REVISTA DE ODONTOLOGIA DA UNESP

ORIGINAL ARTICLE

Rev Odontol UNESP. 2016 Mar-Apr; 45(2): 90-96
Doi: http://dx.doi.org/10.1590/1807-2577.00615

 - ISSN 1807-2577

Effect of dietary sugars on dual-species biofilms of *Streptococcus mutans* and *Streptococcus sobrinus* – a pilot study

Efeito dos açucares da dieta em biofilme dupla espécie de Streptococcus mutans e Streptococcus sobrinus – um estudo piloto

Rosa Virginia Dutra de OLIVEIRA[a*], Yasmin Etienne ALBUQUERQUE[a], Denise Madalena Palomari SPOLIDORIO[a], Cristiane Yumi KOGA-ITO[b], Elisa Maria Aparecida GIRO[a], Fernanda Lourenção BRIGHENTI[a]

[a]Faculdade de Odontologia, UNESP – Universidade Estadual Paulista, Araraquara, SP, Brasil
[b]Faculdade de Odontologia, UNESP – Universidade Estadual Paulista, São José dos Campos, SP, Brasil

Resumo

Introdução: O consumo frequente de açucares e a presença de *Streptococcus mutans* e *Streptococcus sobrinus* estão correlacionados com maior experiência de cárie. **Objetivo:** Elucidar o efeito de diferentes carboidratos fermentáveis na biomassa e acidogenicidade de biofilmes formados por *S. mutans* e *S. sobrinus*. **Material e método:** Biofilmes única e dupla- espécie de *S. mutans* ATCC 25175 e *S. sobrinus* ATCC 27607 em concentrações iguais cresceram no fundo de placas de microtitulação por 24 h a 37 °C em microaerofilia. Maltose, sacarose, glicose e lactose foram adicionados a 2%. BHI caldo (0.2% glicose) foi usado como controle negativo. Acidogenicidade foi avaliada por meio da medição do pH do meio de cultura após 24 h, imediatamente após troca de meio e nas próximas 1 h e 2 h. Coloração por cristal violeta foi usada como indicador do total de biomassa aderida, após 24 h de incubação. Os dados foram analisados por teste ANOVA two way e Teste de Bonferroni. O nível de significância foi de 5%. **Resultado:** Todos os carboidratos resultaram em maior formação de biomassa em ambos os tipos de biofilme (única ou dupla- espécie), quando comparado ao grupo controle. Sacarose, lactose e maltose mostraram maior acidogenicidade que o grupo controle após 24 h nos biofilmes única ou dupla-espécie, apenas após 24 h. **Conclusão:** Os achados indicam que o tipo de biofilme (única ou dupla- espécie) e o tipo de carboidrato usado podem influenciar tanto na quantidade de biomassa formada quanto na taxa de redução do pH.

Descritores: Biofilme; biomassa; *Streptococcus mutans; Streptococcus sobrinus*.

Abstract

Introduction: Frequent consumption of sugars and the presence of *Streptococcus mutans* and *Streptococcus sobrinus* are correlated with higher caries experience. **Objective:** The aim of this pilot study was to elucidate the effect of different fermentable carbohydrates on biomass formation and acidogenicity of *S. mutans* and *S. sobrinus* biofilms. **Material and method:** Single and dual-species biofilms of *S. mutans* ATCC 25175 and *S. sobrinus* ATCC 27607 were grown at the bottom of microtiter plates at equal concentrations for 24 h at 37 °C under micro-aerobic atmosphere. Carbohydrates were added at 2% concentration: maltose, sucrose, glucose and lactose. BHI Broth (0.2% glucose) was used as negative control. Acidogenicity was assessed by measuring the pH of spent culture medium after 24 h, immediately after refreshing the culture medium and for the next 1 h and 2 h. Crystal violet staining was used as an indicator of the total attached biofilm biomass after 24 h incubation. Data were analyzed by two-way ANOVA followed by Bonferroni post hoc test. Significance level was set at 5%. **Result:** All carbohydrates resulted in higher biomass formation in single- and dual-species biofilms when compared to the control group. Sucrose, lactose and maltose showed higher acidogenicity than the control group in both single- and dual-species biofilms after 24 h. **Conclusion:** These findings indicate that the type of biofilm (single- or dual-species) and the carbohydrate used may influence the amount of biomass formed and rate of pH reduction.

