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pH changes of mixed biofilms of *Streptococcus mutans* and *Candida albicans* after exposure to sucrose solutions *in vitro*

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ABSTRACT

Objective: This study aimed to standardize an *in vitro* experimental model able to reproduce the pH changes that occur in dental biofilm under *in vivo* conditions, using a mixed biofilm of *Streptococcus mutans* and *Candida albicans*.

Design: Biofilms were developed for 96 h, and exposed to three different concentrations of sucrose (10, 20 or 30%) during 1, 3 or 5 min. The pH was measured before exposure to sucrose, immediately after its removal from the biofilms, and at 1, 3, 5 and 10 min after removal.

Results: Sucrose solutions at 10 and 20% required 1 min to significantly reduce the biofilm pH, while for 30% sucrose a significant reduction was already seen immediately after its removal, even for the shortest exposure time. For an exposure of 3 min to 20% sucrose, the biofilm pH attained the critical value for hydroxyapatite dissolution when measured 1 min after sucrose removal, followed by a recovery phase.

Conclusions: A mixed biofilm of *S. mutans* and *C. albicans* exposed to a 20% sucrose solution for 3 min exhibited a pattern of pH change similar to that observed *in vivo*, despite at a higher speed when compared to *in vivo* conditions.

1. Introduction

Dental caries is a biofilm- and sucrose-dependent disease (Sheiham & James, 2015), whose etiology, diagnosis, treatment, and control have been extensively studied over the last decades. *Streptococcus mutans* is the main pathogen related to this condition, mainly due to its capacity to ferment carbohydrates, survive in a low pH environment, and produce extra- and intracellular polysaccharides, which facilitate biofilm formation and adherence to dental surfaces (Kleinberg, 2002). Notwithstanding, in cases of early-childhood caries, *Candida albicans* is also found in the cariogenic biofilm (Falsetta et al., 2014), what contributes to its pathogenesis due to collagen degradation produced by proteolytic enzymes (Pereira, Seneviratne, Koga-Ito, & Samaranayake, 2017). These microorganisms thrive better together and in presence of sucrose (Falsetta et al., 2014; Kim et al., 2017).

Caries lesions result from a mineral imbalance between tooth and biofilm on its surfaces (Fejerskov, 2004). In this sense, when the pH of the biofilm fluid decreases (pH < 5.5), it becomes undersaturated in relation to hydroxyapatite, resulting in dissolution of this mineral (Buzalaf, Pessan, Honório, & ten Cate, 2011). Regarding the effects of pH changes on dental biofilm, a time-course pH curve was designed *in vivo* (Stephan, 1944), presenting four distinct phases, as recently revised by Bowen (Bowen, 2013).

Due to the physiological complexity associated to the polymicrobial nature of the oral cavity, besides ethical issues involving clinical studies, there is an increasing interest in the development of laboratory models that mimic clinical conditions related to dental caries (Maske, van de Sande, Arthur, Huysmans, & Cenci, 2017). In this regard, new *in vitro* techniques have been developed to improve the knowledge about biofilm properties (Azeredo et al., 2017). The Stephan curve, for instance, was reproduced using an *in vitro* model of microcosm biofilm, in which the biofilm was exposed to 5 or 10% sucrose solutions by continuous flow, and its thickness was shown to affect the pH recovery (Sissons, Cutress, Faulds, & Wong, 1992).

Considering that the pH of a dual-species biofilm of *S. mutans* and *C. albicans* is neutralized after exposure to sucrose over time (Willems, Kos, Jabra-Rizk, & Krom, 2016), and that its behavior in relation to the Stephan curve remains unknown, the aim of the current study was to

