GISELE PEDROSO MOI

ANALISE DO PERFIL PROTEÔMICO DO BIOFILME DENTAL FORMADO *IN SITU* NA PRESENÇA DE GLICOSE + FRUTOSE E SACAROSE

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas para a obtenção do Título de Doutor em Odontologia – Área de Cariologia.

Orientadora: Profa. Dra. Adriana Franco Paes Leme
Co-orientador: Prof. Dr. Jaime Aparecido Cury

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Aristóteles (384 – 322 A.C.)

RESUMO

A cárie dental é uma doença biofilme-dependente e os carboidratos fermentáveis presentes na dieta são considerados os fatores ambientais chaves envolvidos em sua iniciação e desenvolvimento. Algumas hipóteses baseadas na estrutura, composição e cinética dos íons no biofilme têm sido sugeridas para explicar a maior cariogenicidade do biofilme dental formado na presença de sacarose. Dentre estas, a expressão diferencial de proteínas bacterianas e presença ou ausência de proteínas salivares no biofilme formado na presença e ausência de sacarose tem sido sugerida. A sacarose promove alterações na composição protéica da matriz extracelular (MEC) do biofilme dental e não foram detectadas proteínas ligadoras de cálcio pela eletroforese bidimensional (2-DE) no biofilme formado em sua presença. A ausência destas proteínas na matriz do biofilme poderia ajudar explicar a sua cariogenicidade. Para avaliar se as alterações no perfil de proteínas da MEC são sacarose-dependentes ou ainda, se a presença de seus monossacarídeos poderia interferir similarmente neste perfil, um estudo *in situ*, boca-dividida foi realizado em duas fases de 14 dias. Este modelo experimental possibilitou que as proteínas salivares e bacterianas estivessem presentes no mesmo ambiente, simulando a condição natural da formação do biofilme dental. Cada fase experimental foi realizada em triplicata pelo mesmo voluntário. Assim, um voluntário utilizou um dispositivo palatino contendo 16 blocos de esmalte, sendo oito de cada lado, para acúmulo de biofilme. Foi gotejada extra-oralmente uma solução da sacarose 20% (0,58 M), água destilada e deionizada (H_2Odd) e uma mistura de uma solução de glicose 10,45% (0,29 M) com frutose 10,45% (0,29 M) sobre 8 blocos de esmalte dental 8 vezes/dia. No 14º dia, o biofilme dental foi coletado, as proteínas da MEC do biofilme dental foram extraídas e analisadas por duas abordagens diferentes: (1) 2-DE seguidos pela cromatografia líquida acoplada com a espectrometria de massas em tandem (LC-MS/MS) e (2) a mistura complexa das proteínas extraídas da MEC do biofilme foram analisadas por LC-MS/MS. Entre as proteínas diferencialmente expressas ($p<0,05$) estavam às proteínas ligadoras de cálcio e as proteínas indutoras de prolactina. Estas proteínas possuem um papel peculiar no potencial cariogênico do biofilme, uma vez que são responsáveis pela manutenção da saturação iônica do biofilme e agregação,

respectivamente. Os resultados sugerem que as mudanças do perfil protéico da ECM do biofilme dental não são atribuídas exclusivamente à sacarose, uma vez que seus monossacarídeos são capazes de promover alterações na composição protéica do biofilme, sugerindo que este perfil seja dependente dos carboidratos fermentáveis presentes na dieta.

Palavras-chave: Biofilme dental, Proteínas, Sacarose, Carboidratos, Eletroforese bidimensional, Espectrometria de massas.

ABSTRACT

Dental caries is a biofilm-dependent oral disease, and fermentable dietary carbohydrates are the key environmental factors involved with its initiation and development. Some hypotheses based on the structure, composition and ion kinetic aspects of biofilm have been suggested to explain the cariogenicity of biofilm formed in the presence of sucrose. Among them, the differential expression of bacteria proteins and the presence and absence of salivary proteins in biofilm formed in the presence and absence of sucrose has been suggested. Sucrose promotes changes in the extracellular matrix protein (ECM) of dental biofilm composition and the undetectable levels of calcium-binding proteins by two-dimensional gel electrophoresis (2-DE) in biofilm formed in its presence provide further insight into the unique cariogenic properties of this dietary carbohydrate. To evaluate whether these changes are sucrose-dependent, an *in situ* and split-mouth study was conducted during 2 phases of 14 days. Under these conditions, salivary and bacterial proteins are in the same environment, which is able to mimic natural condition of biofilm formation. Each experimental phase was performed in three distinct times with the same volunteer. A volunteer wore a palatal appliance containing 16 enamel blocks, being 8 in each side for dental biofilm accumulation. 20% (0.58 M) sucrose solution, distilled and deionized water (H_2Odd) and mixture containing 10.45% (0.29 M) glucose and 10.45% (0.29 M) fructose solution were extraorally dripped onto 8 blocks 8 times/day. On the 14th day, dental biofilms were collected, ECM proteins were extracted and analyzed by two different approaches: (1) 2-DE followed by liquid chromatography coupled with mass spectrometry in tandem (LC-MS/MS) and (2) whole extract proteins were also analyzed by LC-MS/MS. Among the differential expressed proteins ($p<0.05$) were the calcium-binding and prolactin-induced proteins. Particularly, these proteins have a unique role in the cariogenic potential of biofilm considering their properties of keep biofilm saturation and bacterial aggregation, respectively. Data show that protein profile changes in ECM of dental biofilm are not exclusively attributed to sucrose, since its monosaccharides are able to modify biofilm protein composition, suggesting that the biofilm proteomic profile is dietary carbohydrate-dependent.

Key-words: Dental biofilm, Proteins, Sucrose, Carbohydrates, Bidimensional electrophoresis, Mass spectrometry.

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INTRODUÇÃO GERAL

A formação de uma comunidade bacteriana embebida em uma matriz extracelular e organizada na forma de biofilme é o meio mais comum de crescimento bacteriano na natureza (Branda *et al.,* 2005), incluindo aquele formado sobre os dentes (Marsh, 2004). A matriz extracelular é tipicamente constituída por água, bactérias, polissacarídeos, proteínas, e muitas vezes por ácidos nucléicos (Sutherland, 2001; Branda *et al.,* 2005). As proteínas e os polissacarídeos representam 75-89% da composição dos polímeros da matriz extracelular do biofilme, indicando que estes são os principais componentes desta matriz (Tsuneda *et al.,* 2003).

A cárie dental é uma doença biofilme-dependente e os fatores ambientais chaves envolvidos em sua iniciação e desenvolvimento são os carboidratos presentes na dieta (Marsh, 2004). Dentre os carboidratos fermentáveis presentes na dieta, a sacarose tem sido considerada o mais cariogênico (Zero *et al*., 1992; van Houte, 1994; Cury *et al*., 2001),pois, além de ser fermentável e promover queda do pH e seleção microbiana (Marsh, 1991) no biofilme, a sacarose ainda serve como substrato para a síntese de polissacarídeos extracelulares (PEC) (Newbrun, 1967; Bowen, 2002). Estes, por sua vez, têm sido considerados importantes fatores de virulência dos microrganismos (Bowen, 2002), pois interferem na aderência e acúmulo de microorganismo, na estrutura, maturação e no pH do biofilme (Rölla, 1989; Schilling e Bowen, 1992; Vacca-Smith *et al*., 1996; Hayacibara *et al*., 2004; Pecharki *et al*., 2005; Ribeiro et al., 2005; Aires *et al*., 2006; Paes Leme *et al.,* 2006). Essas alterações provocadas na matriz do biofilme formado quando da exposição à sacarose estão relacionadas ao aumento da cariogenicidade do biofilme dental. Embora a maior parte do conhecimento esteja limitado ao papel dos PEC, estudos têm mostrado que o biofilme exposto à sacarose apresenta menor concentração de íons cálcio (Ca), fósforo inorgânico (P_i) e fluoreto (F) (Cury *et al.*, 1997; Cury *et al*., 2000). Sugerindo que a maior cariogenicidade do biofilme dental está relacionada também com a menor concentração Ca, P_i, F em sua matriz extracelular.

