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Comparison of Antimicrobial Effects of Stevia Rebaudiana Extract and Xylitol on Dental Biofilm: An In Vitro Study

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Objectives This study aimed to assess the antibacterial effects of xylitol and Stevia rebaudiana (S. rebaudiana) ethanolic extract on oral biofilm.

Methods A total of 96 acrylic discs were divided into two main groups for inoculation with Streptococcus mutans (S. mutans) and Streptococcus sobrinus (S. sobrinus). Each group consisted of 6 subgroups including a positive control subgroup and 5 subgroups of discs immersed in 1% or 3% xylitol solutions, 2 or 4 mg/mL S. rebaudiana, or a combination of 3% xylitol and 4 mg/mL S. rebaudiana. After incubation, the discs were rinsed and transferred to fluid universal medium. The solutions were cultured on specific culture media and incubated. The colony-forming units (CFUs) were counted for each disc. The structure of biofilm in each group was evaluated under a scanning electron microscope (SEM).

Results ANOVA revealed significant differences between the subgroups in both S. mutans and S. sobrinus groups (P=0.03 and P=0.01, respectively). In S. mutans group, the logarithmic mean of colony count in the positive control subgroup was 6.75 while this value was significantly lower in 2 mg/mL (5.81) and 4mg/mL (5.92) S. rebaudiana subgroups using the post hoc Dunnett's test (P=0.01 and P=0.04, respectively). The three other subgroups did not show significant differences. In S. sobrinus group, all five experimental subgroups demonstrated significantly lower colony count than the positive control group (P<0.05).

Conclusion S. rebaudiana extract appears to be more potent than xylitol against dental biofilm.

Keywords Stevia; Xylitol; Biofilms; Streptococcus sobrinus; Streptococcus mutans

Introduction

Bacterial plaque is a yellow, sticky biofilm on tooth surfaces and is formed by the accumulation of microorganisms (mainly bacteria) entrapped in a polymer matrix derived from the saliva. Bacterial plaque plays a primary role in the pathogenesis of caries and periodontal disease.[1] Tooth decay is an inflammatory process with a bacterial origin that results in demineralization and destruction of enamel, dentin, and cementum.[2]

The ability of microorganisms to attach to biomaterials and form biofilm is among the main causes of human infections.[1,2] Streptococcus mutans (S. mutans), Streptococcus sobrinus (S. sobrinus), and lactobacilli are mainly responsible for tooth decay.[3] These bacteria produce acids in presence of carbohydrates such as glucose, sucrose, and fructose, and accelerate the process of demineralization and its dominance over the remineralization process.[4]

Microbial adhesion to tooth surfaces is a prerequisite for bacterial colonization, and is the first step in development of infections. Complex mechanisms are involved in prevention of microbial colonization in the oral cavity. Several methods have been used to discontinue this process and prevent dental caries such as following a low-carbohydrate diet, use of calcium and fluoride, sealants, xylitol, and antibacterial agents such as chlorhexidine mouthwash, which is the most commonly used mouthwash.[5-8] Extensive studies have evaluated the preventive and therapeutic effects of xylitol on tooth caries and have confirmed its antimicrobial efficacy against dental pathogens and its lack of effect on dental biofilm.[7, 9, 10]

Scientists have been in search of new medicinal plants with pharmaceutical and antimicrobial properties. Herbal medicines have a long history. Although a significant portion of medications available in the market is synthetic, one-third of pharmaceutical products are believed to have a plant origin or have been modified after extraction from plants.[11]

According to the World Health Organization, 80% of people living in developing countries believe that medicinal plants are beneficial for primary care.[12] The antimicrobial and pharmaceutical effects of Stevia rebaudiana (S. rebaudiana) have been confirmed. It is commercially used as an herbal sweetener for diabetic patients. It has no adverse effect on normal cells but its anti-tumor and anti-proliferative effects on cancer cells have been well documented, which are attributed to its antioxidant properties.[13] S. rebaudiana has extensive antimicrobial properties and a naturally sweet taste. Given that its antimicrobial effects on dental pathogens are confirmed, it may be used as a substitute for cariogenic carbohydrates. Considering the high prevalence of dental caries worldwide

(30.2%) and the related financial and mental burden of tooth decay, the need for more efficient preventive strategies is obvious.[14] Recently, an increasing number of studies have been investigating the anti-caries effects of S. rebaudiana with few of them investigating this effect on simulated dental biofilm.[15-18] All these studies focused on S. mutans biofilms and no study investigated these effects on S. sobrinus as another significant cause of dental caries. Also, only one study compared the anti-biofilm effects of the recently known S. rebaudiana and the well-known xylitol.[18] Thus, this study sought to assess the antimicrobial effects of S. rebaudiana extract on dental biofilm in comparison with the effects of xylitol.

