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ADRIANA FRANCO PAES LEME

PAPEL DA SACAROSE NA FORMAÇÃO DO BIOFILME DENTAL E NA COMPOSIÇÃO DE PROTEÍNAS DA MATRIZ DO BIOFILME FORMADO *IN SITU*

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.

PIRACICABA
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Orientador: Prof. Dr. Jaime Aparecido Cury
Co-orientadora: Profa. Dra. Cláudia de Mattos Bellato

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A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 27 de Julho de 2005, considerou a candidata ADRIANA FRANCO PAES LEME aprovada.

PROF. DR. JAIME APARECIDO CURY

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PROF. DR. SERGIO ROBERTO PERES LINE

PROFa. DRa. MARIA REGINA LORENZETTI SIMIONATO

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Portanto, teu destino está constantemente sob teu controle.

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SUMÁRIO

RESUMO

A cárie dental é uma doença biofilme-dependente e os carboidratos fermentáveis são considerados os fatores ambientais chaves envolvidos na iniciação e desenvolvimento desse processo. 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. Essa tese é composta de dois artigos. O primeiro discute o papel da sacarose na formação do biofilme dental cariogênico e o segundo avalia as proteínas do biofilme formado *in situ* na presença da sacarose. Entre as várias hipóteses para explicar a menor concentração de íons no biofilme, a hipótese da ausência de proteínas ligadoras de cálcio no biofilme formado na presença de sacarose parece explicar esse fenômeno. No segundo estudo, proteínas ligadoras de cálcio foram identificadas somente no biofilme formado na ausência de sacarose, o que ajudaria a explicar a alta concentração de cálcio na sua matriz. Proteínas de origem bacteriana também foram identificadas e a maioria está associada com funções de manutenção do metabolismo energético, síntese de aminoácidos, tradução e proteínas relacionadas ao estresse. Diferentes proteínas de resposta ao estresse foram expressas nas duas condições avaliadas, sugerindo respostas específicas de adaptação para o biofilme formado na presença e ausência de sacarose. Nossos resultados mostram que a caracterização e estudo da função da proteína no biofilme dental podem ajudar a elucidar importantes aspectos envolvidos na iniciação e desenvolvimento da cárie dental.

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. Thus, this thesis was comprised by two manuscripts. The first discusses the role of sucrose in cariogenic dental biofilm formation and the second evaluates the proteins from biofilm formed *in situ* in the presence of sucrose. Among the hypotheses to explain the low inorganic concentration in the biofilm, the absence of calcium-binding proteins in biofilm formed in the presence of sucrose can help explain it. In the second study, calcium-binding proteins were identified only in biofilm formed in the absence of sucrose and help explain the higher calcium concentration in biofilm matrix. Proteins from oral microorganisms were also identified and most of them were associated to housekeeping functions, such as energy metabolism, amino acid biosynthesis, translation and stress-related proteins. Different stress-responsive proteins were expressed in the two conditions evaluated, suggesting specific adaptive-response in biofilms formed in the presence and absence of sucrose. Our results show that the characterization and the study of protein function in dental biofilm help explain important aspects involved with the initiation and development of dental caries.

1. INTRODUÇÃO GERAL

A formação de uma comunidade bacteriana embebida em uma matriz e organizada na forma de biofilme é o meio mais comum de crescimento bacteriano na natureza (Costerton *et al.*, 1987), incluindo aquele formado sobre os dentes (Marsh, 2004). A transição do biofilme saúde para doença está associada às mudanças na composição e metabolismo das bactérias no biofilme. Assim, a cárie dental é uma doença biofilme-dependente e os carboidratos presentes na dieta são considerados os fatores ambientais chaves envolvidos na iniciação e desenvolvimento desse processo (Marsh, 1991).

A sacarose é considerada o mais importante dos carboidratos, pois além de fermentável, promovendo queda do pH e seleção microbiana (Marsh, 1991) no biofilme, é substrato para síntese de polissacarídeos extracelulares (PEC) (Newbrun, 1967; Bowen, 2002). Os PEC têm sido considerados importantes fatores na virulência dos microrganismos (Bowen, 2002), visto que evidências têm mostrado que interferem na aderência e acúmulo de microrganismos, 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). Esses fatores promovem mudanças microbiológicas, físicas, metabólicas, fisiológicas e químicas provocando aumento da cariogenicidade do biofilme dental.

Entre as mudanças bioquímicas, a baixa concentração de íons, como cálcio, fósforo e fluoreto, observada no biofilme tem sido associada à presença de PEC (Cury *et al.*, 2000; Paes Leme *et al.*, 2004; Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005; Aires *et al.*, 2005). A concentração de íons no biofilme é fator determinante na saturação do biofilme, pois mantém o equilíbrio mineral entre o fluido do biofilme e a superfície dental, e com isso entre o processo de des e remineralização (Pearce, 1998).

Assim, algumas hipóteses baseadas na estrutura, composição e aspectos cinéticos dos íons do biofilme têm sido propostas para explicar a baixa concentração inorgânica do biofilme formado na presença de sacarose. Uma das hipóteses é a baixa concentração de proteínas específicas no biofilme formado na presença de sacarose, pois foi observado perfil distinto de proteínas extracelulares no biofilme formado na presença de sacarose quando comparado com o do biofilme formado na ausência desse carboidrato (Cury *et al.*, 2000). Assim, a expressão diferencial de proteínas salivares e bacterianas no biofilme formado na presença e ausência de sacarose tem sido sugerida.

Estudos recentes têm mostrado a expressão diferencial de proteínas em condições de estresse ácido, revelando novas informações sobre os mecanismos de adaptação, principalmente, de *Streptococcus mutans* nesse ambiente (Svensäter *et al.*, 2000; 2001; Wilkins *et al.*, 2003; Len *et al.*, 2003; 2004). Entretanto, esses estudos foram realizados *in vitro* utilizando uma única espécie.

Assim, o objetivo desse estudo foi discutir o papel da sacarose na formação do biofilme dental cariogênico e analisar a expressão de proteínas do biofilme formado *in situ* na presença e ausência de sacarose e, dessa forma, permitir avaliação no mesmo ambiente da resposta bacteriana e do hospedeiro na formação e acúmulo do biofilme cariogênico.

2. PROPOSIÇÃO

Esta tese será apresentada na forma de 2 capítulos, conforme a deliberação CCPG 001/98 (Anexo 1) e teve como objetivo:

Capítulo 1: Discutir o papel da sacarose na formação do biofilme cariogênico.

Capítulo 2: Identificar as proteínas da matriz do biofilme formado *in situ* na presença e ausência de sacarose.

3. CAPÍTULOS

CAPÍTULO 1: The role of sucrose in cariogenic dental biofilm formation – New insight. AF Paes Leme, H Koo, CM Bellato, G Bedi, JA Cury. Esse artigo foi submetido à publicação no periódico *Critical Reviews in Oral Biology & Medicine* (Anexo 2).

CAPÍTULO 2: Mapping and identification of proteins in dental biofilm formed *in situ* in the presence and absence of sucrose using two-dimensional gel electrophoresis and peptide mass fingerprinting. AF Paes Leme, CM Bellato, H Koo, G Bedi, CPM Tabchoury, AA Del Bel Cury, JA Cury. Esse artigo será submetido à publicação no periódico *Journal of Biological Chemistry.*

CAPÍTULO 1*

The Role of Sucrose in Cariogenic Dental Biofilm Formation – New insight

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ABSTRACT

Dental caries is a biofilm-dependent oral disease and fermentable dietary carbohydrates are the key environmental factors involved in its initiation and development. However, among the carbohydrates, sucrose is considered the most cariogenic, since in addition to being fermentable by oral bacteria, it is a substrate for the synthesis of extracellular polysaccharides (EPS). Therefore, while the low pH environment triggers the shift of the resident plaque microflora to a more cariogenic one, EPS are involved in the adherence of mutans streptococci to tooth surfaces and in changing the structure of the matrix of the biofilm. Furthermore, it has recently been shown that the biofilm formed in the presence of sucrose presents low concentrations of Ca, Pi and F, which are critical ions involved with caries development. Thus, the aim of this review is to explore the broad role of sucrose in the biofilm cariogenicity, and to present a new insight of its influence on the pathogenesis of dental caries.

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INTRODUCTION

Dental caries is a diet-bacterial disease and sucrose is considered the most cariogenic carbohydrate because, it is fermentable, and also serve as a substrate for synthesis of extracellular polysaccharides (EPS) in dental plaque (Newbrun, 1967; Bowen, 2002).

The low pH induced by sucrose fermentation triggers a shift in the balance of resident plaque microflora to a more cariogenic one, according to the ecological plaque hypothesis (Marsh, 1991). This hypothesis has been supported by long-term sugar consumption-diet (De Stoppelaar *et al.*, 1970; Dennis *et al.*, 1975; Staat *et al.*, 1975) and *in situ* experimental studies (Minah *et al.*, 1981; Pecharki *et al.,* 2005; Ribeiro *et al.*, 2005).

Furthermore, the EPS (mainly insoluble glucans) promote bacterial adherence to the tooth surface (Rölla, 1989) and contribute to the structural integrity of the dental biofilms. The EPS also increase the porosity of biofilm formed, allowing sugar diffusion into the deepest part of the biofilm (Dibdin and Shellis, 1988), which would result in low plaque pH values due to microbial catabolism (Zero *et al.*, 1986). There is also evidence showing that sucrose exposure and insoluble EPS are associated with the pathogenesis of dental caries (Johnson *et al.*, 1977; Zero *et al.*, 1986; Cury *et al.*, 1997; 2000; Mattos-Graner *et al.*, 2000; Nobre dos Santos *et al.*, 2002; Paes Leme *et al.*, 2004b; Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005; Aires *et al.*, 2005).

Therefore, it is clear that EPS are critical virulence factors in the dental biofilm formed in presence of sucrose (Bowen, 2002). However, a recent *in situ* data have shown that sucrose, in addition to increasing the EPS content in the biofilm matrix, also induced a significant reduction in the inorganic concentration of calcium (Ca), inorganic phosphorus (P_i) and fluoride (F) (Cury *et al.*, 1997; 2000; 2003; Nobre dos Santos *et al.*, 2002; Paes Leme *et al.*, 2004b; Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005; Aires *et al.*, 2005). These ions are relevant in maintaining the mineral

equilibrium between the tooth and the oral environment (Margolis *et al.*, 1988; Pearce, 1998) and this reduction may increase the cariogenic potential of the biofilm (Margolis and Moreno, 1992; Cury *et al.*, 1997; 2000; 2003; Gao *et al.*, 2001; Ribeiro *et al.*, 2005; Aires *et al.*, 2005). Some hypotheses have been tested experimentally to explain how sucrose reduces the inorganic concentrations in biofilms (Cury *et al.*, 2003), but the phenomenon remains to be elucidated.

Thus, the aim of this review was to discuss the broad role of sucrose in the cariogenic properties of the biofilm and to present a tenable hypothesis to explain the low inorganic concentration found in the matrix of the biofilms formed in the presence of this carbohydrate.

(1) THE "ECOLOGICAL PLAQUE HYPOTHESIS" AND DENTAL PLAQUE AS A BIOFILM

The ecological plaque hypothesis was proposed in an attempt to unify some of the clinical and laboratory observations (Theilade, 1986; Marsh, 1991) by combining elements of the non-specific (Theilade, 1986) and the specific (Loesche, 1976) theories. Thus far, it is the best explanation for the microbial etiology of dental diseases (Theilade, 1996).

With regard to dental caries, and according to this hypothesis (illustrated by Figure 1A), a change in a key environmental factor will trigger a shift in the balance of the resident plaque microflora, which would promote the emergence of more cariogenic bacteria and change the equilibrium toward dental demineralization (Marsh, 1994). Dietary fermentable carbohydrates have been recognized as primary factors responsible for biochemical and physiological changes in dental biofilms. It is well established that after the intake of fermentable sugars (glucose, sucrose or fructose) the pH in plaque falls rapidly, from around neutrality to pH 5.0 or below (Stephan, 1944; Bowen *et al.*, 1966). In addition, the frequent long-term carbohydrate

consumption increase the proportions of mutans streptococci and lactobacilli, with a concomitant fall in levels of the *S. sanguinis*-group (De Stoppelaar *et al.*, 1970; Dennis *et al.*, 1975; Staat *et al.*, 1975). However, it was not known whether the rise in cariogenic bacteria was due to the sudden availability of sugar *per se* or a response to the inevitable conditions of low pH following sugar catabolism (Marsh, 2003). Since these two possibilities cannot be distinguished *in vivo*, Bradshaw *et al.* (1989) demonstrated *in vitro* that when pH was allowed to fall after glucose pulse, the composition of the microflora altered dramatically. After 10 pulses without pH-control, the percentage of viable *S. mutans* and *L. casei* counts increased 19 and 180 times respectively, compared with the condition at constant pH 7.0. Subsequently, it was shown that a fall in pH to values between pH 5.5 and 4.5 may allow the enrichment of potentially cariogenic species, whilst permitting species associated with health to maintain relatively unaffected (Bradshaw and Marsh, 1998). It was also reported that mutans streptococci or lactobacilli are competitive at pH values low enough to demineralize enamel, which inhibited the growth and metabolism of non-cariogenic species (Bradshaw and Marsh, 1998). Collectively, these *in vitro* studies showed conclusively, for the first time, that it was the low pH generated from carbohydrate metabolism rather than carbohydrate availability that leads to the breakdown of microbial homeostasis in dental biofilm. The survival of specific bacteria is probably due to several properties of biofilms when they function as surface-associated microbial communities (Marsh, 2003), and the acid tolerance/adaptation mechanisms of mutans streptococci and lactobacilli (Burne, 1998; Quivey *et al.*, 2000).

The ecological plaque hypothesis, based on *in vitro* studies, has been also supported by *in situ* study showing a clear relationship between mutans streptococci and lactobacilli, and enamel

demineralization or inhibition underneath dental biofilm formed in presence of sugars and antibacterial substances (Pecharki *et al.*, 2005).

However, the low pH generated by sugar metabolism and the subsequent shifts in microbial composition may not be the only factors involved in the pathogenesis of dental caries. A recent study reported that dental biofilm formed *in situ* by frequent exposure to starch displayed 200 times higher numbers of lactobacilli compared with those formed in the absence of the sugar, but this was not enough to induce mineral loss in enamel (Ribeiro *et al.*, 2005). On the other hand, the relationship between the predominance of aciduric bacteria and enamel caries was confirmed in this study when the biofilm was formed in the presence of sucrose.

Therefore, there may be additional factors, besides acidogenicity, to explain the distinct cariogenic potentials among carbohydrates (Carlsson and Egelberg, 1965; Krasse, 1965; Edwardsson and Krasse, 1967; Carlsson and Sundström, 1968; Birkhed *et al.*, 1980; Lingström *et al.*, 1994; Mattos-Graner *et al.*, 1998; Cury *et al.*, 2000; Ribeiro *et al.*, 2005).

(2) THE ROLE OF SUCROSE IN BIOFILM CARIOGENICITY

During the past several years, a causal relationship between sucrose and dental caries has been demonstrated in epidemiological and experimental studies (Edwardsson and Krasse, 1967; Birkhed *et al.*, 1980; Downer, 1999; Cury *et al.*, 1997; 2000; 2001; Nobre dos Santos *et al.*, 2002; Zero, 2004 and references therein). Sucrose causes major biochemical and physiological changes during the process of biofilm formation and accumulation, which in turn enhances its caries-inducing properties.