A concentração de íons no biofilme dental é importante para a manutenção do equilíbrio mineral entre o fluido do biofilme e a superfície dental (Pearce, 1998). Assim, Cury *et al*. (1997) e Paes Leme *et al*. (2004) mostraram *in situ* que a exposição freqüente à

sacarose reduz as concentrações de Ca, P_i e F na matriz do biofilme dental, causando também maior perda mineral no esmalte. Pearce *et al*. (2002) também observaram *in vitro* que a concentração de Ca no biofilme dental diminuiu em função do aumento da freqüência de exposição à sacarose. Baixas concentrações de F, Ca e P_i também foram observadas em biofilme formado com concentrações crescentes de sacarose (5, 10, 20 e 40%), e proporcionalmente, maior desmineralização foi observada (Aires *et al*., 2006). Coletivamente, este fenômeno da baixa concentração de íons no biofilme tem sido mostrado ser dependente da concentração e freqüência de exposição à sacarose, entretanto, ainda não foi completamente elucidado.

A menor concentração de Ca, Pi e F no biofilme formado na presença de sacarose não parece ser causada pela alta quantidade de polissacarídeos insolúveis, uma vez que os monossacarídeos constituintes da sacarose, glicose e frutose, também promovem diminuição da concentração inorgânica do biofilme (Cury *et al.*, 2000), embora não sejam substrato para a produção de polissacarídeos insolúveis. Adicionalmente, a depleção desses minerais para a saliva causada pelas constantes quedas de pH promovidas por esses carboidratos não parece explicar o fenômeno, pois os achados foram obtidos em média 12 horas após a realização dos tratamentos (Cury *et al.*, 1997; 2000), tempo suficiente para a saliva repor os reservatórios minerais do biofilme. Além disso, após a interrupção da exposição à sacarose por 1 ou 2 dias não foi observado aumento na concentração inorgânica do biofilme dental formado por 28 dias (Cury *et al.*, 2003).

Dados recentes de análise proteômica poderiam ajudar a explicar este fato, visto que não foram detectadas proteínas ligadoras de Ca na matriz do biofilme dental formado na presença de sacarose, as quais foram identificadas somente no biofilme formado durante a não exposição a este carboidrato (Paes Leme *et al*., 2008). Assim, a ausência destas proteínas na matriz do biofilme poderia explicar a baixa concentração de íons Ca, P_i e F encontrada, pois poderiam funcionar por exemplo como núcleo de cristalização de minerais. Entretanto, não se sabe se esta alteração na composição protéica da matriz extracelular do biofilme pode ser atribuída exclusivamente à presença da sacarose ou ainda se a presença de seus monossacarídeos poderia interferir no perfil protéico desta matriz. Assim, o objetivo desse estudo foi avaliar se este perfil protéico da matriz do biofilme

dental formado *in situ* é sacarose-dependente e se seus monossacarideos componentes interferem na composição protéica do biofilme.

PROPOSIÇÃO

Avaliar *in situ* o perfil de protéico da matriz do biofilme dental formado na presença de glicose+frutose e sacarose.

Este trabalho foi realizado no formato alternativo, conforme deliberação CCPG 001/98, da Comissão Central de Pós-Graduação da Universidade Estadual de Campinas (UNICAMP), que permite a inclusão de artigos científicos de forma a atingir o objetivo deste trabalho (Anexo 1).

CAPÍTULO

O presente artigo intitulado **"Proteomic Analysis of Extracellular Matrix of Dental Biofilm Formed *In Vivo* under Dietary Carbohydrates"** foi submetido ao periódico **"Proteomics"**.

CAPÍTULO*

Proteomic Analysis of Extracellular Matrix of Dental Biofilm Formed *In Vivo* under Dietary Carbohydrates

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Key-words: Biofilm, Extracellular proteins, Protein expression map, Large scale two-dimensional gel experiments, Liquid chromatography-tandem mass spectrometry, Sucrose.

* De acordo com as normas do periódico ***Proteomics*.**

ABBREVIATIONS

2-DE two-dimensional gel electrophoresis

Ca calcium

ECM extracellular matrix

emPAI exponentially modified protein abundance index

EPS extracellular polysaccharide

F fluoride

H_2Odd distilled and deionized water

LC-MS/MS liquid chromatography coupled with mass spectrometry in tandem

LDH lactate dehydrogenase

P_i inorganic phosphorus

Abstract

Dental caries is a biofilm-dependent oral disease, and fermentable dietary carbohydrates are the key environmental factors involved with its initiation and development. *In vivo* studies have shown that sucrose changes the protein composition of extracellular matrix (ECM) of dental biofilm (DB) and undetectable levels of calcium-binding proteins evaluated by 2-DE provide further insight into the unique cariogenic properties of this dietary carbohydrate. To evaluate whether these changes are sucrose-dependent, an *in vivo* study was conducted during 2 phases of 14 days, in triplicates. A volunteer wore a palatal appliance containing 16 enamel dental blocks and 20% sucrose solution, 10% glucose and 10% fructose solution or H_2Odd were extraorally dripped onto the blocks 8 times/day . On the 14th day, DB were collected, ECM proteins were extracted and analyzed by two approaches: (1) 2-DE followed by LC-MS/MS and (2) whole extract proteins were also analyzed by LC-MS/MS. Among the differential expressed proteins were the calcium-binding and prolactin-induced proteins. These proteins have a unique role in the cariogenic potential of biofilm, considering their properties of keeping biofilm saturation and bacterial aggregation, respectively. These data show that protein profile changes in ECM of DB are not exclusively attributed to sucrose, since its monosaccharides are able to modify biofilm protein composition, suggesting that the biofilm protein profile is dietary carbohydrate dependent.

Introduction

Bacterial community formation embedded in an extracellular matrix (ECM) and organized in a biofilm is the most common form of bacterial growth in nature [1]. ECM is typically composed of water, bacteria, polysaccharides, proteins and sometimes nucleic acids [1, 2]. Proteins and polysaccharides accounted for 75-89% of the ECM polymers composition, indicating that they are its major components [3]. Dental biofilm, a complex microbial biofilm, is the primary etiologic factor in dental caries and dietary carbohydrates are the key environmental factors involved with its initiation and development [4]. Among diet carbohydrates, sucrose is the most cariogenic carbohydrate, because beyond being acidogenic, it serves as substrate for extracellular polysaccharide (EPS) synthesis by cariogenic bacteria [5]. EPS are one of the components of the biofilm matrix and they have an important role on the biofilms' virulence [6]. Previous studies have shown that sucrose promotes biochemical changes in the extracellular matrix (ECM) composition of dental biofilm, such as high EPS concentration, low concentrations of Ca, P_i and F [5], being the most intriguing and a relevant factor in the context of biofilm cariogenicity [7]. There is an inverse relationship among concentrations of Ca and P_i into the biofilm matrix [8] and fluid [9], and caries experience. These ions would be released to the plaque/enamel interface during the fall of pH, thereby maintaining the aqueous phase in a saturated condition [10].

Moreover, differences in the patterns of the matrix proteins extracted from dental biofilm formed under presence/absence of sucrose or glucose and fructose have been shown [5].

Recent findings showed undetectable levels of calcium-binding proteins in 2-DE gels from ECM of biofilm formed in the presence of sucrose [11]. It could provide further insight into the unique cariogenic properties of this dietary carbohydrate and explain the data in which biofilm formed in the presence of sucrose exhibited lower inorganic ion concentrations [5, 11], since calcium-binding proteins could act as organic mineral reservoirs in biofilms [7].

However, considering that it is not known whether the changes in protein profile composition of ECM are exclusively attributed to the presence of sucrose, two different and complementary approaches based on gel and label-free proteomics were used to evaluate the composition of proteins in dental biofilm formed in the presence of sucrose and its monosaccharides. The development of complementary approaches of gel-based and non-gel-based proteomics techniques has provided powerful tools for studying large scale protein expression and characterization in complex biological systems [12, 13, 14].

