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Efeito da infusão de *Stevia rebaudiana* Bertoni na desmineralização do esmalte e na formação do biofilme dental: um estudo *in situ*

Effect of *Stevia rebaudiana* Bertoni infusion on enamel demineralization and dental biofilm formation: an *in-situ* study

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ABSTRACT

Aim: *Stevia rebaudiana* Bertoni (*Stevia)* is a natural non-caloric sweetener that can modify the cariogenicity of biofilms. This study aimed to evaluate the effect of *Stevia* infusion in microbial and biochemical composition of biofilms formed in the presence of sucrose and on enamel demineralization. **Materials and Methods:** In a cross-over design, eleven volunteers wore an intraoral palatal appliance containing 4 slabs of bovine enamel during 3 phases of 7 days each. Sucrose solution (20%) was dripped onto slabs 8 times/day and 0.9% sodium chloride (NaCl), 0.12% chlorhexidine (CHX), or 5% infusion of *Stevia* were dripped 2x/day. Biofilm formed on the slabs was collected and analyzed for counts of microorganisms (total bacteria, *Lactobacilli*, *Candida* spp., and *Streptococcus mutans*) biochemical composition in terms of soluble and insoluble extracellular polysaccharides and qualitative assessment by scanning electron

microscopy. The percentage of surface hardness loss (%SHL) was determined on enamel slabs taken baseline and post-biofilm Knoop surface hardness values. **Results:** The % SHL in the CHX treatment was statistically lower in comparison to NaCl ($p < 0.05$). No differences were found between *Stevia* and CHX and between *Stevia* and NaCl. No other difference was found among the experimental groups with respect to the other outcomes. **Discussion:** Under high cariogenic conditions resembling frequent exposure to sucrose and absence of mechanical disruption, use of *Stevia* can neither modify the counts of cariogenic microorganisms nor the concentration of extracellular polysaccharides on *in situ* formed biofilms. This may have occurred due to the exposure of the biofilm to high sucrose concentration for all treatments and the condition of the microorganism growth *in situ*, which may hinder the diffusion of substances through the thick biofilm. **Conclusion:** Biofilm exposed to a high cariogenic challenge and without mechanical disruption is not affected by an infusion of *Stevia rebaudiana* Bertoni.

Keywords: Stevia. Biofilms. Dental enamel. Hardness. Demineralization.

RESUMO

Objetivo: A *Stevia rebaudiana* Bertoni (Stevia) é um adoçante natural não calórico que pode modificar a cariogenicidade de biofilmes. Este estudo teve como objetivo avaliar o efeito da infusão de Stevia na composição microbiana e bioquímica de biofilmes formados na presença de sacarose e na desmineralização do esmalte. **Materiais e métodos:** Em um desenho cruzado, onze voluntários usaram um aparelho intraoral palatino contendo 4 placas de esmalte bovino durante 3 fases de 7 dias cada. A solução de sacarose (20%) foi gotejada em placas 8 vezes/dia e cloreto de sódio a 0,9% (NaCl), clorexidina a 0,12% (CHX) ou infusão a 5% de Stevia foram gotejados 2x/dia. O biofilme formado nas placas foi coletado e analisado para contagem da composição bioquímica de microrganismos (bactérias totais, Lactobacilos, Candida spp. e Streptococcus mutans) em termos de polissacarídeos extracelulares solúveis e insolúveis e avaliação qualitativa por microscopia eletrônica de varredura. A

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porcentagem de perda de dureza superficial (%SHL) nos blocos de esmalte foi determinada com base nos valores de dureza superficial Knoop tomadas no início e pós-biofilme. **Resultados:** O % SHL no tratamento CHX foi estatisticamente menor em comparação ao NaCl ($p < 0,05$). Não foram encontradas diferenças entre Stevia e CHX e entre Stevia e NaCl. Nenhuma outra diferença foi encontrada entre os grupos experimentais em relação aos outros resultados. **Discussão:** Sob condições cariogênicas elevadas que se assemelham a exposição frequente à sacarose e ausência de disrupcao mecânica, o uso de Stevia não pode modificar as contagens de microrganismos cariogênicos nem a concentração de polissacarídeos extracelulares em biofilmes formados *in situ.* Isso pode ter ocorrido devido à exposição do biofilme à alta concentração de sacarose para todos os tratamentos e à condição de crescimento do microrganismo *in situ*, o que pode dificultar a difusão de substâncias através do biofilme espesso. **Conclusão:** O biofilme exposto a um alto desafio cariogênico e sem disruptucao mecânica não é afetado por uma infusão de Stevia rebaudiana Bertoni.

Palavras-chave: Stevia. Biofilmes. Esmalte dentário. Dureza. Desmineralização.

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INTRODUCTION

Stevia rebaudiana Bertoni is a native plant to South America, specifically found in northeastern Paraguay and Brazil. It occurs as an annual and perennial herb, sub-shrub, and shrub in mountainous regions, open forests, riverbanks, and valleys. Currently, it is cultivated in several regions of the world, such as North America, Asia, and Europe[1-3]. *Stevia rebaudiana* presents higher sweetening potential than sucrose and until now has no reported side-effects[2,4-6]. Many benefits for systemic health have been reported with the use of stevia, as antidiabetic[7-10], antioxidant[11,12], antihypertensive[13,14], antimicrobial[15], anti-inflammatory[16], and antitumor activities[17] as well as benefits in the oral health[15,18,19].