Materials and Methods

This in vitro study was conducted using the Guggenheim's model of dental biofilm formation, which has been used in a similar previous study.[19]

Preparation of S. rebaudiana extract:

Ten flowerpots of S. rebaudiana were purchased from a medicinal plant farm near Tehran. Fresh leaves were separated, washed with water, and dried at room temperature without exposure to sunlight, and were then ground. Ethanolic extract of the plant was prepared in the Medicinal Plants and Drug Research Institute of Shahid Beheshti University of Medical Sciences. Extraction was done using the maceration technique; 50 g of the plant leaves were powdered and immersed in 500 mL of 96% ethanol for 48 h. The obtained extract was paper-filtered and the solvent was vaporized using a rotary distiller (Heidolph, Germany) at 40°C. The solvent was added again to the plant and immersion was continued for 2 more days. All the above-mentioned phases were repeated in triplicate.[20]

Culture, activation, and storage of microorganisms:

All steps of the culture and microbiological assessments were done in the Microbiology Laboratory of Shahid Beheshti University, School of Dentistry. Microbial strains including S. mutans (ATCC 35608) and S. sobrinus (ATCC 27607) were purchased as lyophilized vials from the Center of Industrial and Medical Fungi and Bacteria Collection, Tehran, Iran. To activate strains, vials were opened as instructed aseptically and their contents were transferred to a test tube containing liquid brain heart broth medium for common oral microorganisms (Merck, Germany). The tubes were closed with cotton wool plugs and incubated at 37°C (Memmert, Germany) for 24 h for the microorganisms to proliferate. After 24 h, the microbial suspension was cultured on plates containing a nutritive solid brain heart agar medium for common oral bacteria (Merck, Germany) using a sterile swab. The plates were then incubated at 30°C for 24 h for the colonies to form. The formed colonies were used for the preparation of microbial suspension in the next steps.

Saliva collection:

Paraffin stimulated human saliva samples were collected in several days at least 1.5 h after eating, drinking, or tooth brushing for 1 hour/day from 10 adults with a mean age of 25 years with no known infectious oral disease or immunosuppression. The collected samples were stored at -20°C and when a volume of 200 cc was reached, the samples were centrifuged for 30 min at 4°C at 2700 rpm. The supernatant was pasteurized for 30 min at 60°C and centrifuged again. The final supernatant was stored in 50 cc tubes at -20°C.[19]

Preparation of discs:

Acrylic discs measuring 10 mm in diameter and 1.3 mm in thickness (MeadWay Dental, UK) were fabricated in Teflon molds and used as substrates. After fabrication, acrylic discs were autoclaved at 120°C for 20 min. A total of 96 discs were fabricated.

Preparation of biofilm:

To form an acquired pellicle, discs were immersed in pasteurized saliva for 4 h and shook. The saliva was then extracted and each disc was soaked in a mixture of 200 µL saliva and 200 µL brain heart infusion broth containing 4% sucrose in such a way that the final concentration of sucrose on each disc was 0.075%. Then, 50 µL of S. mutans culture medium was added to one group and the S. sobrinus culture medium was added to another group. Each group of discs containing microorganisms was divided into 6 subgroups: One group without any exposure to xylitol or S. rebaudiana as the positive control group (C+), two groups of discs exposed to S. rebaudiana ethanolic extract at 2 and 4 mg/mL concentrations, two groups of discs exposed to xylitol at 1 and 3% concentrations (Sigma, Germany) and a group exposed to the mixture of 3% xylitol and 4 mg/mL S. rebaudiana extract to investigate the possible synergistic effects of these two agents. For each S. mutans or S. sobrinus arms of the study, one disc was immersed in 200 µL of culture medium and 200 µL of sterile saliva, and it was considered as the negative control for each group to ensure the sterility of the process before the biofilm formation. Figure 1 shows the grouping structure of the study. Discs were anaerobically incubated for 16 h. After incubation, discs were gently transferred to another 24-well plate (Greiner Bio-One, Germany) and rinsed with saline to eliminate the bacteria not participating in the biofilm structure on the discs. The discs were then transferred from the 24-well plate to test tubes containing 0.9% saline one by one for the dilution process, and the test tubes containing discs were vortexed for 1 min. Using a blue point sampler (Socortex, Switzerland), 1000 µL of the solution in tubes was transferred to another tube containing 9 mL saline. This process was repeated 5 times for each disc-containing tube. Biofilm-containing solutions were diluted to 10-5 and were spirally cultured on specific culture media. Culture media containing S. mutans and S. sobrinus were incubated anaerobically at 37°C for 24 h and then colony-forming units (CFUs) were counted by a colony counter (Biocompare, USA) and recorded. Of each group, one disc