Evidence has been shown that sucrose promotes an increase in the proportions of mutans streptococci and lactobacilli and, simultaneously, a decrease in the *S. sanguinis* levels, as a result

of pH fall caused by sucrose fermentation (de Stoppelaar *et al.*, 1970; Dennis *et al.*, 1975; Staat *et al.*, 1975; Minah *et al.*, 1981). It suggests that the acid production from metabolism of sucrose disrupts the balance of the microbial community, favoring the growth of cariogenic species (Marsh, 1991). Recent studies have demonstrated that biofilms formed in the presence of sucrose displayed lower pH and higher mutans streptococci and lactobacilli than those formed in absence of the sugar, which enhanced the cariogenicity of the biofilm (Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005). In addition, the cariogenicity of sucrose has been associated with the frequency of exposure and its concentration; because, as these parameters increase, caries development also increases (König *et al.*, 1968; Hefti and Schmid, 1979; Bowen *et al.*, 1980; Cury *et al.*, 1997; Duggal *et al.*, 2001; Paes Leme *et al.*, 2004b; Aires *et al.*, 2005). By increasing the frequency of exposure to carbohydrates, the plaque would be subjected to prolonged period below the critical pH for enamel demineralization; in addition, a greater decrease in pH is observed when sucrose concentration increases. These conditions would favor the growth and selection of cariogenic bacteria, thus changing the biofilm from a healthy to a diseased one and enhancing demineralization (Marsh, 1991). This suggests that sucrose may act as a typical fermentable carbohydrate source, however, when it is compared to other carbohydrates, sucrose shows enhanced cariogenicity (Bowen *et al.*, 1966; Edwardsson and Krasse, 1967; Birkhed *et al.*, 1980; Horton *et al.*, 1985; Cury *et al.*, 2000; Ribeiro *et al.*, 2005).

Furthermore, two recent *in situ* studies clearly demonstrated that sucrose has additional properties that determine its higher cariogenic potential either in comparison to glucose + fructose (Cury *et al.*, 2000) or starch (Ribeiro *et al.*, 2005). For example, sucrose promoted higher enamel mineral loss when compared with its monosaccharides, glucose and fructose (Cury *et al.*, 2000). Sucrose also promotes lower pH, higher mutans streptococci counts in

biofilm and higher mineral loss when compared to starch. Indeed, when sucrose + starch were used in association, the cariogenic potential of starch was enhanced by promoting lower pH, increased lactobacillus and mutans streptococci counts and higher mineral loss (Ribeiro *et al.*, 2005).

Sucrose is a unique cariogenic carbohydrate because it is fermentable, and also serves as a substrate for extracellular glucan synthesis by glucosyltransferases (GTFs) from mutans streptococci (Newbrun, 1967; Bowen, 2002). Several studies have demonstrated a direct relationship between sucrose exposure, extracellular polysaccharides (EPS) and caries development (Johnson *et al.*, 1977; Cury *et al.*, 1997; 2000; Mattos-Graner *et al.*, 2000; Nobre dos Santos *et al.*, 2002; Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005). Therefore, a number of studies have been conducted to investigate how EPS increase the cariogenicity of biofilm.

(3) EPS ENHANCE THE CARIOGENICITY OF BIOFILMS

The EPS are synthesized mostly by bacterial glucosyltransferases (GTFs) using sucrose primarily as substrate (Hamada and Slade, 1980; Bowen, 2002). These polysaccharides are largely insoluble and has complex structure (Kopec *et al.*, 1997) and promote selective adherence (Schilling and Bowen, 1992; Vacca-Smith *et al.*, 1996) and accumulation of large numbers of cariogenic streptococci on the teeth of human subjects (Rölla, 1989; Mattos-Graner *et al.*, 2000; Nobre dos Santos *et al.*, 2002) and experimental animals (Frostell *et al.*, 1967; Krasse, 1965; Johnson *et al.*, 1977). Furthermore, EPS increase the bulk and porosity of dental plaque matrix and a higher amount of substrate would diffuse to the enamel surface (Dibdin and Shellis, 1988). As a result of enhanced substrate diffusibility, deeper layers of dental plaque would display lower pH values due to sugar metabolism by acidogenic microorganisms (Zero *et*

al., 1992) and, thereby enhancing the development of dental caries (Cury *et al.*, 1997; 2000; Mattos-Graner *et al.*, 2000; Nobre dos Santos *et al.*, 2002; Ribeiro *et al.*, 2005).

The relationship between sucrose exposure, EPS and caries development has been demonstrated in several *in situ* studies. For example, an *in situ* study evaluating the composition of dental biofilm formed in the presence of sucrose showed that there was a tendency towards increasing insoluble polysaccharide (IP) concentration in the biofilm matrix, depending on the frequency of sugar exposure (Cury *et al.*, 1997). In a subsequent study, it was observed that dental biofilm formed in the presence of sucrose exhibited significantly higher IP concentration and higher enamel demineralization levels than that formed in the absence of sugars (control), or in the presence of glucose + fructose (Cury *et al.*, 2000). Furthermore, dental plaque formed in the presence of 40% sucrose solution showed the highest IP concentration in the matrix and higher levels of carious lesions than plaque formed in the presence of 5, 10 or 20% sucrose solutions. These findings suggest that higher EPS content may have influenced the pH of the biofilm matrix, resulting in increased mineral loss of adjacent enamel surface (Aires *et al.*, 2005). Clinical studies have also suggested that synthesis of IP is related to caries-activity in children (Mattos-Granner *et al.*, 2000). In addition, dental plaque samples from nursing caries displayed a higher concentration of IP than those from caries-free children (Nobre dos Santos *et al.*, 2002). Clearly, EPS, especially IP, play a significant role in the pathogenesis of dental caries, and sucrose and GTFs are the key components involved in the synthesis of these complex polysaccharides.

However, other factors may influence the biochemistry and structural integrity of EPS. It was observed that sucrose in the presence of starch, not only increases the synthesis of the EPS by GTFs but also changes their biochemical (Vacca-Smith *et al.*, 1996) and physical structure

(Kopec *et al.*, 1997), when compared to those EPS formed in the presence of sucrose alone. A recent *in situ* study showed that IP concentration in biofilms formed in the presence of sucrose + starch did not differ statistically from those exposed to sucrose only (Ribeiro *et al.*, 2005). In contrast, higher enamel demineralization and lactobacillus counts were observed when the biofilm was formed in the presence of sucrose + starch (Ribeiro *et al.*, 2005). These results indicate that the cariogenicity of sucrose could be enhanced by combining it with starch. In this direction, it has been suggested that the formation of glucans and the adherence of oral microorganisms can be modulated by the interaction of amylase and GTF enzymes adsorbed on to hydroxyapatite surfaces; this may influence the formation of dental biofilm and the pathogenesis of caries (Vacca-Smith *et al.*, 1996).

Furthermore, there is significant evidence showing that glucans structure could be influenced by glucanohydrolases present in the oral cavity. For example, while the synthesis of polysaccharides by plaque bacteria during sucrose-rich diet increases, the levels of dextranase and levanase of plaque bacteria also increase (Gawronski *et al*, 1975). Therefore, a dynamic interaction of the enzymes responsible for glucan synthesis on one hand, with those cleaving the glucosidic linkages (dextranase and mutanase) on the other, could be occurring concomitantly in the biofilm matrix. It has recently been shown that the presence of dextranase and/or mutanase during glucans synthesis by GTFs caused linkage remodeling and branching, which influenced bacterial binding sites of the polysaccharides (Hayacibara *et al.*, 2004). The presence of glucanohydrolases may have an impact on the formation, maturation, physical properties, and bacterial binding sites of the polysaccharide matrix in dental biofilm.

Thus, there is a multitude of evidence showing that EPS interfere with: i) microorganism adherence and further accumulation, ii) structural integrity and bulk of biofilms, and iii)

acidogenicity of the biofilm matrix. The biochemical and physiological changes in the matrix of the biofilm promoted by EPS would increase its ability to induce dental caries.

(4) EPS MAY CHANGE THE INORGANIC COMPOSITION OF BIOFILMS

Among the chemical changes that may be associated with EPS content and, consequently, with the cariogenicity of biofilm, low concentration of ions such as Ca, P_i and F is a relevant factor to be considered. The low concentration of ions is directly related to the saturation level of biofilm and determines the driving force of minerals for the demineralization process (Pearce, 1998).

The concentrations of Ca and P_i in dental plaque are relevant in terms of caries development because there is an inverse relationship between concentrations of these ions in plaque's matrix (Ashley and Wilson, 1977) and fluid (Margolis and Moreno, 1992) and caries experience. Dawes and Jenkins (1962) suggested that the relative caries resistance of the lower anterior teeth is associated with the higher Ca and P_i content of the plaque in this region. These ions would be released to the interface plaque/enamel with a fall in pH, and thereby, maintaining the aqueous phase in a saturated condition.

There are also evidence showing a relationship between sucrose, EPS, ion concentrations and caries development. For instance, Cury *et al.* (1997) showed *in situ* that frequent sucrose exposure significantly increased the concentration of IP, and simultaneously reduced F, Ca and P_i concentrations in the dental plaque matrix, which resulted in higher mineral loss of adjacent tooth enamel. Pearce *et al.* (2002) also observed *in vitro* that the concentration of Ca in plaque decreased and IP content increased as sucrose frequency increased. This finding was later confirmed by Paes Leme *et al.* (2004b). Moreover, it has been shown that the concentrations of

Ca, P_i and F were lower in dental plaque formed in the presence of either sucrose or glucose + fructose when compared to a control group (plaque formed in the absence of carbohydrates) (Cury *et al.*, 2000). It is interesting to note that Nobre dos Santos *et al.* (2002) also found lower concentrations of F, Ca and P_i in dental plaque samples collected from nursing caries children, when compared to those from caries-free children. Recently, it was shown that biofilms formed in the presence of sucrose + starch and sucrose alone displayed lower inorganic concentrations than those formed in the absence of sugar or with starch only, which also resulted in higher enamel demineralization (Ribeiro *et al.*, 2005). Finally, lower concentrations of F, Ca and P_i and higher concentration of IP were found in biofilms formed with increasing concentrations of sucrose solutions (5, 10, 20 and 40%), and proportionally higher enamel demineralization was observed (Aires *et al.*, 2005).

These findings suggest that the cariogenicity of dental biofilm is associated with the lower inorganic concentration found in its matrix. Furthermore, it is likely that the inorganic concentration is directly related to the EPS content because, in all *in situ* studies, the lower inorganic concentration found in biofilm formed in the presence of carbohydrates is associated with higher EPS concentration. It appears that the matrix of the biofilms undergoes biochemical changes in the presence of sucrose affecting its ion binding sites. Nevertheless, as yet, it is unclear how this phenomenon occurs. Therefore, several hypotheses and experimental evidence are discussed next to identify a plausible explanation for the lower inorganic concentration in cariogenic biofilms.

(5) HOW COULD THE LOW INORGANIC CONCENTRATION IN A CARIOGENIC BIOFILM BE EXPLAINED?

Recent studies showing that dental biofilm formed in the presence of sucrose display lower ion concentrations in the biofilm matrix (Cury *et al.*, 1997; 2000; 2003; Paes Leme *et al.*, 2004b; Ribeiro *et al.*, 2005, Aires *et al.*, 2005) provide new insight into the formation and composition of a cariogenic dental biofilm, and an enhanced understanding of the pathogenesis of dental caries. Thus, some hypotheses based on the structure, composition and ion kinetic aspects of biofilm have been suggested to explain the lower inorganic concentrations in the presence of carbohydrates: (1) constant low pH values attained in the biofilm matrix due to persistent sucrose fermentation would release biofilm-bound mineral ions, which could diffuse into saliva; (2) enamel could have taken up ions from dental biofilm; (3) the low pH values caused by sucrose fermentation in biofilm promote the release of the ions bound to bacterial cell walls; (4) low density of bacteria due to high insoluble polysaccharide (IP) content results in lower binding sites for ions; (5) low concentration of specific proteins in biofilm formed in the presence of sucrose.

The first hypothesis is that constant low pH, due to sucrose fermentation, would release biofilm-bound mineral ions (Pearce, 1998), which could diffuse into saliva resulting in a biofilm with lower inorganic concentration. However, dental plaque samples in the studies were collected 10-12 h after the last sucrose exposure (Cury *et al.*, 1997; 2000; 2003; Paes Leme *et al.*, 2004b; Ribeiro *et al.*, 2005; Aires *et al.*, 2005). Thus, there would have been enough time for the minerals ions that had been lost to saliva to be replaced by a simple law of mass action. This hypothesis was not considered any further, because the ion concentrations neither increased nor decreased in dental plaque when control and sucrose treatments were switched for 48 h after 28

days of biofilm formation. It is likely that the F, Ca and P_i concentration in biofilm is a result of changes in the matrix structure, rather than depletion of inorganic pools by organic acids (Cury *et al.*, 2003) (Fig. 2 A/B).

It was also considered that the depletion of ions could be explained by the uptake of ions by enamel. However, the mineral ions that have been taken up by enamel would be replaced since the plaque samples were collected 12 h after the last sucrose exposure. This hypothesis was rejected because the biofilms formed in the presence of glucose + fructose or sucrose still showed lower inorganic concentrations than the control (no sugar) (Cury *et al.*, 2000) (Fig. 2 C/D).

Another hypothesis is related to the ability of bacterial cell walls to bind ions, which could act as another reservoir of ions in dental plaque (Fig. 2 E/F). For example, calcium binding in streptococci is predominantly phosphate group-based and in *L. casei* and *A. naeslundii* is predominantly carboxylate group-based (Rose *et al.*, 1997a). These ions could be released when the pH falls, and reduce enamel demineralization (Rose *et al.*, 1993). This reservoir of ions could explain not only our findings on Ca concentrations in the matrix of the biofilm, but also fluoride, since Zn^{2+}, Mg^{2+} and Ca^{2+} at 5 mmol/l considerably enhance fluoride binding to the cell wall (Rose *et al.*, 1996). Thus, these ions could be released when the pH falls and prevent enamel demineralization, although, a high frequency of acidification due to exposure to sucrose would make the plaque subsaturated and in turn, demineralization would occur. However, even after the pH in dental plaque has increased and again saturated with ions, plaque formed in the presence of sucrose still showed lower inorganic concentrations than the control group (absence of sucrose) (Cury *et al.*, 2000; 2003). It is apparent that a transitory effect of pH releasing calcium (or fluoride)-binding can not explain the lower inorganic concentration in biofilm, considering

that 12 h after sucrose exposure, the ion concentration still remained low. Therefore, this hypothesis could not explain the cariogenicity of dental plaque formed in the presence of sucrose.

