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"AVALIAÇÃO DE CARACTERÍSTICAS FENOTÍPICAS DE CARIOGENICIDADE DE GENÓTIPOS DE *STREPTOCOCCUS MUTANS* ISOLADOS DE BIOFILME DENTAL"

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

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RESUMO

Os estreptococos do grupo mutans, em especial *Streptococcus mutans*, são considerados como um dos principais microrganismos relacionados à doença cárie dental. Clinicamente, esses microrganismos estão presentes na cavidade bucal na forma de diferentes genótipos, que podem apresentar diferentes características fenotípicas. Nesse sentido, foi avaliada diversidade genotípica de *S. mutans* em biofilme dental formado *in vivo* ou *in situ* durante 3 dias sob condições controladas de exposição à sacarose, o mais cariogênico dos carboidratos, e os seus monossacarídeos constituintes (glicose e frutose). Diferentes genótipos de *S. mutans* foram encontrados nessas distintas condições, entretanto, não foi observada seleção de genótipos nos biofilmes dentais formados. Além disso, as características fenotípicas de virulência desses genótipos não foram avaliadas. Sendo assim, seria importante avaliar nesses genótipos previamente isolados de biofilme dental formado *in vivo* e *in situ*, na presença ou ausência de sacarose, as características fenotípicas de cariogenicidade relacionadas à aciduricidade e à acidogenicidade, visando investigar a relação entre um alto desafio cariogênico (exposição frequente à sacarose e acúmulo de biofilme) e a virulência de *S. mutans*. Tanto naqueles genótipos previamente isolados de biofilme dental formado *in vivo,* quanto naqueles isolados de biofilme dental formado *in situ,* a aciduricidade foi avaliada em relação à análise da viabilidade celular em condições ácidas e em relação à atividade da bomba F-ATPase, e a acidogenicidade foi avaliada em relação à análise da curva de queda de pH devido metabolização de glicose. Além disso, oito genótipos previamente isolados do biofilme dental formado *in situ* durante 3 dias foram submetidos a um crescimento na forma de biofilme *in vitro*, condição na qual foram avaliadas a acidogenicidade, a habilidade do genótipos em sintetizar polissacarídeos extracelulares e o potencial desses genótipos para desmineralizar o esmalte dental. Em relação aos genótipos isolados de biofilme dental formado *in vivo*, aqueles isolados de biofilme formado na presença de sacarose foram mais ácido-tolerantes, tanto em pH 5,0 ou 2,8, e mais acidogênicos, uma vez que

apresentaram menores valores de pH final durante a análise da curva de queda de pH e também maior velocidade na produção de ácidos nos primeiros 15 minutos de metabolização da glicose, que aqueles encontrados em biofilme formado na sua ausência de sacarose. Além disso, não foram encontradas diferenças expressivas na atividade da bomba F-ATPase entre essas duas condições distintas. Comportamento semelhante também foi observado para genótipos previamente isolados de biofilme dental formado *in situ,* com maior aciduricidade e acidogenicidade para aqueles genótipos isolados de biofilme formado na presença de sacarose. No modelo de biofilme *in vitro* não foram encontradas diferenças nem na acidogenicidade nem na habilidade dos genótipos de produzirem polissacarídeos extracelulares. Entretanto, os genótipos apresentaram potenciais cariogênicos distintos, não havendo relação entre o potencial cariogênico desses genótipos e a condição na qual esses genótipos foram isolados (presença ou ausência de um alto desafio cariogênico). Os resultados sugerem que as freqüentes quedas de pH decorrentes da exposição à sacarose parecem tornar os genótipos mais de *S. mutans* mais virulentos. Além disso, genótipos distintos de *S. mutans* podem apresentar diferentes potenciais cariogênicos.

Palavras-chave: *Streptococcus mutans,* genótipos, sacarose, aciduricidade, acidogenicidade, biofilme.

ABSTRACT

Mutans streptococci, mainly *Streptococcus mutans*, are considered as the main microorganisms related to dental caries. These microorganisms are present in oral cavity as distinct genotypes, which may show distinct phenotypic traits. In this context, it was evaluated the *S. mutans* genotypic diversity in dental biofilm formed *in vivo* and *in situ* during 3 days under controlled exposure to sucrose, the most carigenic carbohydrate, and its monosaccharides constituents (glucose and fructose). Distinct *S. mutans* genotypes were found under these before mentioned conditions, but no selection of them was found in dental biofilms formed. Moreover, the virulence phenotypic traits of these genotypes were not evaluated. Thus, it would be important to evaluate in these genotypes previously isolated from dental biofilm formed *in vivo* and *in situ* in the presence or absence of sucrose, the phenotypic traits of cariogenicity related to aciduricity and acidogenicity in order to investigate the relationship between a high cariogenic challenge (frequent exposure to sucrose and biofilm accumulation) and their virulence. Either in those genotypes isolated from *in vivo* dental biofilms, or in those isolated from *in situ* dental biofilms, the acidogenicity trait was evaluated through counts of viable cells in acid conditions and F-ATPase activity, and the acidogenicity trait was evaluated through the ability to lower the pH due to glycolysis. Besides, eight genotypes previously isolated from *in situ* dental biofilms formed during three days were grown as *in vitro* biofilms, and these genotypes were evaluated regarding their acidogenicity, cariogenic potential and ability to synthesize extracellular polysaccharides. In relation to genotypes isolated from *in vivo* biofilms, those isolated from biofilms formed in the presence of sucrose were more acid-tolerant, either at pH 5.0 or at pH 2.8, and more acidogenic, since they showed lower values of final pH during the evaluation of the curve of pH fall and also higher ability to produce acids in the first 15 minutes of glucose fermentation, than genotypes isolated from *in vivo* biofilms formed in the absence of sucrose. Besides, no expressive differences regarding F-ATPase activity between these two distinct conditions were found. Genotypes found only in the presence of sucrose were

more acidogenic than those found only in the absence of this carbohydrate. Similar data were found for genotypes isolated from *in situ* dental biofilms formed in the presence of sucrose, which were more aciduric and more acidogenic than genotypes isolated from biofilms formed in the absence of this carbohydrate. In *in vitro* biofilm model, no differences either in the acidogenicity or in the ability of genotypes to synthesize extracellular polyssacharide were found. However, the *S. mutans* genotypes showed distinct cariogenic potential, independent of the fact that these genotypes had been isolated in the presence or absence of a high cariogenic challenge. The results suggest that frequent pH fall due to sucrose exposure may select more virulent *S. mutans* genotypes. Besides, distinct *S. mutans* genotypes may show distinct cariogenic potential.

Key-words: *Streptococcus mutans,* genotypes, sucrose, aciduriciry, acidogenicity, biofilm.

SUMÁRIO

INTRODUÇÃO GERAL

A cárie dental é uma doença biofilme-açúcar dependente relacionada à frequente ingestão de carboidratos fermentáveis (Bowen *et al*., 1980). Dentre os carboidratos da dieta, a sacarose é considerada como o mais cariogênico (Paes Leme *et al*., 2006), pois além de ser fermentada a ácidos, reduzindo o pH do biofilme dental, é substrato para a síntese de polissacarídeos extracelulares (PECs) (Rölla *et al*., 1985; Cury *et al*., 2000). Esses PECs, principalmente os insolúveis, tornam o biofilme mais poroso (Dibdin & Shellis, 1988), facilitando a difusão de ácidos (Zero *et al*., 1986; 1992), que são capazes de provocar desmineralização em esmalte dental (Cury *et al*., 2000; Pecharki *et al*., 2005; Ccahuana-Vásquez *et al*., 2007; Vale *et al*., 2007) e em dentina (Aires *et al*., 2002, 2008). Além disso, essas quedas de pH no biofilme dental são consideradas como um dos principais estresses aos quais os microrganismos orais estão expostos (Lemos *et al*., 2005), podendo alterar a homeostasia microbiana do biofilme, influenciando o crescimento e a sobrevivência dos microrganismos orais (Bowden & Hamilton, 1998; Marsh, 2003).

Nesse contexto, os estreptococos do grupo mutans, em especial o *Streptococcus mutans*, têm sido considerados como um dos principais microrganismos relacionados à cárie dental (Löesche, 1986; Tanzer *et al*., 2001). Isso se deve ao fato desses microrganismos apresentarem algumas características de virulência, tais como, a aciduricidade, que é a capacidade de sobreviverem e crescerem nos ambientes e nos períodos de reduzido pH decorrentes da metabolização dos carboidratos, e a acidogenicidade, que é a capacidade de produzirem ácidos mesmo em condições de baixo pH (Banas, 2004). Essas características conferem vantagem adaptativa aos *S. mutans* em relação aos outros microrganismos do biofilme em períodos de acidificação do meio. Além dessas características de virulência, os *S. mutans* também apresentam a particularidade de sintetizarem PECs, por ação enzimática de glicosiltransferases, utilizando sacarose como substrato (Hamada & Slade, 1980;

Bowen *et al*., 2002), que viabilizam a aderência desses microrganismos à superfície dental (Rölla, 1989).

Sabe-se que os ácidos produzidos pelos *S. mutans* devido à metabolização de carboidratos fermentáveis poderiam acarretar a acidificação do meio intracelular, comprometendo o funcionamento celular, principalmente das enzimas ácido-sensíveis. Para evitar o comprometimento da viabilidade celular, os microrganismos possuem um sistema, denominado "bomba translocadora de prótons" (F-ATPase) localizada na membrana celular, que bombeia os íons hidrogênio para o meio extracelular. Dessa forma, dentre os diversos mecanismos relacionados à aciduricidade dos *S. mutans*, o sistema F-ATPase tem sido considerado como um dos mais importantes (Lemos *et al*., 2005). Como esse transporte é contrário ao gradiente de difusão desses íons, há gasto de energia, que é fornecida pela hidrólise de moléculas de ATP (Deckers-Hebestreit & Altendorf, 1996). Nesse contexto, alguns estudos têm demonstrado o importante papel desempenhado pela bomba F-ATPase na manutenção da viabilidade celular dos *S. mutans* (Belli & Marquis, 1991; Hamilton & Buckley, 1991; Nascimento *et al*., 2004a).

Clinicamente, alguns trabalhos têm demonstrado que existem vários genótipos de *S. mutans*, tanto na saliva quanto no biofilme dental (Caufield & Walker, 1989; Alaluusua *et al*., 1996; Saarela *et al*., 1996; Mattos-Graner *et al*., 2001; Lindquist & Emilsson, 2004; Klein *et al*., 2004; Napimoga *et al*., 2004; Nascimento *et al*., 2004b; Nogueira *et al*., 2005; Cogulu *et al*., 2006; Gou *et al*., 2006; Lembo *et al*., 2007; Tabchoury *et al*., 2008; Alves *et al*., 2009). Em média, de 1 a 5 genótipos distintos de *S. mutans* têm sido identificados por indivíduo (Saarela *et al*., 1993; 1996; Gronroos & Alaluusua *et al*., 2000; Redmo-Emanuelsson & Thornquist 2000, 2001; Redmo-Emanuelsson *et al*., 2003; Klein *et al*., 2004; Lemos *et al*., 2007; Tabchoury *et al*., 2008), sendo que genótipos distintos podem colonizar uma mesma superfície dental, e que um mesmo genótipo também pode colonizar diferentes sítios (Redmo-Emanuelsson *et al*., 2003). Esses diferentes genótipos, principalmente os mais prevalentes, foram

ainda detectados 4, 7, 18, 20, 24, 36, 60 e 84 meses após a genotipagem inicial (Redmo-Emanuelsson & Thornquist 2000, 2001; Redmo-Emanuelsson *et al*., 2003; Klein *et al*., 2004; Lindquist & Emilson, 2004; Alves *et al*., 2009), sugerindo que os mesmos são estáveis na cavidade bucal. Interessantemente, não tem sido encontrada qualquer correlação entre níveis de *S. mutans* na saliva e diversidade genotípica (Gronroos & Alaluusua, 2000; Mattos-Graner *et al*., 2001; Lembo *et al*., 2007; Alves *et al*., 2009) e nem entre diversidade genotípica de *S. mutans* e atividade de cárie (Gronroos & Alaluusua *et al*., 2000; Redmo-Emanuelsson & Thornquist, 2000; Mattos-Graner *et al*., 2001; Lemos *et al*., 2007). Porém, alguns trabalhos têm sugerido que indivíduos cárie-ativos possuem menor diversidade genotípica de *S. mutans* (Kreulen *et al*., 1997; Redmo-Emanuelsson *et al*., 2003), enquanto que outros trabalhos têm sugerido o oposto (Alaluusua *et al*., 1996; Napimoga *et al*., 2004; Alves *et al*., 2009).

Em relação a essa diversidade genotípica presente na cavidade bucal, tem sido demonstrado que genótipos distintos podem apresentar diferentes capacidades de virulência. Mattos-Graner *et al.* (2004) verificaram que diferentes genótipos de *S. mutans* apresentam diferentes atividades de glicosiltransferase. Guo *et al*. (2006) mostraram que diferentes genótipos de *S. mutans* apresentaram diferença na síntese de polissacarídeo extracelular insolúvel, na acidogenicidade e na aciduricidade. Além disso, Napimoga *et al.* (2004) observaram que genótipos de *S. mutans* isolados de sítios com cárie ativa apresentavam maior habilidade para sintetizar polissacarídeo extracelular insolúvel quando comparados àqueles genótipos presentes em sítios livres de cárie. Maior aciduricidade também foi encontrada em genótipos de crianças cárie-ativas quando comparada aos genótipos de crianças livres de cárie (Lembo *et al*., 2007).

De uma forma geral, essas características fenotípicas têm sido avaliadas em *S. mutans* cultivados como células planctônicas, porém pouco se sabe sobre o potencial cariogênico desses genótipos quando cultivados na forma de biofilmes. Nesse contexto, tem sido sugerido que o comportamento de microrganismos aderidos a superfícies pode ser diferente quando comparado ao comportamento

como célula planctônica. Alguns trabalhos têm demonstrado que a expressão protéica e gênica de *S. mutans* em biofilmes é diferente em relação à expressão em culturas planctônicas (Svensäter *et al*., 2001; Marsh, 2004; Shemesh *et al*., 2007). Além disso, tem sido demonstrado que *S. mutans* em biofilmes são mais acidúricos em relação a células planctônicas (Li *et al*., 2001; Welin-Neilands & Svensäter, 2007), o que demonstra que podem ocorrer modificações no fenótipo dos microrganismos por ocasião do crescimento em biofilmes.

No contexto dos estudos de diversidade genotípica na cavidade bucal, Arthur *et al*. (2006; 2007) avaliaram a diversidade de *S. mutans* isolados de um biofilme dental formado *in vivo* e *in situ* na presença de sacarose (indutor da síntese de PEC e carboidrato fermentável a ácido, promovendo queda de pH no biofilme) e de seus monossacarídeos constituintes, glicose e frutose (somente fermentáveis a ácido). Foi encontrada diversidade genotípica no biofilme dental entre os voluntários, entretanto, não foi verificada seleção de genótipos devido ao estresse induzido pelo metabolismo da sacarose nem pela fermentação de seus monossacarídeos constituintes. Genótipos específicos foram encontrados em menores proporções em cada uma dessas condições descritas, entretanto não foram encontradas diferenças expressivas na diversidade genotípica entre biofilmes formados sob diferentes desafios cariogênicos. Porém, as características fenotípicas de cariogenicidade dos genótipos isolados mediante esses diferentes desafios cariogênicos não foram exploradas. Sendo assim, seria importante avaliar as características fenotípicas de aciduricidade e de acidogenicidade dos genótipos isolados em ambos os biofilmes (*in vivo* e *in situ*), visando investigar a relação entre um alto desafio cariogênico (exposição frequente à sacarose e acúmulo de biofilme) e a virulência de *S. mutans*.

Dessa forma, o objetivo do primeiro trabalho (Capítulo 1) foi avaliar as características fenotípicas de cariogenicidade, tais como aciduricidade, por meio da análise de viabilidade celular em condições ácidas e da atividade da bomba F-ATPase, e a acidogenicidade, por meio de análise da habilidade em reduzir o pH devido à glicólise, de genótipos de *S. mutans* previamente isolados de biofilme

dental formado *in vivo* sob um alto desafio cariogênico (Arthur *et al*., 2006). O objetivo do segundo trabalho (Capítulo 2) foi avaliar essas mesmas características de cariogenicidade, relacionadas à aciduricidade e à acidogenicidade, em células planctônicas de genótipos de *S. mutans* previamente isolados de biofilme *in situ* formado durante 3 dias sob um alto desafio cariogênico (Arthur *et al.,* 2007) e também a habilidade de sintetizar polissacarídeos extracelulares e o potencial de desmineralizar o esmalte dental de alguns genótipos num modelo de biofilme *in vitro*.