Materials and Methods

Experimental Design

A model of *in vivo* study was performed to evaluate the protein composition of ECM of dental biofilm formed in the presence of sucrose or glucose and fructose. This study was previously approved by the Research and Ethics Committee of the Piracicaba Dental School (Protocol No. 099/2003) and involved a crossover and split-mouth *in situ* design conducted in two phases of 14 days each. A healthy 32-year-old volunteer wore an acrylic

palatal appliance containing two sets of eight blocks of human dental enamel [21, for details]. In one phase, 20% sucrose (0.58 M) solution was dripped onto the side of the appliance where the mesh was fixed with red resin and the other side was treated with distilled and deionized water (H_2Odd). In the another phase, a mixture of 10.45% glucose (0.29 M) and 10.45% fructose (0.29 M) solution was dripped onto the side indicated with red resin and in another side H_2Odd was used. Treatments were extraorally dripped onto each set of eight blocks 8 times a day, at pre-determined times as carried through for Paes Leme *et al*. (2008) [11]. The dental biofilm formed on the enamel blocks was collected 10 h after the last exposure to treatment and subjected to protein extraction and analyses. Each experimental phase was performed in three distinct times with the same volunteer. The samples were submitted to two-dimensional gel electrophoresis (2-DE), in-gel trypsin digestion of 2-DE differential spots, in-solution trypsin digestion of whole extract and mass spectrometry (MS) analysis.

Extraction of ECM Protein of Dental Biofilm

Before extraction of ECM protein of dental biofilm, some protein extraction procedures from biofilm matrix were previously evaluated to validate a mild alkali protocol conditions that it has been used to extract proteins from biofilm matrix [5] aiming proteomic analysis [11], since the alkali media could also extract proteins that are trapped into extracellular polysaccharides (EPS) net but the bacterial cellular membrane can be lyzed contaminating the extract with intracellular proteins. To validate the method for extracellular protein extraction and guarantee high efficiency of protein extraction without cell lysis, an *in vitro*

experiment was performed before *in vivo* experiments. *S. mutans* UA159 biofilms were grown on glass slides according to Koo *et al*. [15] and subjected to the following extraction procedures (n=5): 1) standard protocol [5, 11]: 0.1 N NaOH [16]/1.0 mM EDTA [17], 0°C/1 h, under agitation; 2) 0.1 N NaOH/1.0 mM EDTA, 100°C/15 min; 3) sonication 1x, 10 s, 7 W, in 0.1 N NaOH/1.0 mM EDTA, 0°C; 4) sonication 1x, 30 s, 7 W, in 0.1 M Tris/1.0 mM EDTA, 0°C; 5) sonication 1x, 60 s, 7 W, in 0.1 M Tris/1.0 mM EDTA, 0°C and 6) mechanical disruption with glass beads in 0.1 M Tris/1.0 mM EDTA, 0°C. Extracts from each tested procedure were centrifuged (12,000 *g)* for 30 min at 4°C and the supernatant was collected to assess the concentration of proteins [18], EPS [19] and the activity of lactate dehydrogenase (LDH) [20] as an indicator of cellular lysis. After this protocol validation, extracellular proteins were extracted according to standard protocol [5, 11] validated by an *in vitro* study. An aliquot of the extracted supernatant containing the extracellular proteins was collected to assess in replicates the LDH activity [20] to confirm the cell membrane integrity.

2-Dimensional Electrophoresis and Image Analysis

The first-dimension (isoelectric focusing, IEF) and second-dimension electrophoresis were performed according to Bellato *et al.* [22] and Paes Leme *et al.* [11, 23], using 20 µg of proteins [24] for each experimental conditions. Briefly, for the first dimension a precast IPG strips (18 cm, linear pH 4-7 gradient, GE Healthcare) was used and for the second dimension the strips were applied on 8-18% SDS-polyacrylamide gels. The gels were silver

147 stained [25]. Each experimental phase was repeated in three distinct times to check the
148 treatment reproducibility.
149 Nine gels were imaged using LabScan (GE Healthcare) with identical parameters and
150 analyzed (ImageMaster 2-D Platinum software version 7.0; GE Healthcare) as described by
151 Paes Leme et al. (2008) [11], except that the analysis were performed with the original
152 images instead of creating master gel. On average, more than 80% of all protein spots on
153 each gel were successfully matched to their respective protein spot in the reference gel.
154 *Tryptic digestion and mass spectrometry analysis of 2-DE spots*
155 Differential protein spots were excised, reduced, alkylated and submitted to in-gel
156 digestion with trypsin [26]. An aliquot (4.5 μL) of the resulting peptide mixture was
157 separated by C18 (75 μm x 100 mm) RP-nanoUPLC (nanoAcquity, Waters) coupled with a
158 Q-Tof Ultima mass spectrometer (Waters) with nano-electrospray source at a flow rate of
159 0.6 μL/min. The gradient was 2-90% acetonitrile in 0.1% formic acid over 45 min. The
160 instrument was operated in the 'top three' mode, in which one MS spectrum is acquired
161 followed by MS/MS of the top three most-intense peaks detected. The spectra were
162 acquired using software MassLynx v.4.1 and the raw data files were converted to a peak
163 list format (mgf) by the software Mascot Distiller v.2.2.1.0, 2008 (Matrix Science Ldt.) and
164 searched against non-redundant protein database (NCBI nr 2009.07.20, 9,298,190
165 sequences) using engine MASCOT v.2.0 (Matrix Science Ltd.), with carbamidomethylation
166 as fixed modification, oxidation of methionine as variable modification, one trypsin
167 missed cleavage and a tolerance of 0.1 Da for both precursor and fragment ions. For each

identified protein spot, the spectral counts [27], the number of unique peptides and exponentially modified protein abundance index (emPAI) [28] were assessed. The peptide was considered as unique when it differs in at least 1 amino acid residue, covalently modified peptides, including N- or C-terminal elongation (i.e. missed cleavages) count as unique and different charge states of the same peptide and modifications were not counted as unique. The emPAI index was automatically calculated by MASCOT engine as emPAI= 10^{PAI} -1, where PAI= N observed/N observable (N observed is the number of experimentally observed peptides and N observable is the calculated number of observable peptides for each protein) as described by Ishihama *et al.* (2005) [28].

Total extract of Tryptic digestion and mass spectrometry analysis

Proteins (30 µg) from each experimental conditions were reduced, alkylated and digested with trypsin (Sigma) (1:50) in 50 mM ammonium bicarbonate for 16 h at 37°C. The analysis was performed as described before; except that it was performed over 60 min with two runs for each sample. The resulting fragment spectra were searched as described above with decoy database with a maximum estimated false discovery rate of 1%.

Statistical Analysis

In vitro study data (n=5) were statistically analyzed by One-way ANOVA, followed by Tukey's test ($p<0.05$). The assumptions of equality of variances and normal distribution of errors were previously checked for all response variables [29].

For statistical analyses of spot volume in image gels, it was considered the spots present at least in two gels of the same treatment with a fold change of 1.5 [11]. One-way analysis of variance was used to compare the nine gels (n=3) followed by Tukey's test ($p<0.05$). Additionally, Pearson correlation coefficients were calculated between emPAI index and percentage of spot volume, spectral count and number of the unique peptides. For all analyses, GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California, USA) was used and the significance level was set at 5%.

Results

In vitro study

The results showed that the mild alkali protocol conditions (1, 2 and 3) extracted higher amounts of extracellular proteins and EPS (Fig. 1) from the matrix biofilm than other tested procedures ($p<0.05$). LDH activity was detected in almost all tested protocols; except for the protocol 1 (standard) and 4 (Fig. 2).

Two-Dimensional Gel Electrophoresis and Image Analysis

After mild alkali extraction of proteins from biofilm matrix using standard protocol [5, 11], LDH activity was not detected in the samples. Then, protein profile of ECM of dental biofilm formed in the presence of sucrose or glucose and fructose was evaluated by IEF within a linear pH gradient ranging from pH 4-7 followed by 8-18% SDS-polyacrylamide gel electrophoresis and silver-stained (Figs. 3A, 3B and 3C). Using Image Master 2D Platinum software v.7.0 (GE Healthcare), 533.3 ± 10.4, 513.7 ± 10.5 and 512.0 ± 9.8 (mean ± SD; n

209 =3) spots were automatically detected in gels formed in the absence of sugar, in the
210 presence of 10% glucose and 10% fructose solution or 20% sucrose one, respectively. It
211 has been matched 502 spots among dental biofilms formed in the presence of different
212 treatment solutions. Sixteen protein spots were statistically different among the gels from
213 dental biofilms formed in the presence or absence of different carbohydrates ($p<0.05$)
214 and they were identified by mass spectrometry. Of these protein spots, seven were
215 exclusively found in ECM of dental biofilm formed in the absence of carbohydrate
216 solution, six were present only in ECM of dental biofilm formed in the presence of mixture
217 of 10% glucose and 10% fructose solution and three appeared in ECM of dental biofilm
218 formed in the presence of H_2Odd and 20% sucrose solution. These three spots showed
219 higher abundance (>1.5-fold, $p<0.05$) in ECM of dental biofilm formed in absence of
220 carbohydrate solution.