was evaluated under a scanning electron microscope (SEM) to assess the biofilm structure.

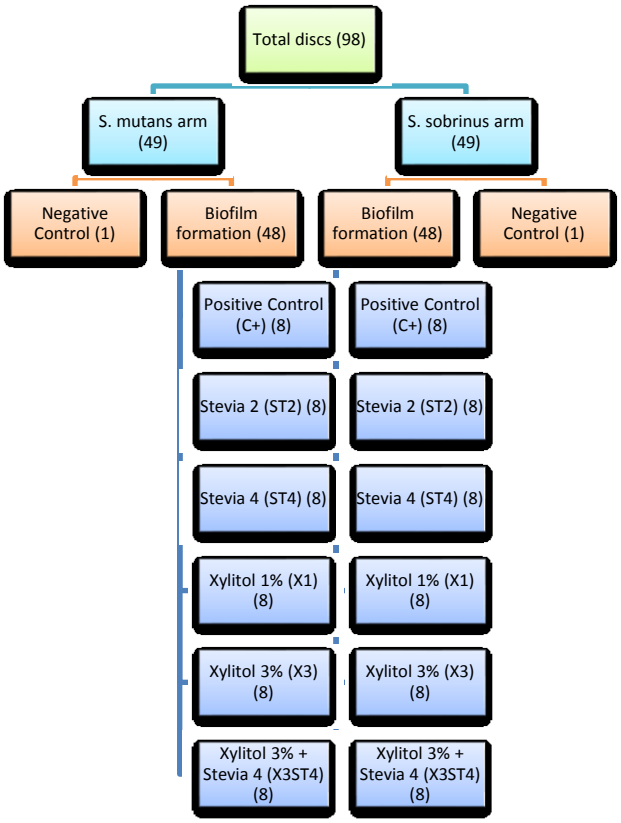

Figure 1- Diagram showing the grouping structure of the study and sample size of each group

Fixation of discs for evaluation under SEM:

Of each group, one specimen was prepared for observation under a SEM as described earlier. Discs were soaked in xylitol and S. rebaudiana extract at the respective concentrations and after 24 h of incubation, solution in the plate was extracted and discs were washed with phosphate-buffered saline (added and immediately removed). Next, 500 µL of 2% glutaraldehyde was added to each disc and refrigerated for 24 h. Next, 2% glutaraldehyde was extracted and the specimens were washed with deionized water. Discs were then immersed in 30, 50, 70 and 80% concentrations of alcohol for 10 min followed by 90% alcohol for 20 min and 100% alcohol for 20 min twice, and the plates were stored under the laminar hood for 24 h for the specimens to completely dry. Discs were placed on stubs and gold sputter-coated in 10 µm thickness. Specimens were evaluated under a SEM (Vega; Tescan, Check Republic) using the secondary electron mode at 12 kV voltage and x10,000 and x20,000 magnifications.

A total of 96 discs (48 for S. mutans and 48 for S. sobrinus) were analyzed. Colony counts of S. mutans and S. sobrinus in the control groups without any antibacterial agent (C+), 1% xylitol (X1), 3% xylitol (X3), 2 mg/mL S. rebaudiana extract (ST2), 4 mg/mL S. rebaudiana extract (ST4) and mixture of 3% xylitol and 4 mg/mL S. rebaudiana extract (X3ST4) were analyzed using SPSS version 22. Eight specimens were evaluated in each group and to facilitate calculations, log transformation of data was performed. Normal distribution of data was ensured using the Shapiro-Wilk test. The equality of variances was confirmed by the Levene's test. One-way ANOVA was used to compare the antibacterial property of materials and the Dunnett's t-test was used as the post hoc test to compare each experimental group with the positive control group.