CAPÍTULO 1

Journal section for manuscript publication: **Microbial ecology**

Genotypic and phenotypic analysis of *S. mutans* isolated from dental biofilm formed *in vivo* under a high cariogenic condition

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RUNNING TITLE: *S. mutans* genotypes and phenotypic traits

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ABSTRACT

Oral cavity harbors several *Streptococcus mutans* genotypes, which could present distinct virulence properties. However, little is known about the diversity and virulence traits of genotypes isolated under *in vivo* controlled conditions of high cariogenic challenge. This study aimed to evaluate the genotypic diversity of *S. mutans* isolated from dental biofilms formed *in vivo* under frequent exposure or not to sucrose*,* and the aciduricity and acidogenicity of these genotypes. Volunteers rinsed with distilled deionized water or 20% sucrose solution for 10 sec, 8 x/day, during 3 days for biofilm formation on upper posterior teeth. *S. mutans* isolates collected from saliva and biofilms were analyzed for their genotypic identity by arbitrarily-primed PCR. Biofilm genotypes were evaluated regarding their acid susceptibility, F-ATPase activity and ability to lower the pH through glycolysis. Most of the volunteers harbored only one genotype in saliva, which was detected in almost all biofilm samples always at higher proportion, but other specific genotypes were also found in a lower proportion in the biofilms. Genotypes from biofilms exposed to sucrose showed higher acid tolerance at pH 5.0 and 2.8 after 60 and 30 min of incubation, respectively. Genotypes exclusively isolated from biofilms exposed to sucrose showed higher acidogenicity than those exclusively isolated from biofilms not exposed to this carbohydrate. Even though the results suggest that there were no expressive differences in genotypic diversity between biofilms formed under exposure or not to sucrose, it seems that biofilms formed under a high cariogenic condition harbored more virulent genotypes.

INTRODUCTION

Dental caries is a dietary and biofilm-dependent disease related to the frequent consumption of fermentable carbohydrates (5) and to the shift in biofilm microbiota induced by the pH fall (18). Among the dietary carbohydrates, sucrose is the most cariogenic because, besides being fermented, it is also the unique substrate for the synthesis of extracellular polysaccharide (EPS) (8, 28), which may improve bacterial adherence to tooth surfaces as well as modify the matrix of dental biofilm (25).

Among the cariogenic microorganisms colonizing the dental biofilm, the one most implicated in dental caries is mutans streptococci, especially *Streptococcus mutans* (17). *S. mutans* produce EPS from sucrose and are acidogenic and aciduric bacteria, that is, they metabolize fermentable carbohydrates, producing acids that decrease the biofilm pH, and also show the ability of surviving, growing and maintaining their metabolism in this acidic condition (18). This acid tolerance trait is partly due to the presence of a membrane-bound protein called F-ATPase, which extrudes protons out of the cells, preventing the intracellular pH fall and a consequent damage to acid-sensitive enzymes, DNA and proteins (26).

Several studies have shown that the oral cavity harbors distinct *S. mutans* genotypes, either in saliva or dental biofilms (15, 27, 29). Additionally, it has already been reported that genotypes could differ in their virulence abilities (20, 22), which may facilitate their ability to colonize and even predominate in a environment under a high cariogenic condition. Genotypic diversity was also observed among volunteers in *in situ* dental biofilms formed under sugar stress exposure (2), but no specific genotypes were selected due to the stress induced by sucrose metabolism or simple fermentation of its monosaccharides. However, little is known about the genotypic diversity of *S. mutans* and their phenotypic traits in a dental biofilm formed *in vivo* under a frequent and controlled exposure to sucrose.

Thus, this study aimed to evaluate the genotypic diversity of *S. mutans* isolates from *in vivo* dental biofilms, formed under high cariogenic challenge

(biofilm accumulation under frequent exposure to sucrose), as well as some virulence traits related to aciduricity and acidogenicity of the genotypes.

MATERIALS AND METHODS

Selection of volunteers and study design

This study was approved by the Research and Ethics Committee of Piracicaba Dental School (protocols n°. 053/2004 and 004/2006). A total of 12 healthy adults were screened for salivary levels of mutans streptococci (MS). From that group, a subset of 6 volunteers (18 to 28 years old) were selected, due to high MS counts in saliva (≥ 10^5 colonies forming units/mL; CFU/mL) and ability to comply with the experimental protocol. The exclusion criteria included antibiotic use for the last 2 months before starting the study, use of any form of medication that modifies salivary secretion, use of fixed or removable orthodontic appliance, periodontal disease or general/systemic illness. Biofilms formed *in vivo* in the absence or in the presence of sucrose on the surfaces of upper pre-molars and molars were collected from these subjects for the analysis of genotypic diversity of *S. mutans* by arbitrarily-primed PCR (AP-PCR). Afterwards, all biofilm *S. mutans* genotypes were phenotypically evaluated regarding their aciduricity, through evaluation of acid susceptibility and F-ATPase activity, and acidogenicity, by evaluation of the ability to lower the pH through glycolysis.

Saliva sampling and microbiological analysis

Stimulated whole saliva samples were collected from the individuals in the morning within fasting condition and without previous teeth brushing. Saliva was diluted in sterile 0.9% NaCl, and inoculated in triplicate onto Mitis Salivarius Agar (Difco, Sparks, MD, USA) plates supplemented with 20% sucrose (Merck, Darmstadt, Germany) and 0.2 U of bacitracin/mL (Sigma, Steinheim, Germany) (MSB), for culturing of mutans streptococci group. All plates were incubated at 37ºC for 48 h in an atmosphere of 10% CO_2. Eight representative morphological types of *S. mutans* colonies were collected from MSB plates previously inoculated

with saliva samples, subcultured on mitis salivarius agar (MSA) and Brain Heart Infusion (BHI) agar (Difco, Sparks, MD, USA), and pure cultures stored at -70ºC in 10% skim milk medium (Difco, Sparks, MD, USA) for further genotypic analysis (2). The purity and identity of the isolates were checked by Gram's stain and colonial morphology on MSA.

In vivo biofilm formation and biofilm collection

An *in vivo* cross-over and blind study was conducted in 2 experimental phases. During 3 days, the six selected volunteers rinsed with 15 mL of 20% sucrose solution or distilled deionised water, for 10 sec, 8 x/day, at predetermined times (13). The volunteers were randomly assigned to the above mentioned conditions of presence or absence of sucrose during biofilm formation and were instructed to neither brush the upper pre-molars and molars nor use dental floss in these teeth, during the experimental phases, for biofilm accumulation. A wash-out period of 15 days was carried out between both experimental phases. Distilled and deionized water and sucrose solutions were handed daily to the volunteers. At the end of each 3-day experimental phase, 10 h after the last exposure to the respective solution, biofilms formed on upper pre-molars and molars were collected with a sterile spatula, in the morning, with the volunteers in fasting conditions and without having brushed their teeth. The biofilm was weighed (± 0.01 mg), suspended in 0.9% NaCl sterile solution (1 mL/mg wet weight), sonicated (8) (Sonics and Materials, Danbury, CT, USA), serially diluted and inoculated in duplicate in MSB. All plates were incubated at 37ºC for 48 h at 10% CO_2.

Isolation of *S. mutans* strains and extraction of genomic DNA

From seven to eight colonies, representing all morphological types of *S. mutans*, were collected from cultures of saliva and *in vivo* biofilm samples on MSB plates and pure cultures were frozen at -70ºC in 10% skim milk (2). For genomic DNA extraction, aliquots from saliva and biofilm samples were collected from skim milk and plated on BHI agar (Difco, Sparks, MD, USA), which was incubated at

37ºC for 24 h at 10% CO_2. The colonies from BHI agar were inoculated into 3 mL of Todd Hewitt Broth (Difco, Sparks, MD, USA) and incubated at 37ºC for 18 h at 10% CO_2. Cells from these cultures were then harvested and genomic DNA was extracted from the cell pellet (21). Integrity of the genomic DNA samples was checked at samples electrophoretically resolved in 1% agarose gel (Invitrogen, Spain) and stained with ethidium bromide (5 μg/mL). PCR reactions with species-specific primers to *gtf*B and *gbp*B genes were adopted in order to confirm the identity of the *S. mutans* isolates (23, 19).

Genotypic analysis

Arbitrarily primed PCR (AP-PCR) assays were performed with the arbitrary primer OPA 02 (5´-TGCCGAGCTG-3´) (16). The amplifications occurred under the following conditions: 95ºC for 2 min, for initial denaturation, and 45 cycles of 94ºC for 30 sec (denaturation), 36ºC for 30 sec (annealing) and 72ºC for 1 min (extension) and a final extension at 72ºC for 5 min. Genomic DNA of *S. mutans* strain UA 130 (kindly provided by Dr. Page W. Caufield, New York University, NY, USA) and distilled deionized water were applied in all PCR baths, as positive and negative controls, respectively. Products of AP-PCR were electrophoretically resolved in agarose gels that were run at 3 V/cm during 3 h in Tris-Borate-EDTA (TBE) running buffer. Gels were stained with ethidium bromide solution (5 μg/mL) (Invitrogen, Carlsbad, CA, USA) for 10 min and their images captured by a digital imaging system (Gel logic 100 Imaging System, Kodak, Japan).

For analysis of the *S. mutans* genotypic profiles from the same volunteer, AP-PCR products from the isolates obtained from saliva and dental biofilms were always resolved side-by-side in the same gel for visual comparisons (2). Thus, samples representative of each genotype were re-run side-by-side in a subsequent gel for direct comparisons of genotypes identified within distinct saliva or biofilms samples. The genotypes found were descriptively analyzed and their proportion, in relation to the number of colonies isolated in each sample and condition, was calculated.

Phenotypic analysis

For the phenotypic analysis, all genotypes identified in dental biofilm were reactivated from frozen stocks on BHI agar plates, which were incubated at 37ºC for 48 h at 10% CO_2. Thus, CFU were transferred to tubes containing BHI broth, which were then incubated at 37ºC for 18 h at 10% CO_2.

The ability of genotypes to withstand acid challenge was evaluated by acid killing assay (15). Briefly, aliquots of the 18-h growth BHI broth were transferred to tubes containing fresh BHI broth medium and grown until mid-exponential phase (OD_{550} = 0.5). Then, the suspension was centrifuged and the pellet was washed once with 0.1 M glycine buffer (pH 7.0) (Fluka, Steinheim, Germany). In addition, the washed pellets were resuspended in 0.1 M glycine buffer pH 7.0 (control) and at pH 5.0 or 2.8. Immediately after the resuspension (T0), after 30 (T30) and 60 min (T60) of incubation at 37ºC, aliquots were serially diluted, plated on BHI agar plates and incubated at 37ºC for 48 h at 10% CO_2. Cell viability at each time was expressed as the ratio between counts of viable cells at pH 5.0 or 2.8 in relation to counts of viable cells at pH 7.0 at each time.

For F-ATPase assay, aliquots of the 18-h growth BHI broth were centrifuged and resuspended in 75 mM Tris-HCl plus 10 mM $MgSO_4$. Then, the cells were permeabilized both with toluene (Merck, Darmstadt, Germany) and by freezing and heating cycles. The permeabilized cells were incubated with 0.5 M adenosine 5´-triphosphate (ATP) (Sigma, Steinheim, Germany) during 10 min in 50 mM Tris-maleate buffer (pH 6.0) (Sigma, Japan) with 10 mM $MgSO_4$ (Fluka, Steinheim, Germany) (3). Inorganic phosphorous released from ATP was determined by the method of Bencini et al. (4). ATPase activity of each genotype was expressed as micromoles of phosphate released from ATP per gram of dry cell per minute of reaction. The standard unit of ATPase activity is 1 μmol of phosphate released per min (3).

In addition, the ability of *S. mutans* genotypes to lower the pH through glycolysis was monitored (3). Aliquots of the 18-h growth BHI broth were

centrifuged and resuspended in 50 mM KCl plus 1 mM $MgCl_2$ solution (Fluka, Steinheim, Germany). The pH of the solution was adjusted to 7.2 and glucose was added in a final concentration of 55.6 mM. Then, the pH drop was assessed during 180 min with glass electrode, previously calibrated with pH standards (pH 4.0 and 7.0). The area under the curve of pH fall after 180 min (AUC) was calculated (considering pH 3.0 as a cut-off point). Also, the pH data were converted into hidrogenionic concentration (cH^+) and the cH^+ area either between time zero and after 15 min or between time zero and 180 min was calculated with the pH Plaque® software (14). The acidogenicity was expressed as AUC and final pH (after 180 min) and cH^+ area (after 15 and 180 min). All the assays described above were conducted in duplicate in 3 distinct experiments, the genotypes were codified and

S. mutans UA 159 was used as control.

Statistical analysis

For statistical analysis, the phenotypic traits of all genotypes isolated from biofilm formed in the absence of sucrose were compared to all genotypes isolated from biofilms formed in the presence of sucrose. Also, the phenotypic traits of genotypes exclusively isolated from biofilms formed in the absence of sucrose were compared to those genotypes isolated only from biofims formed in the presence of this carbohydrate. The assumption of equality of variances and normal distribution of errors were checked for all the response variables tested. Data that violated these assumptions were transformed when necessary (6) and submitted to t-test. When no transformation was adequate to normalize data (ratio between counts of viable cells at pH 2.8 T30 in relation to counts at pH 7.0 T30 for both comparisons and AUC and cH^+ area (after 180 min) for the second comparison), the data were analyzed by Wilcoxon non-parametric test. SAS software system (version 8.02, SAS Institute Inc., Cary, NC, USA) was used, and the significance limit was set at 5%.

RESULTS

Distribution of S. mutans genotypes from saliva and in vivo biofilms

A total of 48 and 95 representative colonies of *S. mutans* were isolated from saliva and biofilms, respectively. All of the isolates were identified as *S. mutans* species, as determined in PCR reactions with species-specific primers for *gtf*B and *gpb*B genes. A total of 19 distinct genotypes were identified in saliva and in *in vivo* biofilm samples (Table 1). AP-PCR genotypic profiles from volunteer F are depicted in Figure 1. In 5 volunteers, only one *S. mutans* genotype was identified in the respective saliva samples, whereas in one volunteer (volunteer B) two genotypes were detected in saliva (Table 1). All genotypes identified in saliva samples were also detected in biofilm samples of the respective volunteer, independently of sucrose exposure. One exception was volunteer F, whose

salivary genotype was not found in the biofilm formed in the absence of sucrose exposure. In addition, besides the salivary genotypes, others were found in the biofilm formed either under sucrose exposure (4 genotypes: 7su, 10su, 11su and 13su) or not (8 genotypes: 4ddw, 6ddw, 9ddw, 16ddw, 17ddw, 18ddw and 19ddw) (Table 1). However, these genotypes were generally detected in lower proportions (12.5%) in biofilm when compared with genotypes also detected in saliva samples (Table 1).

Table 1. Genotypic diversity of *S. mutans* (%; percentage of each genotype in relation to the total numbers of isolated colonies) in saliva and *in vivo* dental biofilm according to the presence or absence of sucrose during biofilm formation:

Volunteer	Saliva	Biofilm formation	
		Absence of sucrose	Presence of sucrose
A	1sa (100)	1ddw (100)	1su (100)
B	2sa (85.7) 3sa (14.3)	2ddw (87.5) 4ddw (12.5)	2su (100)
C	5sa (100)	5ddw (87.5) 6ddw (12.5)	5su (87.5) 7su (12.5)
D	8sa (100)	8ddw (87.5) 9ddw (12.5)	8su (75) 10su (12.5) 11su (12.5)
E	12sa (100)	12ddw (100)	12su (87.5) 13su (12.5)
F	14sa (100)	15ddw (50) 16ddw (12.5) 17ddw (12.5) 18ddw (12.5) 19ddw (12.5)	14su (100)

Distinct numbers represent different genotypes.

sa: genotypes isolated from saliva;

ddw: genotypes isolated from biofilm formed in the absence of sucrose

su: genotypes isolated from biofilm formed in the presence of sucrose.

