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Non-Nutritive Sweeteners Reduce The Adhesion Of *Streptococcus mutans* UA159 On Acrylic Resin Tooth.

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ABSTRACT

This work aimed to verify the influence of aspartame, steviol, sucralose and xylitol on the adhesion of *Streptococcus mutans* UA159 on the surface of artificial teeth composed of acrylic resin. The *in vitro* adhesion test was performed with the teeth immersed in artificial saliva for 48 hours at 37ºC. The biofilm formed was quantified using the violet crystal technique. The control test used 10% sucrose and lactose, major adjuvants of the sweeteners. There was no bactericidal or bacteriostatic activity of the sweeteners. Although the pioneer cells were viable under all conditions assessed, the adhesion was reduced by up to 33%. The highest and lowest percentage of reduction were observed in the presence of xylitol and aspartame, respectively.

Keywords: Oral microbiota. Non-nutritive sweeteners. Dental prosthesis. Biofilm.

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INTRODUCTION

The most recent definition for dental caries concerns a dynamic multifactorial dental condition, which results in the loss of minerals from solid dental tissues. In addition, caries is mediated by biological, behavioural, psychosocial, environmental and dietary factors.[1] Dental caries is characterized as a demineralization process. In the initial stage, it can be reversed either through the natural process of remineralization or through enhanced remineralization due to fluoride therapy.[2] The development of dental caries occurs due to the formation of biofilm on the dental surface, maintained nutritionally by the food that remains in the mouth, giving rise to dental plaque.[3]

The main factors for the development of dental caries are oral microbiota and the presence of fermentable carbohydrates in the diet, in addition to the genetic predisposition.[4] The most prevalent species on the plates are acidophil members of the genera *Streptococcus* and *Lactobacillus*. The latter is more present in more advanced stages of dental caries from the dentin.[5] *Streptococcus* spp. in particular, *Streptococcus mutans* is recognized as one of the main dental caries initiation factors. The bacterium was described at the beginning of the 20th century as a facultative Gram-positive microbe, with an optimal growth temperature of 37°C, not growing at temperatures below 22°C. The ideal growth pH occurs in the range close to 7, with no growth at pH values below 5.6.[6] The preferred adhesion surface of *S. mutans* is on natural teeth. It has already been demonstrated *in vitro*, however, that the bacterium can adhere to artificial teeth similar to what can be seen on natural teeth.[7] In addition, the presence of biofilms on full, upper or lower dentures may represent a health risk, since some important systemic infections may originate in the mouth.[8]

The number of people using fixed or removable dental prosthesis in Brazil is estimated by age group. The percentage of young people aged 15-19 who need dental prostheses is 13.7% and this percentage increases according to the age group: 68.8% in people aged 33-44 years, and an impressive number, 92.7 %, in the elderly aged 65-74 years.[9]. In addition, little is known about proper hygiene practices among people who use removable dental prostheses, nor about the estimated 60% who have the habit of sleeping with their prosthesis.[10] On the other hand, the reduction of fermentable sugars in the daily food in people who use dental prostheses can contribute to oral health, obviously not in terms of cariogenesis, but in the prevention of bacterial plaque, responsible for certain conditions that can develop into important infections.[11]

Although a large portion of the population attributes the need to use non-nutritive sweeteners to a question of diet[12] or with conditions associated with obesity, cardiovascular disorders and metabolic diseases[13], the use of synthetic or natural sweeteners can bring benefits to individuals outside of these groups, since non-nutritive sweeteners can be a strategy for prophylaxis or reduction of dental caries.[14] Substitution by sweeteners in food, beverages, toothpaste and chewing gum is associated with a reduction in caries.[15] This does not imply, however, that the individual using sweeteners is not exempt from maintaining good oral health habits, especially those people who use dental prostheses.

MATERIAL AND METHODS

Strain

The strain *Streptococcus mutans* UA159 was used, kept at 4˚C in nutrient agar supplemented with 10% sucrose, under an anaerobic atmosphere.

