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Full Length Research Paper

Comparative inhibitory effect of xylitol and erythritol on the growth and biofilm formation of oral *Streptococci*

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Our aims were to examine the effects of xylitol and a novel polyol sweetener, erythritol, on growth of oral *Streptococci* and compare their effects. The inhibitory effects of xylitol and erythritol on *Streptococcus* strains, as well as on streptococcal biofilm formation were examined. *Streptococcus mutans*, *Streptococcus sobrinus*, and *Streptococcus sanguinis* were used as representatives of oral *Streptococci*. The effects of these polyols on biofilm formation were determined by microtiter plate assay. The growth was compared at each experiment using analysis of variance of repeated measures (SPSS 16.0 for Windows). Our results indicated that in the presence of 4% xylitol and 4% erythritol the growth of *S. mutans* was decreased by 68 and 71%, respectively. Biofilm formation by *S. mutans* was reduced to 31.32% in the presence of 4% erythritol. Regardless of concentration, in general, erythritol was found more effective than xylitol in inhibiting the growth and biofilm formation of *Streptococci* strains used in this study. Xylitol and especially erythritol both inhibited microplate surface adherence of oral *Streptococci*, which are known to contribute to plaque accumulation.

Key words: Xylitol, erythritol, oral *Streptococci*, biofilm, microtiter plate assay.

INTRODUCTION

Dental caries form through a complex interaction over time between acid-producing or acid-tolerating bacteria and fermentable carbohydrates. Acid production by oral bacteria by hydrolysis of the food debris accumulated on the tooth surface, cause demineralization and destruction of the tooth. Thus, it has been an obvious idea to utilize sugar substitutes in order to prevent the disease (Wennerholm et al., 1994; Selwitz et al., 2007). Sugar alcohols are not utilized by the oral bacteria, and so the absence of fermentation and acid production reduces risk of dental caries (Mattos-Graner et al., 2000; Tanzer, 1995). Xylitol and most of other polyols used as bulk sweeteners may have laxative effects and, therefore, they are only suitable for small size products like chewing gums (Soderling, 2009; Soderling et al., 2010). In contrast to other poly alcohols, erythritol is not a laxative and its food applications could be much broader.

The first study to report that xylitol inhibits growth of *S. mutans* was published in 1975 (Knuuttila and Makinen, 1975). Clinical studies have shown xylitol to decrease the number of *S. mutans*, the amount of plaque, and the incidence of caries in children (Ly et al., 2006; Bradshaw and Marsh, 1994; Soderling et al., 1989). A number of studies have also suggested that some *S. sanguinis* and *S. salivarius* strains may be inhibited by xylitol (Isotupa et al., 1995; Kontiokari et al., 1995). Substitution of xylitol for sucrose in the human diet, totally as well as partially, resulted in more than 85% reduction in the incidence of dental caries (Scheinin, 1976). Most of the *S. mutans* strains transport xylitol into the cell via the phosphotransferase system, and xylitol is then phosphorylated to xylitol-5-phosphate and expelled from the cell (Makinen et al., 2001; Soderling, 2009). This futile energy-consuming pathway is thought to be responsible for the growth inhibition of *S. mutans* (Burt, 2006;

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Takahashi and Nyvad, 2008; Trahan, 1995). None of the predominant bacteria found in dental plaques produce acid from xylitol.

Erythritol has been suggested to be caries preventive, but few studies have so far been published on its effects on dental caries (Makinen et al., 2002, 2005; Burt, 2006). The present study shows the effects of xylitol and erythritol on the growth of oral *Streptococci* and compares their potential advantages against adhesion and biofilm development of these bacteria. Since *S. mutans* are considered the most acidogenic microorganisms in dental biofilm, an important aspect of this study is to find high efficiency of erythritol on inhibition of growth and biofilm formation of this bacterium.

MATERIALS AND METHODS

Microorganisms

The following types of *Streptococci* were used: *S. mutans* ATCC35668, *S. sobrinus* ATCC27607, and a clinical isolate of *S. sanguinis* provided by Pasteur Institute of Iran.

