Brazilian Dental Science
Ciência Odontológica Brasileira
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UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
Instituto de Ciência e Tecnologia
Campus de São José dos Campos

ORIGINAL ARTICLE

doi: 10.14295/bds.2015.v18i2.1100

Influence of different fermentable carbohydrates on dual-species biofilms of *S. mutans* and *A. naeslundii*: a pilot study

Influência de diferentes carboidratos fermentáveis em biofilmes de duas espécies com *S. mutans* e *A. naeslundii* - um estudo piloto

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ABSTRACT

Objective: The aim of this study was to elucidate the effect of presence of different fermentable carbohydrates in the biomass and acidogenicity of biofilm formed by *Streptococcus mutans* ATCC 25175 in association with *Actinomyces naeslundii* ATCC 19039. **Material and Methods:** Single and dual-species biofilms were grown on wells of microtiter plates at equal concentration for 24 h at 37 ºC. Carbohydrates were added at 2%: maltose, sucrose, glucose and lactose and as negative control, BHI Broth (0.2% glucose) was used. The pH of each culture was measured to assess acidogenicity after 24 h, immediately after changing the culture medium and 30 min, 1 h and 2 h after. Crystal violet was used as indicator of the total attached biofilm biomass after 24 h incubation and the absorbance was measured at 590 nm. Tukey Multiple Comparison Test was performed for all the statistical analysis. **Results:** Higher amount of biomass was formed by dual-species than single-species biofilm in the presence of all carbohydrates, except to glucose. *S. mutans* biofilms showed statistically significant higher acidogenicity than control group only after 24 h. In dual-species biofilms the highest acidogenicity were found after 24 h for sucrose, lactose, maltose and the control group. **Conclusion:** The findings indicate that the type of biofilm (single or dual-species) and the carbohydrate used may influence amount of biomass formed and rate of the pH reduction.

KEYWORDS

Actinomyces; Biomass; Biofilm; Streptococcus mutans.

RESUMO

Objetivo: O propósito deste estudo foi analisar o efeito da presença de diferentes carboidratos fermentáveis na biomassa e acidogenicidade do biofilme formado por *Streptococcus mutans* ATCC 25175 em associação com *Actinomyces naeslundii* ATCC 19039. **Material e Métodos:** Biofilmes com uma ou duas espécies cresceram em poços de placas de microtitulação em igual concentração, por 24 h a 37 ºC. Carboidratos foram adicionados em concentração de 2%: maltose, sacarose, glicose e lactose, além disso, como controle negativo, caldo BHI (0.2% de sacarose) foi usado. O pH foi medido individualmente para avaliar a acidogenicidade após 24 h, imediatamente após troca do meio de cultura e 30 min, 1 h e 2 h depois. Cristal violeta foi usado como indicador do total de biomassa formada após 24 h de incubação e a absorbância foi medida a 590 nm. Teste de Tukey foi utilizado para todas as análises estatísticas. **Resultados:** Em geral, maior quantidade de biomassa foi formada por biofilmes dupla-espécie que única-espécie na presença de todos os carboidratos, exceto glicose. Biofilmes formados por *S. mutans* mostraram significativamente maior acidogenicidade que o grupo controle apenas após 24 h. Em biofilmes dupla-espécie, maior acidogenicidade foi encontrada após 24 h na presença de sacarose, lactose, maltose e no grupo controle. **Conclusão:** Esses achados indicam que o tipo de biofilme e o carboidrato usado podem influenciar ambas: formação de biomassa e taxa de queda do pH.

PALAVRAS-CHAVE

Actinomyces; Biomassa; Biofilme; Streptococcus mutans.