On the other hand, the concept of bacterial binding sites would be extremely important, considering the density of bacteria in biofilm (Carlsson and Sundstöm, 1968) (Fig. 2 G/H), which could be influenced by the amount of insoluble polysaccharides (IP). The IP may occupy a large volume of dental plaque reducing the number of bacteria and consequently, ion-binding sites. It was demonstrated that when the frequency of sucrose exposure was increased, a higher concentration of IP (Cury *et al.*, 1997; Pearce *et al.*, 2002) and lower cell biomass content in biofilm (Pearce *et al.*, 2002) were observed. The concentration of IP in plaque formed in the presence of sucrose was higher than exposed to either control or glucose + fructose (Cury *et al.*, 2000). Using an *in vitro* biofilm model, Rose *et al.* (1997b) suggested that a high proportion of calcium-binding sites in biofilm may reduce mineral loss *in vivo*, which is determined by saturations levels in biofilm. However, even though a higher IP concentration was found in the biofilm formed in the presence of sucrose, the mutans streptococci level in the plaque was unaffected irrespective of whether the plaque was formed in the presence or absence of sucrose (Cury *et al.*, 1997; 2001). In contrast, Nobre dos Santos *et al.* (2002) showed higher mutans streptococci levels in dental plaque samples from nursing caries children than that from caries-free children, and the authors related it to the high frequency of sucrose exposure. Thus, it is unclear whether the bacteria density is associated with the lower inorganic concentration in biofilm formed in the presence of sucrose; further studies are needed to elucidate this issue.

The last proposed hypothesis to explain the simultaneous low concentration of Ca, P_i and F would be the protein composition of dental plaque matrix (Fig. 2 I/J). Recent data showed clear

differences in the pattern of the matrix proteins extracted from dental plaque formed under three distinct conditions: 1) in the absence of sugar (control), 2) in the presence of glucose + fructose, and 3) in the presence of sucrose (Cury *et al.*, 2000). Considering the protein profiles and their concentrations in the biofilms, it would be relevant if there were differences in their ability to bind calcium and work as a template for mineral growth.

Recently, it was shown that approximately 33% of the total calcium in dental fluid is free, 17% is bound to phosphate and organic acid anions, and 50% is bound to the other species (such as proteins) (Gao *et al.*, 2001). If proteins are responsible for 50% of calcium concentration, a change in protein profile could result in fewer calcium-binding sites. Thus, this observation may help to explain the findings that biofilm formed in the presence of sucrose exhibit lower inorganic concentration (Cury *et al.*, 1997; 2000; 2003; Paes Leme *et al.*, 2004b; Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005). Whether calcium-binding proteins from saliva or from bacteria can actually serve as a template for mineral growth in dental biofilm awaits further evaluation.

Proline-rich proteins (PRP), statherin, histatins identified in acquired enamel pellicle (Schüpbach *et al.*, 2001), cysteine-containing phosphoproteins in dental plaque (DiPaola *et al.*, 1984) and low-molecular-weight peptides in human parotid saliva (Perinpanayagam *et al.*, 1995) may play significant role as calcium-binding proteins. Studies on calcium-binding properties on acidic PRP indicated that there is an interaction between the calcium binding N-terminal end and the proline-rich C-terminal (Bennick, 1987). PRP and statherin are also potent inhibitors of calcium phosphate precipitation (Moreno *et al.*, 1979). Moreover, the low-molecular weight peptides are likely to be in exchange with dental plaque fluid and may therefore help modulate events, such as demineralization and remineralization, microbial attachment and dental plaque metabolism at the tooth-saliva interface (Perinpanayagam *et al.*, 1995). These proteins bind

preferentially to hydroxyapatite surfaces and possibly can bind to calcium. The protein binding mechanism could be similar to that of casein phosphopeptides (CPP), a protein that stabilizes amorphous calcium phosphate (ACP) forming small clusters, which are able to release calcium to inhibit demineralization and/or enhance remineralization (Rose, 2000). The addition of CPP-ACP to either sorbitol- or xylitol-based sugar-free gum resulted in a dose-dependent increase in enamel subsurface remineralization (Shen *et al.*, 2001). Therefore, the calcium-binding proteins can work as a calcium reservoir and modulate crystal growth, interfering with de-remineralization.

Several studies have identified calcium-binding proteins in saliva, acquired pellicle and gingival crevicular fluid by using two-dimensional gel electrophoresis (2D-PAGE) and peptide mass fingerprinting (Kojima *et al.*, 2000; Ghafouri *et al.*, 2003; Yao *et al.*, 2003; Huang, 2004). Nevertheless, none of them analyzed the protein profile in the matrix of dental biofilms. The protein profile in biofilm formed in the absence or presence of sucrose (Fig. 3 A/B) was recently evaluated by means of 2D-PAGE (Paes Leme *et al.*, 2003) and peptide mass fingerprinting (Paes Leme *et al.*, 2004a). Calcium-binding proteins were identified only in biofilm formed in the absence of sucrose (Paes Leme *et al.*, 2004a). This finding is the first evidence showing that the absence of calcium-binding proteins in a biofilm formed in the presence of sucrose is associated with the low concentration of calcium in its matrix, which would promote conditions of undersaturation and, consequently favor the demineralization process.

The qualitative protein differences observed in dental biofilm formed in the presence of sucrose may also be directly related to the presence of EPS, since they occupy a large volume of plaque, decreasing binding sites for proteins. Moreover, it is not known whether the presence of

ions, such as calcium, is necessary for protein binding, or these specific proteins would serve as a template for mineral binding sites.

The findings of the absence (or undetectable levels) of calcium-binding proteins in biofilm formed in the presence of sucrose offer a promise among the different hypotheses discussed here to identify additional pathways by which this carbohydrate influence the cariogenicity of biofilms. On the other hand, it is related only to calcium concentration; it does not directly explain the low concentration of fluoride and inorganic phosphorus.

(6) CONCLUSION

The structure, composition and physical-chemical properties of cariogenic biofilm need to be explored in greater detail, since these features can reveal new insight into understanding the pathogenesis of dental caries and its prevention. Moreover, further studies on the ability of calcium to bind to bacteria cell walls and salivary proteins may enhance our current understanding of the dynamic process of caries development. Further investigation on the biological and chemical aspects of cariogenic biofilm formation is clearly warranted.

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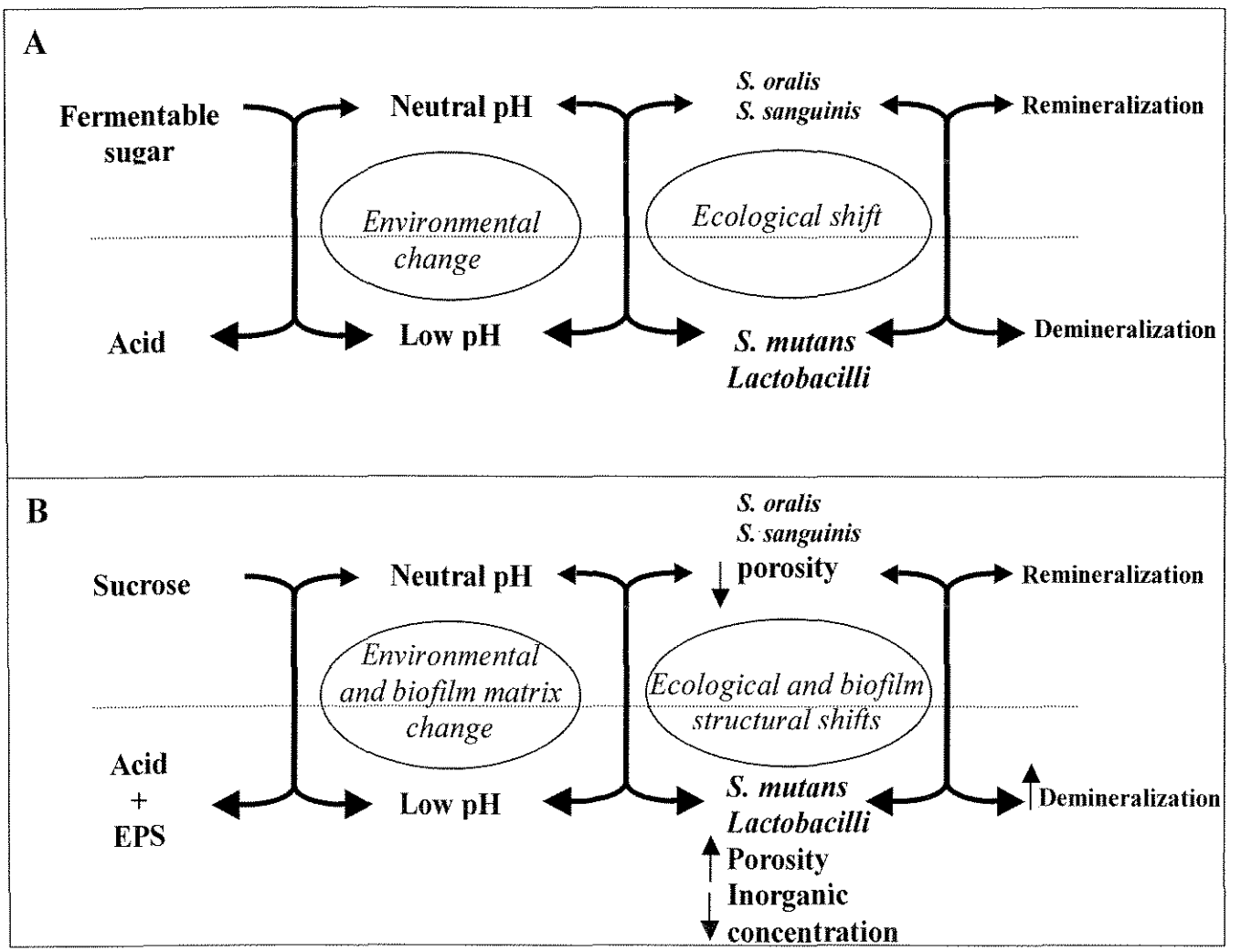

Figure 1

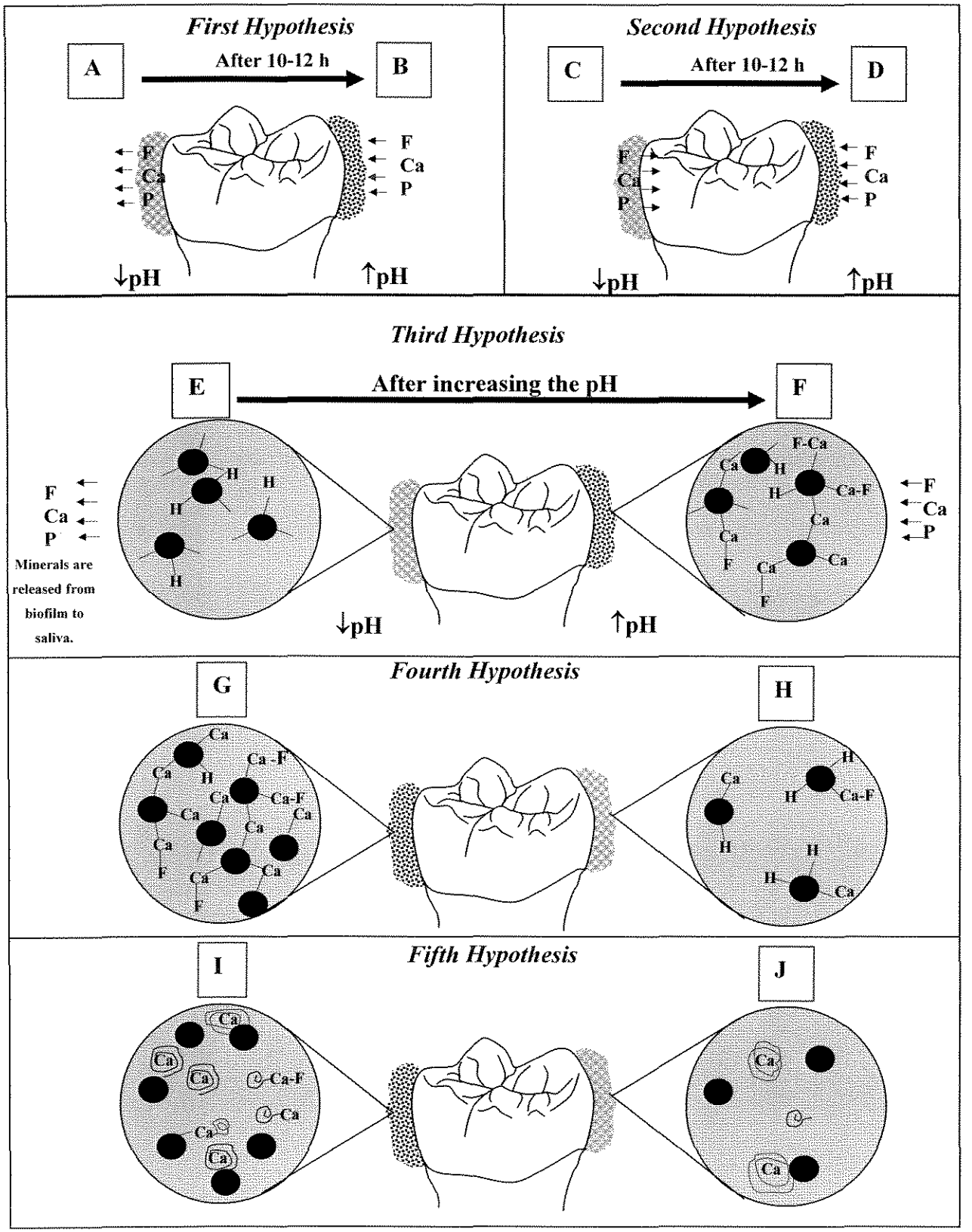
First Hypothesis
A
After 10-12 h
B
F
Ca
P
↓pH
↑pH
Second Hypothesis
C
After 10-12 h
D
Third Hypothesis
E
After increasing the pH
F
Minerals are released from biofilm to saliva.
Fourth Hypothesis
G
H
Fifth Hypothesis
I
J

Figure 2

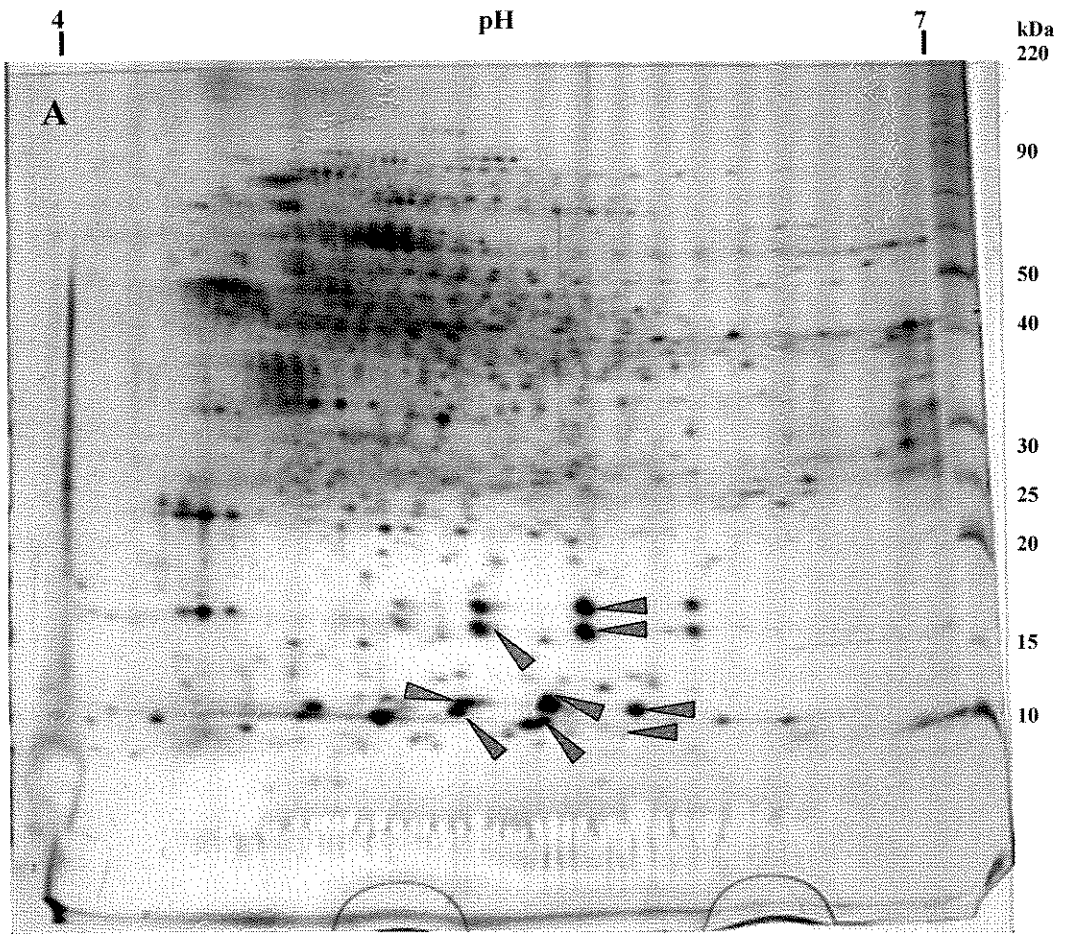

Figure 3 (A)