Results

A summary of descriptive statistical parameters for colony counts of each experimental subgroup is demonstrated separately for S. aureus and S. sobrinus in Table 1.

Table 1- Summary of descriptive statistical parameters for colony counts of each microorganism in the experimental subgroups

	Sub-group	Mean	Median	Min	Max	Std. deviation
S. mutans	C+	6.75	6.94	5.09	7.82	1.03
	ST2	5.81	5.78	5.30	6.80	0.44
	ST4	5.92	5.72	5.52	6.79	0.50
	X1	6.46	6.39	5.67	7.96	0.73
	X3	6.48	6.56	5.96	6.93	0.34
	X3ST4	6.40	6.53	5.88	6.97	0.39
S. sobrinus	C+	5.85	5.88	5.53	5.98	0.15
	ST2	5.45	5.49	4.88	5.94	0.32
	ST4	5.43	5.42	4.90	5.73	0.27
	X1	5.50	5.49	5.32	5.80	0.15
	X3	5.51	5.54	5.00	5.79	0.23
	X3ST4	5.41	5.37	5.00	5.93	0.30

C+: Control positive; ST2: Stevia 2mg/ml; ST4: Stevia 4mg/ml; X1: Xylitol 1%; X3: Xylitol 3%; X3ST4: Combination of Xylitol 3% and Stevia 4mg/ml

S. mutans:

One-way ANOVA demonstrated significant differences between the subgroups for S. mutans colony counts (P=0.03). Using Dunnett's t-test as the post hoc multiple comparisons test, each of the 5 experimental groups was compared with the positive control group. Based on the results, 2 mg/mL S. rebaudiana (ST2) and 4 mg/mL S. rebaudiana (ST4) resulted in a significant reduction in S. mutans biofilm (P=0.018 and P=0.043, respectively) while 1% xylitol (X1), 3% xylitol (X3), and combination of 3% xylitol and 4 mg/mL S. rebaudiana (X3ST4) resulted in relative reduction in S. mutans count compared with the control group (Figure 2), which was not statistically significant (Table 2).

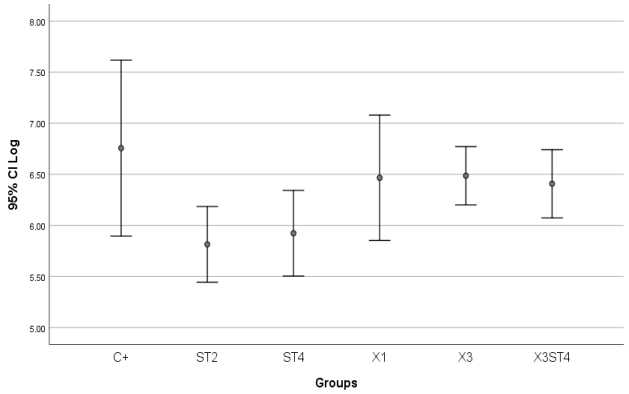

Figure 2- Comparison of the logarithmic means of S. mutans counts and 95% confidence interval for each subgroup

S. sobrinus:

A significant difference was also seen between the colony

counts within the experimental subgroups of S. sobrinus using one-way ANOVA (P=0.01). Using Dunnett's test, comparisons were made between each experimental group and the positive control group, which revealed that ST2, ST, X1 and X3 (P=0.013, P=0.009, P=0.034, and P=0.043, respectively) significantly inhibited biofilm formation. Also, X3ST4 showed significant synergistic effects of 3% xylitol and 4 mg/mL S. rebaudiana on biofilm inhibition (P=0.005, Table 2). The data demonstrated that both xylitol and S. rebaudiana had anti-biofilm effects on S. sobrinus and the combination of them had a synergistic effect (Figure 3).

Table 2- Results of statistical analysis comparing colony counts for each experimental subgroup with the positive control group of the related microorganism.