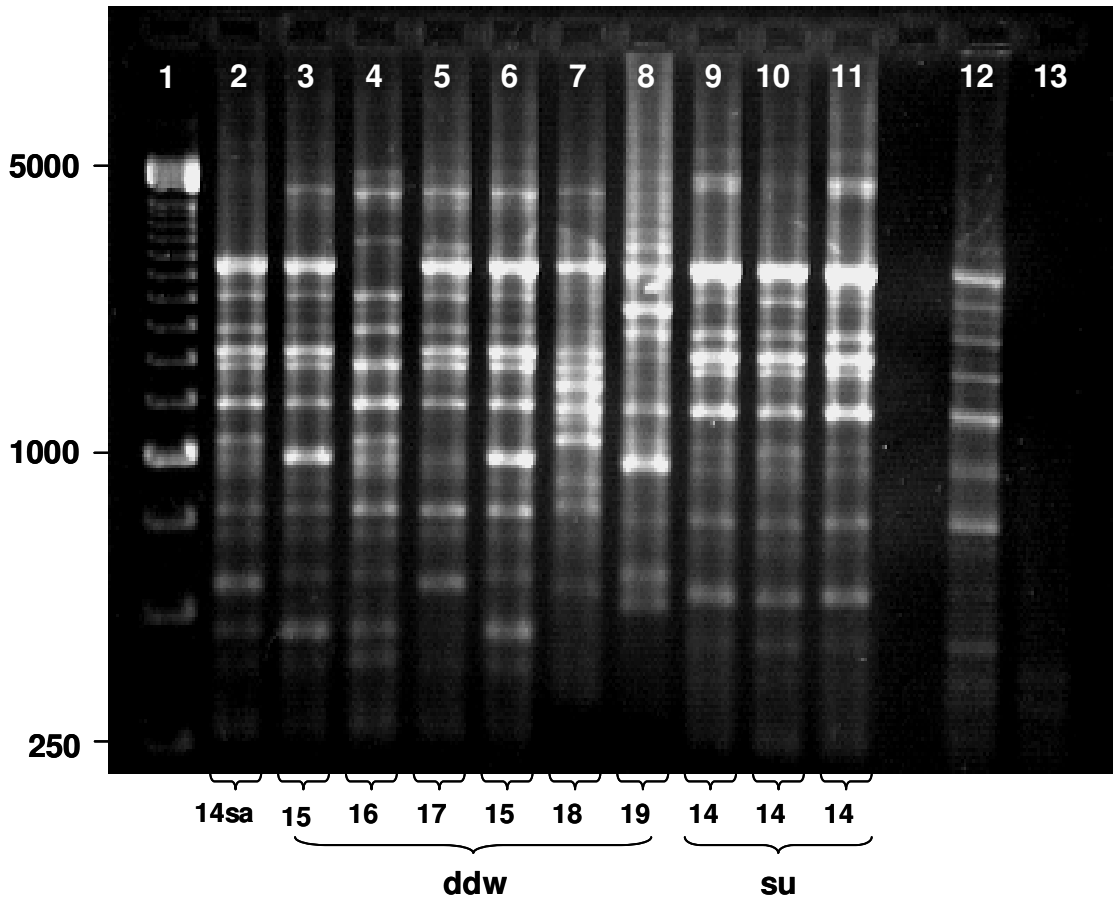

Figure 1. AP-PCR fingerprinting profiles of representative strains of *S. mutans* isolated from volunteer F from saliva and *in vivo* biofilms according to experimental treatments; 250-bp DNA ladder is showed in lane 1; lane 2 corresponds to genotype present in saliva; lanes 3 to 11 correspond to different isolates of *S. mutans* according to the presence or absence of sucrose during biofilm formation; positive control (*S. mutans* UA 130 – Forsyth Institute, Boston, Massachussets) and negative control (water) were present in lanes 12 and 13, respectively. Genotypes and condition of biofilm formation are represented at the bottom of the Figure. ddw: biofilms formed in the absence of sucrose; su: biofilms formed in the presence of sucrose.

Phenotypic Characteristics

All genotypes isolated from biofilms formed in the presence of sucrose showed significantly higher ratio of counts of viable cells at pH 2.8 (T30) in relation to counts of viable cells at pH 7.0 (T30) when compared to genotypes isolated from biofilms formed in the absence of sucrose ($p<0.05$) (Table 2). Also, statistically higher ratio of counts of viable cells at pH 5.0 (T60) in relation to counts of viable cells at pH 7.0 (T60) were also found for genotypes exclusively isolated from biofilms formed under sucrose exposure. In addition, a numerically higher activity of F-ATPase was observed for all genotypes isolated from biofilms formed in the presence of sucrose in comparison with those from biofilms formed in the absence of this carbohydrate, but the differences were not statistically significant ($p=0.06$) (Table 2).

With regard to the acidogenicity traits, genotypes exclusively found in biofilms formed under sucrose exposure (7su, 10su, 11su and 13su) showed higher cH^+ area (after 15 min) ($p<0.01$) and lower final pH ($p<0.05$) than those found exclusively in biofilms not exposed to sucrose (4ddw, 6ddw, 9ddw, 15ddw, 16ddw, 17ddw, 18ddw and 19ddw) (Table 3).

Table 2. Cell viability at pH 5.0 and 2.8 (ratio in relation to cell viability at pH 7.0 at each time), and F-ATPase activity of the genotypes isolated from *in vivo* biofilms (mean ± sd):

Comparison	Condition of biofilm formation	pH 5.0			pH 2.8			F-ATPase activity (µmol/g/min)
		T0	T30	T60	T0	T30 ($\times 10^{-2}$)	T60	
All genotypes	**Absence of sucrose (n=13)**	1.1 ± 0.3	1.1 ± 0.4§	0.9 ± 0.3§	1.0 ± 0.4	0.03 ± 0.09^{B}	0.0 ± 0.0	16.1 ± 8.5
	Presence of sucrose (n=10)	1.1 ± 0.2	1.2 ± 0.6§	1.0 ± 0.2§	1.2 ± 0.6	0.1 ± 0.2^{A}	0.0 ± 0.0	25.1 ± 13.8
Exclusively-isolated genotypes	**Absence of sucrose (n=8)**	1.0 ± 0.2	1.0 ± 0.4§	0.8 ± 0.2^{B}	0.9 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	15.9 ± 8.6
	Presence of sucrose (n=4)	1.2 ± 0.04	1.0 ± 0.2§	1.1 ± 0.2^{A}	1.3 ± 0.2	0.04 ± 0.05	0.0 ± 0.0	23.7 ± 15.1

Means of each type of comparison followed by distinct letters differ statistically ($p<0.05$).

Means of each type of comparison, which are not followed by letters, do not differ from each other.

§: data transformed by $\log_{10}$;

Table 3. Acidogenicity traits of the genotypes isolated from *in vivo* biofilms (means ± sd):

Comparison	Condition of biofilm formation	AUC (after 180 min) (min*pH)	Final pH (after 180 min)	cH^+ area (after 15 min) (µmol/l/min)	cH^+ area (after 180 min) (µmol/l/min)
All genotypes	**Absence of sucrose (n=13)**	315.0 ± 58.7[#]	4.0 ± 0.1	0.3 ± 0.1[§]	131.8 ± 58.7
	Presence of sucrose (n=10)	321.2 ± 105.5[#]	3.9 ± 0.2	0.81 ± 1.3[§]	147.2 ± 60.9
Exclusively-isolated genotypes	**Absence of sucrose (n=8)**	298.1 ± 17.0	3.9 ± 0.06[A]	0.4 ± 0.1[B §]	139.5 ± 16.2
	Presence of sucrose (n=4)	295.0 ± 13.1	3.7 ± 0.1[B] (n=3)[*]	2.3 ± 0.9[A §] (n=3)[*]	167.6 ± 77.2

Means of each type of comparison followed by distinct letters differ statistically ($p<0.05$).

Means of each type of comparison, which are not followed by letters, do not differ from each other.

AUC: Area under the curve of pH fall; cH^+: area of hidrogenionic concentration

§: data transformed by $\log_{10}$; #: data transformed by 1/AUC.

[*]The n value is different due to the exclusion of outliers from genotype 13su (values of 4.22 for final pH and 0.10 for cH^+ area after 15 min)

DISCUSSION

Regarding the data of genotypic diversity, we found that most of the volunteers harbored just one genotype in saliva, which was also identified in the biofilms (2, 15) (Table 1). Even though some studies discuss that saliva does not carry all genotypes present in the oral cavity (27), there are evidences that a positive correlation between mutans streptococci in saliva and those present in dental biofilm exists (31) and we believe that saliva reflects, at least, those genotypes present at higher proportions in teeth biofilms as previously reported by some studies (2, 15) and also observed in the present research.

Our data suggest, in addition, that there were no expressive differences in genotypic diversity among biofilms formed either in the presence or absence of sucrose. In this context, some studies have suggested that frequent exposure to sucrose and even the frequent pH perturbations may be related to an increased genotypic diversity of microorganisms in oral cavity (1, 24). However, it is difficult to correlate our data with these previous one since the experimental conditions evaluated in these studies were different compared to ours. Nevertheless, despite the absence of expressive differences in genotypic diversity, the distinct conditions of biofilm formation allowed that specific genotypes were also isolated from biofilms, although in a lower proportion (Table 1), as previously described (2). These particular genotypes might be present in saliva below the detection limit of the microbiological method used (27), and these specific conditions may have enhanced their proportions in biofilms.

In relation to the phenotypic traits, genotypes isolated from biofilms formed in the presence of sucrose seemed to be more acid tolerant, since they showed higher ratio of counts of viable cells at pH 2.8 T30 than those isolated from biofilms formed in the absence of this carbohydrate (Table 2). Moreover, those genotypes exclusively isolated from biofilms formed in the presence of sucrose showed higher ratio of counts of viable cells at pH 5.0 T60 (Table 2). Also, genotypes exclusively isolated from biofilms formed in the presence of sucrose (7su, 10su, 11su and

13su) were more acidogenic (in terms of cH^+ area after 15 min and final pH) than those exclusively isolated in biofilms not exposed to sucrose (4ddw, 6ddw, 9ddw, 15ddw, 16ddw, 17ddw, 18ddw and 19 ddw) (Table 3). With the exception of data of cH^+ area (after 15 min), which means how fast the biofilm pH decreased due to acid production, the other statistical significant differences showed above might be seen with care as they may not be clinically relevant either considering that the pH drop in oral cavity due to carbohydrate fermentation may not reach values as low as pH 2.8 and the relevance of a higher cell viability after 60 min at pH 5.0 or even considering the final pH evaluation, since in an *in vitro* study all genotypes had chance to decrease the pH and probably the first minutes were the most important to observe. Nevertheless, these findings suggest that genotypes isolated from biofilms formed in the presence of sucrose may be more virulent. Probably, these genotypes might have developed an adaptive response to the frequent pH fall due to sucrose exposure, which may have increased their acid-tolerance. In addition, the higher acidogenicity of genotypes exclusively isolated in the presence of sucrose may suggest that not only the modifications induced by microorganisms in biofilms, but also their virulence traits, are more important than only their relative numbers in biofilms (9). Moreover, as the expression and modulation of virulence factors related to caries development are dependent on environmental conditions (7), this may help explain the higher cariogenicity and acidogenicity of biofilms formed in the presence of sucrose (8).

Since the F-ATPase pump activity has an inverse relationship with the environmental pH (3), we hypothesized that genotypes isolated from biofilm formed under frequent pH fall due to sucrose exposure may show higher activity than those isolated from biofilms not exposed to this carbohydrate. However, in our experimental conditions, the difference did not reach statistical significance ($p=0.06$) (Table 2). We may consider that the absence of difference in F-ATPase activity between these two distinct conditions can be due to some inherent problems regarding the protocol adopted to evaluate the activity of this pump. It has been suggested that the toluene, used during the cell permeabilization step,

may inhibit F-ATPase activity (30). Additionally, the authors discussed that toluene may induce ruptures in the cell membrane causing an outflow of the cytoplasm ATPases. In this case, the values of F-ATPase activity may be a result of not only the membrane-bound F-ATPase activity but also the non-specific activity of intracellular ATPases, which might have interfered with our results, eliminating any differences among genotypes. Thus, it would be interesting in future studies to evaluate a modified protocol, considering these observations.

Besides F-ATPase acitivity, other mechanisms might be involved in acid-tolerance. In this context, under an acidic stress condition, there is a shift in the membrane fatty acid profile of *S. mutans,* which may decrease its permeability to protons (10). In addition, the modulated expression of some proteins and an increased expression of DNA repair enzymes under low pH might be also involved in acid tolerance of *S. mutans* (32, 11). As these mechanisms also play a role in the acid tolerance of *S. mutans*, it would be relevant to study them in future studies in order to clarify their roles in the aciduricity of different *S. mutans* genotypes.

Furthermore, it is shown that distinct genotypes may present distinct phenotypic traits. In the present study, distinct genotypes showed distinct acid tolerances, considering not only the counts of viable cells after 30 min at pH 2.8 or the F-ATPase activity, but also distinct acidogenicities, regarding AUC and cH^+ data, within 5 of 6 volunteers (by Kruskal-Walis test; $p<0.05$; data not shown). These findings are in agreement with previous data which showed that distinct genotypes may show distinct ability to sinthesize insoluble extracellular polysaccharides, different activities of glucosyltransferases or even different cariogenic potential in an animal caries model (12, 20, 22). Perhaps, an evaluation of these phenotypic traits in an *in vitro* biofilm model, additionally, might also better reflect the behavior of the genotypes in a stress condition induced by the frequent pH fall due to sucrose exposure.

Overall, the findings of the present study suggest that, even though there was not a considerable difference in the genotypic diversity between biofilms

formed either in the presence or absence of sucrose, biofilms formed under a high cariogenic condition harbored more aciduric and acidogenic genotypes.

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CAPÍTULO 2

Phenotypic analysis of *S. mutans* genotypes in planktonic and biofilm conditions"

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ABSTRACT

S. mutans genotypes may show distinct phenotypic traits; however little is known about the phenotypic traits of *S. mutans* genotypes isolated from an *in situ* dental biofilm formed in the presence or absence of a high cariogenic challenge. Thus, this study aimed to evaluate the aciduricity and acidogenicity of these genotypes grown in a planktonic condition and also their enamel demineralization potential in an *in vitro* biofilm model. Sixteen genotypes from 3-day *in situ* biofilm, formed in the presence or absence of sucrose exposure, were evaluated regarding acid susceptibility, F-ATPase activity and ability to lower the pH through glycolysis in planktonic conditions. Eight of these genotypes, with the highest or the lowest aciduricity and acidogenicity, were grown during 5 days as an *in vitro* biofilm exposed 8x/day to 10% sucrose solution and the acidogenicity, the ability to synthesize extracellular polysaccharides and the enamel demineralization potential of these genotypes were also evaluated. Distinct genotypes showed distinct F-ATPase activity and distinct acidogenicities, although the difference in the acidogenicity was less pronounced. In addition, genotypes isolated from biofilms formed in the presence of sucrose might be more acid-tolerant in planktonic conditions than those isolated in sucrose absence. In biofilm conditions, the genotypes were not different regarding either their ability to produce EPS or their acidogenicities, but, they showed distinct enamel demineralization potential. Either in planktonic or biofilm conditions, distinct genotypes show distinct phenotypic traits.

INTRODUCTION

Mutans streptococci, mainly *Streptococcus mutans*, are the bacteria most closely associated as causative agents of dental caries (Löesche, 1986; Tanzer et al., 2001). In the oral cavity, some stress factors, such as pH fall, which follows the carbohydrate fermentation, could disrupt microbial homeostasis in dental biofilm and influence growth and survival of oral bacteria (Marsh, 2003). At this point, *S. mutans* present some adaptive advantages in relation to other microorganisms of dental biofilm, like the ability of surviving and growing in this acid condition (aciduricity) and producing acids from carbohydrates even under this low pH environment (acidogenicity) (Marsh, 2003; Lemos et al., 2005). In addition to these before mentioned virulence traits, *S. mutans* also have the ability to synthesize extracellular polysaccharides (EPS) from sucrose, which may enhance the adherence of these microorganisms to tooth surface (Rölla, 1989) and alter the matrix of dental biofilm (Paes Leme et al., 2006 – review).

Several mechanisms have been attributed to *S. mutans* acid tolerance, such as changes in the cell membrane composition (Fozo & Quivey, 2004), the synthesis of some stress proteins and the action of some two-component regulatory systems are also involved with the enhanced aciduricity of *S. mutans* (Li et al., 2002; Len et al., 2004; Lemos et al. 2005). However, the membrane-bound F-ATPase pump is considered the primary determinant of *S. mutans* acid-tolerance, since it pumps protons out of the cells at the expense of ATP hydrolysis (Quivey et al., 2001), maintaining the intracellular pH suitable for enzymatic reactions (Lemos et al., 2005). Moreover, the role of this pumping-protons system in acid tolerance has been well explored (Belli & Marquis, 1991).

It has been shown in some recent studies that distinct clinical isolates of *S. mutans* might present distinct phenotypic traits when grown as planktonic cells (Mattos-Graner et al., 2004; Gou et al., 2006; Arthur et al., 2010). In this context, Arthur et al. (2007) isolated *S. mutans* genotypes from *in situ* biofilms formed either in the absence or presence of a high cariogenic challenge (frequent exposure of

sucrose and biofilm accumulation). However, the phenotypic traits of these genotypes were not evaluated. Besides, it would be important to evaluate, additionally, the enamel demineralization potential and the ability to synthesize EPS in a biofilm condition, which represents a more clinically relevant condition, in order to clarify the role of *S. mutans* genotypes on dental demineralization.