Dental caries is one of the most common problems affecting the oral cavity as a result of interaction among cariogenic microbiota and a rapidly-fermented and sugar-rich diet, especially containing sucrose[20,21]. Thus, biofilms formed in the presence of sucrose are extremely cariogenic, presenting low calcium, phosphate, and fluoride concentrations as well as large amounts of insoluble extracellular polysaccharides[22-24]. Therefore, a reduction of sugar intake and its replacement with no fermentable sweeteners may be a desirable and useful approach for caries prevention and treatment aiming to modify the cariogenicity of biofilms[25,26]. Moreover, non-caloric sweeteners may also benefit overweight and diabetic individuals or those at risk of developing these diseases.

In vitro[27-30] and *in vivo*[31] studies have demonstrated that stevioside can modify the cariogenicity of biofilms, reducing biomass and viable bacterial cells resulting and decreasing extracellular polysaccharide production[29] but the underlying mechanism is not well understood. Thus, this *in situ* study aimed to evaluate the effect of *Stevia rebaudiana* infusion on enamel demineralization and on the microbial and biochemical composition of biofilms formed under a highly cariogenic condition. We hypothesized that frequent use of *Stevia* decreases the concentrations of both soluble and insoluble polysaccharides in biofilms as well as the counts of viable microorganisms, leading to reduced enamel demineralization even under frequent exposure to sucrose.

MATERIALS AND METHODS

Experimental design

This was a cross-over and randomized *in situ* study conducted in three experimental phases of 7 days each, with a wash-out of 1 week between each phase. Due to the preparation and use of an infusion of *Stevia rebaudiana* dried leaves, the infusion stain and flavor prevented the study from being double blind, as the volunteers could identify the *Stevia* solution. For this reason, the study was blinded only in relation to examiners.

Participants

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Eleven adult volunteers aged between18-28 participate in this study. The volunteers are odontology students. Informed written consent was obtained from all subjects before the start of the study, according to the Declaration of Helsinki. Volunteers were recruited from January to March 2018

Eligibility criteria

In order to be qualified for the study, all volunteers were required to present good oral and general health, non-stimulated salivary flow rates of at least 0.25 mL/min, no systemic diseases, no use of any type of orthodontic appliance, and no use of antibiotic drugs for at least 2 months before the start of the *insitu* study. The study protocol was approved (removed for blind peer review).

Sample Size

The sample size of 12 volunteers was calculated based on a previous *in situ* study (22), considering a power of 80% and a confidence interval of 95%.

Preparation of enamel slabs and intraoral appliance

The dental enamel slabs (6 mm diameter × 2 mm thick) were prepared from sound bovine incisors as described previously[23]. The prepared and selected enamel slabs had their baseline surface hardness measured using the SHIMADZU HMV-2T. Microdurometer and indentations in the enamel were made in the center of the planned region, with the long axis of the Knoop diamond perpendicular to the outer surface of the enamel under a load of 50 g for 5s. Five indentations were placed in sequence and 100 µm apart from each other

The removable palatal intraoral appliances were prepared on a plaster model of the upper arch for each volunteer, using self-curing acrylic resin. Each appliance contained 2 cavities, one on each side, measuring 14 × 8 × 3 mm, designed to allocate 2 slabs of enamel in each[24]. Three of the four enamel slabs placed in the device had known surface hardness and were within the overall mean described above. The fourth enamel slab was destined for scanning

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electron microscopy. The slabs were glued inside the cavity and covered with a plastic mesh with a 1-mm distance between the enamel surface and the mesh to allow biofilm accumulation[32].

Preparation of the solutions and treatments

The solutions used for the study were 20% sucrose solution (Dinâmica LTDA), 5% *Stevia rebaudiana* Bertoni infusion, 0.12% chlorhexidine digluconate (Colgate PerioGard; 2580BR121A; Colgate-Palmolive Industrial LTDA) and 0.9% NaCl (Solução Fisiológica Panvel Care; Sodium Chloride 0,9%; Lote:0002; FARMAX). Of these, the researchers prepared the 20% sucrose and the Stevia rebaudiana solution, whereas the chlorhexidine and NaCl solutions were used as the commercial forms found on the market. The 5% *Stevia rebaudiana* Bertoni infusion was prepared using 5g of dry leaves (Batch: 0054, expiration: 01/02/2019, Foco Alternativo) which were macerated and, after adding an 100mL of distilled water, a two-hour protocol of two intercalated boils was performed, with 15 hours of rest for the infusion[31]. The choice of *Stevia* infusion at 5% concentration was based on a previous *in vitro* study[33], which demonstrated antimicrobial effects of the plant against *S. mutans* and *Lactobacillus* spp. It was decided not to use marketed *Stevia* solutions as they may contain unknown components. The 0.12% chlorhexidine digluconate solution was used as a positive control and the 0.9% NaCl solution was used as a negative control