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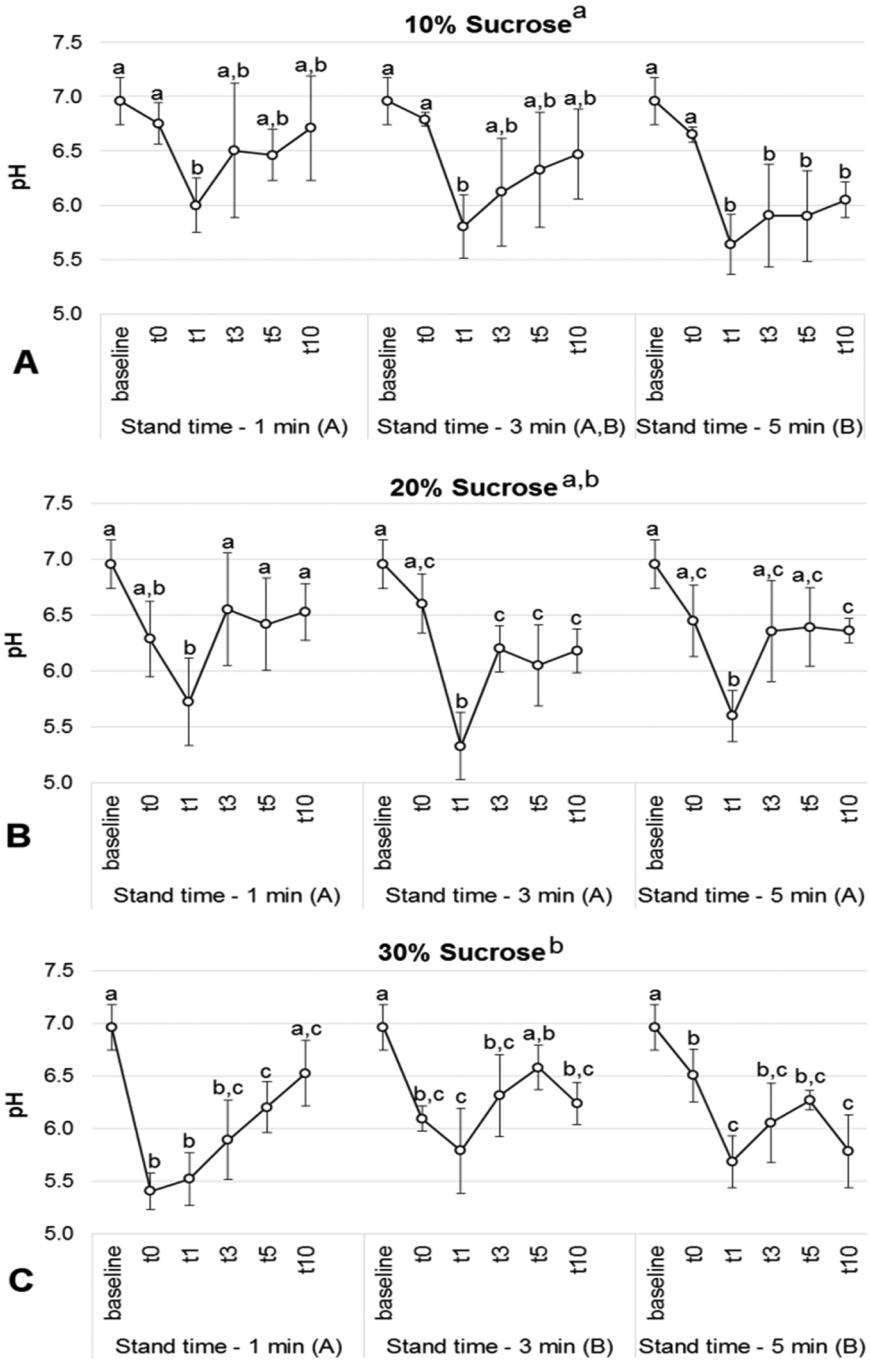

Fig. 1. Mean pH values determined after exposure of the mixed biofilm of *Candida albicans* ATCC 10231 and *Streptococcus mutans* ATCC 25175 to 10% sucrose (A), 20% sucrose (B) and 30% sucrose (C), as a function of the exposure time to sucrose and time of pH determination. Baseline: pH values determined before biofilm exposure to sucrose; t0: pH determined immediately after sucrose removal; t1, t3, t5 and t10: pH determined at 1, 3, 5 and 10 min after sucrose removal, respectively. Different capital letters between parentheses denote significant differences among exposure times to sucrose within the same sucrose concentration (Stand time). Different lowercase letters represent significant differences among pH values at each individual graph, as well as among sucrose concentrations. Vertical bars represent the standard deviation of the means (Student-Newman-Keuls, $p < 0.05$, $n = 3$).

determine the sucrose concentration and its exposure time that would produce pH changes similar to those found *in vivo*, using a mixed biofilm model of the above-mentioned species.

2. Materials and methods

2.1. Experimental design

Mixed biofilms of *S. mutans* and *C. albicans* were developed in sucrose-containing artificial saliva within 6-well plates, during 96 h. After, biofilms were exposed to sucrose solutions at 10, 20 or 30%, which remained in contact with biofilms for 1, 3 or 5 min. Biofilm pH was measured before exposure to sucrose, immediately after its removal from the biofilms, and at 1, 3, 5 and 10 min after removal. Sucrose concentration, time of biofilm exposure to sucrose and time elapsed after exposure were considered as variation factors.