Cultivation of the microorganisms

Streptococci species were initially cultured in 5 ml of Brain Heart Infusion (BHI) broth (Merck, Germany) to produce log phase cells. Cultures were incubated at 37 ◦C for approximately 16 h and then transferred (2%) to fresh BHI supplemented with 1% sucrose. The growth media contained 2% (130 mM) or 4% (260 mM) xylitol (Sigma, St. Louis, MO, USA), or 2% (160 mM) or 4% (330 mM) erythritol (Sigma, St. Louis, MO, USA). The polyol concentrations (w/v %) were chosen based on similar study (Soderling et al., 2010). Stock solutions of xylitol and erythritol were prepared in distilled water and sterilized by filtration through a 0.2 Millipore filter (Axiva, India) and afterwards were added aseptically to the medium at the appropriate concentration. The corresponding control medium was free of polyols. The test cultures were incubated in a 5% CO_2 incubator with slowly shaking at 37°C for 24 h. Growth was monitored by measuring the absorbance at a wavelength of 660 nm. Each test was carried out in three independent experiments. The inhibitory effects of xylitol and erythritol were calculated from the growth curves at late log phase.

Microtiter plate assay for efficacy of Streptococcal biofilm removal

Biofilm production by *Streptococci* strains grown in BHI was measured using a semi-quantitative adherence assay on 96-well tissue culture plates. Each *Streptococcus* strain was grown in 10 ml of BHI supplemented with 1% sucrose at 37°C. Overnight cultures in BHI were transferred (0.1 ml) into polystyrene microtiter plates (Greiner Bio-One, Germany) which previously added 0.1 ml of erythritol and xylitol separately. Final concentration of erythritol and xylitol reached to 2 and 4%. Each plate included eight wells of *Streptococci* as control and eight empty wells as blank (Van Loveren, 2004). The plates were covered and incubated aerobically at 37°C for 24 h. At the end of incubation, the liquid in the wells was poured out and each well was washed three times with 0.25 ml of sterile phosphate buffered saline (PBS). Plates then were stained for 5 min with 0.2 ml of 2% crystal violet per well. Excess stain was rinsed off by washing the plate with PBS. Plates were air-dried and the dye was solubilized from the adherent cells by treatment with 0.2 ml of 33% (v/v) glacial acetic acid per well. The cell turbidity was monitored using a microtiter plate reader at 630 nm optical density. The average OD of the blank wells was subtracted from the OD of experimental samples. Inhibitory effect of these polyols on biofilm production and adhesion of *Streptococcus* strains to polystyrene microplate was assayed and determined using the following formula:

Percentage reduction: [(C-B)-(T-B)/(C-B)] × 100%

Where B denotes the average absorbance for blank wells (no biofilm, no treatment); C denotes the average absorbance for control wells (biofilm, no treatment) and T denotes the average absorbance for treated wells (biofilm and treatment) (Djordjevic et al., 2002; Shakeri et al., 2007).

Disc diffusion test

The antimicrobial assay of these polyols was performed by agar disc diffusion method (Baur et al., 1966). The tested bacterial strains were cultured until the cultures attained a turbidity of 0.5 McFarland units. A 0.1 ml volume of the standard suspension of each test bacterial strain was spread evenly on Mueller Hinton (MH) agar (Merck, Germany) using a sterile glass rod spreader and the plates were allowed to dry at room temperature. Blank sterile discs (0.5mm diameter) (Oxoid, Australia) were saturated with 0.2 ml of each polyol solution and allowed to dry before being placed on the top of the agar plate. The controls included distilled water and the commercial antibiotic tetracycline. After holding the plates at room temperature for 2 h, they were incubated for 48 h at 37C in a carbon dioxide environment and the diameter of growth inhibition zone was determined.

