Effect of Xylitol on an *In Vitro* Model of Oral Biofilm

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Purpose: The aim of the present study was to examine whether xylitol, at different concentrations, inhibits the formation of an experimental model of oral biofilm.

Materials and Methods: Biofilms of six bacterial species (*Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus rhamnosus*, *Actinomyces viscosus*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*) were prepared on hydroxyapatite (HA) discs according to the Zürich Biofilm Model. Xylitol was tested at two concentrations, 1% and 3%. At the end of their designated incubation times, some HA discs were destined for confocal laser scanning microscopy (CLSM) and the others were harvested using a sterile surgical instrument. Aliquots of harvested biofilms were diluted and plated onto specific media. After a 48-h anaerobic incubation at 37°C, the colony-forming units (CFUs) were counted.

Results: CLSM images showed that only a small amount of isolated bacteria was observed on the surface of HA discs. Culture of harvested biofilms showed an inhibition in the growth of different species included in the biofilms.

Conclusions: Xylitol has a clear inhibitory effect on the formation of the experimental biofilms. This study shows that xylitol is not only efficient in inhibiting the acid production of cariogenic bacteria, but also in preventing the formation of a multispecies biofilm; it confirms the relevance of the use of this polyol for the prevention of oral diseases caused by dental plaque.

Key words: adherence, biofilm, oral bacteria, xylitol

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Some oral bacterial species have the ability to adhere to dental surfaces and form a biofilm. The first stage in the formation of this biofilm is the development of a thin layer composed mainly of salivary proteins and cell-free enzymes including glucosyltransferase (GTF) and fructosyltransferase. Following the initial colonisation, the bacteria multiply and form micro-colonies. The proliferation of these micro-colonies results in the development of the biofilms in which the microorganisms are intimately associated with each other and are embedded in a matrix of exopolysaccharides of bacteria found in saliva (Listgarten, 1999).

Dental biofilms are associated with the initiation and the progression of caries and periodontal diseases (Liljemark and Bloomquist, 1996). Hence, control of these biofilms is fundamental to the maintenance of oral health and to the prevention of caries, gingivitis and periodontitis. However, oral biofilms are not easily controlled by mechanical means, and they represent a difficult target for chemical controlling (Socransky and Haffajee, 2002). One probable explanation for this low efficacy is that the microorganisms demonstrate different behaviour when they are organised in a biofilm from when they are organised in the planktonic cells. Moreover, the efficacy of antimicrobial agents against the existing biofilms appears to be limited, and chemicals may be better in preventing bacterial colonisation and biofilm development (Pratten et al, 1998). Several methods of interference with the accumulation of bacteria in biofilm have been tried. The use of anti-adhesion compounds that prevent the bacterial accumulation has given promising results (Kelly and Younson, 2000; Ofek et al, 2003; Steinberg et al, 2005). As sucrose intake appeared to be one of the important risk factors for dental caries,

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substitutes for sucrose have been investigated for many years. The increase of the relative amount of *Streptococcus mutans* in dental plaque in connection with the intake of sucrose appears to be correlated with the production of adhesive polysaccharides (PSs) (Krasse et al, 1967). In the experimental trials, xylitol seemed to be a promising molecule, by being a non-cariogenic sweetener. For example, it inhibits growth and acid production of *S. mutans*, and regular consumption of xylitol has been shown to reduce the incidence of dental caries (Tanzer, 1995; Trahan, 1995). It has also been shown that between-meal intake of xylitol decreases the quantity as well as the adherence of dental plaque (Grenby and Bashaarat, 1982; Makinen and Scheinin, 1982; Birkhed et al, 1983; Topitsoglou et al, 1983). However, in other controversial studies, the use of xylitol-containing chewing gum showed no effect against dental plaque deposits (Scheie et al, 1998).

Less research has been carried out on the *in vitro* effect of xylitol on biofilms; hence, the aim of the present study was to examine whether xylitol, at different concentrations, inhibits the formation of an experimental model of oral biofilm.