	Control Group (B)	Experimental Group (A)	Mean Difference (A-B)	Std. Error	Sig.
S. mutans	C+	ST2	-.9419	.31120	.018*
	C+	ST4	-.8331	.31120	.043*
	C+	X1	-.2902	.31120	.817
	C+	X3	-.2705	.31120	.853
	C+	X3ST4	-.3488	.31120	.692
S. sobrinus	C+	ST2	-.3963	.12522	.013*
	C+	ST4	-.4106	.12522	.009*
	C+	X1	-.3469	.12522	.034*
	C+	X3	-.3351	.12522	.043*
	C+	X3ST4	-.4344	.12522	.005*

C+: Control positive; ST2: Stevia 2mg/ml; ST4: Stevia 4mg/ml; X1: Xylitol 1%; X3: Xylitol 3%; X3ST4: Combination of Xylitol 3% and Stevia 4mg/ml

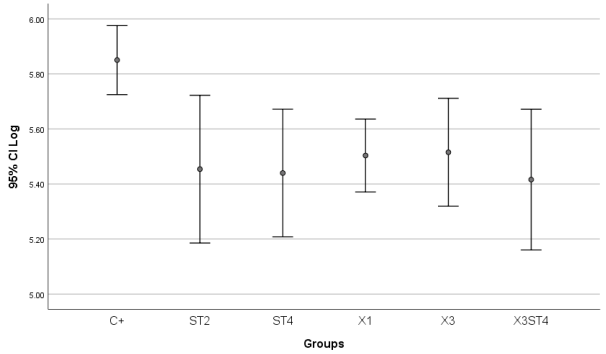

Figure 3- Comparison of the logarithmic means of S. sobrinus counts and 95% confidence interval for each subgroup

SEM results:

Negative control discs, exposed to saliva only, were completely free from bacteria and only the porous disc surface was observed (Figures 4A, 5A). On S. mutans positive control discs (no exposure to xylitol or S. rebaudiana), classic biofilm was seen as aggregates of cocci in extracellular polysaccharide (EPS) along with water channels (Figure 4B). In discs exposed to 1% xylitol, no significant change was noted in biofilm structure in terms of bacterial count, water channels, or EPS (Figure 4C). In discs exposed to 3% xylitol, EPS was more compact and larger than that in the control and 1% xylitol groups (Figure 4D). In discs exposed to S. rebaudiana at 2 and 4 mg/mL concentrations, a significant change in biofilm structure was noted. The bacterial arrangement was filamentous rather than compact, and EPS around the bacteria had significantly decreased and was almost not visible. Structured water channels were not seen (Figures 4E, 4F).

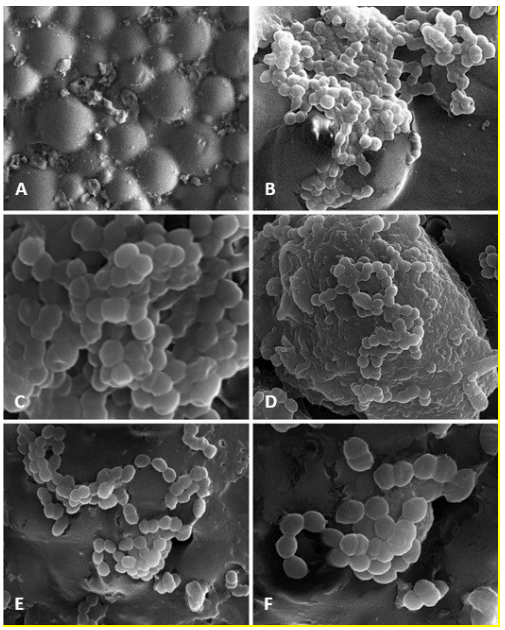

Figure 4- SEM micrograph of negative control discs (A), S. mutans positive control discs (B) and S. mutans discs exposed to 1% xylitol (C), 3% xylitol (D), 2 mg/mL Stevia (E), and 4 mg/mL Stevia (F).

In S. sobrinus positive control discs (no exposure to xylitol or Stevia), classic compact biofilm structure with significant amounts of EPS and numerous water channels were seen (Figure 5B). On discs exposed to 1% and 3% xylitol, bacteria were less compact but showed a similar structure in terms of water channels and EPS to that in the control group. Also, discs exposed to 1% xylitol had almost similar bacterial count to discs exposed to 3% xylitol (Figures 5C, 5D). However, discs exposed to S. rebaudiana extract showed a completely different structure. At x20,000 magnification, limited islets of bacteria along with scarce EPS and no water channels were seen, which was totally different from the structure seen in the xylitol group and control discs (Figures 5E, 5F).

