Decreased Salivary Uptake of [^{14}C]-Xylitol after a Four-week Xylitol Chewing Gum Regimen

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Purpose: The aims were to evaluate a simple method to disclose a microbial shift in saliva and to investigate the short- and long-term effects of daily use of xylitol-containing chewing gums on mutans streptococci (MS) and [^{14}C]-xylitol uptake in saliva.

Materials and Methods: In a pilot set-up, saliva samples were collected from 15 healthy adults and the uptake of xylitol was compared with a specific assay determining xylitol-sensitive MS. The main study consisted of 109 schoolchildren (mean age 9.9 years) who volunteered after informed consent. The children were randomly allocated to a test or control group. The control group was given two pellets containing sorbitol and maltitol 3 times daily for 4 weeks and the test group received identical pellets with xylitol as single sweetener (total dose 6.2 g/day). Saliva samples were collected at baseline, after 4 weeks and 6 months after the intervention. The outcome measures were MS and total viable counts, proportion of MS and salivary uptake of [^{14}C]-xylitol.

Results: The pilot study disclosed a fair positive correlation ($p < 0.05$) between the assays. The proportions of MS and salivary xylitol uptake decreased significantly in the xylitol group by 60% and 30% respectively after 4 weeks compared to baseline which was in contrast to the sorbitol/maltitol group ($p < 0.05$). Six months after the intervention, the outcome measures did not differ significantly from baseline in any of the groups.

Conclusion: A relatively high daily dose of xylitol could alter salivary microbial composition during the intervention period but no long-term impact was observed.

Key words: chewing gum, mutans streptococci, oral microflora, saliva, xylitol

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Different strategies to combat caries have been used over the years. Although it's well known that tooth brushing, use of fluoride and having a diet with less sugar can prevent caries development, the disease is still a global public health problem (Petersen et al, 2005). Xylitol is a sugar substitute claimed to be anti-cariogenic and has been used for more than 30 years (Mäkinen, 2000; Peldyak and Mäkinen, 2002). The pentitol occurs widely in nature and is produced in the human body during normal metabolism. The beneficial effects of xylitol have been demonstrated in several clinical trials with caries increment as outcome measure (Kandelman and Gagnon 1987; Isokangas et al, 1993; Machiulskiene et al, 2001). In some of these studies, the long-term impact has also been investigated, indicating some persisting effects on the oral microflora years after the intervention period (Isokangas et al, 1993; Hujoel et al, 1999). A regular consumption of xylitol may give mutans streptococci (MS) strains that are less sensitive for xylitol, meaning that they are unable to transport xylitol into the cell, an ecological advantage (Beckers 1988; Kakuta et al, 2003; Tanzer et al, 2006). Furthermore, it has been sug-

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gested that such xylitol-resistant mutans streptococci (X^R MS) are less cariogenic (Trahan et al, 1996), but the findings are controversial since other researchers have failed to verify the results (Assev et al, 2002; van Loveren, 2004).

A general idea to optimise the beneficial effects of xylitol is to advocate a daily fractioned intake of 5–10 g/day, and this concept has recently been confirmed by a number of studies (Milgrom et al, 2006; Ly et al, 2006). Milgrom et al (2006) cultured samples of plaque and unstimulated saliva from three test groups with different doses of xylitol and demonstrated reduced counts of MS with doses over 6.4 g/day in plaque samples after 5 weeks and from both plaque and saliva after 6 months. The total cultivable flora, however, remained unchanged in both plaque and saliva. Likewise, Meurman et al (2005) showed a positive relationship between the frequency of xylitol consumption and xylitol-resistant strains; among the children using xylitol several times a day, the mean percentage of X^R MS was the highest.

The detection of X^R MS in saliva is commonly carried out by an autoradiography method described by Dréan and Trahan (1990). Since this assay is technique-sensitive and time-consuming, it was of interest to look for a more rapid and simple method to get a rough estimation on the proportion of salivary bacteria in saliva with the capacity of xylitol uptake. The first aim of the present study was therefore to compare a simplified method for estimating salivary uptake of xylitol with the established and specific assay concerning xylitol-sensitive MS. The second aim was to adopt the simplified method in a field trial designed to investigate the effect of xylitol on saliva during a 4-week period of daily chewing on pellets with a total dose of 6.2 g xylitol and follow-up the findings 6 months after termination of the intervention. The null hypothesis was that at the follow-ups there would be no difference from baseline in either of the groups.