Therefore, the aim of the present study was to evaluate some phenotypic traits of *S. mutans* genotypes, previously isolated from a 3-day *in situ* biofilm, formed under exposure or not to sucrose (Arthur et al., 2007), in a planktonic condition, through the evaluation of the aciduricity and acidogenicity of these genotypes, and in a biofilm condition, through the evaluation of their acidogenicity, enamel demineralization potential and ability to synthesize extracellular polysaccharides.

MATERIALS AND METHODS

Experimental design

This study was approved by the Research and Ethics Committee of Piracicaba Dental School (protocol n°. 004/2006). A total of 16 *S. mutans* genotypes, previously isolated from 3-day *in situ* biofilms (Arthur et al., 2007), were, firstly, evaluated regarding their aciduricity, through evaluation of acid susceptibility and F-ATPase activity, and acidogenicity, by evaluation of the ability to lower the pH through glycolysis, as planktonic cells. Afterwards, eight genotypes, from the 16 described above, were selected and grown in *in vitro* monospecies biofilms during 120 h on the surface of bovine enamel blocks (4 x 6 x 1 mm), with pre-determined surface hardness. Eight times a day, the biofilms were exposed to 10% sucrose solution for 1 min and, at the end of this period, the biochemical and microbiological composition of the biofilms were analyzed and changes in enamel surface hardness were determined.

Selection of *S. mutans* genotypes

S. mutans genotypes were previously isolated from an *in situ* biofilm (Arthur et al., 2007). Briefly, adult volunteers wore an acrylic palatal appliance containing human enamel blocks, which were treated extraorally, 8x/day, with distilled and deionized water or 20% sucrose solution. The biofilms formed onto enamel blocks during 3 days were cultured in Mitis Salivarius Agar (MSA) plus bacitracin (MSB). *S. mutans* morphological types present in biofilm samples were genotyped by arbitrarily primed PCR (AP-PCR) with OPA-02. Eight genotypes isolated from 5 out of 7 volunteers (except volunteers 1 and 7) in the absence of sucrose (B-ddw, E-ddw, I-ddw, J-ddw, K-ddw, Q-ddw, R-ddw and V-ddw) and in the presence of sucrose (B-suc, E-suc, I-suc, M-suc, N-suc, Q-suc, V-suc and W-suc) (Arthur et al., 2007) were selected for this study.

Phenotypic analysis of genotypes as planktonic cells

For the phenotypic analysis as planktonic cells, the genotypes were reactivated from frozen stocks on Brain Heart Infusion (BHI) agar plates (Difco, Sparks, MD, USA), which were incubated at 37ºC for 48 h in 10% CO_2. Then, colonies-forming units (CFU) were transferred to tubes containing BHI broth, which were then incubated at 37ºC for 18 h in 10% CO_2.

The ability of genotypes to withstand acid challenge was evaluated by acid killing assay (Lembo et al., 2007). Aliquots of the 18-h growth BHI broth were transferred to tubes containing fresh BHI broth medium and grown until mid-exponential phase (OD_{550} = 0.5). Then, the suspension was centrifuged and the pellet was washed once with 0.1 M glycine buffer (pH 7.0). In addition, the washed pellets were resuspended in 0.1 M glycine buffer pH 7.0 (control) and at pH 5.0 or 2.8. Immediately after the resuspension (T0), after 30 (T30) and 60 min (T60) of incubation at 37ºC, aliquots were serially diluted, plated on BHI agar plates and incubated at 37ºC for 48 h in 10% CO_2. Cell viability at each time was expressed either as the ratio between counts of viable cells at pH 5.0 or 2.8 in relation to

counts of viable cells at pH 7.0 at each time or as counts of viable cells at each time.

For F-ATPase assay, aliquots of the 18-h growth BHI broth were centrifuged and resuspended in 75 mM Tris-HCl plus 10 mM $MgSO_4$. Then, the cells were permeabilized both with toluene and by freezing and heating cycles. The permeabilized cells were incubated with 0.5 M ATP during 10 min in 50 mM Tris-maleate buffer pH 6.0 with 10 mM $MgSO_4$, as described by Belli & Marquis (1991). Inorganic phosphorous released from ATP was determined by the method of Bencini et al. (1983). ATPase activity of each genotype was expressed as micromoles of phosphate released from ATP per gram of dry cell per minute of reaction. The standard unit of ATPase activity is 1 μmol of phosphate released per min (Belli & Marquis, 1991).

In addition, the ability of *S. mutans* genotypes to lower the pH through glycolysis was monitored, according to Belli & Marquis (1991). Aliquots of the 18-h growth BHI broth were centrifuged and resuspended in salt solution (50 mM KCl plus 1 mM $MgCl_2$). The pH of the solution was adjusted to 7.2 and glucose was added in a final concentration of 55.6 mM. Then, the pH drop was assessed during 180 min with glass electrode, previously calibrated with standards (pH 4.0 and pH 7.0). The area under the curve of the pH fall after 180 min (AUC) for each genotype was calculated (considering pH 3.0 as a cut-off point) with the pH Plaque® software (Larsen & Pearce, 1997). All the assays described above were conducted in duplicate in 3 distinct experiments, and *S. mutans* UA 159 was used as control.

Phenotypic analysis of genotypes as biofilms

Enamel block preparation

A hundred-ten enamel blocks (7 x 4 x 1 mm) were obtained from the middle third of the buccal face of bovine incisor teeth. The dentin of each block was

completely removed by polishing and a 1-mm thickness enamel block was obtained. The enamel surface was polished for baseline surface hardness determination. Fifty-four enamel blocks, with a mean surface hardness of 341.7 ± 18.1 kg/mm^2, were selected and randomized into three independent groups, corresponding to three independent experiments. Within each experiment, the enamel blocks were randomized into the genotypes, whose enamel blocks had approximately the same mean of baseline surface hardness. The enamel blocks whose surface hardness was not within the mean interval showed above were excluded.

Inoculum and salivary pellicle preparation

For the growth of genotypes as biofilms, a monospecies *in vitro* model was adopted (Ccahuana-Vásquez et al., 2009; Cury et al., 2009). Based on the data of planktonic cells, eight from the 16 genotypes (the ones with numerically higher or lower aciduricities and acidogenicities) were reactivated from frozen stocks on BHI agar plates, which were incubated at 37ºC for 48 h in 10% CO_2. Then, from 5 to 10 CFU of each genotype were individually transferred to tubes containing 9 mL of ultra purified tryptone-yeast extract broth plus 1% glucose (Merck, Darmstadt, Germany) and incubated at 37ºC for 18 h in 10% CO_2. After 18 h of incubation (10^9 CFU/mL), 20 µL of each suspension were individually transferred to a “starter” tube containing 9 mL of ultra purified tryptone-yeast extract broth plus 1% sucrose (Merck, Darmstadt, Germany). In order to grow the biofilm, 2 mL of the “starter” suspension was transferred in duplicate to a 24-well plate (TPP, Switzerland), containing enamel blocks previously covered by a salivary pellicle.

In order to form a salivary pellicle on the surface of the enamel blocks, whole stimulated saliva was collected from 1 volunteer. To a total of 25 mL of saliva, collected in a plastic tube, 25 mL of adsorption buffer (100 mM KCl, 17.5 mM K_2HPO_4, 32.5 mM KH_2PO_4, 50 mM $CaCl_2.2H_2O$ and 5 mM $MgCl_2.6H_2O$) and 500 µL of 10 mM PMSF were added to same the tube (Koo et al., 2006). This suspension was centrifuged at 3800 x *g*, 4ºC for 10 min and the supernatant was

vacuum-filtered (in a 0.22 µm PES membrane) (TPP, Switzerland). Aliquots of 2 mL of this filtered suspension were transferred to a 24-well plate. Enamel blocks, sterilized by autoclavation, were individually fixed in an orthodontic wire. An enamel block/orthodontic wire was vertically placed in each well and the plate was incubated at 37ºC, for 30 min under constant agitation (Koo et al., 2006). Afterwards, the enamel block/orthodontic wire was dip-washed three times in 2 mL of filtered adsorption buffer and transferred to a new 24-well plate containing the "starter" suspension described above. This plate was incubated at 37ºC for 8 h in 10% CO_2.

Biofilm acidogenicity

After 8 h, as an indicative of the acidogenicity of the biofilm, the pH of the suspension in each well was evaluated and the blocks were transferred to a new 24-well plate containing fresh ultra-purified tryptone-yeast extract broth plus 0.1 mM glucose, and, additionally, incubated at 37ºC for 12 h in 10% CO_2. After 12 h, the pH of the suspension of each well was measured again and the biofilms were transferred to a 24-well plate containing fresh ultra-purified tryptone-yeast extract broth plus 0.1 mM glucose. This same procedure was followed once a day at 24-h intervals (after 48, 72, 96 and 120 h). All the pH measurements were done with a microelectrode connected to a pH meter. The microelectrode was calibrated against standard buffers (pH 4.0 and 7.0) prior to the measurements.

Biofilm exposure to sucrose solution

After approximately 24 h of biofilm formation, the enamel blocks with the biofilms were dipped in 2 mL of 10% sucrose solution (containing 1.28 mM Ca, 0.74 mM P_i, 0.023 ug F/mL) (Cury et al., 2009) for 1 min, at 90-min intervals, eight times a day, during 4 days. After the exposure to sucrose solution, the biofilms were dip-washed three-times in 0.9% NaCl (Merck, Darmstadt, Germany) and

placed back in the 24-well plate at 37ºC, 10% CO_2. On the fourth day, after the last exposure to sucrose solution, the biofilms were additionally incubated at 37ºC, for 12 h in 10% CO_2. Each experiment was performed in triplicate using *S. mutans* UA 159 as control.

Biofilm collection, dry weight determination and microbiological analysis

After 120 h of biofilm formation, the pH of the suspension of each well was measured. Then, the enamel blocks were dip-washed three-times in 0.9% NaCl, transferred to sterile microcentrifuge tubes containing 1 mL of 0.9% NaCl and sonified at 7 W for 60 sec (Aires et al., 2008). From this biofilm suspension, an aliquot of 300 µL was transferred to a pre-weighed microcentrifuge tube. Three volumes of ethanol were added and the tubes were incubated at -20ºC for 15 min (Cury et al., 2009; Ccahuana-Vásquez et al., 2009). Then, the tubes were centrifuged at 10000 x *g* for 10 min. The pellet was washed with 75% ethanol and centrifuged under the same conditions described above. The supernatant was removed and the pellets were dried over P_2O_5 in vacuum for 4 h for determination of biofilm dry weight. For microbiological analysis, an aliquot of 100 µL of the biofilm suspension was also diluted in 0.9% NaCl in series up to 10^6 and 3 drops of 20 µL from each dilution were dispensed on BHI agar plates, which were incubated at 37ºC, for 48 h in 10% CO_2. The CFU were counted and the results were expressed as CFU per milligram of biofilm dry weight.

Biochemical analysis

The extraction of soluble extracellular polysaccharide (SEPS) and insoluble extracellular polysaccharide (IEPS) were performed according to Aires et al. (2008) and the total carbohydrate was estimated by phenol sulphuric method (Dubois et al., 1956) using glucose as standard. The results were normalized to the dry weight of the biofilm.

Hardness Analysis

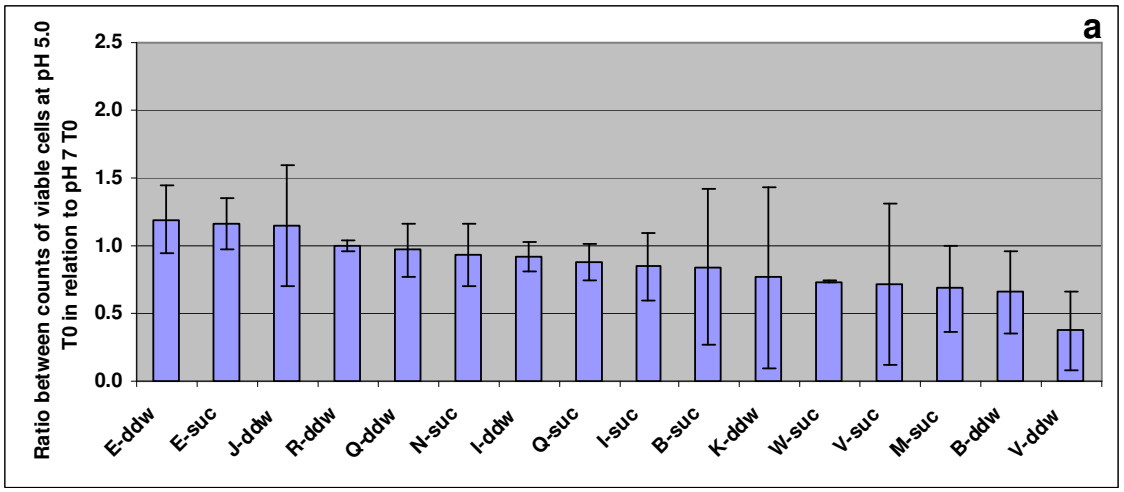
At the end of each experiment, surface hardness of 2 enamel blocks from each genotype was measured again (according to Cury et al., 2000) and an average per genotype was obtained. Three indentations were made at 100 µm from the baseline indentations. The percentage of surface hardness loss (%SHL) was calculated. The hardness tester Future Tech FM, coupled to software FM-ARS (Future-Tech, Tokyo, Japan), was used for these analyses and a Knoop indenter was used with a 25–gram load for 5 s.

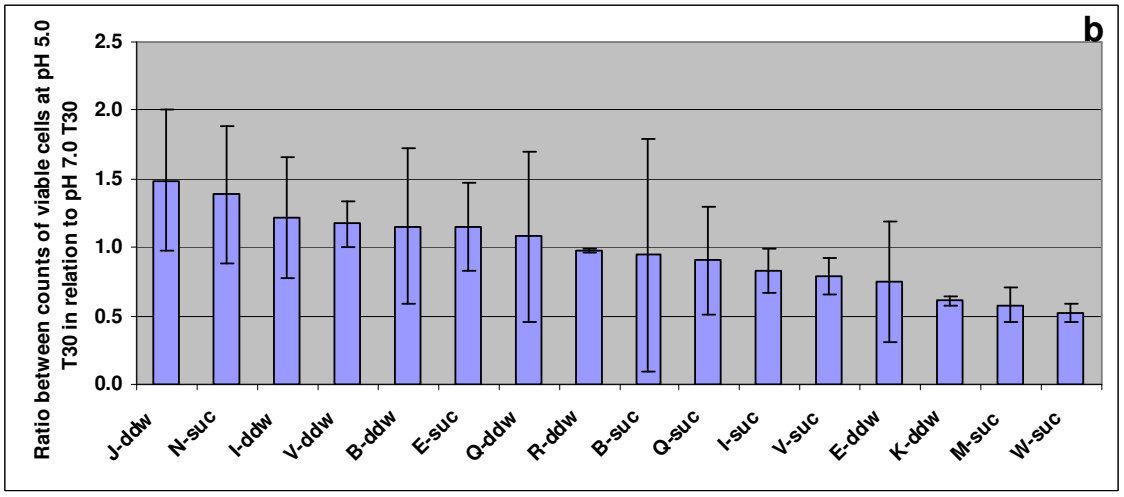
Statistical analysis

For the comparison of phenotypic traits among genotypes, the assumption of equality of variances and normal distribution of errors were checked for all the variable responses tested. Those data that did not satisfy the assumptions were transformed (Box et al., 1978). The data of ratio between counts of viable cells at pH 5.0 T0 in relation to counts of viable cells at pH 7.0 T0 were transformed by square root; data of ratio between counts of viable cells at pH 5.0 T30 in relation to pH 7.0 T30 and IEPS concentration were transformed by $\log_{10}$. The data of ratio between counts of viable cells at pH 5.0 T60 in relation to pH 7.0, acidogenicity in biofilm, SEPS concentration and % SHL were not transformed. All these before mentioned response variables were submitted to ANOVA followed by Tukey test. For the ratio between counts of viable cells at pH 2.8 T0 and T30 in relation to pH 7.0, F-ATPase activity, AUC and counts of viable cells in biofilm a Kruskall-Walis non-parametric test was chosen, since no transformation was adequate to normalize the data. SAS software system (version 8.02, SAS Institute Inc., Cary, NC, USA) was used, and the significance limit was set at 5%.