2.2. Artificial saliva

The composition of artificial saliva (for 1 L of deionized water) was based on the procedure described by Lamfon, Porter, McCullough, and Pratten (2003), with minor modifications: 2 g of yeast extract (Sigma-Aldrich, St Louis, USA), 5 g of bacteriological peptone (Sigma-Aldrich), 1 g of mucin type III (partially purified from porcine stomach; Sigma-Aldrich) 4 g of sucrose (Sigma-Aldrich), 0.35 g of NaCl (Sigma-Aldrich), 0.2 g of CaCl2 (Sigma-Aldrich) and 0.2 g of KCl (Sigma-Aldrich). The pH was adjusted to 6.8 using NaOH.

2.3. Strains, growth conditions and biofilm formation

Two strains from American Type Culture Collection (ATCC) were used: *S. mutans* ATCC 25175 and *C. albicans* ATCC 10231. The media used to seed the cultures of *C. albicans* and *S. mutans* were Sabouraud Dextrose agar (SDA, Difco, Le Pont de Claix, France) and Brain Heart Infusion agar (BHI Agar; Difco), respectively. Colonies of *S. mutans* were suspended in 10 mL of BHI broth (Difco) and incubated for 24 h in 5% CO2 at 37 °C. In turn, *C. albicans* colonies previously cultivated on SDA were suspended in 10 mL of Sabouraud Dextrose broth (Difco) and incubated overnight at 37 °C (120 rpm). After, bacterial and fungal cells were recovered by centrifugation (6,500 × *g* for 5 min at 15 °C) and the cell pellets, washed twice with 10 mL of saline solution at 0.85%. The number of fungal cells was adjusted to a concentration of 10^7 cells/mL in artificial saliva, using a Neubauer counting chamber, while bacterial cells were spectrophotometrically adjusted to 10^8 cells/mL. Mixed biofilms were formed in wells of 6-well plates (Costar, Tewksbury, USA). For this, 4 mL of microbial suspension (1×10^7 cells/mL *C. albicans* + 1×10^8 cells/mL *S. mutans*) were added in the wells, and plates were incubated at 5% CO2 at 37 °C, during 96 h. Artificial saliva was renewed every 24 h.

2.4. Exposure to sucrose and pH measurement

After the biofilm formation period, artificial saliva was completely removed by gentle aspiration, and 2 mL of sucrose were pipetted in the wells containing the resulting biofilms. Sucrose solutions were prepared at 10, 20 or 30%, and remained in contact with biofilms for 1, 3 or 5 min. After each of these periods, the sucrose solution was completely removed and the pH was determined immediately (t0), and at 1 (t1), 3 (t3), 5 (t5) and 10 (t10) minutes after sucrose removal. For the measurements, biofilms were scraped from the wells using a cell scraper (Kasvi), transferred to microtubes (MCT-200-C-Axygen) with the aid of a pipette, and a micro pH electrode (PHR-146 Micro Combination pH Electrode – Fisher Scientific) previously calibrated with standards (pH 7.0 and 4.0) was placed in contact with biofilms. Biofilm pH before exposure to sucrose was also measured (Baseline). All tests were performed in triplicate, on three separate occasions.

2.5. Statistical analysis

The normality of the data was verified by Shapiro-Wilk's test, using the statistical program SigmaPlot version 12.0 (SigmaPlot 12.0 software, Systat Software Inc., San Jose, USA). Data were analyzed by 3-way analysis of variance, followed by Fisher LSD's test. All tests were performed with a significance level of 5%.

3. Results

A marked decrease was observed in pH values measured 1 min after removal of the sucrose solutions (t1) when compared to the other times, being significantly different from the baseline values ($p < 0.033$), regardless of the contact time with biofilms or the sucrose concentration (Fig. 1). Overall, an increasing trend in pH values from t3 was observed, especially for the shortest contact times of the biofilm with the sucrose solutions (1 and 3 min).

Significant differences in pH values were observed for biofilms treated with 10 and 30% sucrose solutions ($p < 0.025$), but not between 10 and 20% ($p = 0.460$) or 20 and 30% ($p = 0.065$). For the 10% sucrose solution (Fig. 1A), pH values observed for 1- and 3-min exposure times were not significantly different from each other ($p = 0.406$), and were significantly higher when compared to 5 min ($p < 0.028$). At this concentration, t1, t3, t5 and t10 did not differ from each other ($p > 0.104$). For sucrose at 20% (Fig. 1B), despite no significant differences were observed among the times of exposure to sucrose ($p > 0.212$), values observed for t1 were significantly different from those seen for t3, t5 and t10 ($p < 0.008$). For sucrose at 30% (Fig. 1C), exposure times of 3 and 5 min did not produce significant differences in the biofilm pH ($p = 0.199$), but were significantly different from t1 ($p < 0.028$). Only for sucrose at 30% the pH analyzed immediately after sucrose removal (t0) significantly differed from the baseline ($p < 0.047$).