MATERIALS AND METHODS

Study groups

The investigation was approved by the regional ethical review board in Umeå, Sweden. The pilot group consisted of 15 healthy non-medicating adults (24–39 years), all employees at the University that voluntarily donated saliva samples. All subjects exhibited a flow rate within normal levels and had detectable levels of salivary MS. The use of xylitol-containing products ranged from never to several times daily.

The main study group comprised 109 children (48 boys, 61 girls). All children were pupils in grades 1–6 at a comprehensive school in a small municipality in northern Sweden. Exclusion criteria were severe allergies or chronic disease with regular drug medication. Informed consent was collected from the children (mean age 9.9 ± 1.7 years) as well as from their parents. The school staff were also informed on the purpose and performance of the project. All children had received a regular preventive-oriented dental care since the age of 3 years at the local Public Dental Clinic and the participants claimed tooth brushing with fluoridated toothpaste at least once daily. The natural fluoride content in the piped drinking water was low (<0.3 ppm F). None of the children were habitual consumers of xylitol- or sorbitol-containing products but all of them had occasional or infrequent intakes.

Study design

Pilot study
In a cross-sectional design, paraffin-stimulated whole saliva samples were collected after a thorough mouthrinse with water approximately 3 hours after breakfast. The samples were immediately handled for determination of X^S MS and uptake of radio-labelled xylitol as described below.

Main study
The study had a randomised double-blind prospective design with two parallel arms. With aid of a computer program, the randomisation was carried out on an individual basis. The participants were instructed to chew on gums sweetened by either sorbitol/maltitol or xylitol three times a day for a period of 4 weeks. Paraffin-stimulated saliva samples were collected at baseline and after 4 weeks and 6 months, respectively. The outcome measures were total viable counts in saliva, the proportion of MS and salivary uptake of radio-labelled xylitol.

Chewing gums and regimen

The test and control gums were produced and supplied by Fennobon Oy (Karkkila, Finland). The ingredients of the control chewing gum A were: sorbitol (63.5% [w/w]), gum base, maltitol (4.5% [w/w]), flavours, humectant (E322), emulsifier (E422), artificial sweetener (E950), food colour (E171), acidity regulator (E296) and glazing agents (E903, E901, E904). The mean weight of one pellet of chewing gum A was

1.08 g, of which sorbitol and maltitol constituted 0.69 g and 0.05 g respectively. The test chewing gum B contained: xylitol (77.0% [w/w]), gum base, flavours, gum arabic, humectant (E322), emulsifier (E422), acidity regulator (E296) and glazing agents (E903, E901, E904). Xylitol was the single sweetener with one pellet of 1.34 g delivering 1.03 g of the polyol. The children were instructed to chew 2 pellets for 10 minutes 3 times a day, after breakfast and tooth brushing, after lunch (weekdays, in school) and in the evening after dinner. The test and the control gums were similar in form and colour and packed in identical boxes and coded as 'A' or 'B'. The code was sealed by an independent monitor and not broken until the statistical calculations were finalised.

Clinical procedures

At baseline, the children were clinically examined in the classroom with the aid of a mirror, a probe and a strong torch. The presence of decayed, missed and filled surfaces was scored in the permanent (DMFS) as well as in the primary (dmfs) dentition according to the World Health Organization (1987). No radiographs were exposed. Thereafter, a sample of stimulated whole saliva was collected by chewing one piece of paraffin (1.0 g) until at least 3 ml of saliva was collected. The stimulated saliva secretion rate was expressed as ml/minute and the samples were immediately transferred to the laboratory for further analyses.

After the clinical examinations, the oral hygiene habits were reinforced and all children received a new toothbrush together with a tube with fluoridated dentifrice. Immediately after the 4-week intervention period, a second sample of stimulated saliva was collected and a third sample was obtained 6 months after the end of the intervention period.

Laboratory and microbial procedures

To determine the bacterial counts, 1.0 ml of saliva was serially diluted in M-DIL (for 500 ml solution: 4.3 g NaCl, 0.42 g KCl, 1.0 g Na_2HPO_4 x $2H_2O$, 1.0 g KH_2PO_4, 10.0 g sodium glycerophosphate x H_2O, 0.1 g $MgCl_2$ x 6 H_2O, 500 ml distilled water) to obtain 5, 200, 4000 and 40,000 times dilutions. Aliquots (50 µl) of the 5x and 200x saliva suspensions were placed on a selective MSB agar plate for determination of the MS counts (Gold et al, 1973), while 50 µl aliquots of the 4000x and 40,000x suspensions were transferred to blood agar plates (Blood Agar Oral) for estimation of the total viable counts (TVC). All plates were cultivated at 37°C in a micro-aerophilic environment in 5% CO_2 for 48 h. The number of colony-forming units (CFU) were counted in a stereo-microscope and expressed as CFU/ml.