RESULTS

In relation to planktonic condition, there were no statistical differences among all genotypes evaluated regarding the ratio between counts of viable cells at pH 5.0 T0, T30 and T60 in relation to pH 7.0 (means of 0.9 ± 0.2; 1.0 ± 0.3 and 0.9 ± 0.2, respectively) (Figure 1). Also, the genotypes were not statistically different regarding the ratio between counts of viable cells at pH 2.8 T0 in relation to pH 7.0 T0 (mean of 0.9 ± 0.2) (Figure 2). Moreover, genotypes isolated from biofilms formed in the presence of sucrose showed numerically higher counts of viable cells at pH 5.0 T0, T30 and T60 and at pH 2.8 T0 than genotypes from biofilms formed in the absence of sucrose, with the exception of genotypes Q-suc and R-ddw, although the data were not statistically significant (Figure 3). In addition, at pH 2.8 T30 genotypes Q-ddw and Q-suc showed higher ratio of counts of viable cells than the other 14 genotypes which were not statistically different among them (mean of 2.2 ± 2.7 and 0.06 ± 0.04 x 10^{-2}, respectively) (Table 1). For all genotypes the ratio between viable cells at pH 2.8 T60 in relation to pH 7.0 was below the detection limit (data not shown). Besides, the genotypes were phenotypically different regarding F-ATPase activity (Figure 4), but no expressive differences were found regarding AUC (Figure 5).


a
Ratio between counts of viable cells at pH 5.0 T0 in relation to pH 7 T0
2.5
2.0
1.5
1.0
0.5
0.0
E-ddw
E-suc
J-ddw
R-ddw
Q-ddw
N-suc
I-ddw
Q-suc
I-suc
B-suc
K-ddw
W-suc
V-suc
M-suc
B-ddw
V-ddw


b
Ratio between counts of viable cells at pH 5.0 T30 in relation to pH 7.0 T30
2.5
2.0
1.5
1.0
0.5
0.0
J-ddw
N-suc
I-ddw
V-ddw
B-ddw
E-suc
Q-ddw
R-ddw
B-suc
Q-suc
I-suc
V-suc
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K-ddw
M-suc
W-suc

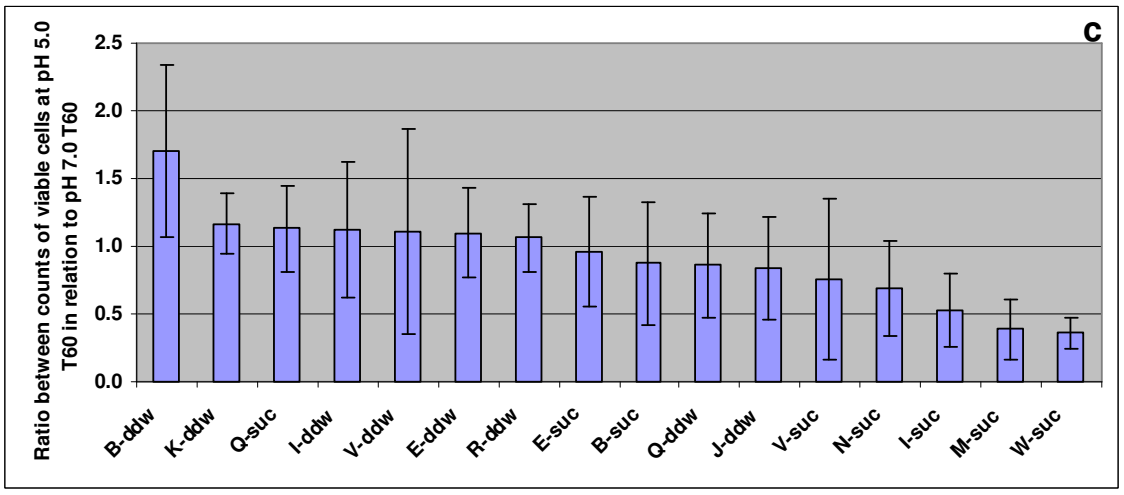

c
Ratio between counts of viable cells at pH 5.0 T60 in relation to pH 7.0 T60
2.5
2.0
1.5
1.0
0.5
0.0
B-ddw
K-ddw
Q-suc
I-ddw
V-ddw
E-ddw
R-ddw
E-suc
B-suc
Q-ddw
J-ddw
V-suc
N-suc
I-suc
M-suc
W-suc

Figure 1. Ratio between counts of viable cells at pH 5.0 in relation to pH 7.0 immediately after the resuspension (fig. 1a), and 30 and 60 min after the resuspension (fig. 1b and fig. 1c) in planktonic condition.

Bars denote standard deviation. Means which are not followed by letters do not differ from each other.

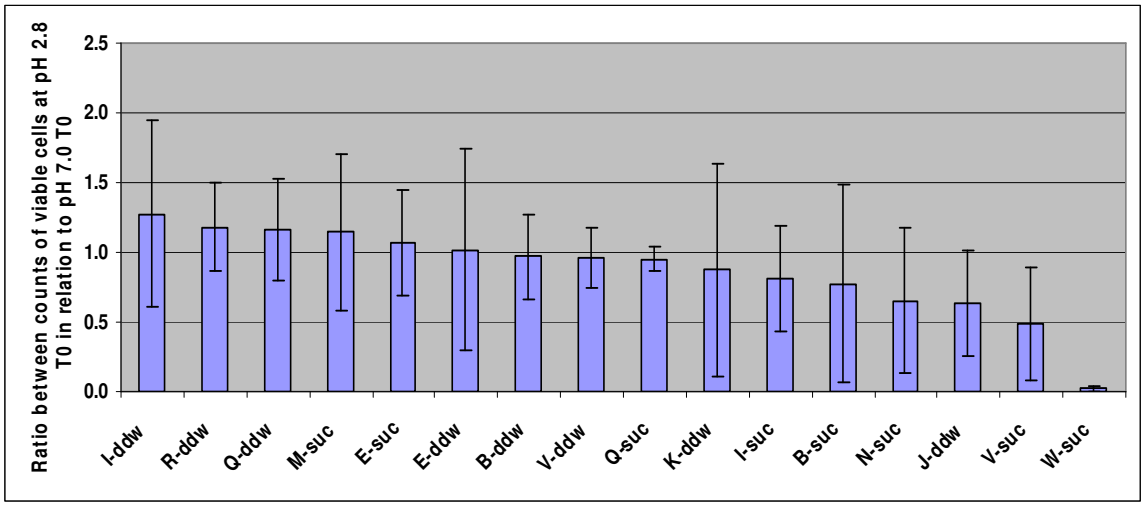
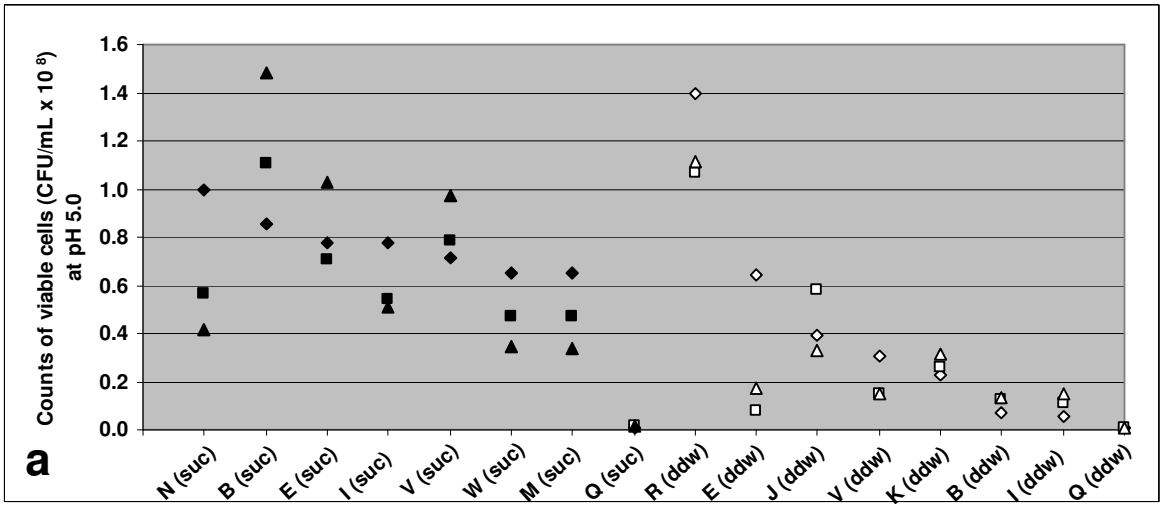


Figure 2. Ratio between counts of viable cells at pH 2.8 in relation to pH 7.0 immediately after the resuspension in planktonic condition.

Bars denote standard deviation. Means which are not followed by letters do not differ from each other.



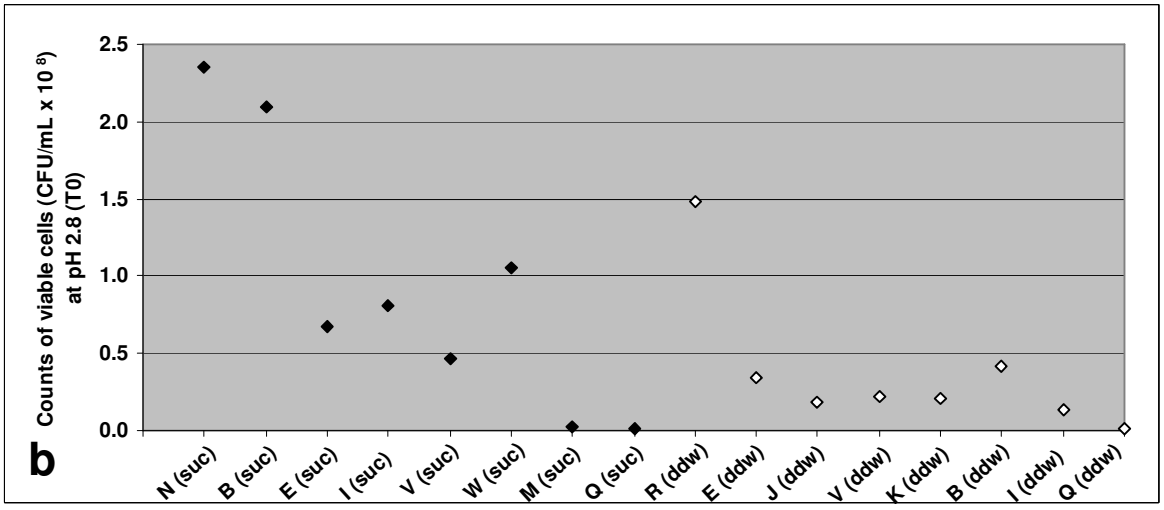


Figure 3. Counts of viable cells at pH 5.0 (Fig. a) immediately after the resuspension (diamonds), after 30 min (squares) and after 60 min (triangles) and at pH 2.8 (Fig. b) immediately after the resuspension of genotypes isolated from sucrose biofilms (solid symbols) and from biofilms not exposed to sucrose (open symbols).

Table 1. Ratio between counts of viable cells at pH 2.8 T30 in relation to counts at pH 7.0 T30 (mean ± sd).

Genotypes	Ratio (x 10^{-2})
Q-ddw	4.1 ± 2.6^{A}
Q-suc	0.3 ± 0.04^{AB}
J-ddw	0.1 ± 0.2^{BC}
B-suc	0.1 ± 0.2^{BC}
I-suc	0.1 ± 0.1^{BC}
N-suc	0.1 ± 0.1^{BC}
E-ddw	0.1 ± 0.05^{BC}
V-ddw	0.1 ± 0.01^{BC}
K-ddw	0.05 ± 0.04^{BC}
B-ddw	0.05 ± 0.04^{BC}
M-suc	0.04 ± 0.02^{BC}
I-ddw	0.04 ± 0.05^{BC}
V-suc	0.03 ± 0.04^{BC}
W-suc	0.01 ± 0.01^{C}
E-suc	0.001 ± 0.001^{C}
R-ddw	0.002 ± 0.001^{C}

Means followed by distinct letters are statistically different by Kruskal-Walis test ($p<0.05$).

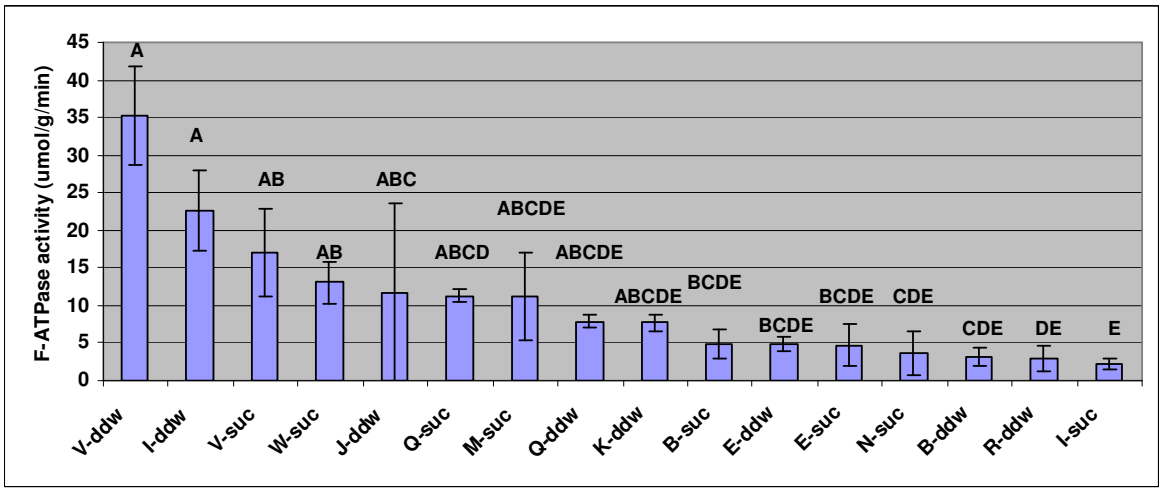


Figure 4. F-ATPase activity (µmol/g/min) of the genotypes in planktonic condition.

Bars denote standard deviation. Distinct letters indicate statistically significant difference among the genotypes by Kruskal-Walis test ($p<0.05$).

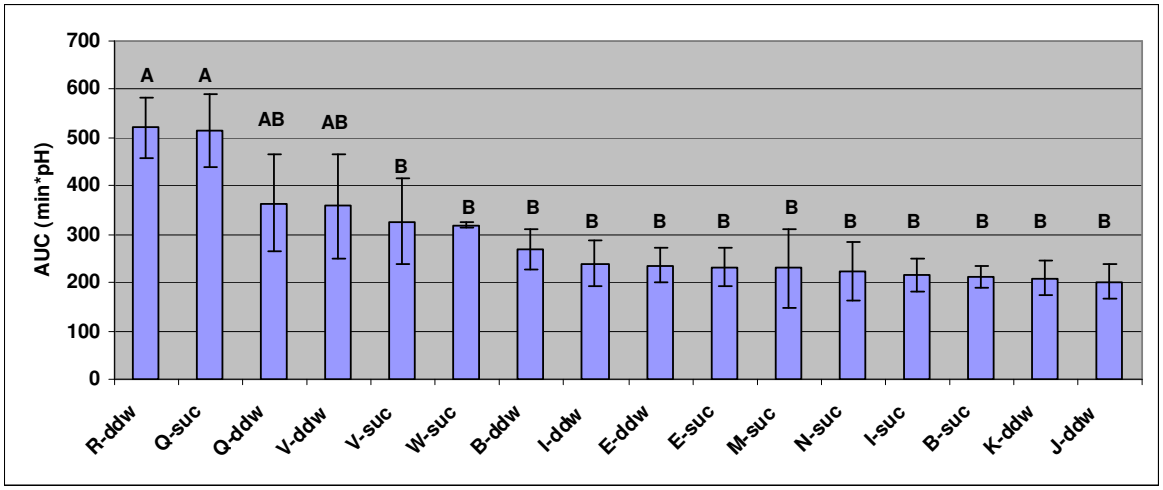


Figure 5. Area under the curve of pH fall (AUC; min*pH) of genotypes in planktonic condition.

Bars denote standard deviation. Distinct letters indicate statistically significant difference among the genotypes by Kruskal-Walis-test ($p<0.05$).

In relation to biofilm condition, considering the acidogenicity, the pH of the suspensions decreased from 6.6 ± 0.08 (after 8 h of biofilm formation) to 4.4 ± 0.05 (after 120 h of biofilm formation), but no statistical differences were found among the genotypes in each time evaluated (data not shown). Higher counts of viable cells in biofilms were found in presence of J-ddw, K-ddw and B-ddw genotypes (Table 2). Also, higher %SHL was found in the presence of genotypes R-ddw, Q-suc, V-ddw and V-suc genotypes when compared with the other four genotypes ($p<0.05$) (Table 1). No differences were found regarding concentration of either SEPS (mean of 24.7 ± 5.1 µg/mg biofilm dry weight) or IEPS (mean of 47.1 ± 9.0 µg/mg biofilm dry weight)(data not shown).