MATERIALS AND METHODS

Biofilm assay

The biofilm was assayed according to the model described by Guggenheim et al (2001). Only a few modifications (mainly changes in bacterial composition) were made.

The bacterial strains used in this study were *S. mutans* ATCC 25175, *Streptococcus sobrinus* ATCC 33478, *Lactobacillus rhamnosus* ATCC 7469, *Actinomyces viscosus* ATCC 15987, *Porphyromonas gingivalis* ATCC 33277 and *Fusobacterium nucleatum* ATCC 10953.

Pre-cultures of each species were incubated at 37°C in fluid universal medium (FUM) and biofilms were developed on hydroxyapatite (HA) discs (Clarkson Chromatography Products, USA) coated with human pasteurised saliva (30 min at 65°C; performed for sterility).

Before the anaerobic incubation, xylitol solution was added into the wells, and the final concentrations were 1% and 3%. Twenty wells for each concentration were tested.

Control biofilms were treated with physiological saline.

Biofilm studies

Harvesting the biofilm

At the end of the incubation time, HA discs that were not destined for confocal laser scanning microscopy (CLSM) were washed with physiological saline to remove the poorly adherent bacteria.

To harvest the adherent cells, each disc was placed in a sterile plastic Petri dish, and the disc surfaces were scraped using a sterile surgical instrument (dental root curette). The surface of the scraped disc and the Petri dish were rinsed with physiological saline (1000 µl), and the cell suspension was vortexed vigorously for 2 min.

Aliquots of harvested biofilms were diluted and spiral-plated onto Mitis Salivarius agar with tellurite (Difco, France) for *Streptococcus*, MRS agar (Merck, France) for *Lactobacillus*, Trypticase Soy agar (Difco, France) for *Actinomyces*, and Wilkins and Chalgren anaerobe agar supplemented with blood and GN supplement (Oxoid, France) for *Fusobacterium* and *Porphyromonas*.

Agar plates were incubated anaerobically at 37°C for 48 h, and the colony-forming units (CFUs) were counted.

CLSM biofilm observation

Non-invasive confocal imaging of the biofilms was accomplished with a Confocal Visible Leica DMR TCS SP2 AOBS fitted with water-immersion dipping lenses (×63).

Specimens were stained with LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit for microscopy according to the manufacturer's instructions.

An excitation wavelength of 488 nm was used and all light rays emitted above 500 nm were collected.

Biofilm structure was analysed by taking a series of horizontal sections, each with a 1-µm thickness.

Digital images were processed using Metamorphe and Leica reconstruction 3D software.

Statistics

CFU per population for triplicate discs inoculated with identical multispecies suspensions were averaged and subjected to logarithmic transformation.

Statistical analysis was performed using the Student *t* test with the Bonferonni corrections. Statistically significant values were defined as $P < 0.05$.

RESULTS

Xylitol has a clear inhibitory effect on the formation of biofilms. Control biofilms, treated with physiological saline, gave results similar to that of intact (non-treated) biofilms.

Confocal microscopy

Intact biofilms (Fig 1)

After 5 days of growth, the biofilm was a confluent of a few single cells and an abundance of galaxies of cells, within which the occasional small lacuna was seen. The firmly adherent stratum of the biofilm had achieved a height of 20–30 μm.

Effect of xylitol (Fig 2)

No structured biofilm was visible under the CLSM.

Only a small amount of isolated bacteria was observed on the surface of the HA discs.