Descriptors: Biofilm; biomass; *Streptococcus mutans; Streptococcus sobrinus*.

INTRODUCTION

Epidemiological studies have shown a correlation between the presence of *Streptococcus mutans* and *Streptococcus sobrinus* with caries incidence[1-4]. In early childhood caries, the presence of both microorganisms promoted a significantly higher caries increment than *S. mutans* alone[5]. Although *S. sobrinus* has been isolated less frequently from carious lesions, it has been associated with active dental caries and may be considered a determinant of caries experience[3], mainly early childhood caries[2].

It is believed that the cariogenic potential of these bacteria is directly related to their ability to generate acids and to tolerate acidic environments[6]. These features provide a competitive advantage over other biofilm bacteria during the periods of acidification[6]. *S. mutans* can grow and carry out glycolysis at pH values below 5.0 and can lower the pH to values below 4.0[7]. At low pH levels, *S. sobrinus* is capable of sustain acid production, whereas other species tend to discontinue or reduce this production[8]. *S. sobrinus* can produce acid more rapidly than *S. mutans* at pH values between 6.5 and 5.0. Thus, *S. sobrinus* may be considered the most acidogenic of the oral streptococci[9].

An association between the consumption of sugar-containing beverages and the presence of mutans streptococci in infants was found[10]. Frequent consumption of sugar-sweetened snacks by school children was also correlated with higher caries experience and with higher isolation of *S. mutans* and *S. sobrinus*[3]. Animals fed with carbohydrates exhibited formation of higher amounts of coronal plaque on the smooth surfaces of the tooth[11]. The fermentation of sucrose produces large amounts of acids within biofilms and serves as a substrate for extracellular and intracellular polysaccharide synthesis[12-14]. Extracellular polysaccharides increase porosity of the biofilm matrix, allowing carbohydrate diffusion through the biofilm. At the tooth-plaque interface these carbohydrates are fermented to acids resulting in pH decrease[14]. Extracellular polysaccharides also increase microorganism adhesion and accumulation, mainly of *S. mutans*[13,15]. Intracellular polysaccharides are reservoirs of carbohydrates that promote pH drop during nutrient deprivation, prolonging the exposure of tooth surfaces to organic acids[12].

Sucrose, which is considered the most cariogenic carbohydrate, needs to be catabolized into glucose and fructose by sucrase before it can be metabolized by *S. mutans*. On the other hand, glucose can be directly metabolized by this microorganism[15]. Glucose also appears to be more efficiently metabolized by *S. sobrinus*, since a higher amount of acids is produced by this microorganism when compared to *S. mutans*[16]. Maltose, a starch derivative, is one of the most abundant carbohydrates in the human diet and is easily fermentable to potentially cariogenic acids by *S. mutans*[17]. Approximately one half of the total amount of acidic end products is produced from glucose and sucrose. However, a lower proportion of acid is produced from maltose by both *S. mutans* and *S. sobrinus*[18]. This fact suggests that glucose and sucrose may provide higher cariogenic potential to biofilms than maltose. In the same way, it has been suggested that lactose is less cariogenic than sucrose, glucose and maltose[19].

The role of sugars in dental caries process has been discussed by numerous studies. However most of them have focused just on sucrose and/or glucose[12-14,20-24]. At present, little is known about the role of maltose and lactose. Since these fermentable carbohydrates are frequently present in children's dietary in their natural form or in processed food and beverages, it is important to evaluate their acidogenic potential and the influence in biomass biofilm formation. Moreover, a contemporary approach is to study not only single-species but also dual-species biofilms, since co-existence of different species provides an advantage in surviving antimicrobial treatment[25]. Therefore, this study was conducted to elucidate the effect of different fermentable carbohydrates on the amount of biomass and acidogenicity of biofilms formed by *S. mutans* and *S. sobrinus*.