Table 2. Counts of viable cells in biofilms (CFU x 10^9/ mg biofilm dry weight) and percentage of surface hardness change (%SHL; mean ± sd) of enamel blocks according to genotypes in *in vitro* biofilm condition:

Genotypes	CFU x 10^9/mg biofilm dry weight	% SHL
V-suc	0.9 ± 0.3^{BC}	49.7 ± 2.9^{A}
R-ddw	0.4 ± 0.08^{BC}	49.7 ± 3.7^{A}
V-ddw	0.7 ± 0.3^{BC}	41.6 ± 5.8^{AB}
Q-suc	0.4 ± 0.05^{C}	39.9 ± 4.9^{ABC}
J-ddw	1.3 ± 0.7^{AB}	36.7 ± 1.9^{BCD}
K-ddw	1.4 ± 0.4^{A}	28.7 ± 6.3^{BCD}
B-suc	0.5 ± 0.2^{BC}	28.0 ± 0.9^{D}
B-ddw	0.8 ± 0.1^{ABC}	26.7 ± 3.1^{D}

Means followed by distinct letters are statistically different ($p<0.05$).

DISCUSSION

The analysis of phenotypic traits in planktonic condition showed that there were no differences among genotypes regarding their acid-tolerance considering the ratio between counts of viable cells in relation to pH 7.0 (Figures 1 and 2). In addition, no expressive differences in AUC were found among these genotypes (Fig. 5). However, genotypes isolated from biofilms formed in the presence of sucrose showed numerically higher counts of viable cells at pH 5.0 and at pH 2.8 T0 than those isolated from biofilms formed in the absence of sucrose (Fig. 3). Although this difference was not statistically significant, these data might indicate that there is a tendency of higher acid-tolerance of sucrose genotypes. In this context, it would be possible that the frequent pH fall in biofilms due to fermentation of sucrose (Marsh, 2003) may have contributed to the adaptation of sucrose-genotypes to acid environments. Also, even under an extreme stress condition, represented by pH 2.8, most of genotypes isolated from biofilms formed in the presence of sucrose still showed numerically higher counts of viable cells than genotypes from biofims formed in its absence (Fig. 3b). Moreover, in the present study, genotypes showed distinct F-ATPase activities (ranging from 2.3 ± 0.7 to 35.3 ± 6.6 µmol/g/min) (Figure 4). Bender & Marquis (1987) suggested that the differences in acid tolerance among microorganisms may be due to their different levels of membrane-bound-ATPases. Although this relationship had been found among different bacteria species, it might explain the distinct behavior of genotypes regarding their F-ATPase activity. Also, in agreement with some previous results (Arthur et al., 2010), it seems that there was no difference in F-ATPase activity between genotypes from biofilms formed in the presence of sucrose and the biofilms formed in its absence.

Regarding the acidogenicity, 4 of the *S. mutans* genotypes were the less acidogenic (R-ddw, Q-suc, Q-ddw and V-ddw), since they had the highest AUC (Fig. 5), which represents a slow fermentation of carbohydrate (in this case, glucose) through the time. All of the other 12 genotypes were not statistically

different among them. In addition, genotypes exclusively isolated from biofilms formed in the presence of sucrose (genotypes M, N and W) showed numerically lower AUC (257.4 ± 53.7 min*pH) than genotypes exclusively-isolated from biofilms formed in the absence of sucrose (K and R) (AUC: 365.22 ± 219.79 min*pH. These findings are in agreement with some previous data (Arthur et al., 2010), suggesting that the biofilms formed in the presence of sucrose exposure may harbor more acidogenic genotypes. The acidogenicity of microorganisms is an important phenotypic trait which could represents their ability to decrease the pH of the environment (specifically, dental biofilm) due to the fermentation of carbohydrates.

Based on these phenotypic traits in planktonic condition, 8 genotypes (B-ddw, B-suc, J-ddw, K-ddw, R-ddw, Q-suc, V-ddw and V-suc), with numerically higher or lower aciduricities (in terms of counts of viable cells at acid conditions and F-ATPase activity) and acidogenicities were selected for evaluation of their enamel demineralization potential, their acidogenicity and their ability to synthesize EPS in a mono-species biofilm designed by Ccahuana-Vásquez et al. (2009) and Cury et al. (2009), in which the biofilms were exposed to sucrose 8x/day. The most important data related to the phenotypic traits in this *in vitro* biofilms was the distinct cariogenicity of these genotypes (Table 2). Higher enamel demineralization potential (%SHL) was found in biofilms containing V-suc, V-ddw, Q-suc or R-ddw genotypes and the lowest %SHL was found in the presence of both B-suc and B-ddw genotypes.

According to Table 2, these above mentioned more cariogenic genotypes showed lower counts of viable cells in biofilms. Besides, based on the data of the acidogenicity of the biofilms, it seems that R-ddw and Q-suc genotypes were more acidogenic than the other ones, since they showed lower pH values after 48 h of biofilm formation, although the values were not statistically different compared with the other 6 genotypes (means of 5.0 ± 0.04 and 5.4 ± 0.11, respectively)(data not shown). Furthermore, the biofilms formed in presence of Q-suc and V-suc genotypes showed numerically higher concentrations of IEPS (63.5 ± 13.9 and

55.8 ± 19.5 µg/mg dry weight biofilm, respectively) (data not shown). The role of these insoluble polysaccharides in dental caries development has been widely discussed (Cury et al., 2000; Aires et al., 2006). Although there was not a statistical difference in IEPS concentrations in biofilms, perhaps, the numerically higher concentration of IEPS in the presence of Q-suc and V-suc genotypes might have been responsible for altering the biofilm matrix structure (Dibdin & Shellis, 1988) in a level which contributed to the higher cariogenicity of these genotypes. These data emphasize that the modifications induced by microorganisms in dental biofilms are more important than their levels in biofilms (Cury et al., 2001).

Furthermore, it is important to point out that in a biofilm condition, opposite to a planktonic one, it seems that genotypes from biofilms formed in the presence of sucrose were not more virulent than those isolated in its absence, since higher cariogenicity was found either in the presence of ddw- or suc-genotypes. In this context, it is well known that the phenotypic traits of microorganisms attached to a surface are different compared with planktonic cells. The altered gene expression of microorganisms and the cell-cell communication in biofilms may change the phenotype of these microorganisms (Li et al., 2001; Marsh, 2004), and this could explain the fact that in a planktonic condition suc-genotypes seemed to be more virulent. Nevertheless, this is the first study to compare these phenotypic traits of *S. mutans* genotypes in planktonic condition and biofilms and probably more analyses would be necessary.

At the present study, it seems that distinct genotypes may show distinct phenotypic traits as ratio of counts of viable cells at pH 2.8 T30 (data not shown), values of F-ATPase activity, AUC, counts of viable cells in biofilm and % SHL (Figures 4 and 5 and Table 2). These finding are in agreement with some previous studies which showed that distinct *S. mutans* genotypes may show distinct phenotypic traits, as the ability to adhere to hydroxiapatite beads, distinct acid-tolerance, acidogenicity, *in vitro* biofilm formation ability and *gtf*B and *gtf*C gene expression (Mattos-Graner et al., 2004; Napimoga et al., 2004; Guo et al., 2006;

Lembo et al., 2007; Arthur et al., 2010) or even distinct cariogenic potential in an animal model (Köhler & Krasse, 1990).

It is interesting to emphasize that, despite some differences have been found among the *S. mutans* genotypes, the lack of statistical significance in counts of viable cells, the ratio of viable cells and AUC may suggest that, probably, the methods used for these phenotypic analyses might lack the sensibility to detect small differences among the genotype. Also, the values of F-ATPase activity found in the present study should be interpreted with care, since the F-ATPase activity may have been inhibited by toluene during cell permeabilization (Thedei et al., 2008). Nevertheless, despite these limitations, it seems that genotypes isolated from biofilms formed in the presence of sucrose are more virulent in planktonic conditions than those isolated from biofilms formed in the absence of sucrose. For future studies, a modified protocol should be tested and adopted.

Overall, the present study suggests that in a planktonic condition distinct genotypes showed distinct acid-tolerance (in terms of counts of viable cells under acid stress condition and F-ATPase activity) and distinct acidogenicities, but the differences in acidogenicity were less pronounced. In addition, it seems that genotypes isolated from *in situ* biofilms formed under frequent pH fall due to exposure to sucrose might be more virulent than those isolated in a low cariogenic condition. In addition, in an *in vitro* biofilm condition, the selected genotypes were not distinct regarding either their ability to synthesize EPS or their acidogenicities, but they showed distinct enamel demineralization potential, which was not related to the conditions of in situ biofilm formation from where the genotypes were isolated.

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DISCUSSÃO GERAL

Os resultados dos Capítulos 1 e 2 sugerem que quedas no pH do biofilme dental induzidas pela frequente exposição à sacarose, de certa forma, modificam o fenótipo dos microrganismos, tornando-os mais ácido-tolerantes e mais acidogênicos. Esses resultados contemplam a hipótese da placa ecológica proposta por Marsh (2003), na qual as quedas de pH a que o biofilme dental está exposto promovem um desequilíbrio da microbiota favorecendo a colonização por microrganismos mais acidúricos e mais acidogênicos. Resultados semelhantes aos da presente tese também foram obtidos no estudo de Lembo *et al*. (2007), que verificaram que crianças cárie-ativas, condição clínica que pode estar relacionada a frequentes quedas de pH no meio bucal, possuíam genótipos mais acidúricos.

Tem sido sugerido que *S. mutans* cultivados em ambientes de reduzido pH se tornam mais ácido-tolerantes em futuros eventos de acidificação do meio (Belli & Marquis 1991; Hamilton & Buckley, 1991), como resultado de uma resposta adaptativa ao baixo pH (Lemos *et al*., 2005). Provavelmente, pode ser que os genótipos de *S. mutans* previamente isolados do biofilme dental formado *in vivo* e *in situ* na presença de sacarose tenham desenvolvido essa adaptação devido às freqüentes quedas de pH aos quais o biofilme dental estava exposto. Isso poderia, de certa forma, explicar a maior aciduricidade desses genótipos apresentada nos Capítulos 1 e 2.

Além da atividade F-ATPase, outros fatores também podem contribuir para resposta adaptativa dos microrganismos ao ambiente ácido. Trabalhos de Quivey *et al*. (2000) e de Fozo & Quivey (2004) demonstraram que, mediante estresse ácido, ocorre um aumento na proporção de lipídeos mono-insaturados e de cadeia longa na membrana celular de *S. mutans* e que a incapacidade de sintetizar esses lipídeos reduz a tolerância à acidificação do meio. Os autores discutem que, embora a bomba F-ATPase transporte prótons para o meio extracelular, ainda ocorrem quedas de pH no citoplasma em virtude do influxo de íons hidrogênio, e que essa acidificação ativa algumas enzimas responsáveis pela biosíntese de lipídeos de membrana. Por isso, foi sugerido que essa alteração na proporção de

lipídeos reduz a permeabilidade da membrana celular á prótons, conferindo maior aciduricidade aos microrganismos. Além disso, também foi verificado que deficiência na síntese de ácido lipoteicóico aumenta a permeabilidade da membrana celular á prótons, reduzindo a tolerância de *S. mutans* ao ambiente ácido (Boyd *et al*., 2000).

Em acréscimo, estudos de proteômica têm sido muito úteis na identificação de proteínas que participam da aciduricidade dos microrganismos. Maior expressão de genes *dnaK* e *groEL* foi verificada em resposta ao estresse ácido (Jayaraman *et al*., 1997; Lemos *et al*., 2001), e mutantes incapazes de sintetizarem DnaK foram mais ácido sensíveis (Lemos *et al*., 2007). Os autores discutem que, provavelmente, a proteína DnaK está envolvida na biogênese do complexo F-ATPase, sendo essencial para o correto funcionamento dessa bomba. Além disso, DnaK e GroEL podem estar envolvidas nos processos relacionados á renaturação ou degradação de proteínas anormais que foram alteradas durante a acidificação do meio (Lemos & Burne, 2008). Em acréscimo, foi verificado que *S. mutans* incapazes de sintetizar uma proteína de membrana, denominada BrpA, tiveram uma expressão reduzida do gene *atpD,* comumente envolvido com a atividade F-ATPase (Wen *et al*., 2006), sugerindo que essa proteína também desempenha um papel importante na aciduricidade desses microrganismos.

Em adição, o sistema de transdução de sinais de dois componentes (do inglês *"two-component signal transduccion system"*), específico de microrganismos procariotos (Kawada-Matsuo *et al*., 2009), também está relacionado com a aciduricidade de *S. mutans.* Esse sistema é responsável pela captura de alterações ambientais e modulação da expressão gênica no intuito de adaptar os microrganismos ao estímulo em questão. Especificamente, alteração nos genes *hk11, CiaHR, LevSR, LiaSR, ScnKR* e *ComDE* produziram fenótipos menos ácido-tolerantes (Li *et al*., 2002; Gong *et al*., 2009). Todos esses mecanismos descritos nesses últimos parágrafos demonstram a complexidade envolvida na aciduricidade de *S. mutans*. A atuação diferencial desses mecanismos pode ter sido responsável pela maior aciduricidade dos genótipos isolados de biofilmes

formado na presença de um alto desafio cariogênico. Entretanto, pesquisas adicionais devem ser conduzidas como forma de se esclarecer e de se avaliar a influência de cada um desses fatores na aciduricidade de diferentes genótipos.

É interessante enfatizar que apesar dos resultados do Capítulo 1 e 2 sugerirem que genótipos isolados de biofilme dental formado *in vivo* e *in situ* durante 3 dias na presença de sacarose serem mais virulentos que aqueles isolados de biofilme dental formado na ausência desse carboidrato, parece que o potencial cariogênico é uma característica fenotípica inerente a cada genótipo, o que poderia explicar as diferenças na severidade da doença cárie entre os indivíduos. Esses resultados confirmam que tanto a freqüente exposição a carboidratos fermentáveis (nesse caso a sacarose) quanto às características de virulência inerente aos genótipos de *S. mutans,* também desempenham um importante papel no desenvolvimento da doença cárie.

Apesar de poucas diferenças terem sido encontradas entre os genótipos numa condição planctônica (Capítulo 2), houve diferença no potencial de desmineralizar o esmalte dental quando esses genótipos foram cultivados na forma de biofilme *in vitro*. Esses resultados podem sugerir que as características fenotípicas expressas em cultura de células planctônicas nem sempre representam o fenótipo dos microrganismos num biofilme. Diversos fatores podem ser responsáveis por esse comportamento distinto, como, por exemplo, a comunicação intercelular ou mesmo diferença na expressão gênica (Marsh, 2004). Estes achados ressaltam a importância de também se adotar modelos de biofilme *in vitro* no estudo de características fenotípicas de microrganismos.

CONCLUSÃO GERAL

Os resultados da presente tese sugerem que:

- Genótipos de *S. mutans* previamente isolados de biofilme dental formado *in vivo* ou *in situ* sob um alto desafio cariogênico (exposição frequente à sacarose e acúmulo de biofilme) parecem ser mais acidúricos e mais acidogênicos em relação àqueles isolados na ausência deste desafio.

- Frequentes quedas de pH no biofilme dental parecem levar à colonização do biofilme por genótipos mais virulentos;

- Quando genótipos previamente isolados de biofilme dental formado *in situ* foram crescidos na forma de biofilme *in vitro*, o potencial desses genótipos de desmineralizar o esmalte dental não mostrou relação com a condição na qual esses genótipos foram isolados, isto é, de biofilme *in situ* formado na presença ou ausência de sacarose.

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Genotypic Diversity of *S. mutans* in Dental Biofilm Formed *In Situ* under Sugar Stress Exposure

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In situ dental biofilm composition under sugar exposure is well known, but sugar effect on the genotypic diversity of *S. mutans* in dental biofilm has not been explored. This study evaluated *S. mutans* genotypic diversity in dental biofilm formed *in situ* under frequent exposure to sucrose and its monosaccharide constituents (glucose and fructose). Saliva of 7 volunteers was collected for isolation of *S. mutans* and the same volunteers wore intraoral palatal appliances, containing enamel slabs, which were submitted to the following treatments: distilled and deionized water (negative control), 10% glucose + 10% fructose (fermentable carbohydrates) solution or 20% sucrose (fermentable and EPS inductor) solution, 8x/day. After 3, 7 and 14 days, the biofilms were colleted and *S. mutans* colonies were isolated. Arbitrarily primed polymerase chain reaction (AP-PCR) of *S. mutans* showed that salivary genotypes were also detected in almost all biofilm samples, independently of the treatment, and seemed to reflect those genotypes present at higher proportion in biofilms. In addition to the salivary genotypes, others were found in biofilms but in lower proportions and were distinct among treatment. The data suggest that the *in situ* model seems to be useful to evaluate genotypic diversity of *S. mutans*, but, under the tested conditions, it was not possible to clearly show that specific genotypes were selected in the biofilm due to the stress induced by sucrose metabolism or simple fermentation of its monosaccharides.

Key Words: sucrose, genotypes, *S. mutans*, arbitrarily primed polymerase chain reaction, *in situ* study.