221 *Tryptic digestion and mass spectrometry analysis of 2-DE spots*

222 Table 1 shows the identification of the sixteen protein spots statistically different among
223 match classes (> 1.5-fold, $p<0.05$). Database search revealed that identified proteins in
224 ECM of dental biofilm were proteins related to binding properties (spots 5, 10, 28, 42, 59,
225 354, 363, 366, 340) and calcium-binding proteins (spots 53, 54, 58, 67, 70, 353, 361). A
226 number of isoforms were identified under the three different conditions and variety in
227 terms of both the number and their abundance. The calcium-binding proteins and some
228 prolactin-induced proteins identified did not have the same isoelectric point or molecular
229 masses in all treatment groups.

A positive linear correlation was found between emPAI index and percentage of spot volume ($r=0.702$; $p=0.003$), emPAI index and spectral counts ($r=0.741$; $p<0.0001$), and emPAI index and number of the unique peptides ($r=0.777$; $p<0.0001$).

Tryptic digestion of whole extract and mass spectrometric analysis

The complex mixture of ECM protein composition of dental biofilm was analyzed using a label-free quantitative proteomics approach based on emPAI index, number of unique peptides and number of spectral counts. Were identified thirty-eight proteins in the all treatments (Table 2) considering Mascot-based score and proteins with at least two or more peptides were listed. A positive linear correlation between emPAI index and number of the unique peptides ($r=0.741$; $p<0.0001$) and between emPAI index and spectral counts ($r=0.792$; $p<0.001$) was also observed in complex mixture. These variables were considered to indicate whether the proteins in whole mixture increased or decreased in the treatments. Calcium-binding proteins (S100 calcium-binding protein A9, S100 calcium-binding protein A8, annexin A2) decreased in dental biofilm formed in the presence of carbohydrate solutions, and proteins originated from saliva or bacteria such as immunoglobulins, prolactin-induced proteins, protein Rei, HRPE773, bactericidal/permeability-increasing protein-like 1, parotid secretory protein, transglutaminase E3, tau-tubulin kinase increased as well as proteins related to biosynthesis (elongation factor Tu) and energy metabolism (glyceraldehyde-3-phosphate dehydrogenase) appeared increased in dental biofilm formed in the presence of carbohydrates.

Discussion

In nature, the majority of bacteria live in close association with surfaces, as complex communities referred to as biofilms [1], and several studies have evaluated the adaptative response in biofilm under stressed conditions to elucidate the mechanisms that allow oral microorganisms to produce acid, survive and grow in such environment and to promote pathogenic effects [2]. However, these reported studies were performed using *in vitro* approaches, which did not mimic the conditions of oral cavity, such as saliva properties (salivary flow, buffer capacity, clearance, minerals and protein content), diversity of species, microorganism selection, succession, nutrient availability and competition. Therefore, in the present study, the dental biofilm was formed in the oral cavity using *in situ* model to analyze the protein expression profile in the presence and absence of dietary carbohydrates. Under these conditions, it is possible to evaluate the expression of salivary and bacterial proteins, which may help explain biofilm pathogenicity.

Many studies have been performed to explain the higher cariogenicity of sucrose considering the microbiological and biochemical composition of biofilm [5, 7, 10, 11, 30]. Although many reports have assembled important information in the last decades, still there are some gaps to explain its higher cariogenicity and how it can change the biofilm organization in the presence of dietary carbohydrates. Studies of our group showed clear differences in the patterns of the matrix proteins extracted from dental biofilm formed under three distinct conditions: (1) in the absence of carbohydrate, (2) in the presence of glucose and fructose, and (3) in the presence of sucrose [5] and recently, undetectable

levels of calcium-binding proteins were observed in ECM of dental biofilm formed in the presence of sucrose [11]. In fact, the protein composition of the dental biofilm matrix may help explain the findings that biofilm formed in the presence of sucrose exhibited lower inorganic ion concentrations [5, 11, 30], since calcium-binding proteins may act as organic mineral reservoirs in dental biofilm [7]. To evaluate more deeply whether the changes in the protein profile of ECM is sucrose-dependent or whether the presence of its monosaccharides could also interfere with the protein profile, the proteomic analysis of ECM of biofilm formed in the presence of sucrose or glucose and fructose was analyzed using complementary approaches of gel-based and label-free analyses.

Considering our results (Figs. 1 and 2), mild alkali extraction was selective and effective to extract proteins from ECM [5], because it showed higher protein and EPS concentrations, without cell lysis, suggesting that it dissolves EPS, allowing the extraction of ECM proteins that could be trapped in the matrix.

Findings showed that the ECM protein composition of dental biofilm formed *in vivo* has distinct protein profiles when it was formed in the absence or presence of the different carbohydrates. The differential expressed proteins identified by 2-DE were mainly calcium-binding proteins and prolactin-induced proteins, both proteins are originated from saliva. Besides, a label-free quantitation approach based on emPAI index, number of unique peptides and number of spectral counts allowed for the first time the global identification of proteins in biofilm formed *in vivo*. Interestingly, most of the proteins present in ECM are salivary proteins, whereas the proteins originated from bacteria

293 showed only 10.5% ± 2.9%. The identified bacterial proteins are mostly associated with
294 biosynthesis energy and energy metabolism and they appeared increased in dental
295 biofilm formed in the presence of carbohydrate solutions, as previously described by Paes
296 Leme *et al.* (2008)[11]. On the other hand, 89.5% ± 12.7% of identified proteins in biofilms
297 are from saliva, playing an important role on oral environment [31], such as, prolactin-
298 induced proteins, immunoglobulins, calcium-binding proteins and annexins.
299 It is interesting to note that it was confirmed the absence of calcium-binding proteins in 2-
300 DE under sucrose exposure [11]. However, calcium binding proteins were identified in all
301 treatments in whole extract using LC-MS/MS. The data of number of unique peptides and
302 number of spectral counts showed that these proteins decreased in abundance in the
303 ECM of dental biofilm formed under carbohydrate exposure, as well as confirmed by
304 emPAI index, which are proportional to relative protein abundance in the complex
305 mixture [34]. In addition, annexins have never been reported in dental biofilm before, but
306 there are previous evidences that annexin I and II are present in whole saliva [35]. These
307 proteins are structurally related family of calcium-binding proteins that contain distinct
308 calcium-binding sites compared with S100 calcium-binding proteins [36].
309 Prolactin-induced proteins were observed in higher abundance in 2-DE and in whole
310 extract of dental biofilm formed in the presence of monosaccharides than control and
311 sucrose groups. For some reason, the mixture of glucose and fructose treatments
312 “favored” the maintenance of this protein either by less processing of this protein by
313 bacteria or due to the presence of new sites in dental biofilm for protein binding. Recent

study confirmed the role of prolactin-induced protein in bacterial aggregation showing significantly higher aggregation in wild-type mice than prolactin-induced protein knockout mice for all the bacterial strains [32]. Several isoforms of prolactin-induced proteins and calcium-binding proteins were identified. However, a great sequence coverage (60-70%) was observed for all the identified proteins in all the treatments, which infers that post-translational modification is not present in the great portion of the molecule, except for the fact that prolactin-induced proteins have a putative site of glycosylation in Asn105 (NetNGlyc 1.0 Server), which was not covered by our mass spectrometry analysis.

Furthermore, many immunoglobulins were identified into whole extract of all the treatments, but in higher abundance in carbohydrate treatments. Not surprisingly, since these proteins are abundant in salivary environment [33] and integrate the oral defense system that protect the oral cavity [12].

In summary, these findings showed that most of the protein composition in ECM of dental biofilm is originated from saliva and the changes in extracellular protein profile of *in vivo* dental biofilm were not exclusively attributed to the presence of sucrose, since its monosaccharides are able to modify this proteomic profile. Additional experiments should be performed to evaluate the effect of other carbohydrates on ECM protein composition.