In order to quantify xylitol uptake, 1.0 ml of saliva and 10 µl of a radio-labelled isotope (ARC 1744 Xylitol, D [1-^{14}C] conc. 0.1 mCi/ml, Larodan Fine Chemicals AB, Malmö, Sweden) were incubated together for 60 min at 37°C. After centrifugation (13,200 rpm for 10 min), the supernatant was carefully separated from the pellet and both were kept frozen until further processing. For the analyses, the thawed pellet was suspended in 100 µl MQ water (ultra-filtrated water, Milli-Q Biocel, Millipore AB, Sundbyberg, Sweden) and placed into a scintillation tube while 100 µl of the supernatant was placed directly into the tubes. Liquid scintillation cocktail (5 ml; Ready Safe, Beckman, Sweden) was added and the disintegration was counted in a β-counter (Rackbeta, LKB Wallace 1214, Turku, Finland) for one minute and expressed as counts per minute (CPM). The xylitol uptake in saliva was calculated as the ratio between the CPM in the pellet and the combined supernatant/pellet counts, expressed as per cent and scored into three categories: 1 = >50%; 2 = 26–50%; 3 = 0–25%.

The quantification of X^S MS was carried out with the autoradiography method described by Dréan and Trahan (1990) with some minor modifications (Stecksén-Blicks et al, 2004). The following X^S MS scores were used: 1 = 76–100%, 2 = 50–75%, 3 = <50%.

Statistical methods

All data were processed by the SPSS software (12.0, SPSS Inc., Chicago Ill, USA). In the pilot study, the scores of the two assays were compared with a chi-square test. In the field study, bacterial and biochemical data were subjected to analysis of variance or Student's *t*-test. A p-value less than 0.05 was considered as statistically significant.

RESULTS

The relationship between the scores of xylitol-sensitive MS and salivary [^{14}C]-xylitol uptake as obtained in the cross-sectional pilot study is shown in Table 1. In general, higher percentage values were found with the specific MS method but a fair statistically significant positive correlation ($p < 0.05$) between the scores was found (contingency coefficient = 0.62).

Table 1 Distribution of xylitol-sensitive mutans streptococci (X^S MS %) in relation to the proportion of salivary uptake of [^{14}C]-xylitol (n = 15)

Simplified method	score 1 (76–100%)	score 2 (50–75%)	score 3 (<50%)
Score 1 (>50%)	2	2	-
Score 2 (26–50%)	-	5	-
Score 3 (0–25%)	1	2	3

Contingency coefficient 0.622; $p < 0.05$ (Chi-square test)

Table 2 Salivary mutans streptococci and total viable count (CFU/ml, mean ± SD, log10) at baseline, after a 4-week period of daily chewing on xylitol-containing gums (group B; 6.2 g/day) or sorbitol/maltitol control gums (group A) and 6 months after the intervention period

Time	Control group (A; n = 54) mean log10 ± SD	Test group (B; n = 55) mean log10 ± SD
TVC		
Baseline	6.1 ± 0.4	6.1 ± 0.4
4 weeks	6.1 ± 0.5	6.1 ± 0.5
6 months	6.2 ± 0.4	6.4 ± 0.3
MS		
Baseline	3.1 ± 1.6	3.1 ± 1.6
4 weeks	3.0 ± 1.5[b]	2.6 ± 1.7[ab]
6 months	3.3 ± 1.4	3.1 ± 1.6

[a] statistically significant difference compared to baseline ($p < 0.05$)
[b] statistically significant difference compared to baseline ($p < 0.05$)

The main study was fulfilled by all 109 participants and no side effects or adverse events were reported. The mean caries prevalence (DMFS+dmfs) for the total study group was 1.2 ± 1.7 and the baseline salivary secretion rate varied from 0.5 to 2.1 ml/min. The majority (75%) had low and medium levels of salivary MS at baseline and only 9% exhibited high counts (>100.000 CFU/ml saliva). The mean values (log10) of salivary MS and TVC are presented in Table 2 and the proportion of original mean values of MS in relation to TVC is shown in Fig 1. No differences between the groups were seen at baseline. In the xylitol group, the mean proportion dropped from 1.2% at baseline to 0.4% after 4 weeks ($p = 0.01$). After 6 months, the mean proportion increased again to 0.7%, which was not significantly different from baseline. The MS/TVC-ratio differed also between groups A and B after 4 weeks in a statistically significant way ($p < 0.05$). The salivary uptake of [^{14}C]-xylitol expressed as percent and scores is given in Table 3 and Fig 2, respectively. The baseline mean values were similar in the test and control groups, 31.4% vs. 27.4%. After 4 weeks, a significant decrease ($p < 0.05$) compared with baseline was disclosed in the xylitol group but not in the sorbitol/maltitol control group. Six months after the termination of the intervention, the proportion of salivary xylitol uptake exceeded the values obtained at baseline although not in a statistically significant way.