INTRODUCTION

Dental caries is a dietary and biofilm-dependent disease related to frequent consumption of fermentable carbohydrates (1) and the low pH produced by sugar fermentation induces changes in microbiota of dental biofilm (2), selecting the most cariogenic bacteria. Among the dietary sugars, sucrose is considered as the most cariogenic (3) because, in addition to decreasing the pH of dental biofilm, it is the substrate for the synthesis of extracellular polysaccharides (EPS) (4), which may either promote bacterial adherence to tooth surface (5) during initial colonization or play a role in the cohesiveness of the bacterial communities in the biofilm.

Among the cariogenic bacteria, mutans streptococci (MS) are acidogenic, survive in acidic environments, and produce EPS from sucrose, which make them the most important cariogenic microorganisms (2). However, there is no direct relationship between the exposure to sugars and the MS counts in dental plaque (6-8), suggesting that the changes induced by these microorganisms in dental biofilm are more important than their levels (6). Thus, EPS have been considered virulence factors of the bacteria present in dental biofilm (9) and it has been shown that caries incidence might be more related to the capacity of specific *S. mutans* strains to synthesize insoluble EPS than to their proportions in dental biofilm (10).

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Therefore, some studies have shown that the oral cavity may present distinct genotypes of *S. mutans*, not only in saliva but also in dental biofilm (11,12). Additionally, under frequent sucrose exposure, clinical findings suggest that there was an increase in genotypic diversity of *S. mutans* in the oral cavity (13). These genotypes could present distinct virulence capacities and different abilities of producing EPS (14). Thus, distinct genotypes could be differentiated by their ability to produce insoluble EPS from sucrose, facilitating their adherence to tooth surface, or simply by their predominance in dental biofilm due to a higher capacity to tolerate the acid environment generated by any fermentable sugar. Nevertheless, studies in this direction have been limited to evaluate the absolute MS counts and not the specific genotypes (7,8).

In this context, the *in situ* model is a recognized experimental design that has been successfully used to evaluate cariogenic dental biofilm formation (3). The biochemical and microbiological composition of dental biofilm has been extensively studied using this model (4,6-8,15). However, the genotypic diversity of *S. mutans* found in dental biofilm formed under exposure to different dietary sugars has not been evaluated.

This research group has hypothesized that genotypes present in saliva would be selectively found in dental biofilm under different stress conditions induced by sugars exposure. Therefore, the purpose of this study was to evaluate *S. mutans* genotypic diversity in dental biofilm formed *in situ* under frequent exposure to sucrose, a fermentable carbohydrate and inductor of EPS synthesis, and, as control, its monosaccharide constituents, glucose and fructose, which are only fermentable.

MATERIAL AND METHODS

Experimental Design

This *in situ* study, approved by the Ethics in Research Committee of the School of Dentistry of Piracicaba, State University of Campinas, (Protocol #053/2004), was crossed and double-blinded. Before the beginning of the experiment, stimulated saliva was collected from 12 volunteers to determine the population of mutans streptococci (MS). Seven healthy volunteers (18 to 28 years old), who fulfilled inclusion criteria (counts of mutans streptococci in saliva $\geq 10^5$ colony-forming units (CFU) *per* mL and ability to comply with the experimental protocol) were selected. Salivary isolates of mutans streptococci, showing *S. mutans* morphological types, were obtained from each volunteer and frozen for subsequent genotyping. Furthermore, the selected volunteers wore acrylic intraoral palatal appliances, containing 6 slabs of sound human dental enamel, which were treated extraorally, 8x/day, with distilled and deionized water (negative control), a mixture of 10% glucose + 10% fructose (only acidogenic) or 20% sucrose solution (acidogenic and EPS inductor) (Merck, Darmstadt, Germany). The biofilm formed onto two enamel slabs was collected after 3, 7 and 14 days of formation, and cultured in Mitis Salivarius Agar (MSA) plus bacitracin (MSB) (Difco, Sparks, MD, USA). *S. mutans* morphological types present in biofilm samples were isolated and DNA from these isolates, and from those colonies cultured from saliva, was extracted. After this, polymerase chain reaction (PCR) with specific primers was conducted for identification of *S. mutans*, and then, these isolates were submitted to a genotyping protocol by arbitrarily primed PCR (AP-PCR) with OPA-02 primer.

Saliva Sampling and Selection of Subjects

A total of 12 healthy adults, who signed a written informed consent form, were screened for salivary levels of MS. Stimulated saliva samples were collected in the morning within fasting condition and without previous teeth brushing. Whole saliva was diluted in sterile 0.9% NaCl and plated on MSB to determine MS counts. CFU were counted and the results expressed as CFU/mL of stimulated saliva. From that group, a subset of 7 volunteers (18 to 28 years old) was selected (counts of mutans streptococci in saliva $\geq 10^5$ CFU/mL). The exclusion criteria were antibiotic use for the last 2 months before starting the study, use of any form of medication that modifies salivary secretion, use of fixed or removable orthodontic appliance, periodontal disease or general/systemic illness. Eight representative morphological types of *S. mutans* colonies were collected from each saliva sample of the selected volunteers, subcultured on MSA and Brain Heart Infusion (BHI) agar (Difco), and pure cultures stored at -70°C in 10% skim milk medium (Difco) (16) for further genotypic analysis. The purity and identity of the isolates were checked by Gram's staining and colonial morphology

on MSA.

In Situ Experimental Phases

An acrylic intraoral palatal appliance containing 6 enamel slabs (4 x 4 x 2 mm) obtained from the buccal and lingual surfaces of sound human impacted third molars, was prepared for each volunteer (4). On the left and right sides of the appliances, 3 cavities of 5 x 5 x 3 mm were made and one slab was placed in each of them, totalizing 3 slabs on each side. Plastic meshes were fixed over the cavities to protect the enamel surfaces from mechanical attrition, leaving a 1-mm space for accumulation of dental biofilm (4, 15).

Seven volunteers wore the intraoral appliances during 3 experimental phases of 14 days each and were randomized into 3 different groups. In these groups, the slabs were treated extraorally 8x/day with: distilled and deionized water (DDW), 10% glucose + 10% fructose solution (G+F), and 20% sucrose solution. The volunteers were instructed to remove the appliances and drip onto the dental slabs one drop of the treatment solutions, at predetermined times. After standing for 5 min, the appliance was replaced in the mouth. The volunteers wore the appliances all the time, removing it for feeding, drinking and oral hygiene (4). All volunteers were subjected to all three treatments, following a double-blind crossover design. At seven days before the beginning of each treatment and throughout the entire experiment, volunteers used a non-fluoridated dentifrice. Washout periods of 15 days were carried out between each treatment. Because all volunteers were subjected to all treatments, no restriction was made with regard to their diet. The treatment solutions were handed every other day to the volunteers.

Collection of Dental Biofilms

On the 3rd, 7th and 14th day of each experimental phase, 10 h after the last exposure to treatments, in the morning and being the volunteers in fasting conditions and without having brushed their teeth, the biofilm formed onto 2 dental slabs selected at random but one from each side of the appliance, was collected. On the 3rd day, biofilm was collected from anterior left and central right slabs; on the 7th day, from central left and posterior right slabs and on the 14th day, from posterior left and anterior right slabs. The biofilm was collected with sterile plastic spatulas, weighed (± 0.01 mg) (Ohaus Analytical Plus, Switzerland), suspended in sterile saline (Merck), sonicated (Sonics and Materials, Danbury, CT, USA), serially diluted and inoculated in MSB.

Isolation of S. Mutans Strains and Extraction of Genomic DNA

Five to eight colonies, representing all morphological types of *S. mutans*, were collected from each saliva and biofilm sample, subcultured on MSA and pure cultures were frozen at -70°C in 10% skim milk. Then, aliquots were collected from skim milk and plated on BHI agar (Difco), which was incubated for 24 h, at 37°C and 10% pCO_2. The colonies from BHI agar were inoculated into Todd Hewitt Broth (Difco) and incubated for 18 h, at 37°C and 10% pCO_2. Cultures were then centrifuged (Jouan, France) at 10,000 g, 4°C for 15 min, genomic DNA was extracted from the cell pellet, using the Master Pure DNA purification kit (Epicentre Technologies, Madison, WI, USA) (16), and stored at -20°C. Integrities of the genomic DNA samples were checked in samples electrophoretically resolved in 1% agarose gel (Invitrogen, Barcelona, Spain) and stained with ethidium bromide (5 µg/mL). Isolates were confirmed for species identity in PCR reactions with primers specific for gtfB, enconding glucosyltransferase B (5'-ACTACACTTTCGGGTGGCTTGG-3' and 5'-CAGTATAAGCGCCAGTTTCATC-3') (17) (Invitrogen) and specific to gbpB, enconding glucan-binding protein B (5'-CAACAGAAGCACAACCATCA-3' and 5'-TGTCCACCATTACCCCAGT-3') (18). The reactions were performed as described elsewhere (17,18) and the PCR products were analyzed by electrophoresis.

Genotypic Analysis of S. Mutans Isolates by AP-PCR

AP-PCR assays were performed with the arbitrary primer OPA 02 (5'-TGCCGAGCTG-3') (16). The amplifications occurred under the following conditions: 95°C for 2 min, for initial denaturation, and 45 cycles of 94°C for 30 s (denaturation), 36°C for 30 s (annealing) and 72°C for 1 min (extension) and a final extension at 72°C for 5 min. Genomic DNA of *S. mutans* strain UA 130 (kindly provided by Dr. Page W. Caufield, New York University, NY, USA) and distilled and deionized water were applied in all PCR baths, as positive and negative controls. Products of AP-PCR

were electrophoretically resolved in 1.5% agarose gels that were run at 3 V/cm during 3 h in TBE running buffer (16). The gel was stained with a 5 μg/mL of ethidium bromide solution (Invitrogen) for 10 min and their images captured by a digital imaging system (Gel logic 100 Imaging System, Kodak, Japan).

For analysis of the *S. mutans* genotypic profiles from the same volunteer, AP-PCR products from the isolates obtained from the 3 periods of biofilm formation under the same treatment were always resolved side-by-side in the same gel for visual comparisons (11). Thus, samples representative of each genotype identified *per* treatment and time were re-run side-by-side in a subsequent gel for direct comparisons of genotypes identified within distinct treatments. Thus, genotypic diversity was compared among isolates from saliva and biofilm samples, obtained from each treatment within the same volunteer. Isolates were considered as having the same genotypic identity when presented identical AP-PCR product-size profiles. The genotypes found were descriptively analyzed and their proportion, in relation to the number of colonies isolated in each sample and condition, was calculated.

RESULTS

A total of 56 and 469 representative *S. mutans* colonies were isolated from saliva and biofilms, respectively. In two samples, only 2 representative colonies were isolated from 7-day DDW biofilm (volunteer 5 and 7), due to absence of more colonies. All isolates were *S. mutans* identified by PCR and a total of 28 distinct genotypes were identified in the saliva and biofilm samples (Table 1). Figure 1 represents the genotypic diversity found in volunteer 2. In the majority of the volunteers, 6 out of 7, just one genotype was observed in saliva samples, with the exception of volunteer 2, who presented two genotypes in saliva with the prevalence of one of them (Table 1). The same genotypes detected in saliva were identified in the respective biofilm samples, independently of the treatment and period of biofilm formation. On the other hand, in addition to the salivary genotypes, others were found in the biofilm samples exposed to DDW (6 genotypes), G+F (5 genotypes) and sucrose (8 genotypes), which were generally detected in lower proportions (Table 1). Regarding the period of biofilm formation, only a discrete shift occurred in the diversity of genotypes, suggesting a lower diversity in the 14-day biofilm.

DISCUSSION

Most volunteers showed a predominance of just one genotype in saliva, which was also identified in the biofilms. This agrees with a previous study that evaluated samples of saliva, tongue and biofilm collected from children (12). This finding suggests that saliva samples may reflect at least those genotypes present at higher proportions in teeth biofilms, independent of the conditions in which the biofilm was formed. In addition, the genotypes present in saliva and biofilm samples were distinct among the volunteers (12). As previously mentioned, we hypothesized that salivary genotypes would be selectively found in dental biofilm under different stress conditions induced by sugars exposure, but this hypothesis cannot be either confirmed or rejected due to the low genotypic diversity found in saliva of each volunteer. Thus, the different genotypes found in the biofilms would be originally in the saliva of each volunteer, but below the detection limit of the microbiological method used (11). Nevertheless, the hypothesis reached in the present can be tested because the biofilm was also formed in the absence of sugars exposure. Therefore,

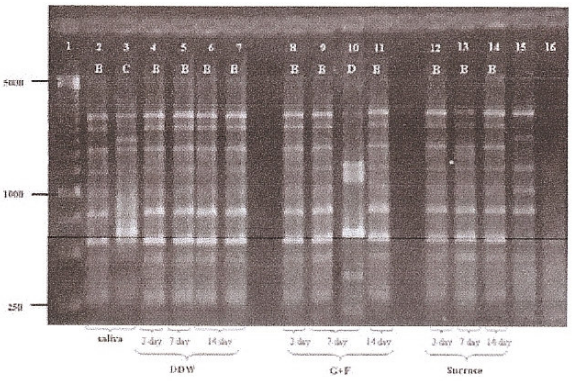

Figure 1. Representative AP-PCR profiles (amplitypes) identified among S. mutans strains isolated from volunteer 2 in saliva and dental biofilm formed under distinct treatments (DDW; G+F and sucrose) and in different periods (3, 7 and 14 days); Lane 1: 250-bp DNA ladder; lanes 2 and 3: corresponds to the amplitypes identified in saliva; lanes 4 to 7 correspond to amplitypes identified in DDW biofilm. Lanes 8 to 11: amplitypes identified in G+F biofilm. Lanes 12 to 14: amplitypes identified in sucrose biofilm. Lane 15: AP-PCR profile of the control S. mutans strain UA130. Lane 16: negative control (water). Different letters represent different amplitypes. Biofilm treatments are indicated at the bottom of the figure.

it was possible to compare the genotypic diversity found in dental biofilm for each volunteer in each evaluation time when biofilm was formed under the tested conditions.

Although the findings were not consistent for all volunteers, Table 1 suggests that different genotypes were selected in the biofilm formed under sugars stress for the volunteers 3, 4, 6 and 7 in comparison with the negative control. Thus, on the 3rd day of biofilm formation in volunteer 4's mouth, the genotypes M and N were only found in the sucrose group. Considering that sucrose differs from G+F only in insoluble EPS production (4), it is possible that these genotypes produce more EPS than J, K and L found in biofilms formed in presence of G+F or in the absence of sugar exposure. The genotypes M and N were not the most prevalent in such biofilm, but specific phenotypic characteristics may be more relevant than the relative counts of *S. mutans* (10) or its absolute counts (6). On the 3rd day of biofilm formation under sucrose exposure, it seems that genotypes W and Z were also selected in the biofilm of volunteers 6 and 7, respectively. The confirmation of the properties of these genotypes should be conducted, evaluating insoluble EPS production and gtfB expression.

On the 7th day of biofilm formation, genotype P was found in the biofilm of volunteer 4 only under sucrose exposure. Furthermore, specific genotypes were also found in 7-day and 14-day sucrose biofilms,

Table 1. Genotypic diversity of S. mutans (%) in saliva and dental biofilm regarding the treatments and time of biofilm formation.

Volunteer	Saliva	3 days			7 days			14 days		
		DDW	G+F	Sucrose	DDW	G+F	Sucrose	DDW	G+F	Sucrose
1	A (100)	NF	A (100)	A (100)	A (100)	A (100)	A (100)	A (100)	A (100)	A (100)
2	B (85.7) C (14.3)	B (100)	B (100)	B (100)	B (100)	B (40) D (60)	B (100)	B (100)	B (100)	B (100)
3	E (100)	E (100)	E (100)	E (100)	E (100)	E (83) F (17)	E (88) G (12)	E (100)	E (100)	E (87.5) H (14.3)
4	I (100)	I (62.5) J (12.5) K (25.0)	I (75) J (12.5) L (12.5)	I (75) M (12.5) N (12.5)	I (60) J (20) O (20)	I (50) L (50)	I (25) L(50) P (25)	I (28.6) O (71.4)	I (42.9) J (57.1)	I (100)
5	Q (100)	Q (87.5) R (12.5)	Q (28.6) S (71.4)	Q (100)	Q (100)	Q (100)	Q (100)	Q (87.5) T (12.5)	Q (87.5) U (12.5)	Q (100)
6	V (100)	V (100)	V (100)	V (87.5) W (12.5)	V (100)	V (100)	V (100)	V (100)	V (100)	V (100)
7	X (100)	X (75) Y (25)	X (100)	X (87.5) Z (12.5)	X (100)	X (100)	X (100)	X (100)	X (87.5) A_1 (12.5)	X (85.7) B_1 (14.3)

The designation of genotypes by letters (A, B, C, etc) is only valid within each volunteer. Distinct letters show different genotypes. The proportion (%) of the genotypes in relation to the number of colonies isolated in each condition is represented within the parenthesis. NF = S. mutans colonies were not found in this sample. DDW = distilled and deionized water group; G+F = glucose and fructose solution group.

as genotypes G and H for volunteer 3. Regarding the role of sucrose on biofilm formation, some *in situ* studies have shown that dental biofilm formed in its presence has high concentrations of insoluble EPS (4,7,15), even after 3 days of formation (8). Insoluble EPS are sticky polysaccharides that could enhance the adherence of *S. mutans* specific genotypes to dental surface (5), which could explain the presence of the described genotypes only in sucrose biofilms.