Statistical analyses

The growth was compared at each experiment using analysis of variance (ANOVA) repeated measures (SPSS 16.0 for Windows). The level of statistical significance was set at $P< 0.01$.

RESULTS

Cultivation of the microorganisms

Both xylitol and erythritol inhibited the growth of all oral *Streptococci* studied in this study. The presence of 4% xylitol and erythritol resulted in 68 and 71% inhibition of *S. mutans* growth, respectively. The same concentrations of xylitol and erythritol also decreased the growth of *S. sanguinis* by 65 and 77%, respectively (Table 1). As shown in Figure 1, after 24 h of incubation, the optical density of the culture reached the lowest in the presence of erythritol than xylitol, indicating erythritol to be more efficient than xylitol in growth inhibitory of *Streptococcus* strains. In addition our results indicated that the degree of inhibition, as well as the inhibitory pattern differs for most *Streptococcus* strains.

Microtiter plate assay

Inhibitory effect of these polyols on biofilm production and

Table 1. Growth inhibition of xylitol and erythritol on oral *streptococci*.

Polyol	Concentration (%, w/v)	Percentage reduction (%) of [a*]		
		S. mutans	***S. sobrinus***	***S. sanguinis***
Xylitol	2	66	71	57
	4	68	72	65
Erythritol	2	69	71	76
	4	71	76	77

[a]Data points: mean values from three independent experiments, [*]significancy level set at P<0.01.