DISCUSSION

In an earlier paper from our group, the proportion of X^S MS was determined with aid of autoradiography (Stecksén-Blicks et al, 2004). In the present study, we evaluated a simple way of estimating the proportion of bacteria with ability to take up and harbour intracellular xylitol, fully aware of the fact that there are other possible ways for xylitol to remain in the saliva. For example, xylitol is hydrophilic and may compete with water molecules for the hydration layers that surround protein molecules in biological environments. Further-

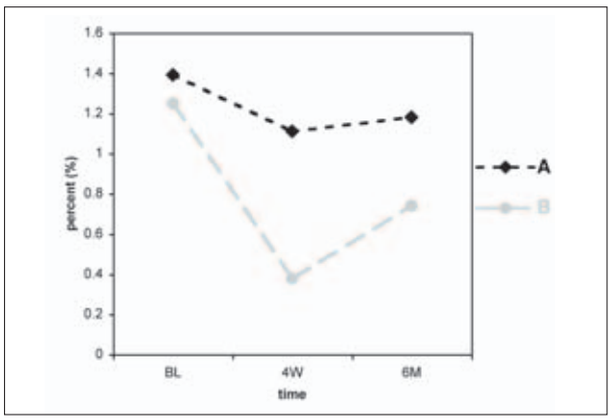

Fig 1 Proportion of salivary mutans streptococci (MS) in relation to the total viable counts (TVC) at baseline, after 4 weeks of daily chewing on xylitol-containing gums (group B) or sorbitol/maltitol control gums (group A) and 6 months after the intervention period (calculated on the original mean values of MS and TVC). One outlier was excluded due to an unrealistic high 4-week value (>25%). There were significant differences between group A and B after 4 weeks ($p < 0.05$) and between baseline and 4 weeks in group B ($p < 0.05$).

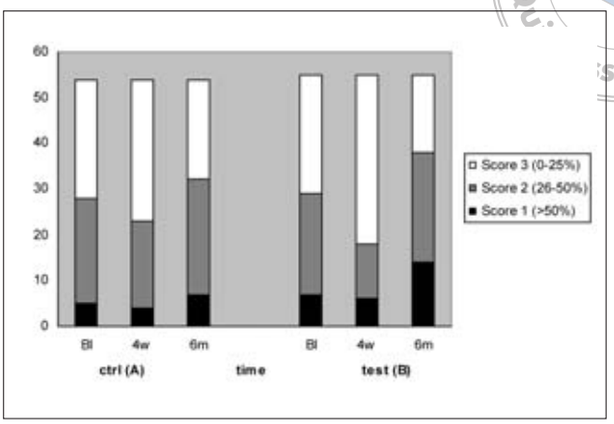

Fig 2 Salivary uptake of [14C]-xylitol at baseline, after 4 weeks of daily chewing on xylitol chewing gums (test) or sorbitol/maltitol gums (control) and 6 months after the intervention period. The uptake was scored into three categories: 1 = >50%; 2 = 26–50%; 3 = 0–25%.

Table 3 Proportion of salivary uptake of xylitol at baseline, after a 4-week period of daily chewing on xylitol-containing gums (group B; 6.2 g/day) or sorbitol/maltitol control gums (group A) and 6 months after the intervention period

Time	Control group (A; n = 54)			Test group (B; n = 55)		
	mean ± SD	median	range	mean ± SD	median	range
Baseline	27.5% ± 17.5	25.6%	62.6%	31.4% ± 19.2	29.8%	61.2%
4 weeks	22.7% ± 17.1	21.8%	62.0%	20.1% ± 17.9[a]	14.0%[a]	64.4%
6 months	31.8% ± 15.2	32.3%	57.5%	34.1% ± 17.9	36.9%	63.9%