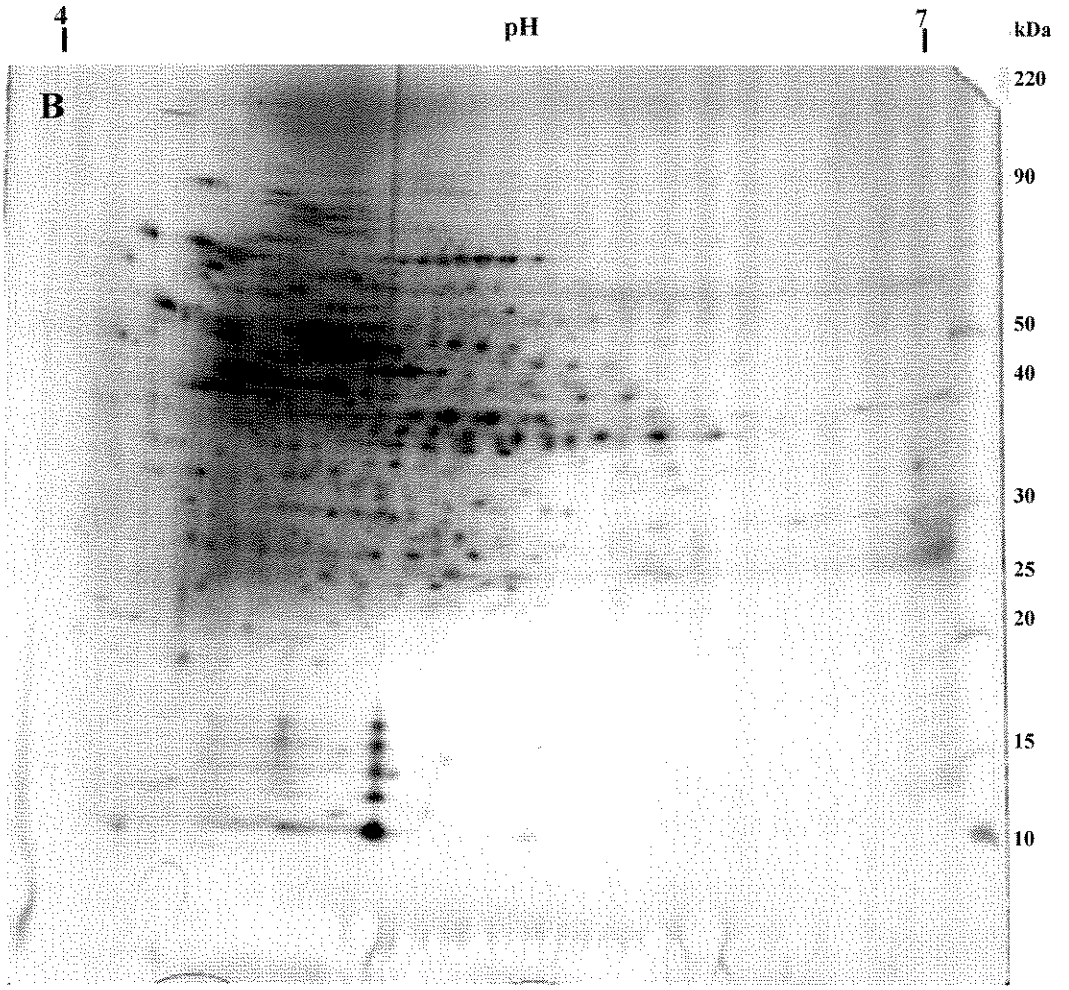

Figure 3 (B)

Figure legends:

Figure 1. Schematic illustration of a cariogenic biofilm formation in the presence of fermentable carbohydrates or sucrose (modified from Marsh, 1994).

Figure 2. Schematic representation of the first, second, third, fourth and fifth hypotheses, respectively. (A) First hypothesis: Constant low pH caused by sucrose fermentation would liberate biofilm-bound mineral ions, which could diffuse to saliva, and promote dental plaque with lower inorganic concentration. (B) However, after 12 h, there would have been enough time for the minerals ions that had been lost to saliva to be replaced by a simple law of mass action. (C) Second hypothesis: Enamel could have taken up ions from biofilm during a pH-cycling. (D) After 12 h, the biofilms would again be saturated with these ions. Third hypothesis: Schematic representation adapted from Rose *et al.*, 1996. Binding to bacteria cell wall is another reservoir of minerals. (E) When the pH falls, the minerals are released from biofilm. (F) After increasing the pH, the biofilm is saturated again with the ions from saliva. Fourth hypothesis: bacteria density. Biofilm formed in absence (G) and in presence of sucrose (H). Note that in the figure H the density of bacteria is lower, since polysaccharides occupy a large volume of the biofilm matrix. Fifth hypothesis: low protein concentration. Biofilm formed in the absence (I) or in the presence (J) of sucrose. It has been suggested that biofilm formed in the presence of sucrose shows fewer calcium-binding proteins.

Figure 3. Two-dimensional gel electrophoresis of dental biofilm formed in the absence (A) and presence (B) of sucrose (20 μg of proteins). Isoelectric focusing with pH range 4-7 and PAGE (8-18%). The gels were silver stained. Spots were excised for in-gel digestion and analyzed using mass spectrometry (MALDI-TOF). Arrows show calcium-binding proteins only in biofilm formed in the absence of sucrose (A) when compared with biofilm formed in the presence of sucrose (B) (Paes Leme *et al.*, 2003; 2004a).

CAPÍTULO 2 *

Mapping and identification of proteins in dental biofilm formed *in situ* in the presence and absence of sucrose using two-dimensional gel electrophoresis and peptide mass fingerprinting

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Running title: Mapping and identification of proteins in dental biofilm

* De acordo com as normas do periódico *Journal of Biological Chemistry*.

Summary

Several *in vitro* studies have shown the expression of proteins from oral bacteria under different stress conditions. However, the protein expression under natural conditions of biofilm formation and accumulation is not known. Thus, the aim of this study was to identify proteins in the matrix of the biofilm formed *in situ* in the presence and absence of sucrose, using two-dimensional gel electrophoresis (2D-PAGE) and peptide mass fingerprinting. A palatal appliance containing eight human dental enamel blocks was used in two-14-day phases for plaque accumulation. A 20% sucrose solution or distilled and deionized water was dripped onto the blocks 8x/day and fluoridated dentifrice was used 3x/day. After 14 days, the biofilm was collected and the extracellular proteins were analyzed through 2D-PAGE. Protein spots were excised, digested with trypsin using in-gel protocol and further analyzed using mass spectrometry. The resulting fingerprints were analyzed in protein database searches. The findings of 2D-PAGE showed that the protein profiles of the biofilms formed in the presence of sucrose were distinct when compared with those formed in its absence. Although the exact role of many proteins in the extracellular milieu is not known, most of the proteins identified were associated with housekeeping functions, such as energy metabolism, amino acid biosynthesis, translation, binding and stress-related proteins, and salivary proteins. Different stress-responsive proteins were expressed in both conditions evaluated, suggesting specific adaptive-response in biofilms formed in the presence and absence of sucrose. Further investigations are required to ascertain protein functions in extracellular location and determine how their expression influences the pathogenicity of biofilm.

INTRODUCTION

The bacterial community formation embedded in an extracellular matrix and organized in biofilm is the most common form of bacterial growth in nature (1), including the biofilm formed on the tooth surface (2). Dental biofilm (known as plaque) is associated with dental caries, which is one of the most ubiquitous oral diseases. The transition from health to disease is associated with compositional and metabolic changes in bacteria population, suggesting that dental caries is a biofilm-dependent oral disease, and dietary carbohydrates are the key environmental factors involved with its initiation and development (3).

The capacity of biofilm to promote mineral loss is directly related to repeated cycles of biofilm acidification, due to carbohydrate fermentation by microorganisms (4-6). Early studies of Stephan (1944) showed that the microflora associated with high caries activity is not only capable of acidification following ingestion of sugar, but is also capable of tolerating the low pH values. This ability to produce acid and grow under low pH conditions is considered to be important in determining the virulence of bacteria associated with caries initiation and progression (8).

A number of recent reports have focused on the proteomics of carbohydrate metabolism and physiological adaptations that allow *Streptococcus mutans*, the major etiologic agent of dental caries, to catabolize multiple fermentable dietary carbohydrates, and to carry out glycolysis (and survive) at low plaque pH values in oral cavity (9-14). These studies analyzed the expression of intracellular proteins (9-11, 15-17), proteins from extracellular milieu (12,18) and those from bacterial surfaces (19, 20). The protein expression profile was determined mostly by two-dimensional gel electrophoresis (2D-PAGE) and revealed differentially expressed proteins at acid pH, which provided new information on the mechanism of acid tolerance mainly of *S.*

mutans. However, all these studies evaluated protein expression under *in vitro* conditions using a single species.

In the present study, protein expression in the biofilm matrix was evaluated *in situ* to mimic the natural conditions of biofilm formation in the oral cavity in the presence of a mixed microbial populations and salivary components, using sucrose to promote biofilm accumulation. Sucrose was used as substrate, since evidence has shown that sucrose increases the cariogenicity of biofilm when compared to other carbohydrates (21-25). In addition to sucrose being able to promote a decrease in pH and bacteria selection, it is also a substrate for extracellular polysaccharides (EPS), which have been associated with low ion concentrations in biofilm, thus enhancing caries development (4, 6, 24-28). Recent study suggested that the higher cariogenicity of sucrose could be associated with the protein expression in biofilm, since it was observed distinct protein profiles in biofilm formed *in situ* in the presence of sucrose, when compared to that formed in the absence of sucrose (24). It would be relevant if there were differences in their ability to bind calcium and work as a template for mineral growth, thus explaining the low inorganic concentration in biofilm formed in the presence of sucrose, and consequently the higher cariogenic potential of the biofilm.

Therefore, we attempt to identify the proteins expressed in the matrix of the biofilm formed in the oral cavity in the presence or absence of sucrose, which would provide new insight on the pathogenesis of dental biofilms related to caries and reveal new approaches to prevent this ubiquitous disease.

EXPERIMENTAL PROCEDURES

Experimental Design

This study was approved by the Research and Ethics Committee of Faculty of Dentistry of Piracicaba-UNICAMP, Piracicaba, SP, Brazil, and involved a crossover design performed in two phases of 14 days each. A healthy volunteer, 26-year-old, wore an acrylic palatal appliance containing eight human dental enamel blocks. The volunteer was instructed to drip a 20% sucrose solution or distilled and deionized water onto the appliance eight times a day during 14 days for biofilm accumulation. The biofilm was collected and its protein profile was evaluated using 2D-PAGE. Protein identification was performed using peptide mass fingerprinting. At least two distinct experiments were performed for each treatment with samples obtained from the same volunteer.

Enamel blocks and palatal appliance preparation

Enamel blocks (4 x 4 x 2 mm) were prepared as previously described (4, 24). The enamel surface was cleaned by professional prophylaxis with non-fluoridated dentifrice containing silica as abrasive, to remove acquired pellicle remnants just before fixed in the acrylic appliance. The volunteer wore a custom-made acrylic palatal appliance containing eight blocks, fixed as closely as possible to the posterior teeth. On the left and right sides of the intra-oral palatal appliances, 8 cavities of 5 x 5 x 3 mm were made, and into each of them 8 blocks of enamel were placed. Plastic meshes were fixed over the cavities to protect the enamel blocks from mechanical attrition, leaving 1.0-mm space for biofilm accumulation (4, 24). During 14 days, dental biofilm was allowed to form on the enamel blocks.

Treatments

The solutions used for the treatments were freshly prepared every two days. The use of 20% sucrose solution was based on results of biofilm analysis found in previous studies (4, 24).

Eight times per day, at pre-determined times (8:00, 9:30, 11:00, 14:00, 15:30, 17:00, 19:00, 21:00 h), the volunteer was instructed to remove the appliance and drip a 20% sucrose solution or distilled and deionized water (control) onto the enamel blocks. After 5 min, the appliance was replaced in the mouth. A washout period of ten days was allowed between the phases. During a 10-day pre-experimental period and during the experimental period, the volunteer brushed the natural teeth with fluoridated dentifrice (silica-based, containing 1100 μg F/g, w:w, as NaF). Oral hygiene was performed 3 times a day and the appliances were brushed, except for the enamel blocks, without disturbing the biofilm. The volunteer consumed fluoridated water (0.6-0.8 mg F/L) and received instructions to wear the appliance all the time, including at night, but to remove it during meals (24). The test subject received oral and written instruction to refrain from using any antibacterial substance during the pre-experimental, experimental and washout periods. Considering that the study followed a crossover design, with the same participant in both steps, no instructions were given regarding daily diet.

Biofilm analysis

The dental biofilm formed on the enamel blocks was colleted ten hours after the last exposure to treatments (4, 24).

Extraction of biofilm matrix proteins

Biofilm was placed in coded, preweighed microcentrifuge tubes and the wet weight was determined. The samples were treated with 50 μL of 0.1 N NaOH (29) containing 1 mM EDTA

(30) for each mg of biofilm (24) for 1 h at 0°C under agitation. Samples were centrifuged (3,000 *g*) for 30 min at 4°C and extracellular proteins in the supernatant were precipitated with 3 volumes of ice-cold acetone, incubating overnight at -20°C and collected by centrifugation (3,000 *g*) at 4°C for 30 min. The resulting pellet was dried to eliminate acetone and resuspended in 0.125 M Tris-HCl, pH 6.8 plus 0.25 mL of protease cocktail-inhibitors (Calbiochem)/g of biofilm and stored at -20°C. At least two distinct experiments were performed for each treatment with samples obtained from the same volunteer.

