In vitro effect of commercial sweeteners on *Streptococcus Mutans* and *Lactobacillus acidophilus* growth.

Abstract

Purpose: To evaluate the effect of two commercial sweeteners based on steviol glycosides or sucralose on *Streptococcus mutans* and *Lactobacillus acidophilus* growth and biofilm formation, as well as the pH change produced by their metabolism.

Materials and methods: 420 bacterial inoculum were assigned to 12 study groups (n=35) according to bacterial type (*Streptococcus mutans* or *Lactobacillus acidophilus*), sweetener type (sucrose, sucralose, steviol glycosides) and incubation time (one hour or 24 hours). Bacterial growth and biofilm formation were measured by spectrophotometry. The pH value was determined using a potentiometer.

Results: ANOVA and student t tests showed statistically significant differences in bacterial growth and biofilm formation with both strains and the three sweeteners.

Conclusions: The sweeteners evaluated do not only present the active substance they promote, they use a mixture of different sweeteners and additives whose impact on the growth of acidogenic bacteria may not be beneficial for the prevention of caries.

Keywords: Dental caries, commercial sweeteners, *Streptococcus mutans*, *Lactobacillus acidophilus*, bacterial growth.

Introduction

Dental caries is one of the most prevalent diseases in the world. In the Global Burden of Disease Study, the World Health Organization (WHO) reports that oral diseases affect half of the world's population (3580 million people). Dental caries in permanent teeth is the most prevalent disorder in the world .[17] Approximately 2.4 billion people have permanent tooth caries and 486 million children have temporary tooth caries.[18]

Dental caries can be prevented with good hygiene habits, moderating the consumption of cariogenic foods and keeping the acidogenic bacteria in balance.[1,9,14,18] Sucrose is considered the most important cariogenic food in the human's diet.[7,16,19] Its abuse promotes diabetes and obesity and provides a favorable oral environment for the proliferation of acidophilus bacteria that include *Streptococcus mutans* and *Lactobacillus acidophilus*.[7,10]

Dietary modification is a useful strategy to decrease the sucrose use.[13] Consumption of commercial sweeteners promises a sweet taste with fewer calories, accessible costs, and claim to be better nutritional supplements. However, most studies conducted to evaluate the effect of sweeteners have focused on measuring the effects of the active substance.[11] Unfortunately, the use of sweeteners at the population level is through commercial presentations and not only is the active substance consumed. Additional components (additives) may influence the results of the active substance.

Materials and methods

An experimental study was carried out. 210 inoculums of *Streptococcus mutans* strains (ATCC 25175) and 210 inoculums of *Lactobacillus acidophilus* strains (ATCC 4356) were used. Twelve study groups (n=35) were formed as indicated in Table 1.

Preparation of inoculums

The bacteria were inoculated in dextrose-free nutrient broth sterile and incubated at 37°C for 24 hrs. Subsequently, the bacterial growth was adjusted with dextrose-free nutritive broth sterile until a level of 0.05 on the McFarland scale, equivalent to 0.08 turbidity at a length of 630 nm was reached so that all inoculums started with the same concentration of bacterial cells.

Sweeteners solutions

Standard sweetener solutions were made, according to the manufacturer's specifications, at a concentration equivalent to 2 teaspoons (common amount used to sweeten beverages) as follows:

Sucrose solution (control group): 5g sachet was dissolved in 250 ml of sterile distilled water. Commercial sweetener solution based on sucralose: one 1g sachet was diluted in 250 ml of sterile distilled water. Commercial sweetener solution based on steviol glycosides: one 1g sachet was diluted in 250 ml of sterile distilled water.

Bacterial growth

8ml of sterile glucose-free soy broth was placed in test tubes. Subsequently, 1ml of the bacterial inoculum and 1ml of the sweetener solution to be evaluated were

added. They were incubated at 37ºC without agitation and 1h and 24 hrs were evaluated after incubation.