INTRODUCTION

Sugars are usually used in food industry not only to sweeten food but also to provide them texture, bulking and to increase their conservation. The most frequently used sugar is sucrose. Lactose, glucose, and maltose, are also used, but do not provide the same sweetening power of sucrose [1]. Sugars may be classified as non-milk extrinsic sugars (NMES), which includes all sugars that are neither components of milk, nor contained within plant cell walls and may be released or added to food or beverages during processing; or intrinsic, when occur naturally and typically reside within the cellular structure [2]. It is believed that foods with NMES are more cariogenic than foods with intrinsic or milk sugars [3]. It has also been suggested that lactose is less cariogenic than sucrose, fructose and maltose [2]. However, lactose is still a cariogenic carbohydrate, since it was observed that children who were overnight fed with any type of milk beyond two years old showed early childhood caries (ECC) [4]. Biofilms formed in the presence of glucose also has been demonstrated to be less cariogenic than that formed in the presence of sucrose [5]. Maltose is a starch derivative carbohydrate and one of the most abundant carbohydrates in human diet [6]. Besides being easily fermentable to acids by many oral microorganisms, its metabolism by *S. mutans* results in intracellular polysaccharides synthesis [7].

It is well-established that these fermentable carbohydrates cause biochemical and physiological changes in dental biofilms [8]. Campbell & Zinner [9] observed that sucrose formed a higher amount of dental biofilm in comparison to fructose, lactose or a mix of glucose and fructose and was considered significantly more cariogenic than other carbohydrates. This is explained because sucrose fermentation produces large amounts of acid and because it serves as a substrate for extracellular and intracellular polysaccharides synthesis [8,10,11].

Extracellular polysaccharides increase biofilm matrix porosity and facilitate carbohydrates diffusion through biofilm. These carbohydrates are then fermented to acids and a decrease on tooth-plaque interface pH as observed by Azevedo et al. [11]. Extracellular polysaccharides are also important to microorganisms' adhesion and accumulation in dental biofilm [8].

On the other hand, Campbell & Zinner [9] also noted dental destruction in animals fed with glucose, lactose and fructose. Sucrose has to be catabolized into glucose and fructose by dextran sucrase before it can be metabolized by *S. mutans* [12]. Glucose can be directly metabolized by this microorganism. Despite other fermentable carbohydrates can also be converted into acids, they are not be able to form the same amount of extracellular polysaccharides produced from sucrose [13]. This may be due to the free energy provided by breaking the strong glycosidic bond between glucose and fructose during sucrose catabolism, which can be used for extracellular polysaccharides production.

As well as *S. mutans*, other microorganisms may also produce acids and contribute to pH decrease. *Actinomyces naeslundii* is an initial colonizer of the tooth surface that coaggregates with *mutans streptococci*, produces acids from various sugars and synthesizes intra- and extracellular polysaccharides [14]. In the early stage of biofilm formation, under aerobic conditions, *A. naeslundii* metabolizes carbohydrates into relatively weak acids, such as acetate. If other cohabitants produce lactate, *A. naeslundii* degrades it into weak acids and neutralizes dental biofilm pH. In addition, under anaerobic conditions, *A. naeslundii* becomes a strict fermenter and produces organic acids that acidify the environment and promote the colonization of more acidogenic and acid-tolerant bacteria, such as *S. mutans* [15].

Commonly studies have focused in the acidogenic potential of fermentable carbohydrates metabolized by *S. mutans*

[6,12,16-18]. However little is known regarding biofilm features when this bacteria is associated with other species in dental biofilm. Therefore, this pilot study was conducted to elucidate the effect of presence of different fermentable carbohydrates in the biomass and acidogenicity of biofilm formed by *S. mutans* in association with *Actinomyces naeslundii*.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Frozen stocks of *S. mutans* ATCC 25175 and *Actinomyces naeslundii* ATCC 19039 were inoculated separately, in a volume of 400 μL 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 in anaerobic atmosphere (BD GasPak EZ Container System Sachets, BD Diagnostics, Sparks MD, United States). Next, microorganisms were inoculated in BHI Agar (HIMEDIA Laboratories, Vadhani Industrial State, LBS MARG, India). After 48 h under anaerobic conditions at 37 °C, one colony of each microorganism was transferred to individual tubes. After incubation at 37 °C for 18 h, a 1% fresh inoculum was prepared in BHI Broth and transferred to microtiter plates. Biofilms were grown on wells of microtiter plates containing either *S. mutans* (single-species biofilm) or a combination of *S. mutans* and *A. naeslundii* at equal concentration (dual-species biofilm). The following carbohydrates were added at 2%: maltose, sucrose, glucose and lactose. As negative control, BHI Broth (contains 0.2% glucose in its composition) was used.