Interventions

Volunteers used a palatal intraoral appliance in each experimental phase, containing 4 slabs of bovine dental enamel (with known surface hardness), 2 slabs on each side of the appliance. All enamel slabs were exposed to 20% sucrose solution that was dripped 8 times/day at pre-established times (08:00, 09:30, 11:00, 14:00, 15:30, 17:00, 19:00, and 21:00 h) and treated 2 times/day at pre-determined times (07:30 and 20:30 h) with one of the following solutions: 0.9% sodium chloride solution (NaCl), 0.12% chlorhexidine digluconate (CHX), or 5% *Stevia* infusion. The volunteers receive instructions to keep the intraoral

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appliance out of the mouth for 5 minutes after dripping the solutions and after that period, the excess solution that might have been present in the appliance was clean with a gauze and the appliance was reinserted into the mouth. A 14 days washout period was adopted in between each experimental phase. New sets of enamel slabs and palatal appliances were used in each experimental phase. The volunteers wore the appliances during the day and at night, removing them only during exposure to sucrose and to treatments, meals, beverages, and oral hygiene. Considering this and considering an average time of 50 minutes for each of the main meals (breakfast, lunch and dinner) and 30 minutes to snack per day, the total time of use was estimated around 20 h / day. The volunteers were instructed to not use fluoride products or any other dentifrice than the one provided for the study. During the study period, for both the experimental and washout phases, the volunteers brushed their teeth twice a day with fluoride dentifrice (Colgate Tripla Ação; 1,450 ppm F, 7335BR122I, Colgate-Palmolive Industrial LTDA). All volunteers were residents of an area that received fluoridated water (0.7 ppm F).

Outcomes

Counts of microorganisms: *Streptococcus mutans*, *Lactobacillus* spp., *Candida albicans* and total microorganisms.

Concentration of soluble (SEPS) and insoluble (IEPS) extracellular polysaccharides in the biofilm

Determination of percentage of surface microhardness loss (%SHL) on enamel slabs

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Analysis

Biofilm collection

At the end of each of the three experimental phases, the dental biofilm formed on the surface of the three enamel slabs was collected approximately 12 h after the last exposure to the sucrose solution. The plastic mesh covering the slabs was removed and all the biofilm formed was collected with a sterile curette

and immediately transferred to pre-weighed sterile micro tubes, identified with the experimental phase, the volunteer number, and the number of the treatment. The wet weight of the biofilm (mg) was determined using a high precision analytical balance (SARTORIUS BP210D). Biofilms were resuspended in sterile saline (0.9% NaCl) in the proportion of 1 mg of wet biofilm per mL of NaCl. One slab with the intact biofilm formed on the surface was processed for scanning electron microscopy analysis.

Dental biofilm microbiological analysis

For microbiological determination, an aliquot of biofilm suspension was sonicated for 20s[34]. The suspension was serially diluted in sterile saline solution. An aliquot of 25uL was inoculated in duplicate by the drop technique in the following culture medium: Mitis Salivarius bacitracin agar (MSB; BD Difco) for the growth of *Streptococcus mutans* (SM); Rogosa SL agar (ROG; Hi Media) for the growth of *Lactobacillus* spp. (LB), Brain Heart Infusion agar (BHI; Kasvi) supplemented with sheep blood at 5% concentration for growth of total microorganisms (TM) and Sabouraud agar (SAB; Hi Media® Mumbai- India) for the growth of *Candida albicans* (CA)[23]. BHI and MSB plates were incubated for 48 h whereas ROG plates were incubated for 72 h. All plates were incubated at 37°C under microaerophilic conditions whereas SAB plates were incubated in aerobic condition at 37°C for 48 h. Colony Forming Units (CFU) were then counted under a stereomicroscope (OLYMPUS SZ51) and the results were expressed as CFU/mg of biofilm wet weight.

Biochemical analysis of dental biofilm

In the biochemical analysis, soluble and insoluble extracellular polysaccharides (SEPS/IEPS) were evaluated through the sulfuric phenol micro-method[35]. This method consists of the dehydration of sugars in a concentrated acid medium and subsequent formation of complexes with phenol. Sugars and their derivatives, when treated with phenol and concentrated sulfuric acid, render the solution yellow-orange, maintaining this staining stable. The colored sample was placed

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in a spectrophotometer (Spectronic 21D, Milton Roy) and compared to a reference in order to present the absorbance value of the solution, which is linearly proportional to the total sugars concentration[35]. The dosages of polysaccharides were determined at a wavelength of 490 nm using a standard glucose curve. All the dosages were analyzed in duplicate, obtaining the absorbance and mean of the samples. After quantification, the results were express as µg/mg

Analysis of microhardness of enamel surface after biofilm formation

After the plastic mesh covering the slabs was removed and all the biofilm formed was collected for the other analyses, the enamel slabs were cleaned and used for the analysis of surface microhardness. At the end of the experiment, the surface microhardness of the enamel slabs was measured. Five indentations were placed 100 µm apart from each other and 100 µm to the right of the baseline indentations under a load of 50 g for 5 s. The percentage of surface hardness loss (% SHL) was calculated considering the average baseline hardness (A) and the average of final surface harness (B) as follows: % SHL = ([B – A]/A) × 100[22].

