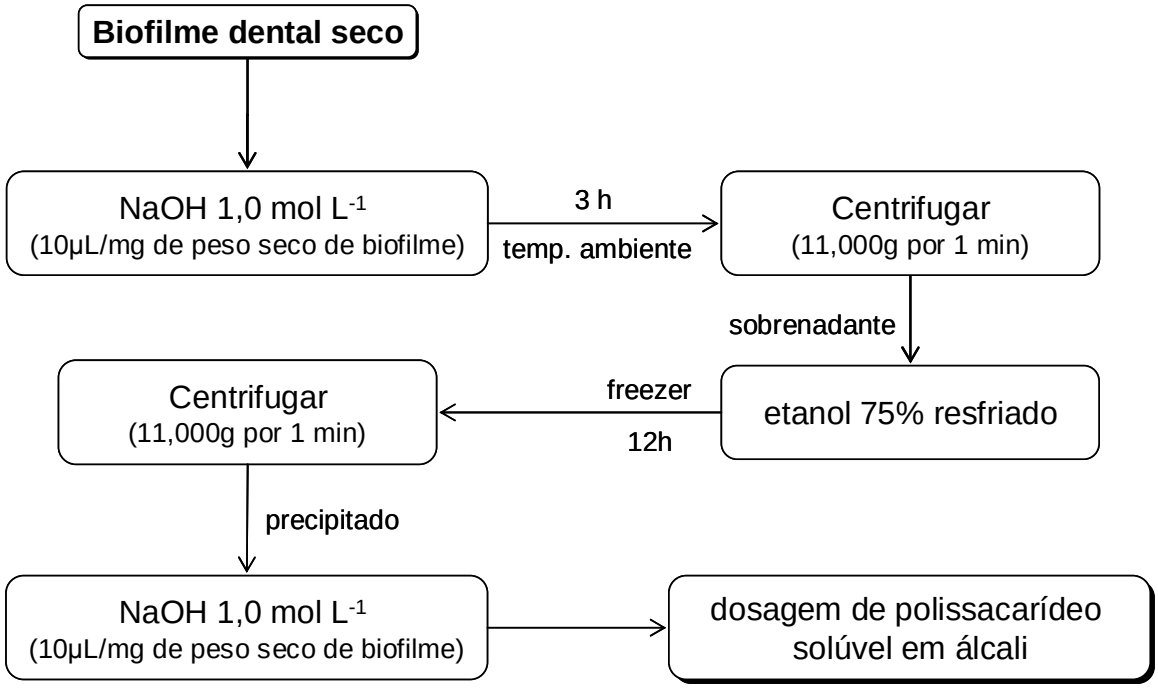


Semeadura (BHI, MS, MSBS)

Incubação 72 h / 37ºC / anaerobiose

Contagem e identificação das colônias

ANEXO 13: EXTRAÇÃO E ANÁLISE DOS CARBOIDRATOS ÁLCALI-SOLÚVEIS



A reação para dosagem dos carboidratos álcali-solúveis foi realizada de acordo com a tabela abaixo, utilizando padrão de glicose 1000,00 µg/mL:

	mL padrão	mL H2O	mL fenol 5%	mL ácido sulfúrico concentrado
Blank	0,00	0,20	0,20	1,00
Padrão 1	0,02	0,18	0,20	1,00
Padrão 2	0,04	0,16	0,20	1,00
Padrão 3	0,07	0,13	0,20	1,00
Padrão 4	0,10	0,10	0,20	1,00
Padrão 5	0,13	0,07	0,20	1,00

As amostras foram acrescentadas na proporção de 10µL amostra para 180 µL água). A coloração da reação foi desenvolvida pela adição rápida do ácido sulfúrico concentrado e agitação imediata. Após 20 min, realizou-se a leitura em espectofotômetro (Hitachi Lts, Tokyo, Japan) com absorbância de 490 nm.

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