Measurement of bacterial growth

Bacterial growth was homogenized by tube inversion. Subsequently, 3 ml of the inoculated culture medium was taken and placed in spectrophotometer cells. Absorbance was measured at a wavelength of 630nm. Uninoculated broth was used as a control. [7]

pH measurement

The pH was measured using a microelectrode coupled to a portable pH meter that was previously calibrated with pH 7 and pH 4 buffer solutions. Initially, the tip of the pH electrode was soaked in KCl solution. Once prepared, the electrode was stored in a reference buffer (pH = 7). Before and after each reading, the electrode was calibrated against the standard pH buffers at pH 4 and 7. Between each reading, the electrode was cleaned in distilled water and dried on absorbent paper.

Study and quantification of biofilm formation.

Staining tests were carried as follows: In sterile Petri dishes, sterile coverslip were placed individually. They were covered with 1 ml of Mc Farland's 0.05 bacterial inoculum, 1 ml of sweetener solution and 8 ml of sterile glucose-free soy tripticasein

broth. They were incubated at 37ºC. 6 groups were incubated for one hour and 6 groups were incubated for 24 hours as specified in Table 1.

After incubation, the coverslip was removed and washed with 5ml of sterile distilled water three times. The biofilm formed on the coverslip was stained with 1ml of crystal violet and allowed to stand for 45 minutes at room temperature. It was then washed 5 times with sterile distilled water to eliminate non-adherent bacteria. Subsequently, 3 ml of 95% ethanol was added to obtain the stained biofilm. It was left to rest for 3 minutes. Finally, the 3 ml of alcohol with colored biofilm were collected from each sample and placed in cells for spectrophotometer. The optical density was measured at 540 nm with a spectrophotometer. Wells without bacterial inoculum were used as negative controls.

Data processing

All experiments were performed in triplicate in independent trials. Quantitative variables generated were analyzed descriptively. Means and standard deviations were calculated. Mean comparisons were made using ANOVA and student t. Statistical analyses were performed using the statistical package stata version 15.

Results

Measurement of bacterial growth

All study groups showed higher bacterial growth at 24 hrs. The groups of the commercial sweetener based on steviol glycosides showed greater bacterial growth from the first hour of incubation. The results are shown in Table 2.

S. mutans had a higher growth both in incubation of one hour and 24 hours in the presence of the commercial sweetener based on steviol glycosides, even with higher growth than the control group added with sucrose (Table 3). *L. acidophillus* growth was higher in the presence of the commercial steviol glycoside based sweetener for all evaluations. The results are shown in Table 3.

pH measurement

No statistically significant differences were found in the pH values. In general, pH values ranged between 5.6 and 7.1 for groups with sucrose, 5.7 and 6.9 for groups with sucralose sweetener and between 5.7 and 7.3 for groups with commercial steviol glycoside sweetener.

Biofilm

Statistically significant differences were observed for all measurements as shown in Table 4.

Discussion

The highest bacterial growth was observed at 24 hours in all study groups, this coincides with the knowledge that people who do not have habits that help them eliminate dentobacterial plaque acidogenic immediately after consuming food are exposed to increased cariogenic activity. The time of formation and maturation of dentobacterial plaque is a process that lasts up to approximately 2 weeks, but the first 48 hours are essential for bacterial colonization.[15]

The hypothesis of this study proposed that the greatest bacterial growth would be found in the sucrose control groups, since it is considered the most cariogenic sweetener. However, the study group supplemented with commercial sweetener based on steviol glycosides (which also contains sucrose and sucralose) exceeded the levels of bacterial growth and acid pH of sucrose.

These results contrast with the publications by Brambilla, Ferrazzano and Siraj[3,6,17] which classify steviol glycosides as an excellent sweetener attributing anti-cariogenic and antiperiodontopathic properties. It is important to note that in these background studies, only the active substance of the sweetener was evaluated. This study, on the other hand, found that the commercial presentation promotes bacterial growth by providing acidogenic bacteria with an optimal environment for their development from the first hour of incubation. It is possible to suggest that the commercial presentation of steviol glycosides evaluated, because it is added with sucrose and sucralose can greatly affect the oral health of the population.

The commercial sweetener based on sucralose was found to be added with dextrose and maltodextrin. It maintained bacterial growth levels like the sucrose control group except after 24 hours of incubation. At this point the bacterial growth was lower. Possibly, in the long term, additives do not provide an optimal environment for bacterial growth such as sucrose.