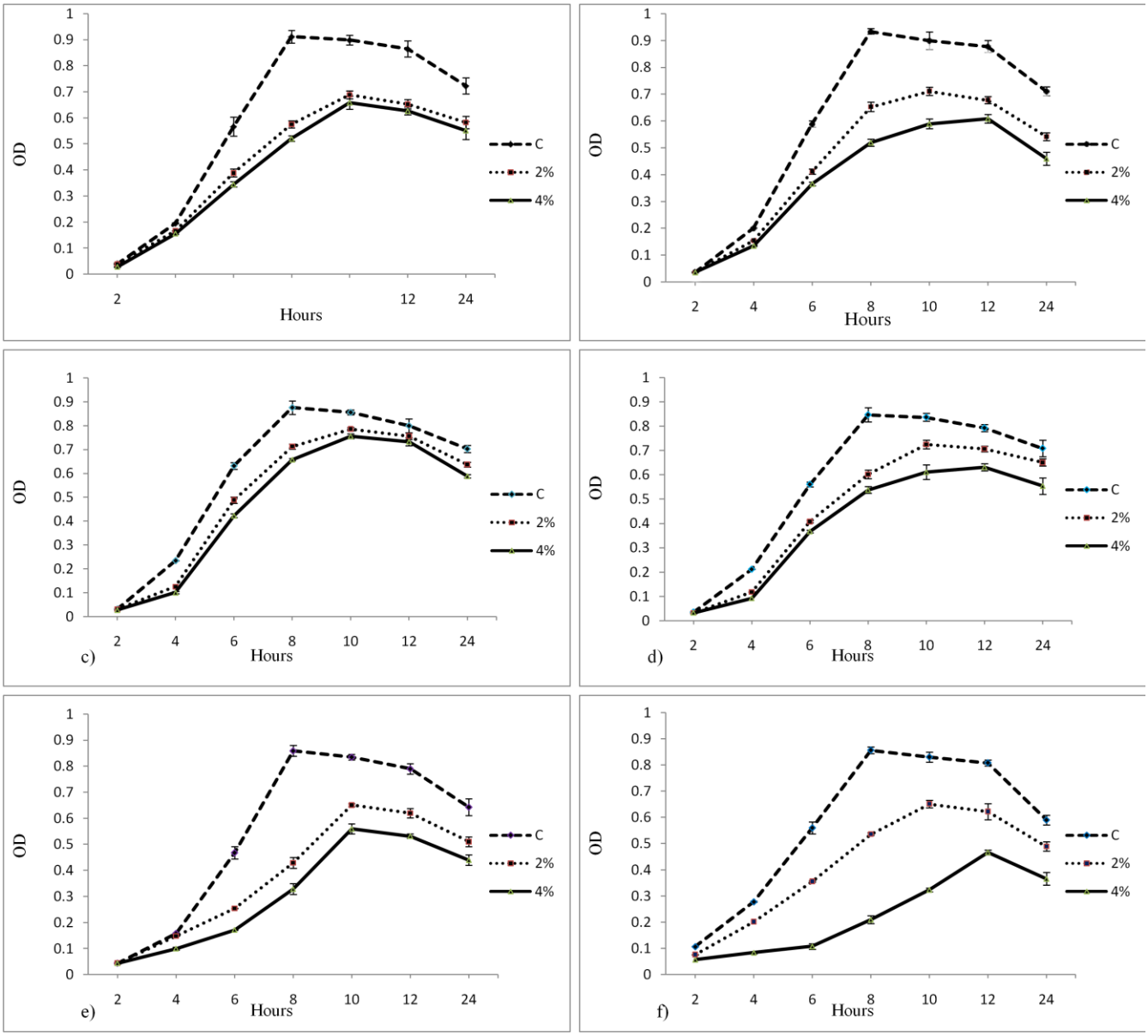

Figure 1. Growth (660 nm) of *S. mutans*, *S. sobrinus* and *S. sanguinis* in the presence of xylitol and erythritol at different concentrations. a) *S. mutans*, xylitol, b) *S. mutans*, erythritol, c) *S. sobrinus*, xylitol, d) S. *sobrinus*, erythritol, e) *S. sanguinis*, xylitol, f) *S. sanguinis*, erythritol (C: Control). Data points: mean values from three independent experiments. Bars represent the standard deviations of means. P < 0.01.

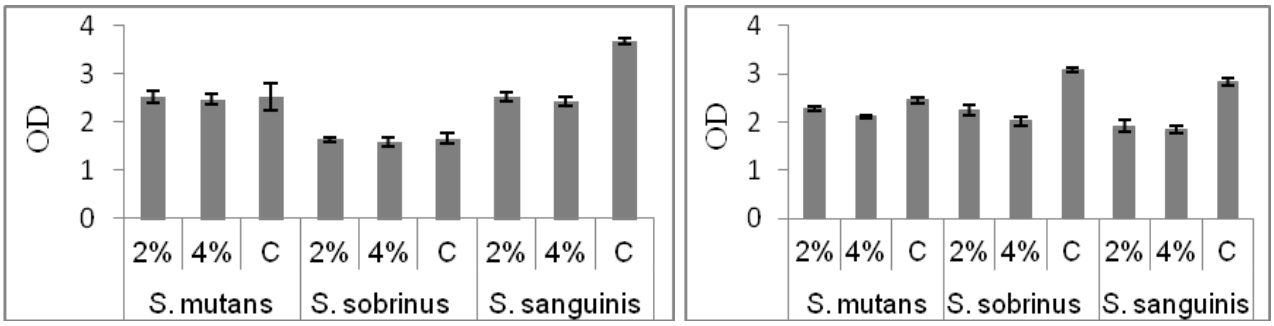

Figure 2. The adhesion (630nm) of three species of *Streptococcus* to polystyrene microplate. (a) The adhesion in presence of xylitol (2%, 4%) b) The adhesion in presence of erythritol (2%, 4%) (C: Control). Data points: mean values from three independent experiments. Bars represent the standard deviations of means.

Table 2. Percent inhibition of biofilm development by xylitol and erythritol on oral *streptococci*.