Analysis of proteins by 2D-PAGE

Before the first dimension, proteins from biofilm were treated with 2D clean up kit (Amersham Biosciences/GE Healthcare Bio-sciences) and protein concentrations were determined by the Bradford method (31). The protocols for first (IEF) and second dimensions (PAGE) were performed according to Bellato *et al.* (2004) with modification. IEF was conducted with Immobiline Dry Strip pH 3-10, 4-7 and 4.5-5.5 (18 cm). Briefly, strips were rehydrated for 8 h at 20°C with 400 µL of IEF solution (8 M urea, 4% CHAPS, 70 mM DTT, 0.8% ampholytes and 0.006% bromophenol blue) containing 20 µg of protein in a IPGphor system (Amersham Biosciences/GE Healthcare Bio-sciences) with current limit 50 µA/strip until focusing reached 70 kVh (30V for 6h, 150V for 2h, 350V for 1h, 500V for 1h, 1000V for 1h, 3000V for 1h and 5000V until the desired focusing time was reached). After focusing, the strips were stored at -20°C prior use or the strips were equilibrated with 50 mM Tris-HCl, pH 8.4; 6 M urea; 30% (vol/vol) glycerol; 2% SDS; 2% DTT for 12 min at room temperature (RT) and, subsequently, in the same buffer but with DTT replaced with 3% iodoacetamide. After 10 min at RT, the strips were sealed with 0.5% agarose in an electrophoresis running buffer (25 mM Tris-HCl, 192 mM glycine and 0.1% SDS) into the top of a 8 to 18% polyacrylamide-gradient gel casted in a

vertical system (20 cm x 20 cm x 1.5 mm, Protean II xi cell, Bio-Rad). Molecular-weight-markers (Invitrogen) covering the 220- to 10-kDa range were applied at the basic end of the IPG strips. Electrophoresis was carried out in the presence of the running buffer at a constant 20 mA at 10°C per gel until the bromophenol blue dye migrated 2 cm followed by a constant 40 V per gel for 12 h at 10°C. Following separation in the second dimension, the gels were washed three times with Milli-Q water (Millipore System) and fixed overnight in 50% methanol/10% acetic acid and proteins were visualized with silver staining (33). The gels were scanned in Fluor-S MultiImager (Bio-Rad). All gels were done in duplicate.

Image analysis

Gel images were analyzed using ImageMaster 2D Platinum software (version 5.0, Amersham Biosciences/GE Healthcare Bio-sciences). The image analyses included eight gels considering the control and treatment groups (two distinct experiments with duplicates for the control and sucrose treatment) and were performed as follow. Observed masses for resolved proteins were calculated by comparing their mobility with that of molecular weight markers, and p*I* values were calculated according to linearity of the IPG strips using the software.

ImageMaster 2D Platinum detection parameters, such as number of smooth, saliency, and minimum area were adjusted for every selected region of each gel to detect protein spots automatically. Subsequently, each protein spots received an identification number. Afterwards, these automatically detected spots were confirmed visually and edited manually when necessary. Edited spots were those found along the edges of the gels and streaked spots.

For each protein spot, the spot volume was calculated, according to the software manual, as above spot border situated at 75% of the spot height (measure from the peak of the spot), which permitted the automatic subtraction of background. The percentage of volume of spot was

determined as function as the total blacking images and number of spots in a gel. In order to analyze the experimental variations between the duplicates, the correlation coefficient was calculated according to the % volume of paired spots (34).

After analyzing the duplicates, one master gel (synthetic gel) was generated for control group and another for sucrose group. The image of the master gel was created through the intersection of four gels (duplicates and gels from distinct experiments) according to the spot positions of the reference gel by averaging shapes and optical densities of matched spots in a given set of gels and showed only the spots found in all gels. The % volume was recalculated according to the total volume of the synthetic gel. The experimental variations were also evaluated through correlation coefficient of the % volume of paired spots between master gel and original gels.

Afterwards, the image master gels produced for control (absence of sucrose) and sucrose treatment (presence of sucrose) were compared through the automatic procedure. Protein spots were considered to be differentially expressed if the % volume of protein in the master gel was up- or down-regulated 1.5-fold or greater under the two conditions evaluated (10, 19).

Protein identification

Proteins were excised from the gel, diced finely and destained according to Gharahdaghi *et al.* (1999), washed with Milli-Q water, followed by washing in 100 mM ammonium bicarbonate (NH_4HCO_3) for 1 hour under agitation. A volume (10 µL) of 41.4 mM DTT was added to 150 µL of 100 mM NH_4HCO_3, and the proteins were reduced for 30 min at 60°C. After cooling to RT, 10 µL of 135 mM iodoacetamide was added. After 30 min incubation in the dark at RT, the gel pieces were washed in a solution of 50 mM NH_4HCO_3-50% acetonitrile (ACN) for 1 h under agitation and dehydrated with 100% ACN. The liquid phase was removed and the gel

pieces were completely dried in a vacuum centrifuge. The gel pieces were re-swollen in a 10 μL of digestion buffer (25 mM NH_4HCO_3) containing 0.01 μg/μL of sequencing-grade trypsin (Roche) and after 5 min, the buffer without trypsin was added to cover the gel and the samples were incubated at 37°C overnight.

Peptides were extracted by two changes in 0.1% trifluoroacetic acid (TFA)-60% ACN (20 min for each change) and one change of 100% ACN (10 min) at RT and dried in a vacuum centrifuge. Peptide extract was resuspended in 10 μL of 1.6% TFA and desalted using Zip Tip_{C-18} (Millipore) according to the manufacturer's instructions. Briefly, the Zip Tips were rinsed three times in a wetting solution (100% ACN and 0.1% TFA-50% ACN) and an equilibration solution (0.1% TFA). The Zip Tip containing peptides was washed three times in 0.1% TFA and eluted three times in the solutions (0.1% TFA-50% ACN and 100% ACN). Samples were dried and resuspended in a 2-μL aliquot of 1% α-cyano-4-hydroxycinnamic acid in 50% ACN-0.1% TFA and were applied to a stainless steel target plate. Samples were allowed to air dry prior to spectra acquisition.

Matrix-assisted laser desorption/ionization-time-of-flight (MALDI/TOF) peptide-mass mapping was performed using a PerSeptive Biosystem Voyager DE-STR (Applied Biosystem, located at the Center for Oral Biology, Protein Core Facility, University of Rochester, USA) with delayed extraction in reflector mode. The laser intensity was maintained up to 1900 units. All spectra were obtained as 200 shot average. Maldi spectra were calibrated by close external calibration using a peptide fragment standard calibration mixture containing bradykinin, angiotensin, glu-fibrinopeptide and neurotensin in close proximity to the sample spot in the plate, and the Data Explorer software (Applied Biosystems) was used to label monoisotopic peaks.

Mass lists were used to screen against database including Mascot (Copyright 2003 Matrix Science Ltd.; www.matrixscience.com) in the first general search and Protein Prospector (Copyright 1995-2005. The register of University of California; www.prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) using National Center for Biotechnology Information non-redundant database (NCBInr 2005.01.06). Current practices for the identification of proteins from 2D gels frequently involve interrogation of the genomic sequence data available for the particular species. Therefore, for those species for which these data are not yet available, protein identification may be more problematic, if analyses are restricted to comparison with a limited number of individually sequenced genes deposited in non-redundant nucleic acid and protein sequence databases. Considering that this study evaluated proteins in the biofilm originated from human saliva and oral microorganisms, and since the genome of some microorganisms is not currently undergoing sequencing, it was necessary to select some phylogenetically similar species (10, 18), or ones that appeared as first candidate and had a high score in the Mascot search, or still some that were associated with dental caries (36-38) and that are present in the NCBI's list of species.

The Mascot search was done without species and molecular mass restrictions; mass tolerance parameter was performed as described for the Prospector program search and with carbamidomethylation as possible variations. All searches in Protein Prospector program were performed against *Actinomyces*, *Homo sapiens*, *Fusobacterium nucleatum*, *Lactobacillus*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Streptococcus anginosus*, *Streptococcus equi*, *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus salivarius*, *Streptococcus sanguinis*, *Streptococcus sobrinus*, *Neisseria subflava*, *Veillonella parvula*, and other species,

such as *Bacillus subtilis*, *Staphylococcus aureus,* and also *Pseudomonas aeruginosa,* when this microorganism was the first candidate according to the general search in the Mascot program and showed a significant score at 200 ppm of mass tolerance.

The parameters for initial database search in the Prospector program were performed with 100 ppm of mass tolerance, one missed cleavage, carbamidomethylation, at least four peptides were required to match; and masses (m/z) of keratin and trypsin and of the gel control without protein were set in the Prospector program as contaminant masses. The proteins and related information listed in Tables 1 and 2 are from searches performed using 100 ppm of mass tolerance. Other searches were conducted by narrowing down the parameters and using more stringent conditions, with 75, 50, 20, 10 and 5 ppm of mass tolerance, and superscript letters in Tables 1 and 2 represent the mass tolerance of the protein that continued as the first candidate in such parameter. This procedure was used to get more reliable results as recommended by Baldwin (2003). The molecular mass entry was based on a 2D gel image with a window between 10 kDa for each search. The chosen protein always had the highest MOWSE (molecular weight search) score (40).

RESULTS

Image analysis and Identification of proteins

Preliminary analyses using IPG strips between the ranges 3 to 10 and 4.5 to 5.5 revealed that the majority of the protein spots were concentrated within the pH range 4 to 7 (data not shown). Based on these results, subsequent analyses for the extracellular proteins extracted from the biofilms were evaluated within the pH 4-7 in two independent experiments with duplicates for each treatment.

Examination of the original 2D protein profiles showed 521.5 ± 7.3 and 512.0 ± 37.4 automatically detected spots for biofilm formed in the absence (control) and presence of sucrose, respectively. The image master gels created by the software showed 445 spots detected in the control and 327 spots detected in sucrose-treated samples. When the data of % volume of spots were compared between gels produced from different experiments, low variability was found between duplicates in absence (0.904–0.957) and presence (0.801–0.855) of sucrose. On the other hand, greater variability in the % of spots volume was observed between the original gel and its respective master gel in absence (0.701–0.964) and presence (0.810–0.940) of sucrose. Since the main source of error associated with this form of quantification is the reproducibility considering the biological variations, the variability in the number and % volume of spots in this study was minimized through repeating the experiment with the same volunteer, and with duplicate gels for the control and sucrose treatment.

The matching analysis of the control and sucrose-treated image master gels revealed 143-paired spots. This analysis also revealed 302- and 184-non-paired spots (hereafter denominated exclusive) in the absence and presence of sucrose, respectively. The variation of protein expression was evaluated according to the % volume of each spot by comparing the two master gels. Considering the paired spots, this analysis showed that 78 proteins were up-, 19 were down-regulated and 46 showed similar abundance in biofilm formed in the presence of sucrose when compared to biofilm formed in the absence of sucrose.

Most of the spots were excised from the gels and subjected to in-gel digestion for identification via mass spectrometry. This protocol was adjusted for low protein abundance. Moreover, low molecular weight proteins, silver staining and the concentration of PAGE (8-

18%) used for the second dimension were factors that interfered in the identification process and therefore, limited the identification of a greater number of proteins.

A total of 33 proteins were identified from the biofilm formed in the absence of sucrose (control) and 23 proteins from the biofilm formed in the presence of sucrose (Tables 1 and 2). Of the 33 spots, 20 proteins were present only in the control group (exclusive), while 5 were up-regulated, 3 were down-regulated and 5 proteins showed similar abundance when compared with the sucrose treatment protein profile. In the biofilm formed in the presence of sucrose, 7 proteins were present only in this group (exclusive), 15 were up-regulated and 1 was down-regulated when compared with the protein profile of the control group.

Database search revealed that the proteins identified from the biofilm formed in the absence of sucrose were (Figure 1 and Table 1) calcium-binding proteins, such as Calcium binding protein 1 isoform 2 (spots 18, 19 and 20), S100 Calcium-binding protein A9 (spots 23, 24, 28, 30, 31 and 32) and Lipase precursor (spot 1); proteins related to bind property, such as putative ATP-binding protein (spot 11), Putative chorismate mutase (spot 22) and Prolactin-induced protein (spots 27, 29 and 33); proteins associated to stress conditions, such as GroEL (spots 3, 4 and 5); proteins associated with maintaining the intracellular pH, such as ATP synthase beta chain (spot 6); proteins related to protein biosynthesis, like Elongation factor Tu (spot 10) and Translation elongation factor Ts (spot 15); and other proteins, whose functions are not clearly established, such as Restriction endonuclease (spot 2), Putative transposase (spot 7), putative transporter protein (spot 8), Putative maltose/maltodextrin-binding protein (spot 9), Thioesterase domain containing 1 (spot 13), GTPase (spot 14), Putative ribonucleotide reductase (spot 17), Nucleoside diphosphate kinase (spot 25) and hypothetical proteins (spots 12, 16, 21 and 26).

Among the proteins identified in the biofilm formed in the presence of sucrose (Figure 2 and Table 2), there were proteins associated with stress conditions, such as DnaK (spot 34); sugar metabolism, such as Enolase (spots 39 and 40), Phosphoglycerate mutase I (spot 50), Phosphotranferase system, mannose-specific EIIAB (spot 45) and Pyruvate kinase (spots 35 and 36); protein biosynthesis, such as Translation elongation factor TU (spot 37) and Tuf (spot 38); amino acid metabolism, such as Putative NADP-specific glutamate dehydrogenase (spots 41 and 42); binding property, such as Prolactin-induced protein (spots 27, 52, 53, 54 and 55); transport capacity, such as Amino acid ABC transporter (ATP binding protein) (spot 49); and other proteins, whose functions are not clearly established, such as Enterotoxin (spot 46), Putative transposase (spot 43), Methionine synthase II (spot 47), Hypothetical protein SMU.373 (spot 51) and hypothetical proteins (spots 44 and 48).

A number of isoforms were identified in biofilm formed in the absence of sucrose, including GroEL, Calcium binding protein 1 isoform 2, S100 Calcium-binding protein A9 and Prolactin-induced protein; and in biofilm formed in the presence of sucrose, including Pyruvate kinase, Enolase, Putative NADP-specific glutamate dehydrogenase and prolactin-induced protein. Comparison of the data indicated that the expression of isoforms, in terms of both the number of isoforms present and their abundance, was altered under the two different conditions.

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 adaptive 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 (9-14).

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 oral cavity using *in situ* model to analyze the protein expression profile in the presence and absence of sucrose, since recent study observed distinct protein profiles in these biofilms (Cury *et al.*, 2000). Under these conditions, salivary and bacterial proteins may be differentially expressed, which may help explain biofilm pathogenicity.

Overall, most of the identified proteins in this study are involved in bacterial energy metabolism, translation, amino acid biosynthesis, present chaperonin activity, and are salivary proteins. Some proteins identified in the biofilm formed in the presence or absence of sucrose, such as, enolase, elongation factors, NADP-specific glutamate dehydrogenase, DnaK and others are known to have intracellular functions (10, 11, 13, 16). However, recent studies have shown that a number of proteins, previously thought to be confined to the cytosol, are also associated with the cell-surface or secreted into the external milieu (12, 18-20, 41, 51, 59). Therefore, the presence of these proteins in dental biofilm may not be resulted from cellular lysis during the extracellular protein extraction process. This hypothesis is supported by the fact that different studies have shown that these proteins have function other than that intracellularly (12, 18-20, 41, 51, 52, 59).