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Figures.

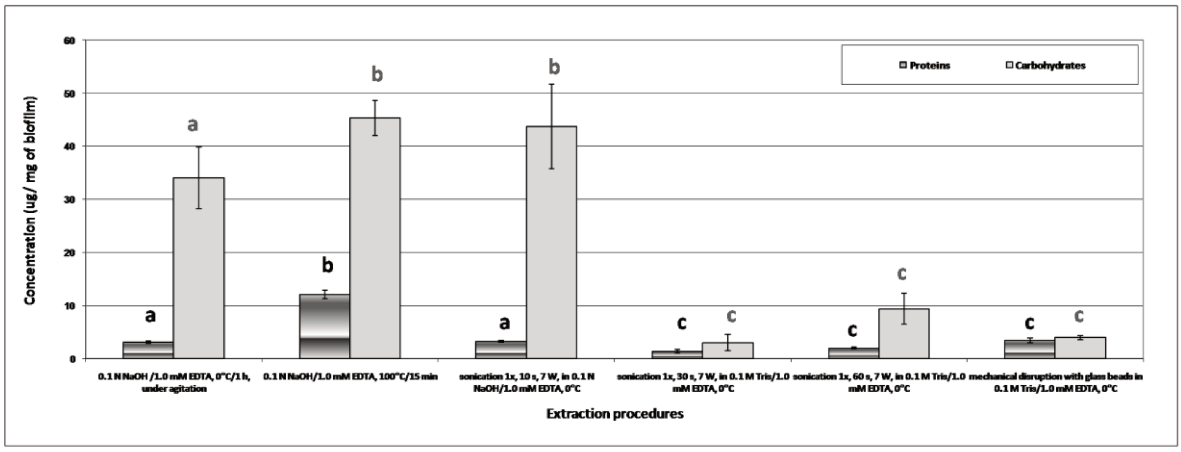

Figure 1. Protein and EPS concentrations according to extraction procedures. Data whose means are followed by distinct letters represent significant differences among the extraction protocols ($p<0.05$).

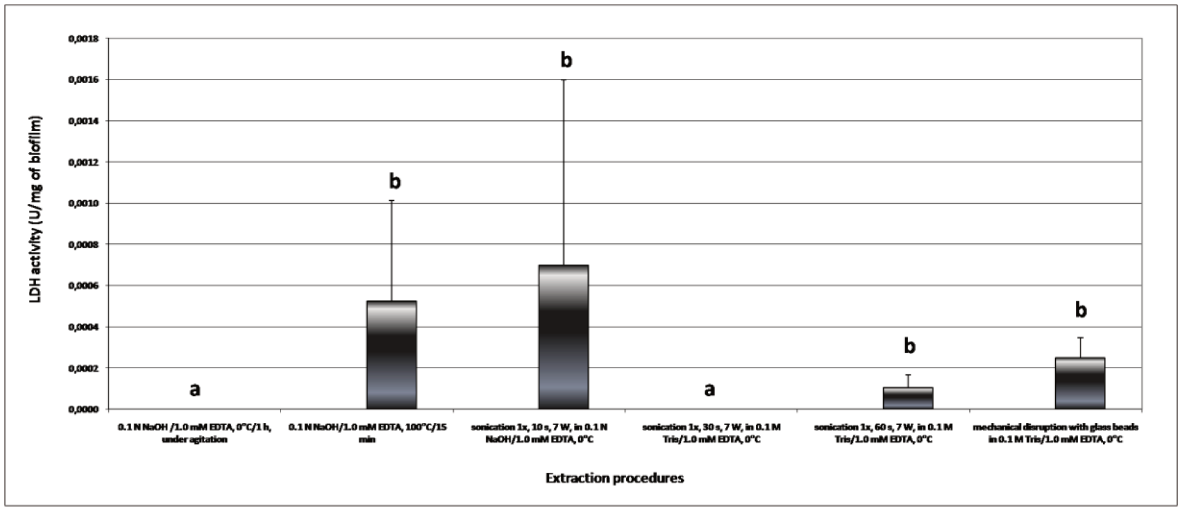

Figure 2. Lactate dehydrogenase activity (LDH) according to extraction procedures. Different letters represent significant differences among the extraction protocols ($p<0.05$).

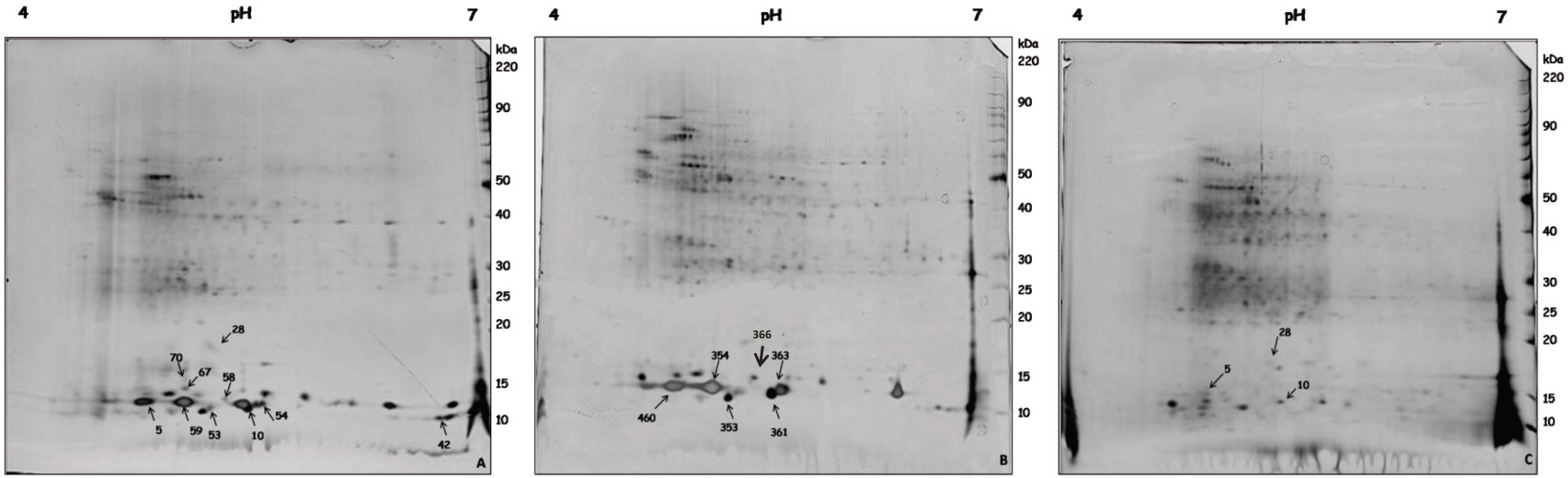

Figure 3. Two-dimensional gel electrophoresis profile of ECM proteins (20 µg) of dental biofilm formed in the presence of H_2Odd (A), mixture of 10% glucose and 10% fructose solution (B) and 20% sucrose solution (C). ECM proteins were applied to 4–7 IPG strips followed by electrophoresis on 8-18% SDS-polyacrylamide gel. The gels were silver-stained. Differential protein spots ($p<0.05$) indicated by numbers were identified by in-gel trypsin digestion and LC-MS/MS analyses.

Tables

Table 1. Identification of differentially expressed spots in the 2-DE by LC-MS/MS analyses.