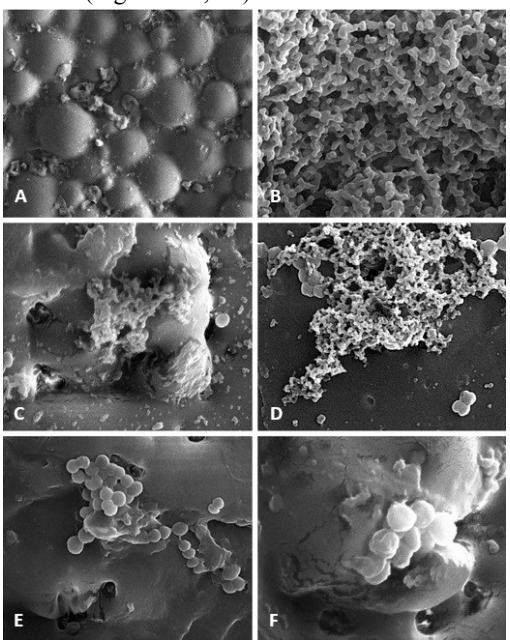

Figure 5. SEM micrograph of negative control discs (A), S. sobrinus positive control discs (B) and S. sobrinus discs exposed to 1% xylitol (C), 3% xylitol (D), 2 mg/mL Stevia (E), and 4 mg/mL Stevia (F).

Discussion

Several studies have assessed the effects of artificial sweeteners and sugar substitutes on oral bacterial counts and dental biofilm. But no consensus has been reached on their anti-biofilm effects. These studies have mainly focused on clinical or laboratory use of xylitol and its effects on S. mutans. On the other hand, recent studies have evaluated the antibacterial effects of S. rebaudiana extract, with a handful of them paying attention to its anti-biofilm properties.[15-18]

In the current study, we demonstrated the significant anti-biofilm activity of S. rebaudiana extract on both S. mutans and S. sobrinus biofilms. We also compared this potential activity to the anti-biofilm characteristics of xylitol and demonstrated the relative superiority of S. rebaudiana extract over xylitol in this regard.

Effects of xylitol and S. rebaudiana on S. mutans biofilm:

The significant inhibitory effect of S. rebaudiana extract with both used concentrations on S. mutans biofilm was an interesting finding. There are few reports of potent inhibitory effects of S. rebaudiana extract on the planktonic form of S. mutans.[17, 21, 22] But, this cannot be necessarily translated to the optimal efficacy of S. rebaudiana extract on S. mutans biofilm, given the fact that bacterial microorganisms will benefit from structural resistance to anti-microbial agents in the form of biofilm. In an attempt to investigate the anti-biofilm activity of S. rebaudiana against multi-species dental biofilm, Abdul Razak et al. found that exposure of dental biofilm to S. rebaudiana extract can lighten the biofilm mass and reduce bacterial adherence to the biofilm.[15] Another study showed that lower concentrations of S. rebaudiana can have inhibitory effects on both biofilm and planktonic forms of S. mutans; but higher concentrations (up to 400 mg/mL) were not able to repress the planktonic form although the effect on biofilm was still present.[17] Therefore, lower concentrations of S. rebaudiana extract may be preferred as we used low concentrations in our study. Contradictory to our results, Kishta et al.[18] showed that low concentration of S. rebaudiana extract was as effective as xylitol in biofilm inhibition. This contradictory result may be due to the higher concentration of xylitol used in their study (5%) compared with ours (1% and 3%). This can also confirm the presence of various S. mutans strains with different genetic infrastructure related to resistance or sensitivity to anti-bacterial effects of xylitol.