The proteome analysis identified two molecular chaperones GroEL and DnaK, which are part of the protective response of bacterial stress (42, 43). GroEL is part of a general stress response and indispensable for cell viability (44); it is able to capture and refold non-native substrate proteins (43). This protein was identified in biofilm formed in the absence of sucrose,

in agreement with Len *et al.* (2003), who also simulated nutrient limiting conditions. According to our results, the image analysis revealed that three identified isoforms showed different expression pattern under the two conditions (absence and presence of sucrose) and one of them showed up-regulation in biofilm formed in the absence of sucrose, where the overnight fasting pH is about 7.8 (25, 28). On the other hand, previous study demonstrated that the isoforms of GroEL showed up-regulation in intracellular compartment in an acidic environment (10, 12, 16). The gene may perhaps be regulated by different pathways under different stress conditions, as in this case, in which the biofilm was formed in a nutrient-limiting condition, where the primary source would be salivary proteins, like glycoproteins (45, 46), and bacterially derived proteins. Studies have shown that both GroEL and DnaK were induced during the acid shock response, whereas acid adaptation maintained elevated levels of DnaK, but not GroEL (47). These results suggested that GroEL could also be regulated by other pathways needed for stress tolerance. An alternative hypothesis would be that this protein might have bifunctional activity in an internal/external location. Indeed, the nature of the modification that gives rise to the different isoforms is unknown and their biological relevance has yet to be clarified.

DnaK protein was identified in the extracellular milieu (18) and according to our study, the abundance of this protein was enhanced by 6.67-fold in biofilm formed in the presence of sucrose. This biofilm was exposed to a sucrose solution eight times a day, and its fermentation promoted acidification and induction of acid tolerance response, which could explain the high levels of DnaK. Our result corroborates with those reported by Jayaraman *et al.* (1997), who found that *dnaK* mRNA and intracellular DnaK increased in response to acid shock, thus suggesting that an increase of gene expression could predict the increase of products, and perhaps the secreted DnaK as well.

Proteins involved in the translation function, such as the translation elongation factors, were identified in this study and they were also previously reported in internal (10), surface (19) and external milieu (12, 18). These proteins are involved in the sorting and amplification of transmembrane signals and the direction of the synthesis and translocation of proteins (49). In addition to their function in translation elongation, these proteins behave like chaperones toward protein folding and protection from stress in *E. coli*. (50). Our study showed that EF-Tu was down-regulated in biofilm formed in the absence of sucrose, and EF-Tu and Tuf were up-regulated in biofilm formed in the presence of sucrose and no change was observed in EF-Ts. This evidence suggested that these proteins were induced by the low pH provoked by sucrose fermentation, and that they may act as chaperone-like manner.

An increase in the expression pattern of translation factors found in our study may enhance the activity of ATP-consuming, which stimulates the carbohydrate metabolism, thus promoting the up-regulation of glycolytic enzymes (17). It was observed the up-regulation of two proteins from glycolysis pathway. Three glycolytic enzymes involved in the final conversion of 3-phophoglycerate to pyruvate were identified, such as phosphoglycerate mutase, enolase and pyruvate kinase (18). According to Len *et al.* (2004b) most of the enolase isoforms showed down-regulation, one phosphoglycerate mutase and all pyruvate kinases were up-regulated in low pH in an internal location, which was explained by the decreased levels of 3-phosphoglycerate and phosphoenolpyruvate and increases of pyruvate, without building-up phosphoenolpyruvate level. As phosphoenolpyruvate is also lactate dehydrogenase inhibitor, low levels of this metabolite would allow higher a concentration of lactate to accumulate at low pH, which could directly interfere in the pathogenesis of biofilm.

Although the intracellular mechanism explains the role and expression of enolase, this protein was identified in biofilm formed in the presence of sucrose in an extracellular compartment as in previous study (18). This protein was found to be part of an anchorless class of proteins, increasingly recognized as bifunctional with plasminogen-binding activity (51, 52). The mechanism by which such a glycolytic protein is secreted, as well as the receptors required for their reassociation with the surface, is still unknown. In the present study, one of the isoforms was down-regulated and the other one appeared only in the presence of sucrose treatment. In contrast, it was demonstrated that it did not change the surface expression in mid-exponential phase cell culture at pH 5.2 or 7.0 (19). These results may reflect the differences in the responses of cells exposed to different conditions. This anchorless protein represents a new class of virulent determinants, since it could function as adhesins and invasions, enhancing microorganism adherence to the host tissue (52).

Phosphoglycerate mutase that appeared only in the presence of sucrose and the two isoforms of pyruvate kinase that showed up-regulation were also identified previously in an extracellular compartment (18). The role of these proteins in glycolysis is recognized, however, it is not known how virulent gene functions are integrated within metabolic pathway networks and globally regulated by some systems (52) to secrete these proteins to extracellular milieu.

Another identified protein in our study that is involved in sugar metabolism and, more specifically, in carbohydrate transport was the Phosphotransferase system, mannose-specific EIIAB (18). This protein was up-regulated 3.75-fold in the presence of sucrose. A recent study showed that mannose-specific EIIAB is involved in sugar uptake and in carbohydrate catabolite repression, and is essential for optimal gene expression of *gtf*BC and *ftf* (53). Its presence helps explain the high concentration of extracellular polysaccharides (EPS) in biofilm formed in the

presence of sucrose (4, 6, 24, 25, 27, 28, 54), since this carbohydrate is a substrate for glucosyltransferases to synthesize EPS, which is considered one of the most important virulence factors in biofilm for caries development (55).

The metabolism of sugar promotes a rapidly producing of acid and the key survive of microorganisms at low pH, it is the ability to maintain a transmembrane pH gradient, with the cytoplasmic pH value higher than that of the environment. One of the key physiological traits for maintaining cellular pH is the membrane-associated H^+ ATPases (56). ATP synthase beta chain, which is part of the ATPase complex, has already been identified in intracellular milieu (16) and associated with cell wall membrane (41). The ability to up-regulate this protein confers a competitive advantage during the growth of an aciduric microorganism in dental biofilm, facilitating the extrusion and active efflux of H^+ ions. Down regulation of ATP synthase beta chain in the absence of sucrose was observed in our study, which is justified by the neutral pH environment in this biofilm, according to previous studies (25, 28). Moreover, the ABC transporter found to be up-regulated in the presence of sucrose, was also previously found in extracellular milieu (18, 19) and associated with cell wall membrane (41). This protein is another family of diverse membrane proteins and uses the energy derived from ATP hydrolysis to fuel the transport of solutes across the membrane (57), but the precise role of this protein in response to acid stress has yet to be elucidated. Cvitkovitch *et al.* (2000) showed that ABC transporters made a significant contribution to the ability of *S. mutans* to grow at a low pH, which could help to explain its up-regulation in biofilm formed in the presence of sucrose.

Another identified protein in our study that is involved in the central and intermediary metabolism of amino acids in an intracellular compartment was the NADP-specific glutamate dehydrogenase (10, 16). This protein was also observed in extracellular milieu in previous study

(18). Our results showed that both isoforms of this protein were up-regulated in the presence of sucrose. The extracellular role of this protein remains open to speculation and a more likely that the surface-located protein may be an adhesin for binding to immobilized host and bacterial proteins through the glutamate-binding domain (59). These properties would enhance bacteria binding capacity in biofilm formed in the presence of sucrose and together with glucans, thus favoring bacteria accumulation and biofilm bulk (60).

Chorismate mutase was also expressed in our study and found only in biofilm formed the absence of sucrose. This protein is involved in amino acid biosynthesis and it is suggested that it is present in pathogenic microorganisms and may aid with colonizing the host (61). Nucleoside diphosphate kinase also found in the present study only in biofilm formed in the absence of sucrose was reported by Shankar *et al.* (1996) as being in membranous and cytosolic forms in *P. aeruginosa.* The cell membrane-associated form synthesizes GTP in preference to other nucleoside triphosphates, but the mechanisms associated with this protein deserve further studies. Regarding the Lipase precursor protein identified in the absence of sucrose, Simons *et al.* (1998) reported that calcium ions stabilize these lipases secreted by *Staphylococcus* (63). It would be interesting if they act as calcium-binding site for keeping saturation levels as ion reservoirs in biofilm to prevent demineralization, but it is a speculative mechanism.

Salivary proteins were also identified in biofilm, such as Calcium binding protein 1 isoform 2, S100 Calcium-binding protein A9 and Prolactin-induced proteins. Calcium-binding protein A9 belongs to the S100 family and it has been reported to be a potent stimulator of neutrophils chemotaxis and adhesion (64) and involved in the metabolism of arachidonic acid in human neutrophils (65), but their function in biofilm warrants clarification. Calcium binding protein A9 was previously identified in human gingival crevicular fluid (66), in whole saliva (67)

and acquired pellicle (68), but it has not been reported in dental biofilm. Considering the ability to bind calcium and that it is present only in biofilm formed in the absence of sucrose, it could help explain the higher calcium concentration found in the biofilm matrix (4, 6, 24-28). One hypothesis could be that these proteins may function as mineral reservoirs in biofilm, maintaining the saturation levels and preventing demineralization. On the other hand, it is still not known what favors the presence of calcium-binding proteins only in biofilm formed in the absence of sucrose, or in lower and undetectable concentration in biofilm formed in the presence of sucrose. Most of the differences found between biofilms, besides selective microorganisms, are the ΔpH values, inorganic concentration and the EPS content (25, 28), and consequently, the bacteria density (69), which all together could modify the sites for proteins, either by favoring or not their binding, however, this mechanism needs to be studied in more details.

Prolactin-inducible protein was identified in biofilm formed in the absence and presence of sucrose and was previously identified in saliva (34, 67). Neither the functional role nor the physiological importance of prolactin-induced protein is known (70). *In vitro* experiment has shown that prolactin-induced protein has higher affinity for streptococci and suggested that it may be involved in non-immune host defense by binding to bacteria (71). Several prolactin-inducible proteins were identified in our study, mainly in the biofilm formed in the presence of sucrose, suggesting that they might be in glycosylated and nonglycosylated forms (70). The precise biological functions of this saliva protein, as well as the implications of different forms, remain to be clarified.

This study identified several isoforms that were expressed in different amounts under each condition tested. These isoforms most probably arose as a result of post-translational modification, since protein phosphatases, which is important in the phosphorylation process and

signals transduction in other organisms (72, 73), were found to be up-regulated in low pH (19), suggesting that it may be involved in the post-translational modification of some secreted proteins. The existence of the many isoforms expressed by a single gene indicates the need to study the full protein complement of the cell to help find useful biological markers (74). The nature of the modifications that give rise to the different isoforms as well as the relatively small differences in mass between the same protein, which maybe resulted of processing by endogeneous bacterial proteases (10) need to be clarified.

Many of the identified proteins in this study showed housekeeping functions that are involved in recognized pathways, and others were related to stress conditions. Different stress-responsive proteins were expressed in biofilm formed under nutrient limiting conditions (absence of sucrose), or when available eight times a day (presence of sucrose), suggesting specific adaptive-response in biofilms formed in the presence and absence of sucrose. Therefore, the proteins expressed in the biofilm formed in the presence of sucrose may help elucidate how the bacterial cells present in the biofilm have the ability to induce acid tolerant response to grow and survive in this complex environment, thus changing it to a pathogenic biofilm that is able to demineralize the enamel. On the other hand, in the absence of sucrose, which is considered to be the healthy biofilm, starvation-induced stress resistance proteins for surviving under such limiting conditions were also observed. It was reported that starvation conditions resulted in the enhanced synthesis of 58 proteins and 11 were specific to starvation (9). Also, Svensäter *et al.* (2001b) reported that 25 proteins that showed enhanced synthesis in the extracellular milieu were common to both the acid environment and under starvation condition (75), suggesting the protective role of stress proteins under carbon-starvation-induced condition as reported in *E. coli* (76).

In the current study, expressed proteins from the dental biofilm were identified under natural conditions and the data obtained reveal new information regarding the physiological mechanisms of the *in situ* interaction between oral bacteria and the host. The significance and role of many identified extracellular and salivary proteins and the intrinsic mechanism for the translocation of the same protein to multiple compartments in response to environmental changes or cellular requirement should be investigated in future studies.

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Tables:

Table 1: Identity of proteins from biofilm formed in the absence of sucrose separated by 2-D PAGE

Spot no.	Protein assigned	Protein information	Specie Related	Observed[h]		Theoretical		Sequence coverage (%)	MOWSE score	% volume of spot [h]	Ratio % vol. Water/sucr[i]
				*p*I	Mass (kDa)	*p*I	Mass (Da)				
1	Lipase precursor [c]	49484866	*Staphylococcus aureus* subsp. Aureus MRSA252	5.4±0.0	84±0.0	7.7	76733	6.0	3.0 +01	0.2±0.0	Exclusive
2	Putative type I restriction-modification system, specificity determinant; restriction endonuclease [b]	24379345	*Streptococcus mutans* UA159	5.6±0.0	67.5±1.4	8.4	69784	7.0	5.1 +01	0.1±0.0	Exclusive
3	GroEL [e]	15599581 576779 [e, g]	*Pseudomonas aeruginosa* PAO1	5.0±0.0	61.8±0.4	5.0	57086 57036 [e, g]	19 19 [e, g]	3.10 +02 118 [e, g]	0.3±0.1	0.85
4	GroEL [b]	15599581 576779 [b, g]	*Pseudomonas aeruginosa* PAO1	5.1±0.0	55.5±0.0	5.0	57086 57036 [b, g]	25 27 [b, g]	1.7 +04 103 [b, g]	0.3±0.0	0.79
5	GroEL [b]	15599581 576779 [b, g]	*Pseudomonas aeruginosa* PAO1	5.1±0.0	55.5±0.6	5.0	57086 57036 [b, g]	14 15 [b, g]	2.41 +02 65 [b, g]	0.3±0.1	6.40
6	ATP synthase beta chain [c]	15600747 9951894 [c, g]	*Pseudomonas aeruginosa* PAO1	4.9±0.0	46.5±0.7	5.0	49500 49469 [c, g]	51 51 [c, g]	4.6 +09 141 [c, g]	0.2±0.0	0.64
7	Putative transposase [b]	56707134	*Lactobacillus salivarius* subsp. salivarius	4.7±0.0	41.5±0.0	8.9	46880	15	2.99 +02	0.3±0.1	1.84
8	Transporter, putative [b]	34398072	*Porphyromonas gingivalis* W83	6.2±0.0	40.5±1.4	9.4	49314	9.0	1.51 +02	0.4±0.1	1.74
9	Putative maltose/maltodextrin-binding protein [a]	28895783	*Streptococcus pyogenes* SSI-1	5.0±0.0	40.8±0.4	7.6	44540	10	3.3 +01	0.3±0.1	0.81
10	Elongation factor Tu [c]	15599473 9950496 [c, g]	*Pseudomonas aeruginosa* PAO1	5.2±0.0	43.8±1.8	5.2	43370 43342 [c, g]	20 20 [c, g]	7.74 +02 73 [c, g]	0.2±0.0	0.38
11	ATP-binding protein, putative [a]	33390966	*Staphylococcus aureus*	5.9±0.0	35.8±0.4	6.1	31808	12	7.3 +01	0.2±0.0	Exclusive
12	Hypothetical protein [a]	34762127	*Fusobacterium nucleatum* subsp. vincentii ATCC 49256	4.6±0.0	35.5±0.0	7.5	30683	17	2.8 +01	0.2±0.1	Exclusive
13	Thioesterase domain containing 1 [b]	8922871	*Homo sapiens*	4.7±0.0	35.5±0.0	6.7	35818	19	2.72 +02	0.3±0.1	Exclusive
14	GTPase [b]	28378511	*Lactobacillus plantarum* WCFS1	5.4±0.0	35.3±1.1	8.9	31783	18	3.9 +01	0.2±0.1	Exclusive