Spot number	Accession number	Protein	Experimental PI value	Theorical PI value	Experimental MW (Da)	Theorical MW (Da)	Percentage of volume spot according to treatment solutions			emPAI index according to treatment solutions			Number of unique peptides/spectral counts according to treatment solutions		
							A	B	C	A	B	C	A	B	C
5	gi|116642259	prolactin-induced protein [Homo sapiens]	4.87	5.25	11000	9232	3.26	-	0.31	2.61	-	0.35	5/6	–	2/3
	gi|4505821	prolactin-induced protein [Homo sapiens]	4.88	8.26	11000	16847	3.26	-	0.31	1.08	-	0.12	5/5	–	2/2
10	gi|4505821	prolactin-induced protein [Homo sapiens]	5.45	8.26	18000	16847	2.00	-	0.03	0.73	-	0.12	4/6	–	2/2
28	gi|4505821	prolactin-induced protein [Homo sapiens]	5.19	8.26	21000	16847	0.15	-	0.07	0.73	-	0.14	3/3	–	1/3
42	gi|4505821	prolactin-induced protein [Homo sapiens]	6.69	8.26	10000	16847	0.19	-	-	0.44	-	-	2/2	–	–
	gi|116642259	prolactin-induced protein [Homo sapiens]	6.69	525	10000	9232	0.19	-	-	0.90	-	-	2/2	–	–
53	gi|4506773	S100 calcium-binding protein A9 [Homo sapiens]	5.28	5.71	10000	13291	1.00	-	-	0.12	-	-	3/4	–	–
54	gi|4506773	S100 calcium-binding protein A9 [Homo sapiens]	5.56	5.71	11000	13291	2.26	-	-	0.12	-	-	2/2	–	–
58	gi|4506773	S100 calcium-binding protein A9 [Homo sapiens]	5.39	5.71	12000	13291	2.26	-	-	0.12	-	-	2/2	–	–
59	gi|116642259	prolactin-induced protein [Homo sapiens]	5.12	5.25	11000	9232	4.38	-	-	2.61	-	-	5/6	–	–
67	gi|4506773	S100 calcium-binding protein A9 [Homo sapiens]	5.13	5.71	12000	13291	0.35	-	-	0.12	-	-	2/2	–	–
70	gi|4506773	S100 calcium-binding protein A9 [Homo sapiens]	5.14	5.71	12000	13291	0.20	-	-	0.12	-	-	2/4	–	–
353	gi|4506773	S100 calcium-binding protein A9 [Homo sapiens]	5.23	5.71	10000	13291	-	1.51	-	-	0.12	-	–	3/3	–
354	gi|4505821	prolactin-induced protein [Homo sapiens]	5.16	8.26	11000	16847	-	5.34	-	-	1.99	-	–	9/16	–
	gi|116642259	prolactin-induced protein [Homo sapiens]	5.16	5.25	11000	9232	-	5.34	-	-	8.46	-	–	6/13	–
361	gi|4506773	S100 calcium-binding protein A9 [Homo sapiens]	5.53	5.71	12000	13291		2.82			0.12		–	3/3	–
363	gi|4505821	prolactin-induced protein [Homo sapiens]	5.57	8.26	11000	16847	-	2.40	-	-	0.44	-	–	2/2	–
	gi|116642259	prolactin-induced protein [Homo sapiens]	5.57	5.25	11000	9232	-	2.40	-	-	0.90	-	–	2/2	–
366	gi|4505821	prolactin-induced protein [Homo sapiens]	6.41	8.26	12000	16847	-	1.48	-	-	0.73	-	–	5/6	–
	gi|116642259	prolactin-induced protein [Homo sapiens]	6.41	5.25	12000	9232	-	1.48	-	-	1.62	-	–	5/6	–
460	gi|116642259	prolactin-induced protein [Homo sapiens]	4.95	5.25	12000	9232	-	3.13	-	-	1.62	-	–	4/6	–
	gi|4505821	prolactin-induced protein [Homo sapiens]	4.95	8.26	12000	16847	-	3.13	-	-	0.73	-	–	4/5	–

Identified protein spots statistically different among gels ($p<0.05$) of dental biofilm formed in the presence of H_2Odd (A), a mixture of 10.45% glucose and 10.45% fructose (B) and 20% sucrose solution (C) . The spot numbers were indicated in the figure gels. The (-) means that there was not match spots in the gels. emPAI index: exponentially modified protein abundance index; MW: molecular weight.

Table 2. Identification of proteins from complex mixture by LC-MS/MS analyses.

Accession number	Eukaryotes	emPAI index according to treatment solutions						Number of unique peptides/spectral counts according to treatment solutions					
		A		B		C		A		B		C	
		Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
gi|178585	alpha-amylase	0.74	0.86	0.47	0.39	0.47	0.73	8/11	9/13	7/8	7/8	7/10	10/14
gi|16306978	Annexin A2 [Homo sapiens]	0.45	0.45	0.28	0.18	0.18	0.09	3/4	3/4	3/3	2/2	2/2	1/1
gi|4757756	annexin A2 isoform 2 [Homo sapiens]	0.45	0.45	–	–	–	–	3/4	3/4	–	–	–	–
gi|4502101	annexin I [Homo sapiens]	1.09	0.90	0.63	0.50	0.5	0.5	8/11	7/8	5/7	4/6	5/6	4/7
gi|15055535	bactericidal/permeability-increasing protein-like 1 [Homo sapiens]	–	–	0.07	0.07	0.07	0.07	–	–	1/1	1/2	1/2	1/1
gi|225632	casein alphaS1	–	–	0.14	0.14	0.29	0.29	–	–	2/2	2/2	2/2	2/2
gi|157830361	Chain A. Human Serum Albumin In A Complex With Myristic Acid And Tri- Iodobenzoic Acid	–	–	0.14	0.20	0.33	0.39	–	–	4/4	4/5	8/8	10/11
gi|493869	Chain L. Crystal Structure Of A Chimeric Fab' Fragment Of An Antibody Binding Tumour Cells	0.16	0.16	0.14	0.14	0.14	0.14	2/7	2/7	2/9	2/9	2/11	2/10
gi|14250058	Chromosome 20 open reading frame 114 [Homo sapiens]	–	–	–	–	0.13	0.06	–	–	–	–	3/3	3/3
gi|37183160	HRPE773 [Homo sapiens]	1.46	1.94	1.60	0.89	2.04	2.04	6/11	7/10	6/37	6/30	8/38	8/31
gi|229536	Ig A L	–	–	0.72	0.72	0.72	0.50	–	–	4/9	4/8	6/12	6/13
gi|229585	Ig A1 Bur	0.16	0.16	0.35	0.41	0.35	0.41	4/7	3/4	7/11	9/14	10/16	11/18
gi|70058	Ig alpha-2 chain C region - human	0.21	0.21	0.19	0.29	–	–	2/6	2/6	3/7	5/8	–	–
gi|229601	Ig G1 H Nie	–	–	0.07	0.21	0.07	0.14	–	–	1/1	3/4	3/3	3/4
gi|87890	Ig lambda chain precursor - human	–	–	–	–	0.65	0.46	–	–	–	–	6/13	6/13
gi|50301689	immunoglobulin alpha 1 heavy chain constant region [Homo sapiens]	0.20	0.20	–	–	–	–	3/7	3/7	–	–	–	–
gi|115394412	immunoglobulin alpha heavy chain constant region [Cercocebus torquatus]	–	–	0.30	0.30	0.30	0.42	–	–	3/6	4/7	4/5	5/8
gi|15418970	immunoglobulin alpha heavy chain constant region [Macaca mulatta]	0.10	0.10	0.30	0.30	0.3	0.42	3/5	3/5	4/7	5/8	4/5	5/8
gi|184761	immunoglobulin alpha-2 heavy chain [Homo sapiens]	–	–	0.41	0.41	0.41	0.53	–	–	6/8	6/8	6/6	7/9
gi|225625764	immunoglobulin kappa light chain [Macaca mulatta]	–	–	–	–	–	–	1/6	1/6	1/5	1/6	–	–
gi|6176308	immunoglobulin kappa light chain constant region [Aotus nancymaae]	–	–	–	–	–	–	–	–	2/2	2/2	1/1	2/2
gi|33700	immunoglobulin lambda light chain [Homo sapiens]	0.15	0.15	0.85	0.85	0.18	0.25	2/4	2/4	4/10	4/10	5/8	6/10
gi|9295295	immunoglobulin light chain variable region [Homo sapiens]	0.79	0.79	–	–	–	–	2/9	2/9	–	–	–	–
gi|16755850	parotid secretory protein [Homo sapiens]	–	–	0.41	0.26	0.12	0.12	–	–	3/3	2/3	1/1	1/1
gi|73746767	prolactin inducted protein [Pongo pygmaeus]	0.65	0.65	0.94	1.43	0.94	0.94	3/14	3/14	5/30	4/32	4/16	4/13
gi|116642259	prolactin-induced protein [Homo sapiens]	5.20	7.93	12.1	8.46	8.46	12.04	3/8	4/9	5/26	6/26	5/22	4/12
gi|4505821	prolactin-induced protein [Homo sapiens]	1.29	1.29	1.49	1.49	1.08	1.08	4/15	4/15	6/36	8/33	5/15	6/17
gi|229526	protein Rei, Bence-Jones	–	–	0.30	0.48	0.48	0.48	–	–	2/11	2/12	2/14	2/14
gi|190668	S100 calcium binding protein A7 [Homo sapiens]	–	–	–	–	0.30	0.30	–	–	–	–	1/1	1/1
gi|21614544	S100 calcium-binding protein A8	2.45	2.45	1.27	1.27	1.98	1.98	8/14	8/13	6/9	6/8	6/8	7/9
gi|4506773	S100 calcium-binding protein A9 [Homo sapiens]	5.12	5.12	0.98	0.58	3.95	2.13	7/11	7/11	5/5	3/3	6/11	5/11
gi|27451602	tau-tubulin kinase [Homo sapiens]	–	–	–	–	–	–	–	–	2/2	2/2	–	–
gi|307504	transglutaminase E3 [Homo sapiens]	–	–	0.13	0.04	–	–	–	–	3/3	2/2	–	–
gi|238236	transmembrane secretory component; poly-Ig receptor; SC [Homo sapiens]	–	–	0.16	0.16	0.16	0.12	–	–	3/4	4/5	4/6	6/6
	Prokaryotes												
gi|24379182	elongation factor Tu [Streptococcus mutans UA159]	–	–	0.08	0.08	0.16	0.24	–	–	1/1	1/1	3/3	3/3
gi|15674691	elongation factor Tu [Streptococcus pyogenes M1 GAS]	–	–	0.08	0.08	0.24	0.16	–	–	1/1	1/1	3/3	3/3
gi|21666599	glyceraldehyde-3-phosphate dehydrogenase; GapC [Streptococcus agalactiae]	–	–	0.09	0.09	0.09	0.09	–	–	1/1	1/1	1/1	1/1
gi|416943	RecName: Full=Elongation factor Tu; Short=EF-Tu	–	–	0.08	0.08	0.16	0.08	–	–	1/1	1/1	3/3	1/1