Biofilm Acidogenicity

Microtiter plates with 12 wells were used. Four milliliters of the 1% fresh inoculum was transferred to each well and 400 μL of each carbohydrate was added in duplicate. As negative control, wells with BHI Broth were used. The plates were incubated at 37 °C in anaerobic 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. The pH was observed after 24 h incubation, immediately after change 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) and 30 min, 1 h and 2 h after. Tukey Multiple Comparison Test was applied to analyze acidogenicity of both biofilms by different sugars and different times.

Biomass

Crystal violet assay was used as 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. A 1% fresh inoculum was transferred to each well in a volume of 1.5 mL and 150 μL of each carbohydrate was added in duplicate. Two wells with just BHI Broth were used as negative control. The plates were incubated statically at 37 °C in anaerobic atmosphere. After 24 h of biofilm growth, the supernatant was removed and biofilms were washed with 2 mL of sterile water. 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 was 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. Next, 2 mL of 33% acetic acid were added to dilute the stain. A volume of 200 μL of each well was transferred in triplicate to a 96 wells microtiter plate. The absorbance

of the crystal violet solution was measured at 590 nm. Tukey Multiple Comparison Test was also applied to analyze the biomass formed after 24 h. GraphPad Prism was employed for all the statistical analyses.

RESULTS

Figure 1 shows that the type of biofilms (single or dual-species) and the carbohydrate used influenced the amount of biomass formed. In general, higher amount of biomass was formed by dual-species biofilms of *S. mutans* and *A. naeslundii* than in single-species biofilms of *S. mutans* in the presence of all carbohydrates, except to glucose. Sucrose, glucose and lactose caused higher biomass formation in *S. mutans* single species biofilms. Dual-species biofilms formed the highest amount of biomass when sucrose and lactose were used; however the amount of biomass produced by these two carbohydrates were the same as the control group.

The rate of the pH fall was dependent on carbohydrates and time. The pH of fermentation medium in the presence of carbohydrates with single-species biofilms of *S. mutans* during different periods of incubation can be observed in Table 1. Statistically significant differences were observed only after 24 h, when all carbohydrates tested showed a higher acidogenicity when compared to the control group (BHI broth only). Sucrose, lactose and maltose showed the highest acidogenicity, followed by glucose.

Table 2 shows the effect of carbohydrates on acidogenicity (pH) of dual-species biofilms of *S. mutans* and *A. naeslundii*. Statistically significant differences were found only after 24 h. The highest acidogenicity were found for sucrose, lactose, maltose and the control group. Glucose showed the lowest acidogenicity.

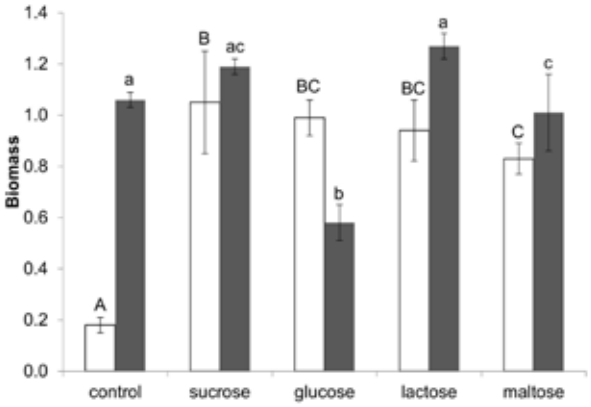

Figure 1 - Biomass quantification (mean ± sd) in single-species biofilms of *S. mutans* (white bars) and dual-species biofilms of *S. mutans* and *A. naeslundii* (grey bars) after 24 h of incubation. Means followed by different uppercase letters show statistically significant differences for single-species *S. mutans* biofilms (Tukey test $p < 0.05$). Means followed by different lowercase letters show statistically significant differences *S. mutans* and *A. naeslundii* dual-species biofilms (Tukey test $p < 0.05$).