15	Translation elongation factor Ts [d]	46164365 9949816 [d, g]	*Pseudomonas aeruginosa* UCBPP - PA 14	5.2±0.1	30.0±0.0	5.3 5.2 [d, g]	29966 30634 [d, g]	51 52 [d, g]	7.9 +05 180 [d, g]	0.3±0.2	0.84
16	Hypothetical cytosolic protein [a]	50914941	*Streptococcus pyogenes* MGAS10394	6.1±0.0	26.8±1.1	5.0	22077	27	2.92 +02	0.2±0.0	Exclusive
17	Putative ribonucleotide reductase (NrdI protein) [a]	28896618	*Streptococcus pyogenes* SSI-1	4.5±0.0	16.3±0.4	5.9	17723	25	3.51+02	0.3±0.0	1.95
18	Calcium binding protein 1 isoform 2 [b]	13929434	*Homo sapiens*	5.7±0.0	17.3±0.4	4.7	19430	18	6.25 +02	1.0±0.4	Exclusive
19	Calcium binding protein 1 isoform 2	13929434	*Homo sapiens*	5.3±0.0	16.0±0.0	4.7	19430	18	1.62 +02	0.7±0.4	Exclusive
20	Calcium binding protein 1 isoform 2 [b]	13929434	*Homo sapiens*	5.7±0.0	16.0±0.0	4.7	19430	14	8.0 +01	1.0±0.3	Exclusive
21	Hypothetical protein [a]	51467140	*Homo sapiens*	5.2±0.0	12.3±0.4	9.4	15581	38	2.5 +01	0.2±0.1	Exclusive
22	Putative chorismate mutase [b]	24379707	*Streptococcus mutans* UA 159	4.8±0.0	10.8±0.4	6.6	10327	32	2.99 +02	0.6±0.0	Exclusive
23	S100 Calcium-binding protein A9	4506773	*Homo sapiens*	5.3±0.0	11.0±0.0	5.7	13242	39	4.92 +02	1.3±0.6	Exclusive
24	S100 Calcium-binding protein A9 [c]	4506773	*Homo sapiens*	5.6±0.0	10.8±0.4	5.7	13242 13234 [c, g]	48 47 [c, g]	5.4 +03 71 [c,g]	1.8±0.4	Exclusive
25	Nucleoside diphosphate kinase [c]	15599002 9949980 [c, g]	*Pseudomonas aeruginosa* PAO1	5.8±0.0	12.0±0.0	5.5	15592 15582 [c, g]	56 66 [c, g]	2.0 +03 89 [c, g]	0.3±0.0	Exclusive
26	Hypothetical protein SA V0447 [c]	15923437	*Staphylococcus aureus* subsp. Aureus Mu50	4.7±0.0	10.0±0.0	5.4	13467	30	3.25 +02	0.4±0.1	Exclusive
27	Prolactin-induced protein [a]	51094526	*Homo sapiens*	5.0±0.0	10.0±0.0	8.3	16573 16562	45 65 [a, g]	3.70 +03 81 [a, g]	1.2±0.1	0.31
28	S100 Calcium-binding protein A9 [c]	4506773	*Homo sapiens*	5.3±0.0	10.0±0.0	5.7	13242 13234 [c, g]	47 46 [c, g]	9.6 +03 68 [c, g]	0.7±0.0	Exclusive
29	Prolactin-induced protein [a]	51094526	*Homo sapiens*	5.5±0.0	9.8±0.4	8.3	16573 16562 [a, g]	36 44 [a, g]	1.5 +03 50 [a, g]	0.7±0.1	0.84
30	S100 Calcium-binding protein A9 [a]	4506773	*Homo sapiens*	5.6±0.0	10.0±0.0	5.7	13242 13234 [a, g]	42 42 [a, g]	1.02 +03 60 [a, g]	0.9±0.6	Exclusive
31	S100 Calcium-binding protein A9 [e]	4506773	*Homo sapiens*	5.9±0.0	10.5±0.7	5.7	13242 13234 [e, g]	31 35	4.31 +02 56 [e, g]	1.6±1.1	Exclusive
32	S100 Calcium-binding protein A9 [b]	4506773	*Homo sapiens*	5.8±0.0	9.3±0.4	5.7	13242 13234 [b, g]	31 35	6.09 +02 53 [b, g]	1.0±1.1	Exclusive
33	Prolactin-induced protein [b]	51094526	*Homo sapiens*	6.4±0.0	10.0±0.0	8.3	16573 16562 [b, g]	45 58 [b, g]	3.7 +03 71 [b, g]	0.7±0.4	2.67

Table 2: Identity of proteins from biofilm formed in the presence of sucrose separated by 2-D PAGE

Spot no.	Protein assigned	Protein information	Specie Related	Observed[h]		Theoretical		Sequence coverage (%)	MOWSE score	% volume of spot [h]	Ratio % vol. Sucrose/ water[i]
				pI	Mass (kDa)	pI	Mass (Da)				
34	dnaK protein [c]	15900431	*Streptococcus pneumoniae* TIGR4	4.4±0.0	73.5±0.7	4.6	64842 64802 [c, g]	15 14 [c, g]	4.07 +02 51 [c, g]	0.6±0.2	6.67
35	Pyruvate kinase [c]	42519006	*Lactobacillus johnsonii* NCC 533	5.4±0.0	64.3±3.9	5.5	63567 63527 [c, g]	27 24 [c, g]	3.6 +04 138 [c, g]	0.3±0.2	3.63
36	Pyruvate kinase [f]	42519006	*Lactobacillus johnsonii* NCC 533	5.5±0.0	64.5±3.5	5.5	63567 63527 [e, g]	20 20 [e, g]	6.2 +03 78 [e, g]	0.2±0.2	2.67
37	Translation elongation factor TU [c]	15903386	*Streptococcus pneumoniae* R6	4.8±0.0	51.8±0.4	4.9	43971	31	8.6 +04	0.4±0.1	5.40
38	Tuf [b]	38606905 38606877 [b, g]	*Lactobacillus johnsonii*	4.9±0.0	46.3±0.4	4.8 4.6 [b, g]	43665 25741 [b, g]	32 42 [b, g]	2.2 +05 107 [b, g]	0.2±0.1	1.56
39	Enolase [b]	15900994 15900994 [b, g]	*Streptococcus pneumoniae* TIGR4	4.6±0.0	43.8±1.1	4.7	47103 47074 [b, g]	21 23 [b, g]	3.6 +03 86 [b, g]	0.1±0.1	0.61
40	Enolase [a]	15900994	*Streptococcus pneumoniae* TIGR4	4.6±0.0	43.8±0.4	4.7	47103	14	2.80 +02	0.2±0.1	Exclusive
41	Putative NADP-specific glutamate dehydrogenase [c]	24379360	*Streptococcus mutans* UA159	5.0±0.0	44.8±1.1	5.4	48233 48203 [c, g]	29 29 [c, g]	2.3 +03 78 [c, g]	0.5±0.0	3.94
42	Putative NADP-specific glutamate dehydrogenase [b]	24379360 24377287 [b, g]	*Streptococcus mutans* UA159	5.1±0.0	44.8±1.1	5.4	48233 48203 [b, g]	38 37 [b, g]	6.6 +04 91 [b, g]	0.7±0.1	36.33
43	Putative transposase [a]	19746041	*Streptococcus pyogenes* MGAS8232	5.1±0.0	38.3±0.4	10.2	41110	17	1.78 +02	0.5±0.3	6.25
44	Hypothetical protein lp_0493 [a]	28377385	*Lactobacillus plantarum* WCFS1	5.5±0.0	38.3±0.4	8.1	46211	11	2.9 +01	0.2±0.1	Exclusive
45	Phosphotransferase system, mannose-specific EIIAB [b]	15902305	*Streptococcus pneumoniae* R6	5.6±0.0	38.3±0.4	5.1	35789	14	6.4 +01	0.4±0.0	3.75
46	Enterotoxin [c]	49484068	*Staphylococcus aureus* subsp. Aureus MRSA252	5.5±0.0	35.5±0.7	6.7	30548	22	1.6 +02	0.5±0.4	Exclusive
47	Methionine synthase II (cobalamin-independent) [a]	23002637	*Lactobacillus gasseri*	5.6±0.0	38.0±1.4	5.5	42440	15	3.0 +01	0.2±0.1	2.43
48	Hypothetical cytosolic protein [a]	19704518	*Fusobacterium nucleatum* subsp. Nucleatum ATCC 25586	4.7±0.0	27.8±0.4	8.6	29481	21	2.40 +02	0.1±0.1	Exclusive

49	Aminoacid ABC transporter, ATP-binding protein [b]	15900712	*Streptococcus pneumoniae* TIGR4	5.1±0.0	26.5±0.7	5.1	26876	9.0	3.1 +01	0.4±0.1	3.50
50	Phosphoglycerate mutase I [c]	23003585	*Lactobacillus gasseri*	5.4±0.0	26.5±0.7	5.2	26550 26533 [c, g]	21 24 [c, g]	1.1 +03 63 [c, g]	0.2±0.2	Exclusive
51	Hypothetical protein SMU.373 [c]	24378870	*Streptococcus mutans* UA159	4.9±0.0	25.0±0.7	4.7	28834	23	1.49 +02	0.3±0.1	1.65
52	Prolactin-induced protein [c]	51094526	*Homo sapiens*	5.0±0.0	15.3±0.4	8.3	16573	21	5.0 +01	0.8±0.1	6.94
53	Prolactin-induced protein [c]	51094526	*Homo sapiens*	5.0±0.0	13.8±0.4	8.3	16573	32	1.53 +02	0.8±0.1	3.03
54	Prolactin-induced protein [a]	51094526	*Homo sapiens*	5.0±0.0	11.8±0.4	8.3	16573 16562 [a, g]	22 42 [a, g]	3.9 +01 62 [a, g]	0.8±0.1	Exclusive
27	Prolactin-induced protein [c]	51094526	*Homo sapiens*	5.0±0.0	10.0±0.0	8.3	16573 16562 [d, g]	34 54 [d, g]	3.98 +02 74 [d, g]	2.8±1.5	3.26
55	Prolactin-induced protein [e]	51094526	*Homo sapiens*	4.7±0.0	10.0±0.0	8.3	16573 16562	39 58 [e, g]	8.27 +02 49 [e, g]	0.8±0.3	Exclusive

Superscript letters mean searches with mass tolerance of 100 ppm (a); 100 and 75 ppm (b); 100, 75 and 50 ppm (c); 100, 75, 50 and 20 ppm (d); 100, 75, 50, 20 and 10 ppm (e); 100, 75, 50, 20, 10 and 5 ppm (f), except for spot 19 (102 ppm) and spot 23 (115 ppm).

[g] Search in Mascot program.

[h] Mass and p*I* observed and the % volume of spot are means of two distinct experiments (n=2).

[i] Ratio of %volume of biofilm formed in the presence of sucrose/absence of sucrose (and vice versa) in master gel. It was considered to have altered expression if the % spot volume was up- or down-regulated 1.5-fold or greater (10, 19).