[a] significantly different from baseline, $p < 0.05$ (Students t-test)

more, xylitol may form complexes with inorganic ions such as Ca^{2+}, thus stabilising the calcium phosphates in saliva (Mäkinen and Söderling, 1984). The thinking was that this assay possibly could be useful as a 'surrogate' measure of anticipated xylitol-induced alterations of the salivary microflora. The theory behind was that fructose phosphotransferase-defect MS without capacity of the futile xylitol-circle may have an ecological advantage on behalf of xylitol-sensitive strains and consequently, increase their proportion in plaque and saliva during a period of regular exposure of xylitol. The advantages of the present assay were that it was rapid and eliminated elements of subjectivity in the intermediate step of MS colony identification. On the negative side, the obtained values displayed a considerable intra- and inter-individual variation, which in part could be dealt with through the scoring system. Although a fairly good association between the simple method and established assay was found, it should be emphasised that the two methods by no means should be compared with each other. Furthermore, it should be underlined that these measurements not were linked to any clinical event and that the clinical significance of a temporary alteration detected by any of the two methods still must be considered as speculative.

The main results of the study reinforced the findings of several recent publications that xylitol can affect oral ecology (Söderling et al, 1989; Roberts et al,

2002; Ly et al, 2006; Milgrom et al, 2006). The null hypothesis was rejected, as a relatively high daily intake of xylitol decreased the proportion of salivary MS temporarily without affecting the TVC in a significant way, and this decrease was mirrored by a similar event concerning xylitol uptake by inorganic/organic material in saliva and salivary microorganisms as assessed with the novel method. A 'high daily dose' was in this context a dose exceeding 5 g/day since earlier clinical studies collectively have indicated that this is a threshold-value for gaining an anti-cariogenic effect from xylitol (Ly et al, 2006; Milgrom et al, 2006). In fact, Milgrom and coworkers (2006) have recently demonstrated a dose-response relationship for daily doses below 6 g followed by a plateau effect between 6 g and 10 g. The higher the amount of xylitol in a single dose, the higher is the risk for gastric upset and the fractioned intake is a way to minimise the risk of side effects. On the other hand, the number of intakes must be balanced towards the willingness to comply with the regimen and the administrations of gums three times a day in this trial seemed to be satisfying since we experienced a good compliance and no adverse reactions.

The significant difference between the test and control groups concerning the proportion of MS obtained after 4 weeks was in harmony with several previous studies (Loesche et al, 1984; Mäkinen et al, 1989; Söderling et al, 1989; Trahan, 1995; Roberts et al, 2002), indicating a small advantage of xylitol over sorbitol/maltitol. It is well known that sorbitol also may affect oral streptococci by hampering its growth and metabolism (Birkhed et al, 1984) but the use of sorbitol/maltitol in the control gums was a prerequisite for the double-blind design and a necessity to secure the compliance as the children in both groups were chewing at the same time at school. For those reasons and the limited amount of children available in the selected school, it was not possible to include a 'true' control group that chewed neutral non-sweetened gum base.

A notable finding was that all outcome measures were similar to baseline 6 months after the interventions. Thus, the results did not indicate a long-term or persisting effect, a fact that has been observed in previous trials (Trahan et al, 1992; Isokangas et al, 1993). The diverging findings can probably be explained by the relatively short duration of xylitol exposure in the present trial. It is possible that the exposure must be prolonged for months or years in order to get a permanent bacterial shift and stable microbial community and a clinical consequence would be that xylitol-containing products must be ingested more or less continuously. The finding of reduced bacterial xylitol uptake in the xylitol group was interesting and in harmony with the assumption that a shift to a more xylitol-resistant bacterial flora may have occurred (Dréan and Trahan, 1990). As the children in this study were non-habitual consumers of xylitol and probably were colonised with a predominantly xylitol-sensitive oral flora at baseline, the present results may indicate that xylitol-resistant strains had an ecological advantage during the intervention period. Such strains are suggested to be less adhesive (Trahan et al, 1992), which may be one factor behind the diminished plaque levels that commonly have been demonstrated in connection with xylitol gum use (Söderling et al, 1989; Trahan, 1995). One should, however, keep in mind that the present children had generally low caries prevalence and that the findings may not be valid for children with very high counts of MS and/or high caries activity.

In conclusion, a one-month daily use of xylitol-containing gums may interfere with the microbial composition and decrease the proportion of MS. The paper supports that something happens with the salivary C^{14}-uptake during habitual xylitol consumption and we have tried to describe this event as precisely and neutrally as possible. These findings should not be over-interpreted, especially not from a clinical point of view, until further investigations have been carried out.

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