The (-) means that there was not identified protein in complex mixture of dental biofilm formed in the presence of H_2Odd (A), a mixture of 10.45% glucose and 10.45% fructose (B) and 20% sucrose solutions (C). emPAI index: exponentially modified protein abundance index; MW: molecular weight.

CONCLUSÃO GERAL

Os resultados do presente estudo demonstram que o perfil protéico da matriz extracelular do biofilme dental formado na ausência ou na presença de glicose + frutose ou sacarose é distinto, sugerindo que as mudanças na composição de proteínas da matriz extracelular do biofilme dental formado *in situ* não podem ser atribuídas exclusivamente à presença de sacarose durante a formação do biofilme dental e que a presença de seus monossacarídeos (G+F) também interfere na composição de proteínas do biofilme, podendo assim também modificar o potencial cariogênico do biofilme.

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

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ANEXOS

ANEXO 1

Deliberação CCPG – 001/98

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

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

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

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

Artigo 2° - A critério do orientador, os Capítulos e os Apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

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

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

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

ANEXO 2

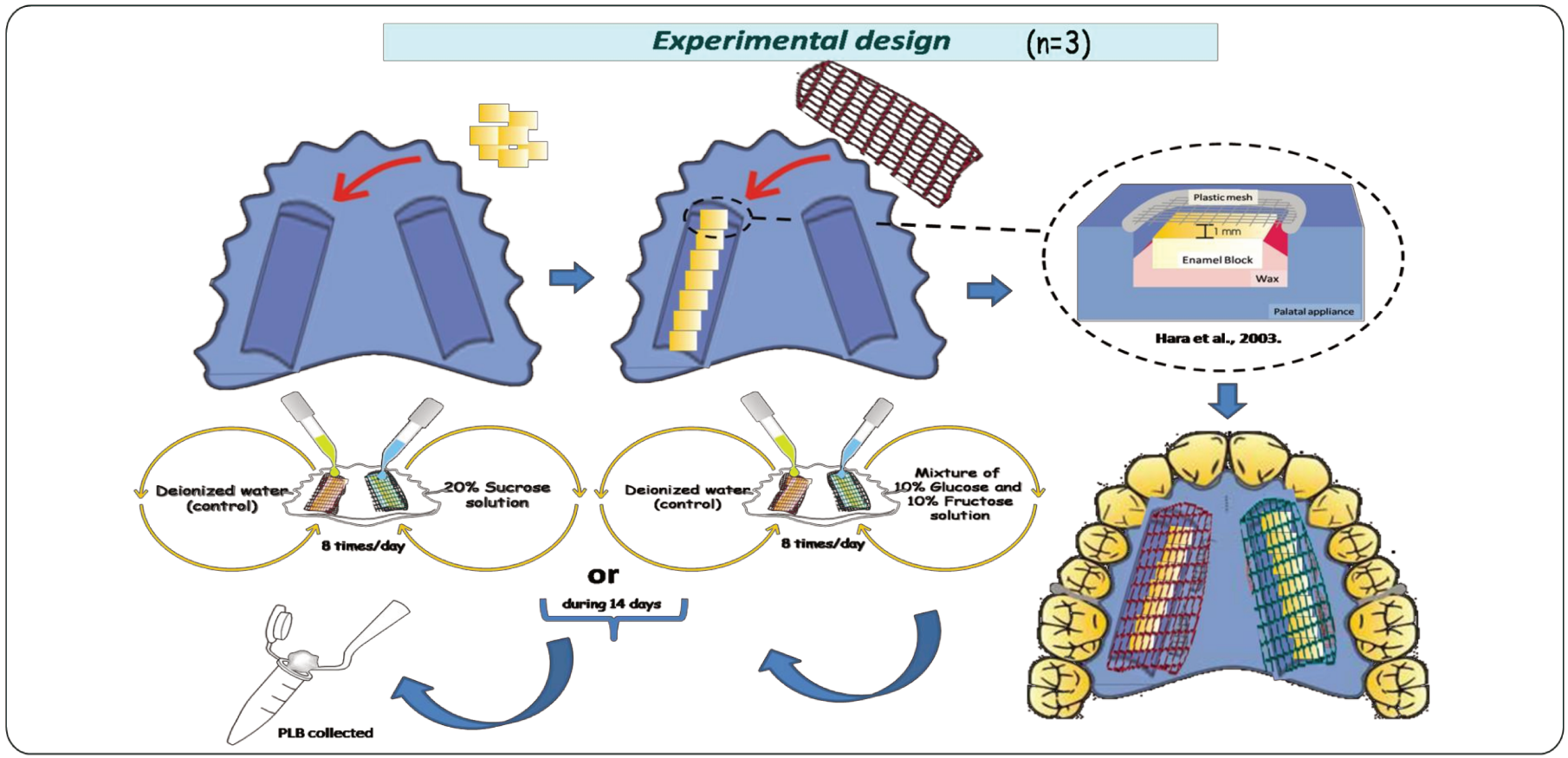
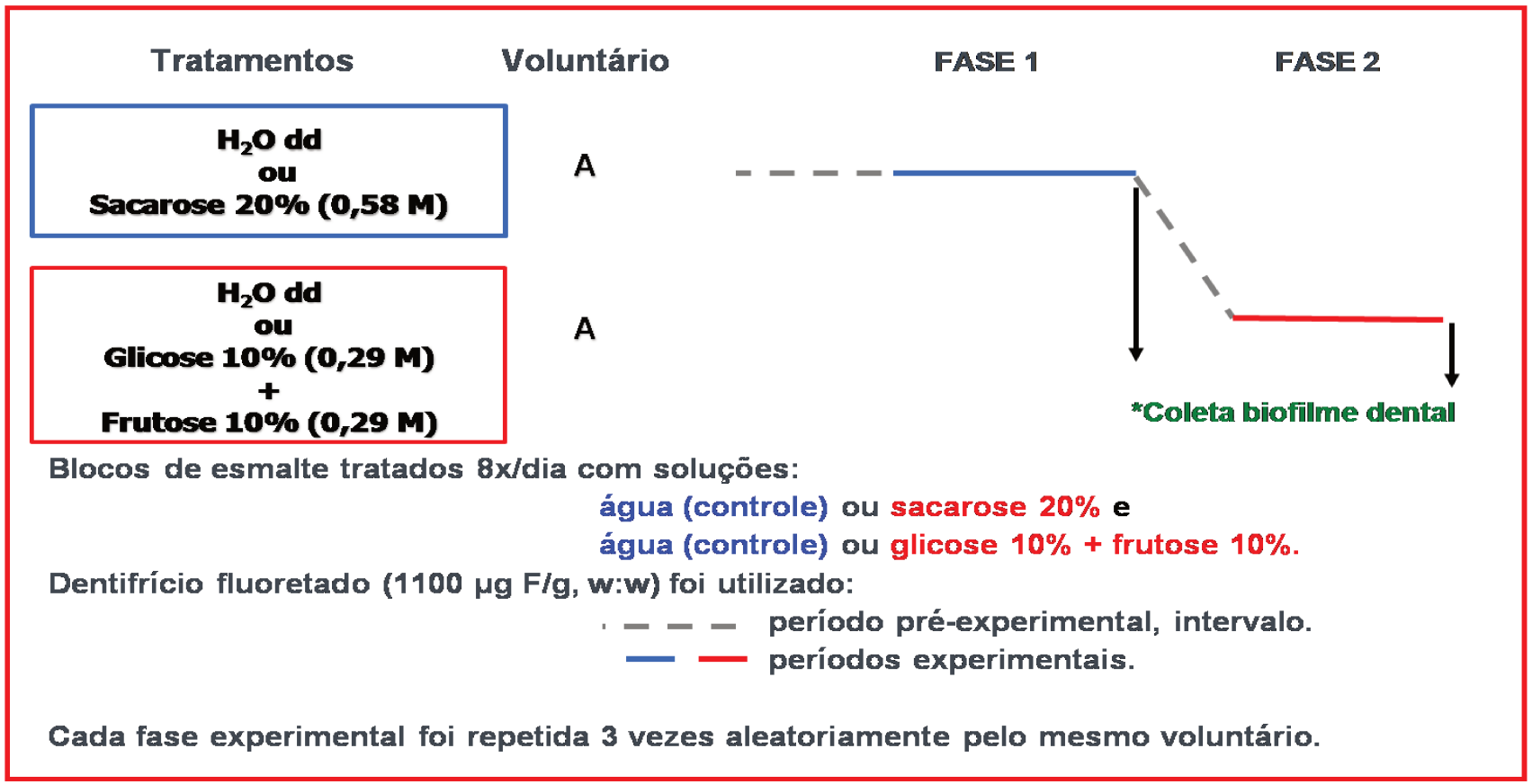