As previously mentioned, xylitol had no significant inhibitory effect on S. mutans biofilm in our study. This finding is in line with the results of some previous studies.[23-26] Also, 3% xylitol and 4 mg/mL S. rebaudiana had no synergistic effect and their combination did not significantly inhibit the biofilm. In a study by Modesto and Drake[23], xylitol at 0.5% concentration did not inhibit S. mutans biofilm but a combination of xylitol and 0.12% chlorhexidine had a synergistic effect in this regard. Giersten et al.[24] showed that 7.5% xylitol did not inhibit the dental biofilm model of supragingival plaque comprising of 6 bacterial strains. Marttinen et al.[25] compared anti-biofilm effects of 5% xylitol on xylitol-sensitive and xylitol-resistant S. mutans strains and concluded that xylitol had an inhibitory effect on 8-h xylitol-sensitive streptococci but had no effect on 24-hour biofilm or biofilm of xylitol-resistant streptococci. In an in-depth study on this topic, Decker et al.[26] demonstrated that exposure to 1% xylitol did not inhibit 24-h S. mutans biofilm. Badet et al.[27] evaluated the anti-biofilm effects of 1% and 3% xylitol on multispecies biofilm consisted of S. mutans, S. sobrinus, and four other non-streptococcal species and demonstrated the significant inhibitory effect of xylitol on the biofilm formation; but their results, due to the mixed nature of experimental biofilm and presence of non-streptococcal species and even multiplicity of streptococcal strains, were not comparable to ours.

The reason for the lack of inhibitory effect of xylitol on S. mutans biofilm was thoroughly explained by Decker et al.[26] In their study, the number of CFUs, biofilm structure, the viability of microorganisms, glucose metabolism, cellular respiration, and expression of different genes were compared among the bacteria exposed to xylitol, exposed to sucrose and a glucose-exposed control group. They found no significant difference in terms of the number of CFUs, biofilm structure, rate of glucose consumption, viability of microorganisms, cellular respiration, and formation of extracellular matrix among the groups. But, groups exposed to xylitol or sucrose had significant differences with the glucose control group in terms of expression of genes. S. mutans has 4 groups of genes related to biofilm formation. Group 1 genes are responsible for microbial adhesion. The second group includes genes responsible for the synthesis of the extracellular matrix. The third and fourth groups include genes related to uptake of carbohydrates and acid resistance, respectively. In their study, the first three groups of genes were up-regulated in the xylitol group compared with the control group. In general, the majority of mechanisms responsible for cell resistance and biofilm formation were enhanced in presence of xylitol and thus, obviously, xylitol could not exert inhibitory effects on biofilm formation.[26] In another study in 2011 on Streptococcus pneumonia, it was shown that xylitol alone in the absence of glucose inhibited biofilm formation while simultaneous exposure to xylitol and glucose resulted in no such inhibition. Thus, it may be concluded that controversial results reported regarding the effects of xylitol on S. mutans biofilm might be related to the presence or absence of xylitol and glucose simultaneously in the environment. In our study, the presence of glucose in the culture media justifies the absence of the inhibitory effect of xylitol. It should be mentioned that simultaneous presence of different carbohydrates in the environment better simulates the oral environment.

In our study, 1% and 3% concentrations of xylitol were used, which was in accord with a study by Aires et al.[28] who showed that 10 min after the consumption of a material

containing xylitol, its salivary concentration reached 1%.

Statistical analysis found no dose-dependent difference between the effects of 1% and 3% xylitol, which confirms the results of previous studies. It should be noted that not observing the anti-biofilm effects of xylitol in the current study does not deny its confirmed antibacterial effects on S. mutans, and xylitol has a high inhibitory effect on the proliferation of streptococci outside the biofilm, which was not evaluated in this study.[27-29]

Effects of xylitol and S. rebaudiana extract on S. sobrinus biofilm:

The results of the current study showed that all concentrations of xylitol and S. rebaudiana significantly inhibited S. sobrinus biofilm (P=0.013 for 2 mg/mL S. rebaudiana and P=0.009 for 4 mg/mL S. rebaudiana, P=0.034 for 1% xylitol and P=0.043 for 3% xylitol). The inhibitory effect of S. rebaudiana was stronger than that of xylitol. These results are in accord with the findings of many previous studies on the effects of xylitol on S. sobrinus biofilm.[30-32] In the majority of studies, xylitol at different concentrations alone or in conjunction with other antibacterial agents had a significant inhibitory effect on S. sobrinus biofilm; and this finding was confirmed in our study. Lee et al.[34] evaluated the effects of xylitol alone and in combination with ribose on S. sobrinus biofilm and showed that xylitol alone had an inhibitory effect on biofilm and this effect was enhanced in presence of ribose. In another study, Zou et al.[33] showed that the same was also true for xylitol and ursolic acid.