Figures

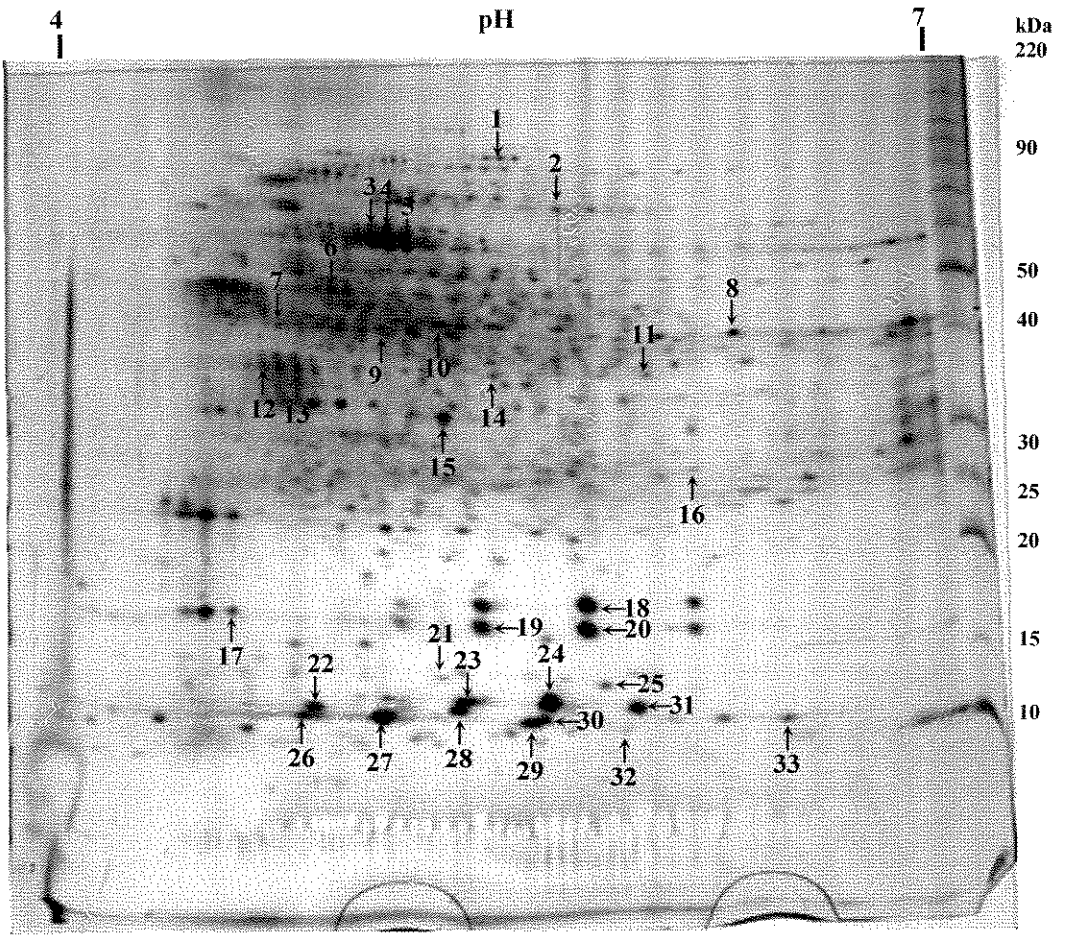

FIG 1

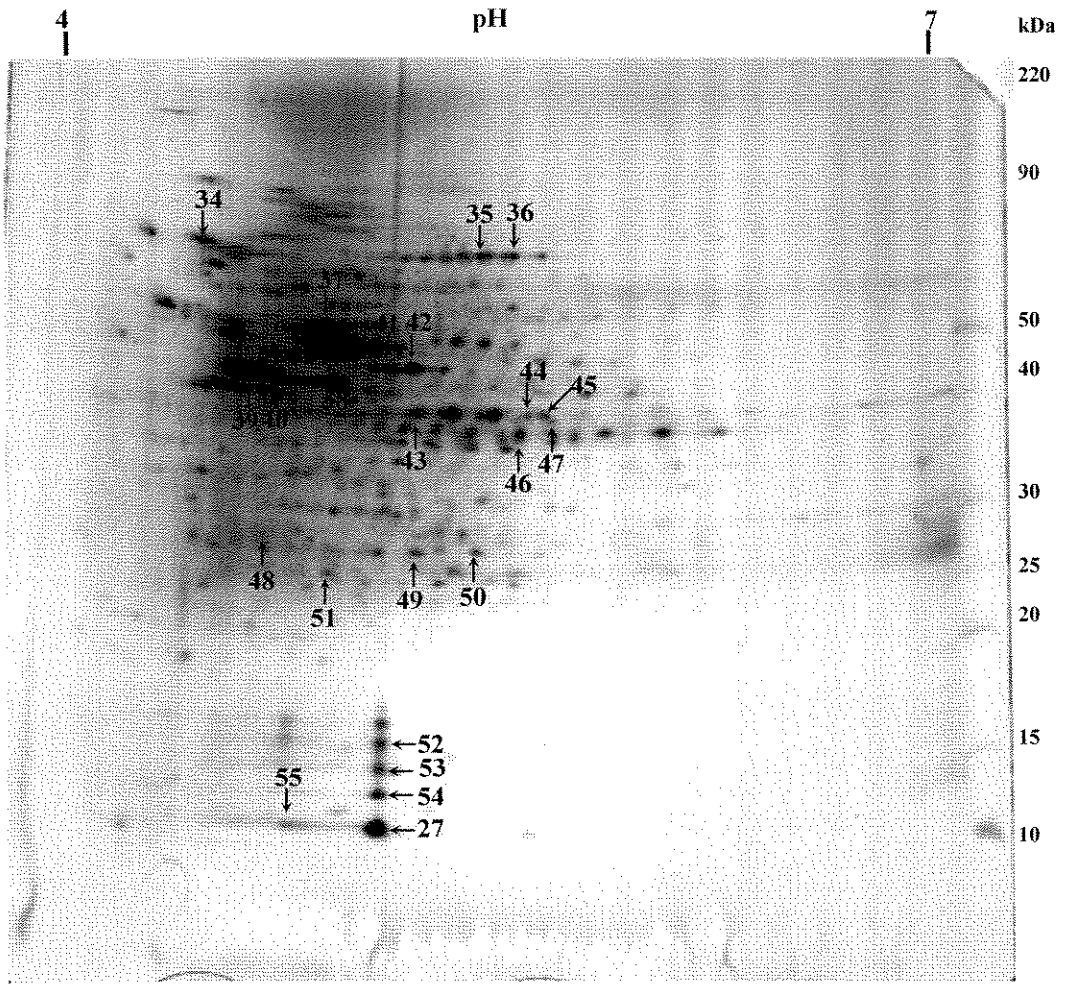
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FIG 2

Figure legends

FIG 1. Resolution of extracellular proteins of dental biofilm formed in the absence of sucrose (20 μg). Isoelectric focusing with pH between 4-7 and PAGE (8-18%). The gel was silver stained. Spots were excised for in-gel digestion and analyzed by MALDI-TOF. Numbers show the identified proteins according to Table 1.

FIG 2. Resolution of extracellular proteins of dental biofilm formed in the presence of sucrose (20 μg). Isoelectric focusing with pH between 4-7 and PAGE (8-18%). The gel was silver stained. Spots were excised for in-gel digestion and analyzed by MALDI-TOF. Numbers show the identified proteins according to Table 2.

4. DISCUSSÃO GERAL

A cárie dental é uma doença bacteriana dieta-dependente (Loesche, 1985), e sua etiologia tem sido explicada pela hipótese da placa ecológica, que propõe que fatores ambientais promovem desequilíbrio da microflora residente promovendo o desenvolvimento da doença (Marsh, 1991).

Estudos têm mostrado que a sacarose apresenta maior cariogenicidade em relação a outros carboidratos (Edwardsson e Krasse, 1967; Birkhed *et al.*, 1980; Horton *et al.*, 1985; Cury *et al.*, 2000; Ribeiro *et al.*, 2005), pois além de fermentável, é substrato para síntese de polissacarídeos extracelulares (PEC) (Newbrun, 1967; Bowen, 2002). Os PEC promovem aderência seletiva (Schilling e Bowen, 1992; Vacca-Smith *et al.*, 1996) e acúmulo de microrganismos cariogênicos (Frostell *et al.*, 1967; Krasse, 1965; Johnson *et al.*, 1977; Rölla, 1989; Mattos-Graner *et al.*, 2000; Nobre dos Santos *et al.*, 2002), aumentam a porosidade do biofilme e difusão de substrato para superfície do esmalte (Dibdin e Shellis, 1988), aumentando a queda de pH na interface dente-biofilme (Zero *et al.*, 1992). Assim, os PEC promovem mudanças no biofilme potencializando a sua cariogenicidade, e estão entre os principais fatores de virulência do biofilme dental.

Entre essas mudanças, a baixa concentração de íons no biofilme pode estar associada à presença de PEC, e é um fator relevante na determinação da saturação do biofilme e, conseqüentemente, no desenvolvimento de cárie (Ashley e Wilson, 1977; Margolis e Moreno, 1992; Pearce, 1998). Tem sido observado *in situ* que o aumento da freqüência de exposição à sacarose promove maior concentração de PEC, menor concentração de íons no biofilme e maior desmineralização do esmalte (Cury *et al.*, 1997; Paes Leme *et al.*, 2004). Também foi demonstrado *in vitro* que o aumento da freqüência de sacarose, promove aumento da

concentração de PEC e diminuição da concentração de cálcio (Pearce *et al.*, 2002). Outros estudos também observaram maior concentração de PEC, menor concentração de íons e maior desmineralização na presença de sacarose quando comparado aos seus respectivos controles negativos (Cury *et al.*, 2000; Nobre dos Santos *et al.*, 2002; Ribeiro *et al.*, 2005). Recentemente, foi demonstrado que o aumento da concentração de sacarose também promove maior concentração de PEC, menor concentração inorgânica e maior perda mineral do esmalte (Aires *et al.*, 2005). Essas evidências sugerem que a maior cariogenicidade do biofilme, além da presença de PEC, também está relacionada com a menor concentração inorgânica. Entretanto, não há explicação para esse fenômeno no biofilme dental.

Assim, algumas hipóteses foram propostas, e duas delas parecem ser as mais prováveis para explicar a baixa concentração de íons no biofilme formado na presença de sacarose: (1) a baixa densidade de bactérias e (2) a baixa concentração de proteínas específicas no biofilme. Com relação à densidade de bactérias no biofilme, foi observado que o biofilme formado na presença de sacarose apresenta um maior espaço intercelular que é preenchido por PEC (Carlsson e Sundstöm, 1968), e se essas bactérias têm habilidade de se ligarem ao cálcio (Rose *et al.*, 1993; 1996), a diminuição da densidade celular levaria a diminuição dos sítios de ligação para o cálcio. Essa hipótese será testada em estudos futuros.

Outra hipótese seria que a expressão diferencial de proteínas salivares e bacterianas no biofilme formado na presença de sacarose resultaria em baixas concentrações de proteínas específicas. Evidências revelaram padrão distinto de proteínas no biofilme formado na presença de sacarose quando comparado com o biofilme formado na ausência de sacarose, entretanto, essas proteínas não foram identificadas (Cury *et al.*, 2000). Esses resultados sugeriram que a

expressão diferencial de proteínas nos biofilmes poderia estar relacionada com o maior potencial cariogênico do biofilme formado na presença de sacarose.

Assim, um estudo experimental foi realizado para avaliar a expressão de proteínas em biofilme formado *in situ* na presença e ausência de sacarose através de eletroforese em duas dimensões e espectrometria de massa. Nesse estudo foi observado um padrão distinto de proteínas no biofilme dental formado na presença e ausência de sacarose. As principais proteínas identificadas que podem estar diretamente relacionadas com a hipótese proposta são as proteínas ligadoras de cálcio. Essas proteínas já foram encontradas na saliva, no fluido crevicular e na película adquirida (Kojima *et al.*, 2000; Yao *et al.*, 2003; Ghafouri *et al.*, 2003; Huang *et al.*, 2004), entretanto, ainda não foram relatadas no biofilme dental. De fato, essas proteínas foram identificadas somente no biofilme formado na ausência de sacarose e não foi identificada na presença de sacarose, seja por ausência ou por concentrações não detectáveis. Esse resultado ajuda a explicar a maior concentração de cálcio no biofilme formado na ausência de sacarose, a maior saturação do biofilme e, conseqüentemente, sua menor cariogenicidade. A razão da ausência dessas proteínas no biofilme formado na presença de sacarose não está clara, e uma possibilidade seria que os PEC presentes nesse biofilme promovam mudanças nos sítios de ligação dessas proteínas. Além disso, não se sabe se a presença de cálcio *per se* seria um sítio de ligação para as proteínas, ou se essas proteínas favoreceriam a ligação desses minerais. Assim, outros estudos são necessários para decifrar esses fenômenos.

As prolactinas também são proteínas encontradas na saliva (Ghafouri *et al.*, 2003; Huang *et al.*, 2004) e foram identificadas nos biofilmes, principalmente, em um maior número no biofilme formado na presença de sacarose. Tem sido sugerido que elas podem ter função na resposta não

imune do hospedeiro por apresentar afinidade a estreptococos (Lee *et al.*, 2002), mas essa função ainda não está claramente definida.

Outras proteínas identificadas no biofilme estão associadas com funções de manutenção bacteriana, como metabolismo energético, síntese de aminoácidos, tradução, ligação e também relacionadas ao estresse. Diferentes proteínas de estresse foram expressas nas duas condições testadas, sugerindo que houve respostas específicas de adaptação na presença e ausência de sacarose.

Na ausência de sacarose, as bactérias poderiam metabolizar constituintes da saliva, como glicoproteínas, em que as enzimas bacterianas clivam os carboidratos dessas proteínas para manter a microflora oral (Beighton *et al.*, 1986). Por outro lado, o limitante de nutrientes poderia induzir a expressão de proteínas específicas (Svensäter *et al*, 2000; 2001) em respostas à condição de ausência de sacarose.

Na presença de sacarose, a indução de proteínas ocorreu tanto em resposta a tolerância ácida, como chaperoninas e ATPase sintase, como também nas vias relacionadas à metabolização do açúcar, como sistema fosfotransferase manose-específico EIIAB, phosphoglicerato mutase, enolase e piruvato quinase. Entretanto, muitas proteínas identificadas ainda não apresentam funções bem definidas no meio extracelular. Diferente dos estudos relatados (Wilkins *et al.*, 2003; Black *et al.*, 2004; Len *et al.*, 2003; 2004), nessa condição testada, além do biofilme ter sido formado e acumulado *in situ*, o meio extracelular apresenta alta concentração de PEC que promovem aumento da porosidade do biofilme (Dibdin e Shellis, 1988), aumentando e prolongando a queda de pH na interface dente-biofilme (Zero *et al.*, 1986; 1992), e com isso, podendo interferir no comportamento bacteriano e na expressão de proteínas.

Assim, esse estudo sugere que a sacarose tem um papel determinante na cariogenicidade do biofilme dental e que a presença de reservatórios de íons no biofilme como proteínas e paredes bacterianas pode ajudar a explicar a maior concentração de íons no biofilme formado na ausência de sacarose. Além disso, observou-se respostas específicas de adaptação na presença e ausência de sacarose, sendo possível avaliar no mesmo ambiente a expressão de proteínas bacterianas e do hospedeiro na formação e acúmulo do biofilme cariogênico. As funções de muitas proteínas identificadas no meio extracelular e das proteínas salivares precisam ser estudadas para revelar novos alvos para o controle da formação do biofilme cariogênico e da cárie dental.

5. Conclusão Geral

Além da presença dos polissacarídeos insolúveis no biofilme formado na presença de sacarose, a menor concentração de íons no biofilme parece ter papel no desenvolvimento de cárie. A hipótese da ausência de proteínas ligadoras de cálcio no biofilme formado na presença de sacarose parece explicar a menor concentração desses íons no biofilme formado. Proteínas expressas na matriz do biofilme formado na presença de sacarose podem ser alvos de novas estratégias para o controle da formação do biofilme cariogênico.

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

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ANEXO 1

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

Detailed Status Information of submission in Critical Reviews in Oral Biology & Medicine.

Manuscript #	05-0240
Current Revision #	0
Submission Date	2005-06-26
Current Stage	Under Consideration
Title	The Role of Sucrose in Cariogenic Dental Biofilm Formation - New insight
Running Title	New insight in cariogenic biofilm formation
Manuscript Type	Other
Special Section	0
Category	Biological
Manuscript Comment	No comments
Corresponding Author	Jaime Cury (UNICAMP)
Contributing Authors	Adriana Paes Leme, Hyun Koo , Claudia Bellato , Gurrinder Bedi
Abstract	Dental caries is a biofilm-dependent oral disease and fermentable dietary carbohydrates are the key environmental factors involved in its initiation and development. However, among the carbohydrates, sucrose is considered the most cariogenic, since in addition to being fermentable by oral bacteria, it is a substrate for the synthesis of extracellular polysaccharides (EPS). Therefore, while the low pH environment triggers the shift of the resident plaque microflora to a more cariogenic one, EPS are involved in the adherence of mutans streptococci to tooth surfaces and in changing the structure of the matrix of the biofilm. Furthermore, it has recently been shown that the biofilm formed in the presence of sucrose presents low concentrations of Ca, Pi and F, which are critical ions involved with caries development. Thus, the aim of this review is to explore the broad role of sucrose in the biofilm cariogenicity, and to present a new insight of its influence on the pathogenesis of dental caries.
Associate Editor	Not Assigned
Key Words	biofilm, sucrose, cariogenic
Author Disclosure	Acknowledgement Section properly discloses sponsor remuneration - no.

Stage	Start Date
Under Consideration	2005-06-26
	2005-06-26
Submission	2005-06-26

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UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

CERTIFICADO

Certificamos que o Projeto de pesquisa intitulado "Caracterização de proteínas ligadoras de cálcio na matriz da placa dental formada na presença sacarose in situ", sob o protocolo nº **016/2002**, da Pesquisadora ***Adriana Franco Paes Leme***, sob a responsabilidade do Prof. Dr. **Jaime Aparecido Cury**, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa – FOP.

Piracicaba, 19 de abril de 2002

We certify that the research project with title "Caracterization of calcium-binding protein in dental plaque matrix formed in the presence of sucrose *in situ*", protocol nº **016/2002,** by Researcher ***Adriana Franco Paes Leme,*** responsibility by Prof. Dr. **Jaime Aparecido Cury**, is in agreement with the Resolution 196/96 from National Committee of Health/Health Department (BR) and was approved by the Ethical Committee in Resarch at the Piracicaba Dentistry School/UNICAMP (State University of Campinas).

Piracicaba, SP, Brazil, April 19 2002

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

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