MATERIAL AND METHOD

Bacterial Strains and Growth Conditions

An aliquot of 400 µL of frozen stocks of *S. mutans* ATCC 25175 and *S. sobrinus* ATCC 27607 were inoculated into 5 mL in Brain Heart Infusion (BHI) Broth (HIMEDIA Laboratories, Vadhani Industrial State, LBS MARG, India) and incubated at 37 °C for 24 h under micro-aerobic atmosphere (4-5% of CO_2 and low O_2 tension)[26]. Next, microorganisms were inoculated in BHI Agar (HIMEDIA Laboratories, Vadhani Industrial State, LBS MARG, India). After 48 h under micro-aerobic conditions (4-5% of CO_2 and low O_2 tension), one colony of each microorganism was transferred to individual tubes containing 5 mL of BHI Broth. After incubation under micro-aerobic atmosphere (4-5% of CO_2 and low O_2 tension) at 37 °C for an additional 18 h, bacterial suspensions were prepared using McFarland scale, which yielded a cell density of 1×10^8 CFU mL^{-1} for each inoculum. The bacterial suspension was used to prepare a 1% fresh inoculum in BHI Broth, which was transferred to microtiter plates. Initial medium pH before experiments was 7.4 ± 0.2 and no buffer was added. Biofilms of either *S. mutans* (single-species biofilms) or a combination of *S. mutans* and *S. sobrinus* (dual-species biofilms) were grown at the bottom of microtiter plates (n=2 wells) at equal concentrations. Stock solutions of carbohydrates were prepared at 20%: maltose, sucrose, glucose and lactose (Lab Synth, Diadema-SP, Brazil). The carbohydrates were added at 2% final concentration as negative control, BHI Broth (contains 0.2% glucose) was used.

Biofilm Acidogenicity

Microtiter plates with 12 wells were used. An aliquot of 4 mL of the 1% fresh inoculum were transferred to each well and 400 µL of each carbohydrate was added in duplicate. The plates were incubated at 37 °C under micro-aerobic atmosphere. Biofilm acidogenicity was assessed by pH measurements of culture medium using a microelectrode connected to a pH meter in combination with a glass reference electrode (Orion Res Inc., Cambridge, Mass., USA). The microelectrode was calibrated using standard pH buffers (pH 4.0 and 7.0) prior to and after each test as well as during tests if necessary. The pH determinations were made in duplicate for all carbohydrates studied and performed on two different days. The pH was measured after 24 h incubation, immediately after

refreshing the culture medium and for the next 1 h and 2 h. To refresh the culture medium, 3 mL was removed from each well, then it was added 3 mL of fresh BHI Broth and 400 µL of each carbohydrate were added.

Biomass

Crystal violet assay was used as an indicator of the total attached biofilm biomass. The advantage of this analysis is that it can be used directly, without disrupting the biofilm. Microtiter plates with 24 wells were used. The 1% fresh inoculum was transferred to each well in a volume of 1.5 mL and 150 µL of each carbohydrate was added. Experiments were performed in two different days. Two wells with just BHI Broth were used as negative controls. The plates were incubated at 37 °C under micro-aerobic atmosphere (4-5% of CO_2 and low O_2 tension). After 24 h of biofilm growth, the supernatant was removed and biofilms were washed two times with 2 mL of sterile water, to remove loosely attached cells. The 2 mL of sterile water was removed carefully with the aid of pipettes and biofilms were immersed in 2 mL of ethanol for 15 min (to fix the biofilm). Ethanol was removed and the plates were dried at room temperature (approximately 20 min). A volume of 2 mL of 1% crystal violet were added to each well and incubated at room temperature. After 5 min, crystal violet was removed and 2 mL of sterile water were added. The water was removed and the biofilms were allowed to dry at room temperature. Then, 2 mL of 33% acetic acid were added to dilute the stain and 200 µL from each well was transferred in triplicate to a 96 wells microtiter plate. The absorbance of the crystal violet solution was measured at 590 nm wavelength (BioPhotometer Plus, Eppendorf, São Paulo, Brazil). Biomass assay was performed twice, in different moments.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism Version 3.02 (GraphPad Software Inc., San Diego, CA, USA). Data showed equality of variances (Bartlett's test) and normal distribution (Kolmogorov–Smirnov test). Data were analyzed by two-way ANOVA followed by Bonferroni post hoc test. The significance limit was set at 5%.