The critical pH of hydroxyapatite dissolution (5.5) was attained in biofilms exposed during 3 min to sucrose at 20% (pH measured 1 min after sucrose removal; Fig. 1B), as well as in those exposed during 1 min to sucrose at 30% (pH determined immediately after sucrose removal; Fig. 1C).

4. Discussion

Changes in biofilm pH are paramount for the development of caries lesions (Islam, Khan, & Khan, 2007), so that *in vitro* models that are able to reproduce the *in vivo* changes in biofilm pH may aid researchers in investigating such changes, as well as possible factors that might affect this pattern. The present study showed that biofilms of *S. mutans* and *C. albicans* exposed to 20% sucrose solution for 3 min (Fig. 1B) exhibited pH drop and recovery similar to that described *in vivo*, despite at a much higher speed.

Sucrose is metabolized by *S. mutans*, leading to a decrease in the pH of the medium caused by the production of lactic acid (Marsh & Martin, 2009). Biofilms exposed to 30% sucrose had significantly lower pH values when compared to those exposed to sucrose at 10% (Fig. 1). In fact, such trend was somehow expected, given that a greater sugar availability would lead to a higher degree of fermentation, thus decreasing the biofilm pH. Interestingly, pH values related to longer exposure times (3 and 5 min) to 30% sucrose were not as low as that observed for the shortest exposure time (1 min). Although the present protocol does not provide data to explain the above-mentioned trend, it is possible that the longest exposure times might have favored a higher degree of neutralization of the acids by *Candida albicans*. In fact, a previous study reported that this microorganism has the ability to metabolize lactic acid as a carbon source, and thus rapidly neutralizes acidic environments (Danhof et al., 2016).

An interesting aspect of the model was the possibility to observe the four phases described by Bowen (2013) regarding the pH changes in dental biofilm under *in vivo* conditions. Thus, exposure of the mixed biofilm to 20% sucrose during 3 min allowed the observation of (1) the baseline pH prior to exposure to sugar; (2) the initial pH drop after exposure to sucrose; (3) the time at which the critical pH for hydroxyapatite dissolution was reached; and (4) the recovery phase. Nonetheless, one major difference between the present *in vitro* data in relation to *in vivo* conditions (Stephan, 1944) is the time that each phase lasted. Despite an initial pH drop occurred after sucrose exposure both *in vivo* and *in vitro*, the fall and permanence of biofilm pH to values below 5.5 were faster *in vitro* compared to *in vivo* conditions. Furthermore, the *in vivo* recovery phase was shown to take longer than the 3 min observed *in vitro*. These differences in phase time intervals, almost 10-fold faster *in vitro*, may be related to intrinsic features of the *in vitro* model used, including biofilm thickness, surface for biofilm growth (polystyrene plates), lack of salivary flow and limited microbiota.

The high mucin levels might also have played an important role in pH recovery, since this protein is likely to be the main buffer present in artificial saliva (Cheaib & Lussi, 2013). This protein is also found in natural saliva, but at lower concentrations (0.0027 g/L) than that used in the present study (1 g/L). Such high mucin concentrations were used in order to compensate the lack of continuous nutrient renewal in the *in vitro* model. Although the high mucin levels may have influenced the pH recovery time, it is worth mentioning that the biofilm had no further contact with saliva after exposure to sucrose, so that any buffering effect of mucin would be related to its residual levels in the biofilm.

The *in vitro* biofilm was probably thinner and more porous compared to that naturally developed on tooth surfaces, which tends to

facilitate the diffusion of sugars and buffers. Regarding the composition of these biofilms, a greater variety of microorganisms is observed *in vivo*, and there may be other microorganisms that also contribute to acid production, thus affecting the resulting pH and the time for neutralization. The use of biofilm microcosm models could partially overcome the limitations of the current protocol, while keeping the advantages of the well-controlled *in vitro* conditions.

To conclude, sucrose concentration and exposure time are important variables affecting the biofilm pH. In this sense, the use of a 20% sucrose solution in a dual-species biofilm of *S. mutans* and *C. albicans* during three minutes allowed the observation of pH changes resembling those seen in the dental biofilm *in vivo*, despite at a higher speed when compared to *in vivo* conditions. This experimental model may assist in preclinical research assessing changes in biofilm pH and the effects of therapeutic agents on this important variable.

Conflict of interests

There is no conflict of interest that could influence the authors' actions.

Ethical approval

This study did not involve experiments with animals or humans, so that no Ethical Approval was needed.

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