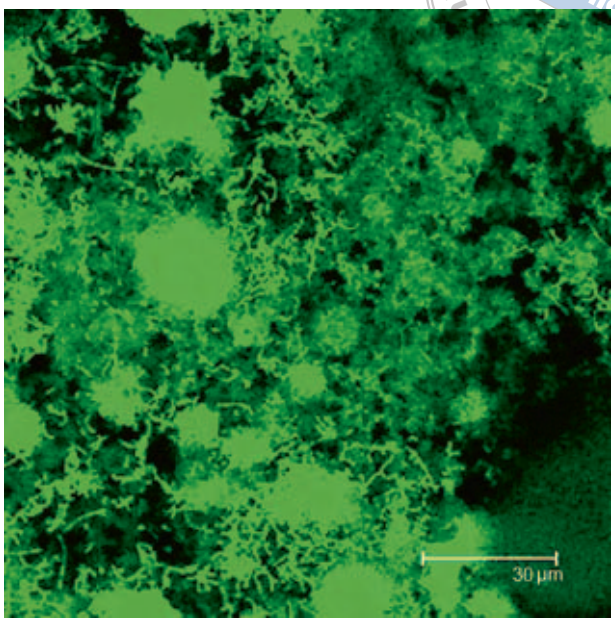

Fig 1 CLSM image of intact biofilm.

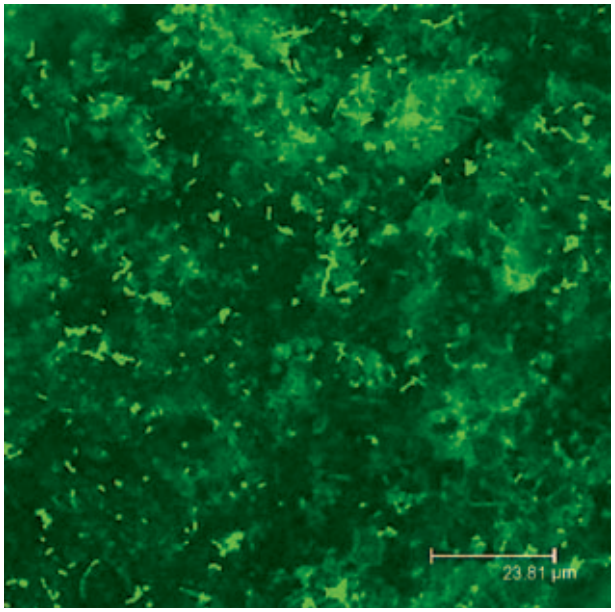

Fig 2 CLSM image of biofilm treated with xylitol at 3% concentration.

Harvesting the biofilms

Intact biofilms

Cell recoveries on different media for discs harvested at 5 days were $(11.5 \pm 0.5) \times 10^8$ CFU per scratched disc (average of triplicate harvests ± SD).

Effect of xylitol

Responses of each species of the biofilm model to the treatment with xylitol are given in Table 1.

Some species were not recovered, whatever the xylitol concentration: *S. mutans*, *S. sobrinus*, *F. nucleatum* and *P. gingivalis*.

L. rhamnosus was only recovered at 1% concentration, but the average number of CFUs was very low and statistically different from that of the control.

At 1% concentration, *A. viscosus* was found at a quantity statistically different from that of the control. At 3% concentration, only small quantities $(4.67 \pm 3.7) \times 10^4$ were recovered.

For *L. rhamnosus* and *A. viscosus*, a dose-dependent effect was found.

DISCUSSION

Various multispecies models of dental plaque have been described and applied to problems of clinical relevance. These systems usually consist of either flow cells (Christersson et al, 1987; Larsen and Fiehn, 1995) or chemostats modified to allow for insertion and removal of colonisable surfaces (Bradshaw et al, 1996; Kinniment et al, 1996; Bowden, 1999). These devices are cumbersome to construct and difficult to maintain over long periods. For this reason, we have chosen the Zürich Biofilm Model that is based on a batch culture approach and is easy to set up. This model was validated by

Table 1 Effect of xylitol (xyl) at two concentrations (1% and 3%) on biofilm bacteria. Results are presented as average CFU/ml subjected to logarithmic transformation

	Lactobacilli			Streptococci			Actinomyces			Anaerobes		
	Control	Xyl 1%	Xyl 3%	Control	Xyl 1%	Xyl 3%	Control	Xyl 1%	Xyl 3%	Control	Xyl 1%	Xyl 3%
Average	7.23	1.40	NR*	7.30	NR*	NR*	7.74	5.90	2.46	7.32	NR*	NR*
SD	0.22	1.68	0.00	0.07	0.00	0.00	0.05	0.15	1.23	0.03	0.00	0.00

NR*: Not recorded.

