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Influence of sucrose and xylitol on an early *Streptococcus mutans* biofilm in a dental simulator

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ABSTRACT

Objectives: In vitro methods to study dental biofilms are useful in finding ways to support a healthy microbial balance in the oral cavity. The effects of sucrose, xylitol, and their combination on three strains of *Streptococcus mutans* and one strain of *Streptococcus sobrinus* were studied using a dental simulator.
Methods: A simulator was used to mimic the oral cavity environment. It provided a continuous-flow system using artificial saliva (AS), constant temperature, mixing, and hydroxyapatite (HA) surface in which the influence of xylitol was studied. The quantities of planktonic and adhered bacteria were measured by real-time qPCR.
Results: Compared against the untreated AS, adding 1% sucrose increased the bacterial colonization of HA ($p < 0.0001$) whereas 2% xylitol decreased it ($p < 0.05$), with the exception of clinical *S. mutans* isolate 117. The combination of xylitol and sucrose decreased the bacterial quantities within the AS and the colonization on the HA by clinical *S. mutans* isolate 2366 was reduced ($p < 0.05$). Increasing the concentration (2%–5%) of xylitol caused a reduction in bacterial counts even in the presence of sucrose.
Conclusions: The continuous-culture biofilm model showed that within a young biofilm, sucrose significantly promotes whereas xylitol reduces bacterial colonization and proliferation. The results indicate that xylitol affects the ability of certain *S. mutans* strains to adhere to the HA. Clinical studies have also shown that xylitol consumption decreases caries incidence and reduces the amount of plaque. This study contributes to the understanding of the mechanism behind these clinical observations.

1. Introduction

Dental caries is a common multifactorial biofilm disease affecting about 60%–90% of children, and people are susceptible to this condition throughout their life (Bagramian, Garcia-Godoy, & Volpe, 2009). The oral cavity is a complex environment with many different surfaces, a constant salivary flow and frequent exposure to material from the external environment; mainly in the form of food. Caries is initiated within dental plaque. Bacterial biofilms develop on oral surfaces due to the interactions between micro-organisms, host factors (salivary flow rate and buffering capacity) and a diet containing fermentable carbohydrates. The commensal oral microbiota is important for preventing colonization by harmful bacteria. The bacterial composition of the biofilm reacts to changes in the local environment and these changes can lead to an imbalance in composition of the biofilm, change the overall metabolic activity of the biofilm, and subsequently lead to disease (Marsh, 2012).

The first step in the development of the biofilm is the formation of acquired salivary pellicle; a thin layer composed mainly of salivary proteins and bacterial enzymes (e.g. glycosyltransferases; Gtfs) (Bowen & Koo, 2011; Siqueira, Helmerhorst, Zhang, Salih, & Oppenheim, 2007). Gtfs are able to directly bind some oral bacteria. Gtfs are also able synthetize glucan on the pellicle which further increases the adherence of bacteria (Bowen & Koo, 2011). The main modulators of the development of cariogenic biofilms are considered to be *Streptococcus mutans* and sucrose (Paes Leme, Koo, Bellato, Bedi, & Cury, 2006). *S. mutans* Gtfs can produce extracellular polysaccharide (EPS) from sucrose and starch and *S. mutans* is well-adapted to tolerate an acidic environment and other stresses (Bowen & Koo, 2011; Klein et al., 2012; Xiao et al., 2012). Sucrose is fermented by oral bacteria creating an acidic microenvironment which leads to the demineralization of tooth enamel (Klein et al., 2012; Matsui & Cvitkovitch, 2010). Natural products are a potential source for therapeutic agents that can be used for caries prevention. Xylitol is a naturally occurring five-carbon polyol sweetener that is used as a sugar substitute in confectionary and in many oral care products. It is non-cariogenic and it has favorable

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effects on oral health (Maguire & Rugg-Gunn, 2003). Clinical studies have demonstrated that xylitol consumption reduces the incidence of caries and caries related issues (Söderling, 2009). Xylitol decreases biofilm acidogenicity, plaque volume and it is able to inhibit the growth of mutans streptococci (MS) (Söderling, 2009; Söderling & Hietala-Lenkkeri, 2010).

Various model systems have been developed over time to evaluate caries related issues *in vitro*. As the bacteria within a biofilm inhabit a highly structured, hierarchical system, their features differ from planktonic bacteria (Marsh, 2012). The complexity of the bacterial caries biofilm models vary depending on a number of factors such as the quantity of bacterial species present, equipment type, growth media used, biofilm formation time, etc. (Salli & Ouwehand, 2015).

The aim of the study was to examine how adhered and planktonic bacterial counts of three strains of *S. mutans* and one strain of *Streptococcus sobrinus* were affected by artificial saliva (AS) containing sucrose, xylitol, or a combination of the two within a dental simulator that provides a continuous flow system to reproduce the rinsing effect of saliva in the oral cavity (Björklund, Ouwehand, & Forssten, 2011; Forssten, Björklund, & Ouwehand, 2010).

2. Materials and methods

Microorganisms

The type strains *S. mutans* DSM 20523 (ATCC 25175), *S. sobrinus* DSM 20381, and two clinical *S. mutans* isolates 2366 and 117 were used in the study. The clinical isolates were kindly provided by Dr. Eva Söderling, University of Turku, Finland. The origin, isolation, and identification of the clinical isolates were described earlier (Söderling, Isokangas, Pienihäkkinen, & Tenovuo, 2000; Söderling, Ekman, & Taipale, 2008; Söderling & Hietala-Lenkkeri, 2010).