RESULT

The type of biofilm (single- or dual-species) and the carbohydrate used influenced the amount of biomass formed (Figure 1). All carbohydrates resulted in higher biomass formation in single- and dual-species biofilms when compared to the control group.

The rate of pH reduction was carbohydrate and time-dependent. For both single- and dual-species biofilm, statistically significant differences were observed only after 24 h, when sucrose, lactose and maltose showed higher acidogenicity when compared to the control group (BHI broth only) (Table 1).

DISCUSSION

There are a considerable number of studies suggesting that sucrose is the most cariogenic carbohydrate[11-13,15]. Nevertheless, this study shows that other fermentable carbohydrates may also influence biomass formation and biofilm acidogenicity. Glucose, lactose and maltose were able to produce the same amount of biomass than sucrose in both single- and dual- species biofilms. Moreover, despite glucose resulted in higher pH values than sucrose, lactose

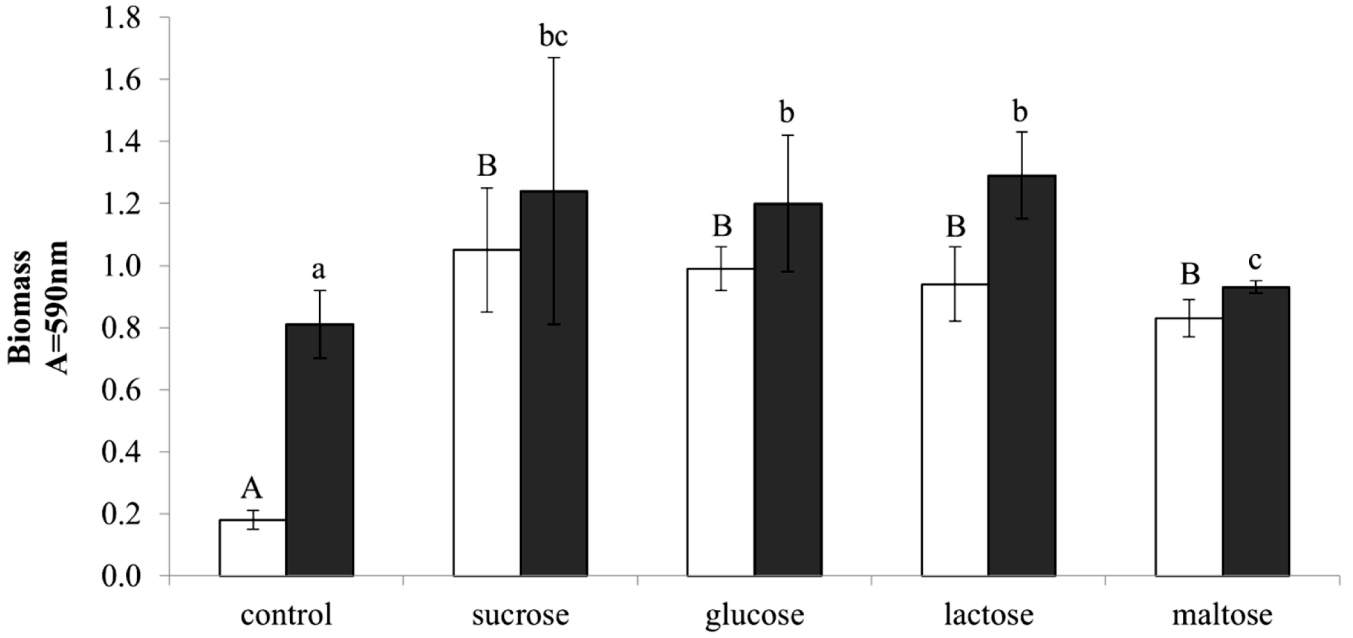

Figure 1. Biomass quantification (mean ± sd) in *S. mutans* single-species biofilms (white bars) or *S. mutans* and *S. sobrinus* dual-species biofilms (grey bars) after 24 h of incubation. Means followed by different uppercase letters show statistically significant differences for *S. mutans* single-species biofilms. Means followed by different lowercase letters show statistically significant differences for *S. mutans* and *S. sobrinus* dual-species biofilms (two-way ANOVA followed by Bonferroni pos roc test, $p<0.05$).

Table 1. Acidogenicity (mean pH ± sd) of single-species (*S. mutans*) biofilms and dual-species (*S. mutans* + *S. sobrinus*) biofilms

Sugars	24 h		Immediate		1 h		2 h	
	single	dual	single	dual	single	dual	single	dual
control	5.52±0.17[a]	5.55±0.04[a]	7.23±0.04	6.62±0.07	6.55±0.43	7.06±0.01	6.62±0.49	6.75±0.32
sucrose	4.72±0.02[b]	4.75±0.05[b]	7.15±0.35	6.51±0.59	6.55±0.40	6.49±0.15	6.83±0.00	6.67±0.88
glucose	5.25±0.11[a]	5.30±0.00[a]	7.10±0.02	6.70±0.39	6.88±0.45	5.96±0.06	6.63±0.27	6.71±0.58
lactose	4.73±0.02[b]	4.75±0.00[b]	6.89±0.42	6.81±0.24	6.90±0.12	6.49±0.12	6.54±0.31	6.11±0.08
maltose	4.69±0.00[b]	4.61±0.10[b]	6.93±0.13	6.51±0.21	6.51±0.09	6.39±0.33	6.40±0.04	6.74±0.09