Scanning electron microscopy after formation of biofilm

The preparation of biofilm samples formed on the fourth slab started immediately after collection by immersion in a 25% glutaraldehyde solution for one week. After this fixation period, the material was passed through three washes of 30 minutes each with 0.2 M phosphate buffer and distilled water in a ratio of 1: 1. Dehydration of the samples was performed by a sequence of 7 immersions in 30%, 50%, 70%, and 90% acetone for 10 minutes each, after re-immersion in 90% acetone for 20 minutes, 100% acetone for 10 minutes, and again in 100% acetone for 20 minutes. Dissection of the samples was performed in a Critical Point Dryer (Critical Point Dryer, BALZERS CPD030). The samples were metallized (Sputter Coater, BALZERS SCD050) and analyzed in the Scanning electron microscope (JEOL JSM 6060; Akishima) at different

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magnifications, at the Center of Microscopy and Microanalysis of the Federal University of Rio Grande do Sul (CMM-UFRGS).

Randomization, allocation and blinding

Enamel slabs were averaged and 129 slabs (329.27 ± 65.85 kg/mm^2) were selected and randomized to the treatments (CHX, NaCl, or *Stevia*) and to the volunteers so that the average surface hardness was similar among the volunteers and treatments in each experimental phase. Volunteers were also randomized in relation to the treatments considering a crossover design. Randomization was carried out using a randomization list generated by Excel software. The randomization was done for one researcher, other make the enrollment of participants and the interventions. All participants are blind in relation to treatment. All solutions were distributed in dropper bottles and the 20% sucrose bottles were identified by the letter "S", whereas the test treatments were codified (1, 2 or 3). Solutions were provided to the volunteers every 2 days. The researcher that collected and processed all the samples was blind in relation of treatments.

Study settings

Preparation of enamel slabs, intraoral palatal appliance and solutions, as well as microbial and biochemical analysis were performed in the Oral Biochemistry and Oral Microbiology laboratory; surface hardness evaluation was carried out in the Dental Materials laboratory; Scanning electron Microscopy was carried out at the Center of Microscopy and Microanalysis. All the study settings belong to the Federal University of Rio Grande do Sul.

Statistical analysis

The mean, standard deviation, minimum and maximum values, and p-values for the variables under study [%SHL, SEPS, IEPS and counts of *Streptococcus mutans* (SM), *Lactobacillus* spp. (LB), *Candida albicans* (CA) and total microorganism (TM)] were calculated in each of the tested conditions and

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were analyzed statistically. The data were analyzed using the analysis of variance for repeated measurements (ANOVA) adjusted with Bonferroni's and Tukey`s *post hoc* tests. Data of microbial counts were log-transformed. The software Stata 13.1 was used for statistical analysis and the level of significance was 5%. All analyses were done based on the data obtained from each volunteer in each phase of the experiment.

RESULTS

The experimental phases were carried out from April to May 2018. Eleven of twelve enrolled volunteers completed the study. One volunteer withdrew participation during the first experimental phase.

Figure 1 shows biofilm images according to the treatments. Overall, biofilms were complex and formed by yeasts, rods and cocci enmeshed in extracellular polysaccharides (EPS) like matrix.

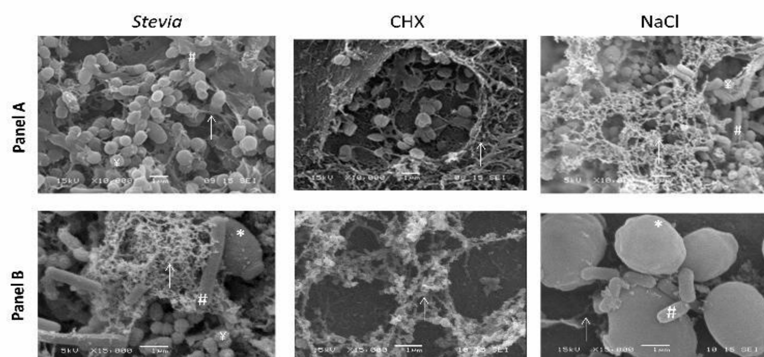

Figure 1. Representative scanning electron microscopy images of *in situ* biofilms according to the treatments. 10,0000x magnification (Panel A) and 15,000x magnification (Panel B).

White arrows indicate the EPS-like material. * Indicates yeasts; # indicates rods; ¥ indicates cocci.

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The results in table 1 shows no differences for soluble extracellular polysaccharide (SEPS), insoluble extracellular polysaccharide (IEPS) ($p > 0.05$). In relation to counts of *Streptococcus mutans* (SM), *Lactobacillus* spp. (LB), *Candida albicans* (CA) and total microorganisms we not observed differences among the experimental groups ($p > 0.05$).

In relation of percentage of surface hardness loss (% SHL) the CHX treatment was statistically lower in comparison to NaCl ($p < 0.05$). In treatment with Stevia no differences were found between Stevia and CHX and between Stevia and NaCl.

Table 1 Comparison among the treatment groups (mean ± sd) for percentage of surface hardness loss (%SHL), soluble extracellular polysaccharide (SEPS); insoluble extracellular polysaccharide (IEPS) and counts of *Streptococcus mutans* (SM); *Lactobacillus* spp. (LB); *Candida albicans* (CA); total microorganisms.