Sweeteners

Four non-nutritive sweeteners from three commercial brands were used: aspartame, steviol, sucralose and xylitol. These were purchased at a supermarket in João Pessoa, state of Paraíba, Brazil. Each envelope of steviol and sucralose contained 0.8 g of the product, with a recommendation to dilute the contents in 160 mL of liquid (one glass of juice). The amount in an envelope of xylitol for the same volume of liquid was 5.0 g. The adjuvants reported were mostly lactose (0.79 g) and silicon dioxide.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Initially, concentrated sweetener solutions were prepared in 160 mL of distilled water, using the content of 4 envelopes, and pH was ascertained (Quimis, Q400AS). The MIC was determined using the microdilution method in culture broth. Aliquots of 10µL of microbial suspensions in NaCl 0.9% with turbidity standardized with tube No. 1 on the MacFarland scale were added to wells containing 100 µL of Müeller-Hinton broth (MH), supplemented with 10% sucrose and 100 µL of the sweetener solution. The microplates were incubated for 24 hours at 37±1°C in an anaerobic jar (Ozion, JA0400), under an oxygen-poor atmosphere.[16]. MIC was defined as the lowest concentration in which no turbidity was observed by visual inspection.[17]. MBC was identified by adding a drop of 1% resazurin solution to the wells containing cell growth and defined as the lowest concentration in which no colour change from blue to pink or lack of colour was observed within 2 hours at room temperature.[18]. The test was performed in triplicate. The control considered the sterility of the modified MH broth, the sweetener solutions and the viability of the strain in modified MH broth.

***In vitro* assay of biofilm formation**

The violet crystal test was used, with adaptations.[19]. Microplates with 24 wells with a capacity of 3 mL were filled with 1200 µL of a mixture containing 300 µL of the four times concentrated sweetener solution and 900 µL of artificial saliva, with the following composition (in mg/L): NaCl (0.125), KCl (0.964), (KH_2PO_4), urea (0.2), Na_2SO_4 (0.763), NH_4Cl (0.178), $NaHCO_3$ (0.631), pH = 6.9.[20] Aseptically, an incisive tooth of methyl polymethacrylate (PMMA) acrylic resin, double pressing, measuring 10 mm wide x 11 mm long (Vipi Dent Plus, 266) was immersed in each well. Then 10 µL of a 0.1% yeast extract solution and 10 µL of the *S. mutans* UA 159 suspension were added. The system was incubated for 48 hours, under the same conditions as the MIC and MBC determination test. Afterwards, the tooth was carefully removed and washed vigorously on thoroughly in tap water, to remove planktonic cells. Then, the tooth was placed in a sterile microdilution tube with a capacity of 2.0 ml, filled with 1.5 ml of 1% violet crystal.

After 20 min of incubation at room temperature, the content was removed and the excess dye on the tooth rinsed off under tap water. The tooth was dried and again taken to another sterilized microdilution tube, adding 1.5 ml of absolute ethanol. After 15 min, the optical density (OD) of the crystal violet-ethanol solution was measured at 590 nm (Zuzi 4251/50). Test control was performed separately in the solutions of sucrose 10% and lactose 4.875 g/L, equivalent to the content of lactose in an envelope. The tests were performed in triplicate.

Percentage of adherence and interpretation criteria

The percentage of adherence was calculated using the formula [(OD_{590} of the treatment – OD_{590} of the control) ÷ OD_{590} of the control) x 100]. The value found, classified the adhesion of *S. mutans* UA 159 as weak (<40%), moderate (40-80%) or strong (> 80%).[21] To identify the viability of pioneer planktonic cells, a cutoff equivalent to three times the value of the optical density of the medium used in the test (0.049) was used. Adherence was considered weak when OD was close to or less than 0.147.[22]

Statistical analysis

The results were expressed as the mean and standard deviation of the replicates of each assay.