Guggenheim et al (2001) and was found to be applicable for the testing of many chemicals (Guggenheim et al, 2001; Shapiro et al, 2002).

Changes in bacterial composition were made from the original description by Guggenheim. We included three species that are involved in caries (*S. mutans*, *L. rhamnosus* and *A. viscosus*) and two species that are implicated in periodontal diseases (*F. nucleatum* and *P. gingivalis*).

It has been demonstrated that xylitol has a variety of cariostatic effects such as inhibition of growth and acid production of *S. mutans* (Mühlemann et al, 1977; Waaler et al, 1985; Tanzer, 1995). However, controversial results have been obtained in *in vivo* studies using xylitol-containing chewing gum against dental plaque deposit (Mühlemann et al, 1977; Topitsoglou et al, 1983; Waaler et al, 1985; Söderling et al, 1989; Assev et al, 1996; Scheie et al, 1998). Less research has been carried out on the effect of xylitol on an *in vitro* model of oral biofilm.

Similar to samples obtained by scraping, bacteria were not recovered in culture, and our findings indicate that xylitol affects the formation of multispecies biofilms *in vitro* in the presence of sucrose.

Although some authors have shown that pentitol interferes with the metabolism of C-6-based sugars and, for example, that xylitol-5-P inhibits the growth of various bacteria (Vadeboncoeur et al, 1983), during preliminary testing we observed that xylitol did not affect the growth of the species used in our biofilm in the presence of glucose (data not shown). These results agreed with those of other authors, showing that the growth was retarded, but not inhibited, after 24 h in the presence of xylitol (Gauthier et al, 1984; Vacca-Smith et al, 1996).

Hence, we can hypothesise that the lack of biofilm development in the present experiment could be due to the inhibition of the adhesion of microorganisms, and this is in accordance with previous studies.

For example, Söderling and co-workers showed that xylitol affects the production of PS by *S. mutans*. However, if the production of soluble PS increases, the amount of insoluble PS (which is a key factor in the adherence of dental plaque), simultaneously decreases (Söderling et al, 1987). Moreover, Sato and co-workers have shown that xylitol has other target sites in addition to sugar transport systems in *S. mutans*. This may be one of the possible mechanisms induced by this polyol to clear *S. mutans* from the oral cavity (Sato et al, 2000).

Other authors have demonstrated that oral microorganisms produced less lipoteichoic acids, which are involved in adhesion mechanisms, in the presence of xylitol when compared with sorbitol or sucrose (Jacques et al, 1979; Rolla et al, 1980; Hardy et al, 1981).

It is now well known that GTF plays a significant role in the development and growth of dental plaque (Hamada et al, 1984; Kuramitsu, 1993). Hence, inhibiting the function of this important plaque-building enzyme could be of great interest in the prevention of bacterial colonisation and in the accumulation of plaque. Many reagents that inhibit streptococcal GTFs have been identified (Marsh, 1993; Vacca-Smith et al, 1996). However, according to Wunder and Bowen (1999), xylitol had no appreciable effect on the GTF activity. This is not an unexpected finding as this polyol never appears to act as a substrate for the enzyme.

Numerous drugs have been tested for their effect on dental biofilm formation and maturation, and those most commonly occurring contain antibacterial agents (Gilbert et al, 1997; Baehni and Takeuchi, 2003). Despite the great benefit of this bactericidal approach, the use of antiseptic molecules can be accompanied by side effects such as disturbance of the microbial balance of the oral ecosystem.

For this reason there is a continuing search for active ingredients that could prevent the dental plaque formation without affecting the biological equilibrium within the oral cavity.

According to our results, xylitol acts as an antiplaque agent by affecting the biofilm formation.

More studies need to be done to examine how xylitol inhibits the biofilm formation: this property,

in addition to the inhibition of acid production, could enhance the interest of the use of this polyol in the prevention of oral diseases.

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