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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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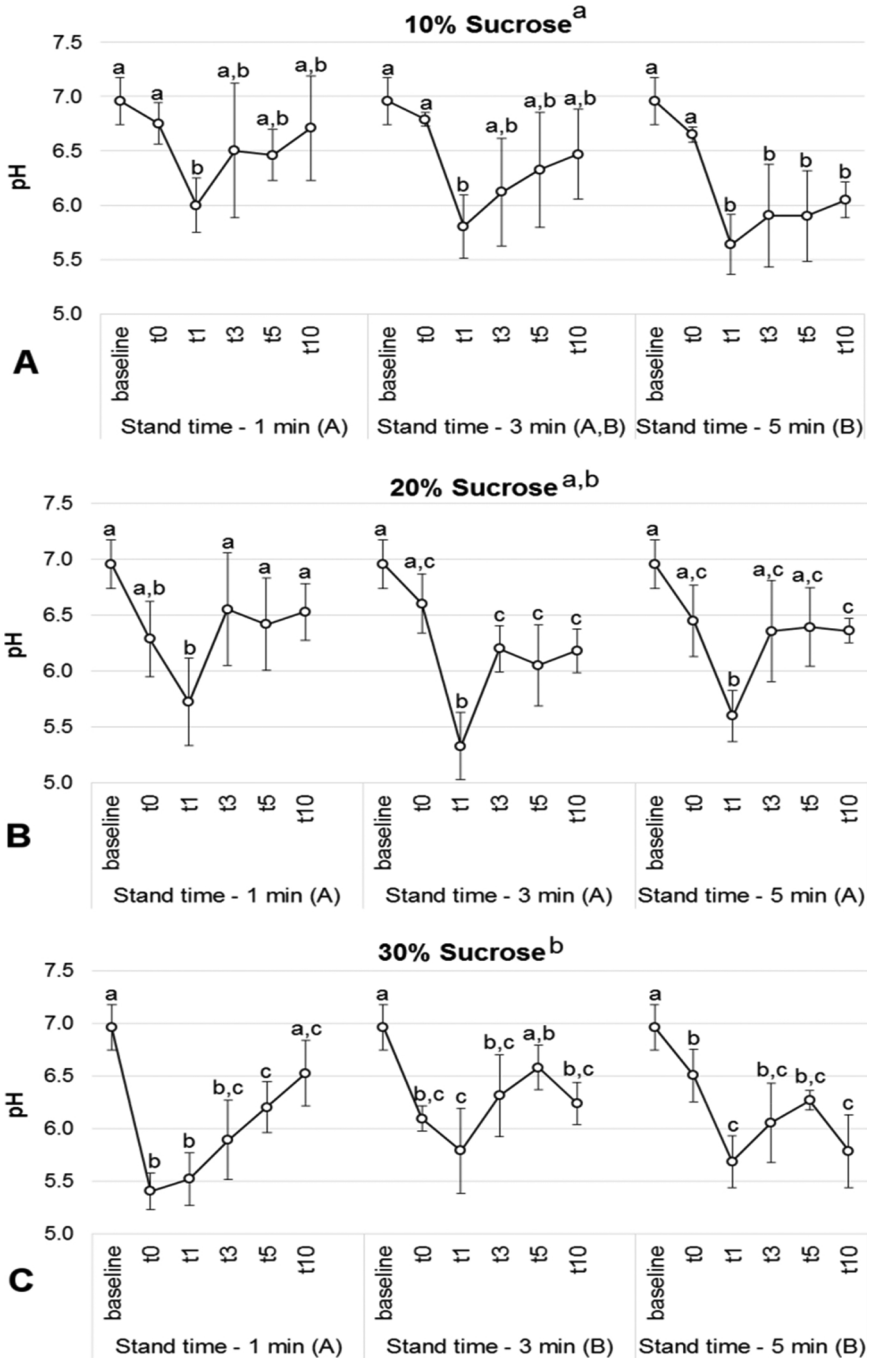

Fig. 1. Mean pH values determined after exposure of the mixed biofilm of *Candida albicans* ATCC 10231 and *Streptococcus mutans* ATCC 25175 to 10% sucrose (A), 20% sucrose (B) and 30% sucrose (C), as a function of the exposure time to sucrose and time of pH determination. Baseline: pH values determined 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.