RESULTS

None of the sweetener solutions promoted bactericidal or bacteriostatic activity on the *S. mutans* UA159 strain (Fig. 1). In the *in vitro* assays of biofilm formation, viable pioneer cells of *S. mutans* UA159 were identified in all tested concentrations. The OD_{590} was about 3.5 to 5.0 times greater than the cutoff, resulting in the formation of robust biofilms within 48 hours.

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Figure 1 - Aspects of *S. mutans* UA159 developed in tubes containing nutrient broth supplemented with 5 and 10% sucrose (left) and MIC and MBC test results (right)

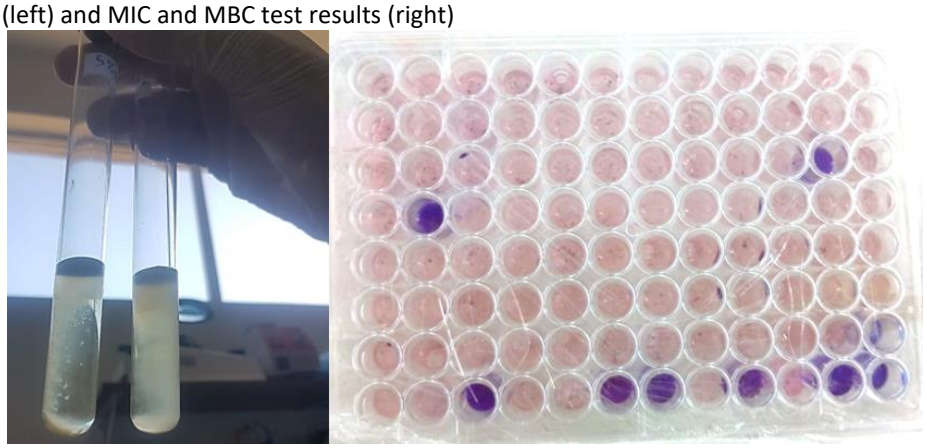

Table 1 summarizes the values of pH and optical density in the *in vitro* test of biofilm formation. The presence of sweeteners, as well as lactose, promoted reductions between 8 and 33% in the adhesion of *S. mutans* UA159. The activity of sweeteners was more pronounced in this order: xylitol, sucralose, steviol and aspartame.

Table 1 - pH, optical density, and percentage of adhesion in the *in vitro* assay of biofilm formation of *S. mutans* UA159 *

Sweetener	pH	OD_{590}	Adhesion reduction (%)
Aspartame	4.4±0.1	0.681±0.241	8.2±0.2
Steviol	6.4±0.1	0.662±0.132	10.8±0.1
Sucralose	6.4±0.1	0.581±0.081	21.7±0.1
Xylitol	6.5±0.1	0.498±0.004	32.9±0.1

OD_{590} > 0.147 indicated presence of pioneer cells. Control with sucrose 10% (pH = 6.3±0.1; OD_{590} = 0.742±0.185), lactose (pH=5.2±0.1; OD_{590} = 0.390±0.251)

DISCUSSION

This work aimed to verify the biofilm formation by *S. mutans* UA159 on the surface of acrylic resin teeth in the presence of the four most commercialized non-nutritive sweeteners in Brazil. Biofilms are sessile microbial communities organized in a complex association surrounded by an adhesive polymeric matrix.[23] It is believed that most bacteria show a preference for the sessile stage, rather than planktonic life style, especially as it offers an advantage over remaining in environments with physical, chemical and biological stresses, to which they may be exposed.[24]

The slightly acidic medium favours the metabolism of *S. mutans* UA159, as well as allowing chemical stability for the sweeteners.[10] The pH variation in the *in vitro* tests of biofilm formation was between 5.2-6-5±0.1. This means that under two conditions evaluated, the acidity state in the medium was not conducive to the development of *S. mutans* UA159, that is, aspartame and lactose (control). When comparing the other pH values of the sweetener solutions with aspartame, it is suggested that the smallest reduction in bacterial adhesion may have been influenced by pH, in terms of electrostatic double-layer interactions and changes in other physical and chemical properties.[25] This hypothesis is strengthened by the fact that the OD_{590} value was similar to that obtained with steviol. The pioneer cells in both conditions were clearly viable, being about 4.5 times higher than the cutoff value making them very close in percentage of adhesion reduction.