		Treatments			
Variables	**N**	***Stevia***	**CHX**	**NaCl**	**P-Value**
%SHL	11	22.1 ^{ab} ± 28.8	7.4 ^{a} ± 12.0	33.4 ^{b} ± 29.5	0.03
SEPS (µg/mg)	11	13.7 ^{a} ± 14.9	15.1 ^{a} ± 15.0	13.2 ^{a} ± 10.8	0.94
IEPS (µg/mg)	10	28.6 ^{a} ± 27.3	22.0 ^{a} ± 21.1	24.8 ^{a} ± 17.4	0.84
SM (CFU/mg x 10^4)	11	2.8 ± 7.0 ^{a}	0.4 ± 1.2 ^{a}	0.7 ± 1.8 ^{a}	0.40
LB (CFU/mg x 10^6)	11	0.8 ± 1.2 ^{a}	0.3 ^{a} ± 0.7 ^{a}	0.8 ± 1.7 ^{a}	0.40
CA (CFU/mg x 10^5)	11	8.9 ± 2.0 ^{a}	0.2 ± 0.4 ^{a}	1.9 ± 2.6 ^{a}	0.20
TM (CFU/mg x 10^7)	11	8.1 ± 1.5 ^{a}	3.9 ± 6.6 ^{a}	1.2 ± 1.7 ^{a}	0.27

Means followed by distinct letters differ statistically among the treatments.

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Abbreviations: %SHL, percentage of surface hardness loss; SEPS, soluble extracellular polysaccharide; IEPS, insoluble extracellular polysaccharide; SM, *Streptococcus mutans*; LB, *lactobacillus* spp.; CA: *Candida albicans*; TM, total microorganisms.

DISCUSSION

This is the first study to test the antimicrobial potential of *Stevia rebaudiana* solution in biofilms formed using an *in situ* model. The effects of natural plant extracts on dental biofilm have been studied in recent decades and some of these studies show that *Stevia* may present anti-cariogenic[19,33] and antimicrobial effects[15]. *Streptococcus mutans* showed higher growth suppression in stevioside-containing medium than in medium containing sucrose, glucose, or fructose[36]. Other *in vitro* studies compared the effect of *Stevia* extracts with different solvents on *Streptococcus mutans* and *Lactobacillus acidophilus*. In these studies, *Stevia* extracts inhibited the growth of *S. mutans* and *L. acidophilus*, indicating the antibacterial activity of *Stevia* against these bacteria[15,27,37-39]. Giacaman et al.[29], in an experimental work with commercial sweeteners, observed that *Stevia*, sucralose, and saccharin leave significantly less viable cells (*S. mutans*) in biofilms compared to other sweeteners. In addition, *Stevia* and sucralose tend to induce the formation of less biomass[29]. Another *in vitro* study also evaluated the antimicrobial activity of *Stevia rebaudiana* solution and non-caloric sweeteners on *Streptococcus mutans* and *Lactobacillus casei* and showed that the solution of the *Stevia rebaudiana* plant presents inhibition of bacterial growth[33]. Although the results found in literature show that the solution of the *Stevia* plant presents negative effects on bacterial growth *in vitro,* it is not known if these properties are maintained in biofilms formed in the oral cavit. In our study, we observed no significant difference in the counts of microorganisms between the treatments. This may have occurred due to the exposure of the biofilm to high sucrose concentration for all treatments and the condition of the microorganism growth *in situ*, which may hinder the diffusion of substances

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through the thick biofilm. Although the thickness of the biofilms was not quantitatively assessed in our study, all biofilms formed in the presence of sucrose are thick due to the presence of insoluble extracellular polysaccharides (IEPS). Previous studies have also showed the role played the IEPS limiting molecules diffusion and reducing biofilm susceptibility to antimicrobials[40]. We hypothesize the same might have happened in respect to *Stevia rebaudiana*. Further studies should address the diffusion of such compound across distinct thickness biofilms and the role played by the thickness on the biofilms on *Stevia rebaudiana* antimicrobial effect.

In relation to hardness loss (% SHL), an *in vitro* study[29] performed with a commercial sweetener containing other sugars such as lactose in its composition, observed that a sweetener containing *Stevia* resulted in a lower percentage of loss of surface hardness compared to the sucrose positive control. In our study, we observed an intermediate loss of hardness in the presence of *Stevia* when compared to the positive and negative controls. Indeed, for this study, we used a *Stevia* infusion prepared from dried leaves to preserve its properties, and not sweeteners available on the market as they can have other sugars and components that influence the results in their composition. Another *in vitro* study evaluating the effect of *Stevia* on enamel microhardness when incorporated into a cariogenic diet (3 times/day) showed that the highest concentration group of *Stevia* (Stevia 5%) induced the highest hardness loss. In that study, the biofilm was formed only with *S. mutans* during 4 days indicating that a higher concentration of *Stevia* in the presence of sucrose and *S. mutans* could present a cariogenic effect[30]. Our study is the first to use an *in-situ* model for evaluating the effect of an infusion of *Stevia rebaudiana* on enamel demineralization and dental biofilms in condition of high cariogenic challenge (8 times/day). Thus, the present study tested an even more intense cariogenic challenge condition compared to other[30] which may have masked the antimicrobial effect of *Stevia* and its anticariogenic properties. We would like to highlight that enamel mineral loss under the tested conditions was indirectly assessed by surface hardness assay (expressed as %SHL). Although

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transversal microradiography assessment (TMR) is considered the gold-standard method for the quantitative assessment of tooth mineral loss (in terms of lesion depth [LD] and integrated mineral loss [IML]), previous studies have shown a positive and significant correlation between %SHL and IML (ρ=0.8)[41] and between %SHL and LD (ρ =0.76) (41). Therefore, the magnitude of the mineral loss found in our study and represented by %SHL is likely to be similar to the one assessed by TMR.