Polyol	Concentration (%, w/v)	Percentage inhibition of biofilm development (%) of [a]		
		S. mutans	***S. sobrinus***	***S. sanguinis***
Xylitol	2	0.33	3.19	43.80**
	4	3.55	9.9*	47.26**
Erythritol	2	11.65*	38.7**	47.76**
	4	31.32**	50.92**	50.88**

[a]Data points: mean values from three independent experiments, * Significancy level set at P<0.05.** Significancy level set at P<0.01.

adhesion of *Streptococci* strains to polystyrene microplate are shown in Figure 2. The results showed that the inhibitory effect of erythritol is stronger than that of xylitol. Biofilm formation by *S. mutans* was reduced to 3.55% and 31.32% in the presence of 4% xylitol and erythritol, respectively (Table 2).

Disc diffusion test

Solutions of xylitol (2%, 4% w/v) and erythritol (2%, 4% w/v) in distilled water did not cause zones of inhibition in any of the three bacteria plated. The tetracycline that served as control caused observed zones of inhibition.

DISCUSSION

It is considered that high sugar intake and low pH are the most primary mechanisms for disrupting homeostasis, leading to the development of dental caries. Strategies that aim to prevent the disease should include the inhibition of acid production, decrease in sugar consumption and use of non-fermentable sugars. Substitution of sugar by xylitol or erythritol is not only non-acidogenic but also may be considered as anti-cariogenic (Fraga et al., 2010). This study demonstrates higher performance of erythritol than xylitol in inhibiting the growth and biofilm formation of *Streptococci* strains and suggests that erythritol could be a good replacement for xylitol for the future of food industry.

Effect of xylitol and erythritol on growth and biofilm formation of oral *Streptococci*

The objective of this study was to compare the inhibitory effect of erythritol and xylitol on *Streptococcus* strains and a polystyrene microtiter plate assay to compare the inhibitory effect of these polyols on biofilm formation. Our results show that both xylitol and erythritol reduce the growth of oral *Streptococci*, but erythritol has a stronger inhibitory effect on biofilm formation than xylitol. An in vivo study by Makinen et al. (2001) also showed which xylitol, and especially erythritol, inhibited the growth of several strains of *S. mutans*. In addition, this study showed that erythritol significantly inhibited the microplate surface adherence of *S. mutans*, as well as other strains of oral *Streptococci*.

Similar to xylitol, erythritol is not catabolized by

Streptococci species. Xylitol entered to oral *Streptococci* by PEP-PTS system and changed to xylitol 5-phosphate, and then dephosphorylated and expelled as xylitol. The xylitol-5-phosphate inhibits glycolytic enzymes, resultingin the inhibition of both growth and acid production. This futile energy consuming xylitol cycle is thought to be responsible for the growth inhibition of these bacteria when the bacteria are exposed to xylitol. But no theories on the mechanism of growth inhibition by erythritol have so far been published (Soderling et al., 2010; Ly et al., 2008; Fraga et al., 2009) and more studies are clearly needed on this topic. Although ‘‘resistance’’ phenomenon demonstrated for xylitol in many oral *Streptococci*, but this phenomenon did not report for erythritol. Since erythritol is a new polyol and doesn’t have laxative effect therefore; use of erythritol and other same biological sweeteners products are the best way for control of dental caries in future.

Antibacterial effect of xylitol and erythritol on growth of oral *Streptococci*

The purpose of disc diffusion test study was to determine if xylitol or erythritol inhibit the *in vitro* growth of oral *Streptococci*. The results indicated that xylitol and erythritol did not inhibit the growth of bacteria in a carbon dioxide environment on MHA. Furthermore, this study revealed that inhibition is not due to anti-microbial activity but the mechanism of growth inhibition of these polyols is futile energy-consuming in metabolism pathway. Therefore, the main mode of inhibition by xylitol, and probably erythritol, appears to be the replacement of the carbohydrate source.

Some studies established that xylitol decreases the synthesis of insoluble polysaccharides by *S. mutans* (Soderling et al., 1989). This study also demonstrated that not only xylitol but also erythritol decrease the polysaccharide production and consequently decrease the microplate surface adherence of oral *Streptococci*. Hereby, xylitol and especially erythritol are considered as important inhibitors of microplate surface adherence of oral *Streptococci* which are known to contribute to plaque accumulation.

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