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

Sugars	Immediate	30 min	1 h	2 h	24 h
control	7.23±0.04 ^{A}	7.15±0.02 ^{A}	6.55±0.43 ^{A}	6.62±0.49 ^{A}	5.52±0.17 ^{A}
sucrose	7.15±0.35 ^{A}	6.76±0.09 ^{A}	6.55±0.40 ^{A}	6.83±0.00 ^{A}	4.72±0.02 ^{B}
glucose	7.10±0.02 ^{A}	6.79±0.21 ^{A}	6.88±0.45 ^{A}	6.63±0.27 ^{A}	5.25±0.11 ^{C}
lactose	6.89±0.42 ^{A}	6.75±0.18 ^{A}	6.90±0.12 ^{A}	6.54±0.31 ^{A}	4.73±0.02 ^{B}
maltose	6.93±0.13 ^{A}	6.72±0.04 ^{A}	6.51±0.09 ^{A}	6.40±0.04 ^{A}	4.69±0.00 ^{B}

Means followed by different letters within a column are statistically different (Tukey's test $p < 0.05$).

Table 2 - Acidogenicity (mean pH ± sd) of *S. mutans* and *A. naeslundii* dual-species biofilms

Sugars	Immediate	30 min	1h	2h	24h
control	7.09±0.05 [a]	6.96±0.00 [a]	6.97±0.00 [a]	6.94±0.08 [a]	5.44±0.05 [ab]
sucrose	6.60±0.35 [a]	6.59±0.09 [a]	6.35±0.40 [a]	6.56±0.00 [a]	4.29±0.02 [a]
glucose	7.15±0.04 [a]	7.12±0.12 [a]	6.71±0.59 [a]	6.68±0.71 [a]	5.85±0.61 [b]
lactose	6.39±0.17 [a]	6.37±0.24 [a]	6.23±0.28 [a]	6.21±0.05 [a]	4.69±0.12 [ab]
maltose	6.89±0.64 [a]	6.33±0.41 [a]	6.60±0.38 [a]	6.24±0.55 [a]	4.68±0.01 [ab]

Means followed by different letters within a column are statistically different (Tukey's test $p < 0.05$).

DISCUSSION

The role of sugars in dental caries process has been discussed by some studies [1,5,11,19]. Carbohydrates are utilized as an energy source by microorganisms and may contribute to the virulence of the microbiota [20]. The present study shows that not only sucrose but other fermentable carbohydrates present in diet may be important in dental caries process. Biofilms supplemented with lactose and maltose produced the same amount of biomass than biofilms supplemented with sucrose and were able to decrease pH to a value lower than enamel critical pH. The presence of *Actinomyces naeslundii* may enhance the expression of *S. mutans* gtfB/gtfC genes that mediate the establishment of an EPS-rich matrix and form more biomass [21].

The amount of biomass formed in the presence of all fermentable carbohydrates by single-species biofilms of *S. mutans* was significantly higher than control group. These results were expected because lower amount of biomass is formed when there are limited nutrients in culture media [5].

According to Renye et al. [17], in glucose-starved situations, such as the control group used in the present study (0.2% glucose), the cells tend to entry in stationary-phase. On the other hand, excess of sugar allows bacterial growth and lactic acid production. Surprisingly, control group from dual-species biofilms was able to produce the same amount of biomass than those with higher concentrations of sucrose and lactose. Moreover, dual-species biofilms formed with a ten-fold increase in glucose concentration (2%) showed the lowest amount of biomass. These results correlate with the highest pH values, which did not reach critical values (5.5). Thus, we suggested that there may be a potential interaction between *S. mutans* and *A. naelsundii*, which affect biofilms' glucose metabolism and it seems to be dose-dependent. The mechanisms involved in this interaction should be further studied.