Regarding the production of SEPS and IEPS, we observed no differences among the 3 groups tested. However, an *in vitro* study revealed a decrease in the production of intracellular polysaccharides (IPS) and IEPS in the the presence of *Stevia*[29]. Besides, stevioside is not metabolized by *S. mutans* as a carbon source, and it does not allow acid formation and IEPS synthesis[28]. Other study have also shown that stevioside, aspartame, xylitol and saccharine sweeteners are not metabolized by *S. mutans* and that *Stevia rebaudiana* leaf extract is a potent inhibitor of IEPS synthesis, which plays an important role in the formation of dental biofilm[29]. Alternative sweeteners such as *Stevia*, were equally effective as xylitol in reducing the amount of the extracellular matrix in *S. mutans* biofilms[42]. In our study, we used an *in-situ* model and a cariogenic challenge induced by daily exposure to sucrose (8 times/day). Yet, biofilms remained undisturbed over the enamel slabs, which also contributes to create a highly cariogenic environment resembling the one found in caries-active individuals. Although some effects of stevioside or even CHX on microbial counts and on SEPS/IEPS synthesis were expected, this highly cariogenic condition might be the reason for the lack of an effect in the experimental groups and might be the reason why our data differ from the studies mentioned above.

In the images obtained by scanning electron microscopy, we noticed the presence of many cocci, rods, filaments, yeasts, and EPS. The EPS matrix, visible in all treatments, appears as a network involving and connecting microorganisms. There were no morphologic differences between the different volunteers with different treatments in relation to the biofilm structure. This may be justified by the high cariogenic challenge of the study with eight applications

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of 20% sucrose per day, which might have overcome any antimicrobial effect of *Stevia* and chlorhexidine. Thus, with this amount of sucrose, selection of microorganisms that are characteristic of cariogenic biofilms was observed, due to the high synthesis of acids due to the fermentation of this sugar. In addition, the study methodology uses enamel slabs protected by a screen in an intraoral appliance, made with self-curing acrylic resin, a porous material. This set of factors makes the *in situ* palatal appliance a biofilm retentive factor, which stimulates the proliferation not only of *Lactobacillus* spp, but also of *Candida albicans*¸being the latter abundant in the SEM images as well as viable cells numbers (Figure 1 and Table 1)[43,44]. A noteworthy point is that we assessed the effect of the tested treatments only on the counts of well-known cariogenic microorganisms. We recognized though this may have a limited clinical impact taking into account the diverse and complex microbial communities found on cariogenic biofilms[45,46] and others. A microbial characterization in depth (using next generation sequencing platforms) would reveal other microbial robust data, which could help us to clarify the effect played by both CHX and *Stevia* overall microbial communities. Nevertheless, although we are unable to show such detailed microbial composition, if there was any anticipated antimicrobial effect decreasing the abundance of some microbial taxa, it was not able to reduce the cariogenicity of the formed biofilms (Table 1).

The vast majority of studies found in the literature are *in vitro* models, performed under ideal standards and conditions, unlike the present study, performed *in situ* with the use of intraoral appliances by volunteers. These appliances are exposed to the oral environment, where there is a high variability of microorganisms, under the action of the saliva and the temperature of the oral cavity. Moreover, in the *in-situ* model, a plastic screen that protects the biofilm and prevents mechanical removal of the biofilm covers the enamel slabs. This biofilm, exposed to a high cariogenic challenge (8 times/day), without mechanical action, protected by the plastic screen, might have been structured in such a way that the treatments did not have the effect observed by many authors in *in vitro* studies

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CONCLUSION

Under high cariogenic conditions resembling frequent exposure to sucrose, use of *Stevia* cannot modify the counts of some cariogenic microorganisms, neither the porosity of *in situ* formed biofilms. Additional studies in different cariogenic conditions are necessary to evaluate possible modifications in biofilms followed by the use of *Stevia* infusion.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

REFERENCES

1. Ferrazzano G, Cantile T, Alcidi B, Coda M, Ingenito A, Zarrelli A, et al. Is stevia rebaudiana bertoni a non cariogenic sweetener? A review. Molecules. 2015 Dec 26;21(1):38.

2. Lemus-Mondaca R, Vega-Gálvez A, Zura-Bravo L, Ah-Hen K. Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: a comprehensive review on the biochemical, nutritional and functional aspects. Food Chem. 2012 Jun;132(3):1121–32.

3. Ruiz-Ruiz JC, Moguel-Ordoñez YB, Segura-Campos MR. Biological activity of *stevia rebaudiana* bertoni and their relationship to health. Crit Rev Food Sci Nutr. 2017 Aug 13;57(12):2680–90.