With respect to the pH obtained in the study, we can observe that all the groups include critical pH values. It has been documented that lower pH levels from 5.5 demineralize dental enamel.[5] After 24 hours the pH increased and we can observe that the levels are close to the optimal pH levels in the mouth (6.7). This aspect is important because according to Carter, Marsh and Zambrano,[4,12,20] the effect of cariogenic foods, especially sucrose, resides in the lowering of pH levels. The microorganisms found in the dentobacterial plaque metabolize the sugar and acidify the oral environment, which causes demineralization of the dental enamel and results in caries.

Conclusions

- The sweeteners evaluated do not present only the active substance they promote. They use a mixture of different sweeteners and additives.

- The commercial sweetener based on steviol glycosides (added with sucrose and sucralose) promotes bacterial growth of *Streptococcus mutans* and *Lactobacillus acidophillus*.
- The commercial sweetener based on sucralose (added with dextrose and maltodextrin) showed a lower growth than the control group sucrose and the commercial sweetener based on steviol glycosides.
- The biggest promoter of biofilm was the sweetener based on sucralose. Its use could impact on the dentobacterial plaque formation at a level that could be detrimental to the prevention of caries.
- The sweeteners evaluated generate a critical pH, which could increase the risk of caries.
- The use of commercial sweeteners for the prevention of caries or to promote oral health may not be appropriate.

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Table 1. Study groups distribution

Group	n	Bacteria	Commercial sweetener	Incubation time
1	35	*Streptococcus mutans*	Sucrose	1hr
2	35	*Streptococcus mutans*	Sucrose	24hr
3	35	*Streptococcus mutans*	Commercial sweetener based on sucralose (additives: dextrose and maltodextrin)	1hr
4	35	*Streptococcus mutans*	Commercial sweetener based on sucralose (additives: dextrose and maltodextrin)	24hr
5	35	*Streptococcus mutans*	Commercial sweetener based on steviol glycosides (additives: sucrose and sucralose)	1hr
6	35	*Streptococcus mutans*	Commercial sweetener based on steviol glycosides (additives: sucrose and sucralose)	24hr
7	35	*Lactobacillus acidophillus*	Sucrose	1hr
8	35	*Lactobacillus acidophillus*	Sucrose	24hr
9	35	*Lactobacillus acidophillus*	Commercial sweetener based on sucralose (additives: dextrose and maltodextrin).	1hr
10	35	*Lactobacillus acidophillus*	Commercial sweetener based on sucralose (additives: dextrose and maltodextrin).	24hr
11	35	*Lactobacillus acidophillus*	Commercial sweetener based on steviol glycosides (additives: sucrose and sucralose)	1hr
12	35	*Lactobacillus acidophillus*	Commercial sweetener based on steviol glycosides (additives: sucrose and sucralose)	24hr

Table 2. Mean difference in bacterial growth between groups of sweetener with different incubation time

Group	Comercial sweetener	Bacteria	Incubation time	Mean value	SD	*p*
1	Sucrose	*S. mutans*	1hr	0.497	0.037	**≤0.01**
2			24hr	**1.288**	0.133	
3	Commercial sweetener based on sucralose (additives: dextrose and maltodextrin)	*S. mutans*	1hr	0.515	0.044	**≤0.01**
4			24hr	**1.105**	0.134	
5	Commercial sweetener based on steviol glycosides (additives: sucrose and sucralose)	*S. mutans*	1hr	1.281	0.089	**0.02**
6			24hr	**1.319**	0.0393	
7	Sucrose	*L. acidophillus*	1hr	0.485	0.039	**≤0.01**
8			24hr	**0.691**	0.189	
9	Commercial sweetener based on sucralose (additives: dextrose and maltodextrin)	*L. acidophillus*	1hr	0.485	0.039	**≤0.01**
10			24hr	**0.691**	0.189	
11	Commercial sweetener based on steviol glycosides (additives: sucrose and sucralose)	*L. acidophillus*	1hr	0.532	0.045	**≤0.01**
12			24hr	**1.540**	0.129	

Abs: Absorbance. SD: Standar deviation. Student T test for mean difference $p \geq 0.05$.

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