In this study, sucrose, glucose and lactose promoted the highest biomass formation in *S. mutans* biofilms. Sucrose and glucose biofilms formation by *S. mutans* is well documented by previous studies. Daneo-Moore et al. [16] showed that although *S. mutans* grows at similar rates in both sugars, bacterial growth in the presence of sucrose produce large amounts of extracellular glucans, which results in formation of large aggregates of cells. On the other hand, a thin but mostly homogenous accumulation of bacterial cells adherent may be observed in the presence of 1% glucose [21]. It was suggested that carbohydrate availability influences the expression of physiologic and biochemical pathways of *S. mutans* and contribute directly to the virulence [22]. However, there are no data about biomass formation by other fermentable sugars in either *S. mutans* single-species biofilms or in *S. mutans* and *A. naeslundii* dual-species biofilms. It is known that *A. naeslundii* produces acids from various sugars and synthesize intra- and extracellular polysaccharides [14]. Direct comparisons between *S. mutans* and *A. naeslundii*

single-species biofilms were not possible in the present study because *A. naeslundii* failed to form biofilms at the bottom of microtiter wells.

Biomass formation is a better indicative of caries activity than the amount of bacteria found, once a previous study showed that sucrose supplementation did not increased the *mutans streptococci* abundance [23]. Biomass obtained by crystal violet staining is a useful technique that has been used in different studies due to its high reproducibility and fast analysis [24]. In the present study, the use of this methodology showed that other fermentable carbohydrates present in diet may be as cariogenic as sucrose and also showed the existence of an important interaction between *S. mutans* and *A. naeslundii*. These results provide an insight that will guide future studies about bacterial interactions in dental biofilm.

Dental biofilm acidogenicity is considered as a crucial factor in the development of carious lesions, since pH below of 5.5 can cause enamel dissolution [17]. After 24 h of growth, pH values were bellow critical value (5.5) for all fermentable carbohydrates in single-species biofilms. For dual-species biofilms, glucose was not able to reduce the pH bellow critical levels. Moreover, there were no differences in pH levels between control group and sucrose or between control group and the other carbohydrates (glucose, lactose and maltose). These findings may show different glycolytic regulation between *A. naeslundii* and streptococci. No considerable shifts in *A. naeslundii* metabolism are found in excess or limited concentrations of glucose. Moreover, optimal pH for glucose fermentation by *A. naeslundii* is 7.0, a higher pH value than those found in the present study [15]. The exact mechanisms involved between *S. mutans* and *A. naeslundii* interaction is not fully understood and should be further evaluated.

There is a considerable amount of studies suggesting that sucrose is the most cariogenic sugar [8-10,12]. Nevertheless, the results of the present study show that other fermentable carbohydrates may be as important as sucrose in caries development. These carbohydrates are frequently ingested in their natural form (such as lactose in milk, glucose in honey, maltose in cereals and sucrose in beets) or in processed food and beverages. To understand the behavior of potentially cariogenic microorganisms in the presence of these sugars is important to guide caries prevention strategies.

There is evidence that lactose is the least cariogenic sugar [2] and that milk may have caries-protective properties [1]. However, in the present study, lactose cariogenicity was similar to sucrose. Considering that human milk has a higher concentration of lactose (7%) than that used in the present study [25], the impact of these findings is important in dental caries etiology.

This study offers valuable insights about interactions between cariogenic bacteria and contributes to our general understanding of carbohydrates metabolism in biofilms.

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 the pH reduction.

ACKNOWLEDGEMENTS

The authors thank Fundação de Amparo à Pesquisa do Estado de São Paulo—FAPESP (grants: 2012/17236-4 and 2013/11799-0, São Paulo Research Foundation - FAPESP) for financial support. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Date submitted: 2014 Jan 22

Accept submission: 2015 Jun 15