Means followed by different letters show statistically significant differences within the same type of biofilm (*S. mutans* single-species biofilms or *S. mutans* and *S. sobrinus* dual-species biofilms; two-way ANOVA test and the significance was examined by the Bonferroni post hoc test, $p<0.05$).

and maltose, all fermentable carbohydrates tested resulted in a pH drop below critical values (5.5) after 24 h in both types of biofilms.

The control group had ten-fold lower carbohydrate than other groups (0.2% glucose). Nutrient limitation contributed to the lower amount of biomass formed by the control group than by other carbohydrates, which is in agreement to Cury et al.[20] Besides, under this low glucose condition, the cells tend to enter stationary-phase[27]. On the other hand, high carbohydrate availability influences the expression of physiologic and biochemical pathways of bacteria[28], allows bacterial growth and increases lactic acid production[27]. It is known that *S. mutans* produces elevated amounts of extracellular polysaccharides (EPS) when sucrose is available, which provides support to development and accumulation of microcolonies and increase the cohesiveness and structural integrity of the biofilm[29]. Intracellular polysaccharides (IPS) are also synthesized and provide bulk to biofilms[12,13].

Despite previous studies showing that lactose is the least cariogenic sugar[19], the present study indicates that lactose produced the same amount of biomass and it was as acidogenic as the other carbohydrates. It was suggested that the regular consumption of carbohydrates may predispose to early colonization of mutans streptococci and influence caries risk in the primary dentition[10]. Despite the recommendation of exclusive breast feeding for at least 6 months[30,31], an association between breast feeding for at least 6 or 7 months and early childhood caries was found[32]. Perera et al.[33] also showed that ECC was present in children older than two years who were fed overnight any type of milk. Moreover, the incidence of ECC was higher in children who harbored both species studied (*S. mutans* and *S. sobrinus*)[2]. Considering that human milk has a higher concentration of lactose (7%)[34] than that used in the present study, these findings may contribute to the understanding of the metabolism of carbohydrates in biofilms and the relationship between carbohydrates and dental caries. On the other hand, milk may also have caries-protective properties[35] and its role in caries development should be further evaluated.