Pihlanto-Leppälä et al.[32] reported that uptake of xylitol by S. sobrinus following its addition to the culture medium decreased over time and it served as a negative feedback response to penetration of xylitol into the bacterial cells. In our study, xylitol or S. rebaudiana were added to cultures from the beginning and the biofilm formation process occurred in their presence; thus, this possible negative feedback response could not prevent the anti-biofilm effects of xylitol. S. sobrinus similar to S. mutans has xylitol-sensitive and xylitol-resistant strains. Based on the results, the strains used in our study were xylitol-sensitive.

Investigations related to the antibacterial effect of S. rebaudiana extract on S. sobrinus available in the literature are limited to the planktonic form of the bacteria. In an attempt to find the minimum inhibitory concentration of different types of S. rebaudiana extracts on different bacterial strains, Gamboa et al. reported that all types of S. rebaudiana extracts including ethanolic, methanolic, and hexane extracts were almost equally effective in reducing the growth of S. sobrinus planktonic from.[22] Our study showed considerably stronger effects of S. rebaudiana on S. sobrinus biofilm compared with xylitol; which can show the superiority of S. rebaudiana to xylitol. Considering the lack of a similar study on the effect of S. rebaudiana on S. sobrinus biofilm, this result cannot be confirmed or refuted, and future studies are required to better elucidate this subject.

Another important finding of the current study was the synergistic effect of S. rebaudiana and xylitol since the combination of the two had a more significant inhibitory effect on biofilm. This finding along with the SEM findings regarding the discs exposed to S. rebaudiana or xylitol indicates different mechanisms of action of these two agents on the biofilm and that they exert a more significant inhibitory effect when combined.

Effects of xylitol and S. rebaudiana on the biofilm structure:

SEM results in our study were in line with the results of bacterial colony counts in biofilms. S. mutans biofilm exposed to both concentrations of S. rebaudiana showed a significantly changed structure and loss of typical pattern, indicating the effect of S. rebaudiana on the reduction of EPS and destruction of the regular orientation of bacteria around water channels. Also, decreased bacterial concentration was in line with the results of colony counting. Discs exposed to 1% xylitol were not significantly different from controls while discs exposed to 3% xylitol showed higher concentration of bacteria compared with the control group. This finding confirms the results of studies that reported no significant effect of xylitol on the mature S. mutans biofilm.[26, 33, 34] However, S. sobrinus biofilm underwent significant changes in structure and was inhibited when exposed to xylitol and S. rebaudiana at any tested concentration. This finding was in accordance with many previous studies reporting the significant inhibitory effect of xylitol on S. sobrinus biofilm.[35-37] The change in biofilm due to the effect of xylitol is mainly limited to the decreased concentration of bacteria in the biofilm and not much change occurred in EPS or water channels compared with the control group. However, in discs exposed to S. rebaudiana, a significant change in structure was noted in addition to the reduction in bacterial concentration. Considering the lack of similar studies on S. sobrinus biofilm structure exposed to S. rebaudiana, a definite conclusion cannot be reached in this regard. But it appears that the mechanism of action of S. rebaudiana on the biofilm is different from that of xylitol. This theory must be confirmed by genetic and molecular studies.

One major limitation of our study was the relative novelty of investigating S. rebaudiana extract as an anti-biofilm agent and the deficiency of significant technical guidelines in the literature regarding extract preparation methods and dosages to be used in the microbiological studies. We also did not find any way to quantify the findings of SEM evaluations and therefore opted to the present qualitative descriptions of SEM micrographs. Future studies with a more in-depth evaluation of biofilm structure can yield interesting results and a better understanding of the anti-biofilm effects of alternative sweeteners.

Conclusion

Based on our results, S. rebaudiana extract appears to have more potent anti-biofilm effects compared with xylitol, and

considering the availability of commercial products containing S. rebaudiana extracts, these products can be recommended as preventive measures alternative to xylitol. However, clinical studies can better assess the effect of S. rebaudiana extract on complex biofilm present in dental plaque and demonstrate the outcomes on caries prevention.

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Conflict of Interest

None Declared ■

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