On the other hand, the least adhesion of *S. mutans* UA159 to the teeth occurred in the presence of lactose. Lactose, it should be noted, is not a sweetener and was tested because it was the major additive to the composition of the sweeteners. The pH value in the lactose solution is also not favourable for the growth of *S. mutans* UA159. In addition, as it is an easily assimilable substrate, acid is generated through lactic fermentation, in the presence of oxygen-poor atmosphere,[26] and the lactic acid formed can also be toxic to cells.[27] On the other hand, it is also possible that the strain may exhibit some *lac* operon failure and the accumulation of galactose metabolism intermediates promoted failure to grow.[28]

It should be noted that with pH below 5.5, no plaque is formed.[29] However, *in vivo* conditions, the buffering capacity of the salivary secretion rate results in an increase in pH, favouring the accumulation of plaque.[30] The denser the plaque, the less oxygen and saliva will penetrate and more damage to the tooth may occur.[31]

While nutritive sweeteners accelerate microbial growth, non-nutritive sweeteners can slow the growth or suppress the virulence of bacteria[32] and fungi[33] in the oral microbiota. In addition, natural or synthetic bioactive compounds promote different and important cellular changes, which can significantly impact the formation of biofilms.[34]

Significant reductions of *S. mutans* biofilms in the presence of pure aspartame, steviol and sucralose was observed in a previous study using bovine bones simulating human teeth.[35] The authors analysed parameters such as biomass, quantification of viable cells, determination of intracellular polysaccharides and characterization of the polymeric adhesive matrix. They attributed the reduction of biofilms to the fact of the poor amount of assimilable substrate being available in the broth. Even so, as observed in this work, aspartame had no significant impact on the formation of the *S. mutans* biofilm, while sucralose and steviol promoted reductions in the formation of biofilm. Additionally, the authors also did not detect any bactericidal effect of the sweeteners.

Although xylitol antimicrobial activity has not been reported, xylitol has been described as a potential way to reduce the formation of oral biofilms in a study evaluating the association of xylitol with steviol.[36] Despite the reduction of the constituent biomass in the biofilm, the authors observed thinner and porous biofilm walls even after 24 hours of exposure to the two compounds. In a recent review of xylitol, anti-carcinogenic activity was described, however occurring by a different mechanism of antimicrobial action, i.e., xylitol stimulates the secretion of saliva, maintaining stable oral pH,[37] as well as inhibiting glycolysis in *S. mutans*.[38]

Dental injuries that cause partial or total loss of teeth are mostly repaired with PMMA acrylic resin, given the characteristics of the resin, such as malleability, tensile strength and impact resistance. Despite this, given the little flexibility of the resin, there is a risk of cracks or breaks.[39] The formation of bacterial plaque in mobile and fixed prostheses can lead to the risk of wear of materials, due to bacterial proliferation and production of organic acids from the metabolism of residual fermentable substrates, stemming from bad hygiene habits, in a process similar to what occurs in materials such as concrete[40] and mortar,[41] called microbiologically induced corrosion.

In addition, plaque formation poses health risks. This work alerts to the fact that non-nutritive sweeteners can favour the appearance of *S. mutans* biofilms in dental prostheses. Based on this observation, it is assumed that many other important oral pathogens can do the same, opening new proposals for investigations. In addition, careful cleaning of the prostheses must be strictly adhered to, as recommended and encouraged by professionals.

CONCLUSION

In the experimental conditions evaluated, the sweeteners did not produce death or stasis of *S. mutans* UA159. The results suggest, however, that the adhesion of planktonic cells is disturbed by some mechanism other than antimicrobial action. When considering that the experiments were carried out *in vitro*, it can be inferred that the effect on the inhibition of the development of cavities is more efficient *in vivo* due to the existence of a constant flow of saliva, favouring the regulation of pH, as well as reducing the demineralization of the dental tissue.

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