In addition to these sucrose-specific genotypes, other genotypes were found only in the presence of G+F when compared with sucrose or DDW biofilms within the same volunteer, such as genotype F from 7-day biofilm of volunteer 3, genotype U from 14-day biofilm of volunteer 5 and genotype A_1 from 14-day biofilm of volunteer 7. All these genotypes were less prevalent than the salivary ones. However, other genotypes (genotype D in 7-day biofilm of volunteer 2 and genotype S from 3-day biofilm of volunteer 5) were only found in the presence of G+F, but were more prevalent than the salivary genotypes of each of these volunteers in the biofilm. The fermentation of sucrose or G+F could create an acid condition that influences growth and survival of bacteria (2) and, perhaps, enhances the colonization of more acid tolerant genotypes. In addition, this could explain the finding of genotype L either in presence of G+F or sucrose biofilms of volunteer 4. The acid tolerant properties of genotypes found later in biofilm formed in presence of sucrose or G+F should be evaluated.

There is evidence that *S. mutans* strains, representing distinct AP-PCR patterns, show significant different virulence capacities (12,14) and, this might probably explain the higher ability of sucrose genotypes in producing EPS. Also, these distinct virulence abilities of *S. mutans* genotypes could explain the higher cariogenicity of a biofilm formed in presence of sucrose (4,8), emphasizing that the modifications induced by microorganisms in dental biofilm are more important than their levels in biofilm (6). Therefore, some virulence characteristics of these genotypes should be experimentally explored, which might help explain their distinct colonization.

Regarding the period of biofilm formation, apart from those genotypes that appeared in biofilm selected by sucrose or G+F treatment (as discussed before), no relation between genotypic diversity and aging of dental biofilm was observed in the volunteers, in agreement with (19). However, a direct comparison of our data with other studies is difficult since this was the first one to evaluate genotypic diversity *in situ* and under experimentally controlled conditions.

Considering some limitations in the reproducibility of the patterns observed (20), the AP-PCR products from the same volunteer at the same treatment and period of biofilm formation were always resolved side-by-side in the same gel and representative samples of each genotype identified *per* treatment were re-run side-by-side in a subsequent gel for direct comparisons of genotypes identified within distinct treatments. Afterwards, all representative genotypes were submitted to another AP-PCR in order to confirm the pattern of the amplicons.

In summary, the findings of the present study suggest that the genotypes found in saliva seems to reflect those genotypes present at higher proportion in dental biofilms, irrespective of the tested conditions. Moreover, a selection of genotypes could not be observed in dental biofilm formed either or not in the presence of sucrose, but other genotypes, in addition to the salivary ones, were observed in both biofilms, most of them in a lower proportion. Although the phenomenon was not general, it was not possible to show for some volunteers that specific genotypes were selected in the biofilm formed by the stress induced by sucrose metabolism or simply by the fermentation of its monosaccharides.

RESUMO

A composição do biofilme dental *in situ* exposto a açúcares é bem conhecida, mas o efeito dos açúcares na diversidade genotípica de *S. mutans* no biofilme dental ainda não foi explorada. Este estudo avaliou a diversidade genotípica de *S. mutans* no biofilme dental formado *in situ* sob frequente exposição à sacarose e seus monossacarídeos constituintes (glicose e frutose). Primeiramente, saliva de voluntários foi coletada para isolamento de *S. mutans* e os mesmos voluntários usaram um dispositivo intraoral palatino, contendo blocos de esmalte, que foram submetidos 8x/dia aos seguintes tratamentos: água destilada e deionizada (controle negativo), solução de glicose 10% + frutose 10% (carboidratos fermentáveis) e solução de sacarose 20% (fermentável e indutor de PEC). Após 3, 7 e 14 dias, os biofilmes foram coletados e colônias de *S. mutans* foram isoladas. A técnica de reação em cadeia de polimerase usando primers arbitrários (AP-PCR) demonstrou que o genótipo salivar foi detectado em quase todas as amostras de biofilme, independente do tratamento, e parece refletir aqueles genótipos presentes em maiores proporções no biofilme. Além do genótipo salivar, outros foram encontrados nos biofilmes, mas em uma menor proporção e foram distintos

entre os tratamentos. Os dados sugerem que o modelo *in situ* é útil para a avaliação da diversidade genotípica de *S. mutans*. Porém, nas condições do presente estudo, não foi possível demonstrar que genótipos específicos foram detectados no biofilme devido ao estresse induzido pelo metabolismo da sacarose ou fermentação de seus monossacarídeos.

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ANEXO 1. CERTIFICADO DO COMITÊ DE ÉTICA

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

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Influência da sacarose na diversidade genotípica de estreptococos do grupo mutans e na composição do biofilme dental formado in situ em diferentes tempos e in vivo**", protocolo nº **053/2004**, dos pesquisadores **CÍNTHIA PEREIRA MACHADO TABCHOURY, ADRIANA FRANCO PAES LEME, ALTAIR ANTONINHA DEL BEL CURY, GLÁUBER CAMPOS VALE, JAIME APARECIDO CURY, RENATA DE OLIVEIRA MATTOS GRAEER e RODRIGO ALEX ARTHUR**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 07/07/2004.

Piracicaba, 27 de outubro de 2005.

The Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas, certify that project "**Influence of sucrose on genotypic diversity of mutans streptococci and on biofilm composition formed in situ at different periods and in vivo**", register number **053/2004**, of **CÍNTHIA PEREIRA MACHADO TABCHOURY, ADRIANA FRANCO PAES LEME, ALTAIR ANTONINHA DEL BEL CURY, GLÁUBER CAMPOS VALE, JAIME APARECIDO CURY, RENATA DE OLIVEIRA MATTOS GRAEER and RODRIGO ALEX ARTHUR**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for researching in human subjects and was approved by this committee at 07/07/2004.

Piracicaba – SP, Brazil, October 27 2005.

Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Prof. Dr. Thales Rocha de Mattos Filho
Diretor
FOP/UNICAMP

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

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

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Avaliação das características de células planctônicas e de biofilme formados a partir de diferentes genótipos de Streptococcus mutans prevalentes em biofilme dental cariogênico formado in situ e in vivo**", protocolo nº **004/2006**, dos pesquisadores **RODRIGO ALEX ARTHUR, ALTAIR ANTONINHA DEL BEL CURY, CÍNTHIA PEREIRA MACHADO TABCHOURY, GLÁUBER CAMPOS VALE, JAIME APARECIDO CURY, LENITA MARANGONI LOPES, PEDRO LUIZ ROSALEN e RENATA DE OLIVEIRA MATTOS GRANER**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 08/02/2006, com aprovação de adendo em 09/11/2007.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Evaluation of characteristics of planctonic and biofilm cells formed by different genotypes of Streptococcus mutans prevalent in cariogenic dental biofilm formed in situ and in vivo**", register number **004/2006**, of **RODRIGO ALEX ARTHUR, ALTAIR ANTONINHA DEL BEL CURY, CÍNTHIA PEREIRA MACHADO TABCHOURY, GLÁUBER CAMPOS VALE, JAIME APARECIDO CURY, LENITA MARANGONI LOPES, PEDRO LUIZ ROSALEN and RENATA DE OLIVEIRA MATTOS GRANER**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 08/02/2006, having an addendum approved at 09/11/2007.

Profa. Cínthia Pereira Machado Tabchoury
Secretária
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
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ANEXO 2. COMPROVANTE DE SUBMISSÃO DE ARTIGO CIENTÍFICO PARA PUBLICAÇÃO

Manuscript submission (AEM00066-10 Version 1)

---------- Mensagem Original ----------
Assunto: Manuscript submission (AEM00066-10 Version 1)
De: journalsrr@asmusa.org
Data: Dom, Janeiro 10, 2010 2:10 pm
Para: cinthia@fop.unicamp.br

Dr. Cinthia Tabchoury
Faculty of Dentistry of Piracicaba
Dept. of Physiological Sciences
Av Limeira 901
Piracicaba, São Paulo 13414-903
Brazil

Re: Genotypic and phenotypic analysis of S. mutans isolated from dental biofilm formed in vivo under a high cariogenic condition (AEM00066-10 Version 1)

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You have successfully submitted your manuscript via the Rapid Review system. The control number of your manuscript is AEM00066-10 Version 1. Take note of this number, and refer to it in any correspondence with the Journals Department or with the editor. You may log onto the Rapid Review system at any time to see the current status of your manuscript and the name of the editor handling it. The URL is http://www.rapidreview.com/ASM2/author.html, and your user name is tabchoury. To find contact information for the editor handling your manuscript, go to the following URL:

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Piracicaba, 20 de janeiro de 2010.

Rodrigo Alex Arthur

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ANEXO 4. SÚMULA CURRICULAR

Rodrigo Alex Arthur, natural de Piracicaba, São Paulo, nasceu em 18 de maio de 1980. Na mesma cidade, completou o ensino fundamental em 1994 e o ensino médio em 1997. Iniciou o curso de Odontologia em fevereiro de 2000 na Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas. Durante os quatro anos de graduação, desenvolveu atividades de Iniciação Científica no Laboratório de Bioquímica Oral da mesma instituição, sob orientação da profa. Dra. Cínthia Pereira Machado Tabchoury e foi bolsista da FAPESP durante 2 anos, além de ter recebido bolsas do SAE (Serviço de apoio ao Estudante – UNICAMP), e do CNpQ-Pibic. Em fevereiro de 2004 foi graduado Cirurgião-Dentista. No mesmo ano ingressou no Programa de Pós-graduação em Odontologia – Área de concentração Cariologia – Nível Mestrado sob orientação da profa. Dra. Cínthia Pereira Machado Tabchoury. Durante esse período recebeu bolsa e auxílio pesquisa FAPESP, e obteve título de Mestre em Odontologia em fevereiro de 2006. Nesse mesmo ano, foi aprovado no exame de seleção e iniciou o doutoramento em Odontologia – Área de concentração Cariologia sob orientação da profa. Dra. Cínthia Pereira Machado Tabchoury. Recebeu bolsa e auxílio pesquisa FAPESP. Foi também bolsista CAPES durante 12 meses, período em que realizou Estágio de Doutorado no Exterior (PDEE), desenvolvido na Universidade de Zurique (Suíça) sob orientação do prof. Dr. Bernhard Guggenheim. Durante esse estágio, aprendeu diversos modelos de formação de biofilme multiespécie *in vitro*, bem como a utilização da hibridização *in situ* e da microscopia confocal na identificação de microrganismos e das estruturas do biofilme, e também a utilização do *"Quantitative light induced fluorescense"* (QLF) como alternativa para se avaliar a desmineralização de espécimes de esmalte dental.

Atividades desenvolvidas durante a pós-graduação:

Cursos de Extensão Universitária:

Participou do Curso de Extensão Universitária intitulado "V Curso de Verão: Manipulação de Ácidos Nucléicos: do DNA ao Produto Gênico" e "Análise da Expressão Gênica Utilizando-se PCR Quantitativo em Tempo Real", no período de 11 a 16 de fevereiro de 2008, com duração de 48 horas, no Instituto de Biociências da UNESP – Botucatu – SP.

Disciplinas extracurriculares:

"Estatística experimental", cursada no departamento de Ciências Exatas da Escola Superior de Agricultura Luiz de Queiros (ESALQ), da Universidade de São Paulo (agosto a dezembro de 2006).

"Genes e genômica funcional", cursada no departamento de Biologia na Agricultura e do Ambiente, no Centro de Energia Nuclear na Agricultura (CENA), da Universidade de São Paulo (março a junho de 2006)

"Métodos experimentais em Biologia Molecular", cursada no departamento de Biologia na Agricultura e do Ambiente, no Centro de Energia Nuclear na Agricultura (CENA), da Universidade de São Paulo (março a junho de 2004)

Programa de Estágio Docente (PED):

Participou do PED na disciplina de Pré-Clínica II, do curso de Odontologia, da Faculdade de Odontologia de Piracicaba, no período de agosto a dezembro de 2005 e de 2007 sob orientação da profa. Dra. Cínthia Pereira Machado Tabchoury.

Participou do PED na disciplina de Biociências II, do curso de Odontologia, da Faculdade de Odontologia de Piracicaba, no período de agosto a dezembro de 2006 sob orientação da profa. Dra. Cínthia Pereira Machado Tabchoury.

Participação em projetos de pesquisa (como colaborador)

Título: "Influência do fluoreto na acidogenicidade, concentração de polissacarídeos e diversidade genotípica de estreptococos do grupo mutans detectados por AP-PCR no biofilme dental formado *in situ*" (concluído em 2009)

Título: "Development of a local demineralization biofilm model to study the cariogenic potential of oral bacteria". (desenvolvido durante estágio de doutoramento no exterior – concluído em 2009)

Título: "*Veillonella dispar* biofilm colonization depends on *Streptococcus mutans* GTFB. (desenvolvido durante estágio de doutoramento no exterior – concluído em 2009)

Título: "Effects of xylitol on survival of mutans streptococci in mixed-species *in vitro* biofilms modeling supragingival plaque" (desenvolvido durante estágio de doutoramento no exterior – concluído em 2009)

Título: "Cariogenicidade de fórmulas infantis contendo amido e sacarose" (concluído em 2008)

Titulo: "Diversidade genotípica de *Streptococcus mutans* detectados com diferentes primers arbitrários por AP-PCR" (concluído em 2007)

Título: "Efeito do gel de clorexidina na recolonização da cavidade bucal por estreptococos do grupo mutans" (concluído em 2007)

Título: “Influência da sacarose na composição do biofilme dental e na desmineralização – estudo *in vivo* e *in situ*” (concluído em 2006)
Título: “Influência do gel e verniz fluoretado no desenvolvimento de cárie em ratos” (concluído em 2005)

Apresentação de trabalhos:

“Development of a local demineralization model to study the cariogenic potential of oral bactéria”. 56ª Reunião da European Organization for Caries Research (ORCA), Budapeste, Hungria. 2009

“Acidogenicities and aciduricities of *Streptococcus mutans* isolated from dental biofilm exposed to sucrose *in situ*” 54ª Reunião da European Organization for Caries Research (ORCA), Helsingor, Dinamarca. 2007

“Diversidade genotípica de *Streptococcus mutans in vivo* e *in situ* na presença de sacarose ou de glicose + frutose. 23ª Reunião Anual da Sociedade Brasileira de Pesquisa Odontológica (SBPqO), Atibaia, SP, Brasil. 2006

“Efeito do tempo de formação do biofilme dental na sua composição bioquímica e relação com cárie dental – estudo *in situ*”. I Reunion Anual de La Federacion Latinoamericana e XXXVIII Reunion Anual de la Sociedad Argentina de Investigation Odontologica. Mar del Plata, Argentina, 2005.

Publicações:

Effect of milk and soy-based infant formulas on *in situ* demineralization of human primary enamel. Papa AMCM, Tabchoury CPM, Del Bel Cury AA, Tenuta LMA, **Arthur RA[*],** Cury JA. *Pediatric Dentistry* (*in press*)

Evaluation of genotypic diversity of *Streptococcus mutans* using distinct arbitrary primers. Tabchoury CPM, Sousa MCKS, **Arthur RA**[*], Mattos-Graner RO, Del Bel Cury AA, Cury JA. *Journal of Applied Oral Science* 2008;16(6):403-407.

Temporal relationship between sucrose associated changes in dental biofilm composition and enamel demineralization. Vale GC, Tabchoury CPM, **Arthur RA***, Paes Leme AF, Del Bel Cury JA, Cury JA. *Caries Research* 2007;41(5):406-12.

Genotypic diversity of *Streptococcus mutans* in dental biofilm formed *in situ* under sugar stress exposure. **Arthur RA***, Tabchoury CPM, Mattos-Graner RO, Del Bel Cury AA, Paes Leme AF, Vale GC. *Brazilian Dental Journal* 2007;18(3):185-191.

In vitro study of dose-response relationship of fluoride with dental enamel. **Arthur RA*,** Tabchoury CPM, Giancristófaro M, Cury JA. *Revista Odonto Ciência* 2007;22(55):10-15.

Effect of preservatives on enamel fluoride uptake. **Arthur RA***, Tabchoury CPM, Giancristófaro M, Del Bel Cury AA, Cury JA. *Revista Gaúcha de Odontologia* 2007;55(4):375-379.