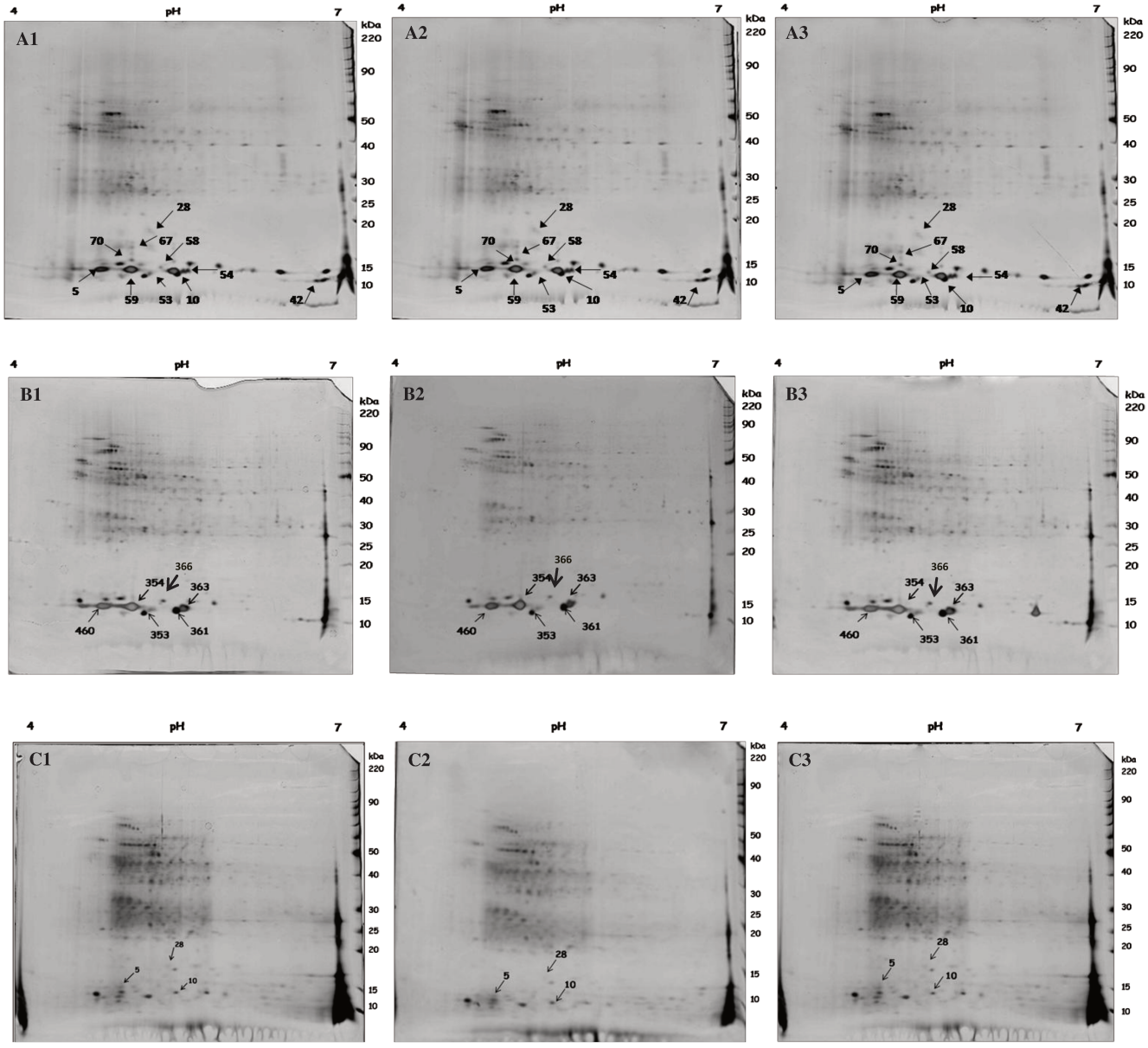
Ilustração do delineamento experimental.

ANEXO 3



Fluxograma dos tratamentos

ANEXO 4



Perfil protéico da matriz extracelular do biofilme dental formado na presença de água destilada e deionizada (A1, A2, A3), glicose 10% e frutose 10% (B1, B2, B3) e sacarose 20% (C1, C2, C3), obtido à partir dos géis bidimensionais corados com prata. Os spots diferencialmente expressos ($p<0,05$) estão representados numericamente nas triplicatas de cada tratamento.

ANEXO 5

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Análise do perfil proteômico do biofilme dental formado na presença de glicose + frutose e sacarose in situ"**, protocolo nº 099/2003, dos pesquisadores Jaime Aparecido Cury, Adriana Franco Paes Leme, Gisele Pedroso Moi e Tatiana Meulman Leite da Silva, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 11/11/2009.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Proteomic analysis of dental biofilm formed in the presence of glucose + fructose and sucrose in situ"**, register number 099/2003, of Jaime Aparecido Cury, Adriana Franco Paes Leme, Gisele Pedroso Moi and Tatiana Meulman Leite da Silva, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at .

Prof. Dr. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

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

ANEXO7

PROTEOMICS

Proteomic Analysis of Extracellular Matrix of Dental Biofilm Formed In Vivo under Dietary Carbohydrate

Journal:	*PROTEOMICS*
Manuscript ID:	Draft
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	
Complete List of Authors:	Moi, Gisele; Piracicaba Dental School, Physiological Sciences Dombroski, Thais; Brazilian Biosciences National Laboratory Pauletti, Bianca; Brazilian Biosciences National Laboratory Cury, Jaime; Piracicaba Dental School, Physiological Sciences Paes Leme, Adriana; Brazilian Biosciences National Laboratory
Key Words:	Biofilm, Extracellular proteins, Protein expression map, Large scale two-dimensional gel experiments, Liquid chromatography-tandem mass spectrometry