4. Nikiforov AI, Rihner MO, Eapen AK, Thomas JA. Metabolism and toxicity studies supporting the safety of rebaudioside D. Int J Toxicol. 2013 Jul;32(4):261–73.

5. Wheeler A, Boileau AC, Winkler PC, Compton JC, Prakash I, Jiang X, et al. Pharmacokinetics of rebaudioside A and stevioside after single oral doses in healthy men. Food Chem Toxicol Int J Publ Br Ind Biol Res Assoc. 2008 Jul;46 Suppl 7:S54-60.

6. Uçar A, Yılmaz S, Yılmaz Ş, Kılıç MS. A research on the genotoxicity of stevia in human lymphocytes. Drug Chem Toxicol. 2018 Apr;41(2):221–4.

VERSÃO DE PROVA

7. Purkayastha S, Kwok D. Metabolic fate in adult and pediatric population of steviol glycosides produced from stevia leaf extract by different production technologies. Regul Toxicol Pharmacol RTP. 2020 Oct;116:104727.

8. Abudula R, Jeppesen PB, Rolfsen SED, Xiao J, Hermansen K. Rebaudioside A potently stimulates insulin secretion from isolated mouse islets: studies on the dose-, glucose-, and calcium-dependency. Metabolism. 2004 Oct;53(10):1378–81.

9. Gu W, Rebsdorf A, Anker C, Gregersen S, Hermansen K, Geuns JMC, et al. Steviol glucuronide, a metabolite of steviol glycosides, potently stimulates insulin secretion from isolated mouse islets: Studies in vitro. Endocrinol Diabetes Metab. 2019 Oct;2(4):e00093.

10. Myint KZ, Chen JM, Zhou ZY, Xia YM, Lin J, Zhang J. Structural dependence of antidiabetic effect of steviol glycosides and their metabolites on streptozotocin-induced diabetic mice. J Sci Food Agric. 2020 Aug;100(10):3841–9.

11. Prata C, Zambonin L, Rizzo B, Maraldi T, Angeloni C, Vieceli Dalla Sega F, et al. Glycosides from *Stevia rebaudiana* bertoni possess insulin-mimetic and antioxidant activities in rat cardiac fibroblasts. Oxid Med Cell Longev. 2017;2017:1–13.

12. Rotimi SO, Rotimi OA, Adelani IB, Onuzulu C, Obi P, Okungbaye R. Stevioside modulates oxidative damage in the liver and kidney of high fat/low streptozocin diabetic rats. Heliyon. 2018 May;4(5):e00640.

13. Ferri LAF, Alves-Do-Prado W, Yamada SS, Gazola S, Batista MR, Bazotte RB. Investigation of the antihypertensive effect of oral crude stevioside in patients with mild essential hypertension. Phytother Res PTR. 2006 Sep;20(9):732–6.

14. Hsieh MH, Chan P, Sue YM, Liu JC, Liang TH, Huang TY, et al. Efficacy and tolerability of oral stevioside in patients with mild essential hypertension: a two-year, randomized, placebo-controlled study. Clin Ther. 2003 Nov;25(11):2797–808.

15. Gamboa F, Chaves M. Antimicrobial potential of extracts from stevia rebaudiana leaves against bacteria of importance in dental caries. Acta Odontol Latinoam. 2012;25:5.

16. Cho BO, Ryu HW, So Y, Cho JK, Woo HS, Jin CH, et al. Anti-inflammatory effect of austroinulin and 6-O-acetyl-austroinulin from Stevia rebaudiana in lipopolysaccharide-stimulated RAW264.7 macrophages. Food Chem Toxicol Int J Publ Br Ind Biol Res Assoc. 2013 Dec;62:638–44.

17. Chen J, Xia Y, Sui X, Peng Q, Zhang T, Li J, et al. Steviol, a natural product inhibits proliferation of the gastrointestinal cancer cells intensively. Oncotarget. 2018 May 29;9(41):26299–308.

18. Vandana K, Reddy VC, Sudhir KM, Kumar K, Raju SH, Babu JN. Effectiveness of stevia as a mouthrinse among 12-15-year-old schoolchildren in Nellore district,

VERSÃO DE PROVA

Andhra Pradesh - a randomized controlled trial. J Indian Soc Periodontol. 2017;21(1):37–43.

19. Contreras MS. Anticariogenic properties and effects on periodontal structures of Stevia rebaudiana Bertoni. Narrative review. J Oral Res. 2013;2(3):158-66.

20. Marsh PD. Are dental diseases examples of ecological catastrophes? Microbiology. 2003 Feb 1;149(2):279–94.

21. Marsh PD. In sickness and in health—what does the oral microbiome mean to us? An ecological perspective. Adv Dent Res. 2018 Feb;29(1):60–5.

22. Cury JA, Rebelo MAB, Del Bel Cury AA, Derbyshire MTVC, Tabchoury CPM. Biochemical composition and cariogenicity of dental plaque formed in the presence of sucrose or glucose and fructose. Caries Res. 2000;34(6):491–7.

23. Rezende G, Arthur RA, Grando D, Hashizume LN. Cariogenic potential of sucrose associated with maltodextrin on dental enamel. Caries Res. 2017;51(2):129–35.