As previously stated, few studies investigated the metabolism of maltose. Kilic et al.[17] found that *S. mutans* cells were able to transport and metabolize maltose two-fold more efficiently than in the presence of glucose. Our findings (Table 1) support these data, since maltose was more acidogenic than glucose and as acidogenic as sucrose and lactose after 24 h, in either single- or dual-species biofilms. Maltose is a starch derivate and one of the most abundant fermentable carbohydrate in the human diet. It is suggested that this carbohydrate enhances *S. mutans* competitiveness[36]. However, it is still not known how maltose is taken up by *S. mutans* and *S. sobrinus*. Further studies are required to better understand the behavior of these bacteria in the presence of this carbohydrate.

Although 2% glucose fed biofilms formed similar amount of biomass compare to other carbohydrates, surprisingly, pH of the culture media after 24 h was higher for glucose than for other fermentable carbohydrates. Sucrose, lactose and maltose are disaccharides and have the same molecular weight (342.3 g/mol). On the other hand, glucose was the only monosaccharide studied and its molecular weight is about 50% the molecular weight of the disaccharides (180.2 g/mol). Thus, although culture media had 2 g carbohydrates per 100 mL, the number of glucose molecules present in the culture media was different than the number of molecules present in disaccharides (sucrose, lactose and maltose). This may explain why glucose showed a statistically significant lower acidogenicity than the other carbohydrates after 24 h in both biofilms.

It is important to note that the carbohydrates used in this study present different composition and thus yield different substrates after hydrolysis: sucrose, lactose and maltose are disaccharides composed by, respectively, glucose + fructose; glucose + galactose and glucose + glucose. These disaccharides also differ in the glycosidic bond between the monomers: while the monomers from sucrose and maltose are alpha-linked, lactose has a beta-link between them. Glucosyltransferases (Gtfs) are related to the synthesis of extracellular polysaccharides (EPS), which promote adhesion, microorganisms accumulation and biofilm matrix establishment, that is responsible for the structural integrity of dental biofilms[15,37,38]. *S. mutans* has been indicated as the main source of Gtfs in biofilms[39]. However, *S. sobrinus* also produces glucosyltransferases for the production of water-soluble glucans and water-insoluble glucans[40,41]. Thus, both species are able to cleave the glycoside bond between disaccharides' monomers.

It was suggested that the presence of bacteria other than *S. mutans* in biofilms may influence acid production[42]. Of particular interest, *S. sobrinus* is able to produce large amounts of acid end products from sugar metabolism[8]. In addition, the higher isolation of *S. sobrinus* from caries-active children indicates that this microorganism may

be actively associated with dental caries and may be considered a determinant of caries experience[3]. Thus, it was decided to study this species in association with *S. mutans*.

The idea of this pilot study was to select fermentable carbohydrates of interest using not only single-species biofilms but also a contemporary approach with dual-species biofilms. The results of the present study will be the basis to further analyze other parameters, such as metabolic activity, susceptibility to chlorhexidine of the biofilms, cell viability and matrix composition.

In this context, this study offers valuable insights about interactions among cariogenic bacteria and contributes to our general understanding of carbohydrate metabolism in biofilms and the relationship of carbohydrates to dental caries.

CONCLUSION

These findings of this pilot study indicate that the type of biofilm (single- or dual-species) and the carbohydrate used may influence the amount of biomass formed and rate of the pH reduction.

ACKNOWLEDGEMENTS

The authors thank São Paulo Research Foundation - FAPESP (grants: 2012/17236-4 and 2013/12326-8) for financial support. They are also grateful to Laura Boegli and Elinor Pulcini for the English reviewing.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

*CORRESPONDING AUTHOR

Fernanda Lourenção Brighenti, Departamento de Clínica Infantil, Faculdade de Odontologia de Araraquara, Rua Humaitá, 1680, Centro, 14801-903 Araraquara - SP, Brasil, e-mail: fbrighenti@foar.unesp.br

Received: January 29, 2015
Accepted: August 27, 2015