Growth conditions

The bacterial strains were subcultured twice from frozen stocks before the experiments began. The bacteria were first cultured in a brain-heart-infusion medium (BHI, LAB049, LabM Limited, Lancashire, United Kingdom) overnight in aerobic conditions at 37 °C. Before simulation, a fresh culture was prepared in a BHI medium and it was grown to a mid-exponential phase (OD_{600} = 0.4-0.6, approximately 6×10^7 colony forming units (CFU)/ml). The culture was centrifuged, washed once with AS and diluted to 1/4 of the original suspension. The AS was prepared according to Björklund et al. (2011). Each simulation vessel was inoculated with 0.5 ml of the diluted culture.

Test compounds and saliva for pellicle formation

A 20% (w/v) stock suspension of sucrose (Suomen Sokeri Oy, Kantvik, Finland) and xylitol (DuPont, Kotka, Finland) was prepared under aerobic conditions in sterile water and sterilized by filtration (0.2 μm Minisart®, Sartorius AG, Göttingen, Germany). For this study the following solutions were prepared in advance: AS with 1% (w/v) sucrose; AS with 2%–5% (w/v) xylitol; AS with 1% sucrose and 2%–5% xylitol (w/v), and plain AS. Stimulated saliva was collected as described earlier (Björklund et al., 2011).

In vitro *biofilm model*

To study the effects of sucrose and xylitol on different bacterial strains, a dental simulator model was used as described by Forssten et al. (2010). In short, the model consists of a chamber system of 16 bottles with a continuous flow of AS that simulates the oral cavity (Fig. 1). A continuous flow of AS was maintained with peristaltic pumps (Minipuls 3, Gilson®, Villiers le Bel, France and 202U, Watson-Marlow Ltd, Falmouth, Cornwall, England) and the growth vessels were kept at 37 °C (Certomat® HK, B.Braun Biotech International, Sartorius AG). Hydroxyapatite (HA) discs with a diameter of 7 mm (Clarkson Chromatography Products Inc., South Williamsport, PA, USA) were used to mimic teeth and to offer an adhesive surface for the bacteria. The HA discs were sterilized by autoclaving and attached to a stainless steel support with nail polish (Maybelline, L'Oreal, Paris, France) to keep the discs fixed at odd angles in the vessel during simulation. Prior to inserting the HA discs into the simulation vessel they were coated with stimulated whole human saliva and kept at 37 °C for 1 h to form a pellicle. A bacterial suspension was inoculated into the culture vessels (AS volume of 15 ml), immediately prior to the start of the simulation. The content of the bottles was well agitated to imitate the concept of mixing within the oral cavity. At the start of the simulation process 10 ml/h AS with no additives was pumped through the system for 0.5 h. Test compounds were then added to the AS for 3 h at 20 ml/h, followed by 0.5 h of incubation. The simulation ended with a final rinsing with 10 ml/h AS for 1 h. The HA discs were collected and samples of AS from the growth vessels were taken. Samples were stored at −20 °C until they were analyzed.

Sample analysis

DNA was extracted from the HA discs by using a modified method from Wilson (2001). In short, the HA discs were rinsed with ultrapure water and then a solution of 12% sucrose in 25 mM Tris-HCl (pH 8.0) was added. Following that, lysozyme was added and the mixture was incubated for 2 h at 37 °C. Then 10% SDS, 250 mM EDTA (pH 8.0) and proteinase K were incrementally added, and again the mixture was incubated for 2 h at 37 °C. After the addition of 5 M NaCl, the solution was thoroughly mixed, cetyl

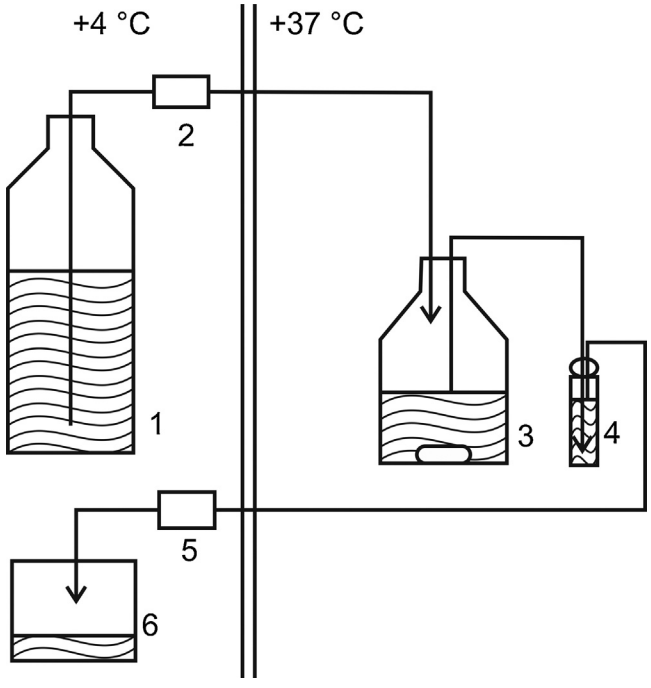

Fig. 1. A schematic diagram of the dental simulator used in the study to evaluate biofilm formation (reproduced from Salli & Ouwehand, 2015) 1. AS 2. Pump 3. Simulation vessel 4. Sample collection during the simulation 5. Outlet pump 6. Waste.