24. Sanchez AY, de Oliveira CL, Negrini TC, Hashizume LN, Hara AT, Maltz M, et al. In situ effect of arginine-containing dentifrice on plaque composition and on enamel demineralization under distinct cariogenic conditions. Caries Res. 2018;52(6):588–97.

25. Kim NC, Kinghorn AD. Highly sweet compounds of plant origin. Arch Pharm Res. 2002 Dec;25(6):725–46.

26. van Loveren C, Broukal Z, Oganessian E. Functional foods/ingredients and dental caries. Eur J Nutr. 2012 Jul;51(S2):15–25.

27. Sapuyes GRV, Vargas SE, Jaimes FOG, Bolaños NC, Scarpetta RÁG. Actividad inhibitoria de la stevia rebaudiana sobre el lactobacillus acidophillus y el streptococcus mutans. Rev Nac Odontol. 2010;8.

28. Ikeda T, Okada A, Motada R. Effects of stevioside on certain metabolism of streptococcus mutans. J Nihon Univ Sch Dent. 1978;4:24–7.

29. Giacaman RA, Campos P, Muñoz-Sandoval C, Castro RJ. Cariogenic potential of commercial sweeteners in an experimental biofilm caries model on enamel. Arch Oral Biol. 2013 Sep;58(9):1116–22.

30. Kishta-Derani M, Neiva GF, Boynton JR, Klm YE, Fontana M. The antimicrobial potential of stevia in an in vitro microbial caries model. Am J Dent. 2016;29(2):87–92.

31. de Slavutzky SMB. Stevia and sucrose effect on plaque formation. J Für Verbraucherschutz Leb. 2010 May;5(2):213–6.

32. Hara AT, Queiroz CS, Paes Leme AF, Serra MC, Cury JA. Caries progression and inhibition in human and bovine root dentine in situ. Caries Res. 2003;37(5):339–44.

VERSÃO DE PROVA

33. Henz SL, Arthur RA, Colvara BC, Severo R, Felten V. Atividade antimicrobiana de Stevia rebaudiana Bertoni e de adoçantes não calóricos sobre bactérias cariogênicas: estudo in vitro. Rev Fac Odontol - UPF. 2018 Aug 15;23(1).

34. Aires CP, Del Bel Cury AA, Tenuta LMA, Klein MI, Koo H, Duarte S, et al. Effect of starch and sucrose on dental biofilm formation and on root dentine demineralization. Caries Res. 2008;42(5):380–6.

35. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem. 1956;28(3):350–6.

36. Grenby TH. Advances in non-caloric sweeteners with dental health advantages over sugars. Proc Finn Dent Soc Suom Hammaslääkäriseuran Toim. 1991;87(4):489–99.

37. Debnath M. Clonal propagation and antimicrobial activity of an endemic medicinal plant Stevia rebaudiana. J Med Plants Res 2008;2(2):45-51.

38. Mohammadi-Sichani, M. Effect of different extracts of Stevia rebaudiana leaves on Streptococcus mutans growth. J Med Plants Res. 2012 Aug 22;6(32).

39. Ajagannanavar S, Shamarao S, Battur H, Tikare S, Al-Kheraif A, Ayed MA. Effect of aqueous and alcoholic Stevia (Stevia rebaudiana) extracts against Streptococcus mutans and Lactobacillus acidophilus in comparison to chlorhexidine: An in vitro study. J Int Soc Prev Community Dent. 2014 Dec;4(5):116.

40. Arthur RA, Waeiss RA, Hara AT, Lippert F, Eckert GJ, Zero DT. A defined-multispecies microbial model for studying enamel caries development. Caries Res. 2013;47(4):318–24.

41. Eidt G, Andrade CG de, Negrini T de C, Arthur RA. Role of candida albicans on enamel demineralization and on acidogenic potential of streptococcus mutans in vitro biofilms. J Appl Oral Sci. 2019;27:e20180593.

42. Abdul Razak F, Baharuddin BA, Akbar EFM, Norizan AH, Ibrahim NF, Musa MY. Alternative sweeteners influence the biomass of oral biofilm. Arch Oral Biol. 2017;80:180–4.

43. Nett JE, Marchillo K, Spiegel CA, Andes DR. Development and validation of an in vivo candida albicans biofilm denture model. Infect Immun. 2010 Sep;78(9):3650–9.

44. Salerno C, Pascale M, Contaldo M, Esposito V, Busciolano M, Milillo L, et al. Candida-associated denture stomatitis. Med Oral Patol Oral Cirugia Bucal. 2011;16(2):e139–43.

45. Hurley E, Barrett MPJ, Kinirons M, Whelton H, Ryan CA, Stanton C, et al. Comparison of the salivary and dentinal microbiome of children with severe-early childhood caries to the salivary microbiome of caries-free children. BMC Oral Health. 2019 Dec;19(1):13.

VERSÃO DE PROVA

46. de Jesus VC, Shikder R, Oryniak D, Mann K, Alamri A, Mittermuller B, et al. Sex-based diverse plaque microbiota in children with severe caries. J Dent Res. 2020 Jun;99(6):703–12.

VERSÃO DE PROVA