UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

CAREN AUGUSTINHO DO NASCIMENTO

Effect of sweetener containing Stevia on the development of dental caries in enamel and dentin under a microcosm biofilm model

Efeito de adoçante contendo Stevia no desenvolvimento da cárie dentária em esmalte e dentina sob um modelo de biofilme de microcosmo

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"A tua palavra é lâmpada que ilumina os meus passos
e luz que clareia o meu caminho."
Salmos 119:105

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que o Senhor, o teu Deus, te dá."
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Professores fascinantes ensinam para a vida."
Augusto Cury

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Chico Xavier

RESUMO

Efeito de adoçante contendo Stevia no desenvolvimento da cárie dentária em esmalte e dentina sob um modelo de biofilme de microcosmo

Este estudo comparou o efeito de um adoçante comercial e puro contendo Stevia ao do aspartame (comercial e puro), sacarose (açúcar comum), xilitol (adoçante natural conhecido por ser anti-cariogênico) no desenvolvimento de cárie dentária em um modelo de biofilme microcosmo formado sobre o esmalte e a dentina. Para isso, foram preparadas 228 amostras de esmalte bovino e 228 amostras de dentina radicular bovina (4 mm x 4 mm). Em placas de 24 poços, cada amostra de esmalte ou dentina foi exposta a 1,5 mL de inóculo (saliva-glicerol humana + saliva de McBain, 1:50), por 8 h. Após as 8 h iniciais, o inóculo foi retirado, as amostras foram lavadas com PBS (5 s), receberam 1,5 mL de meio fresco (saliva artificial McBain) por 16 h, completando as 24 h iniciais. Do 2º ao 5º dia de cultivo do biofilme, as amostras foram expostas diariamente à saliva de McBain suplementada com 0,2% dos respectivos adoçantes/açúcar: Stevia, aspartame (ambos na forma pura ou comercial, marca Finn), xilitol, sacarose e saliva (McBain) controle sem suplementação (n=3/placa, quadriplicada biológica). O cultivo foi realizado a 5% CO_2 e 37ºC. A produção de ácido lático (g/L) e as unidades formadoras de colônia (UFC) para microrganismos totais, lactobacilos totais, estreptococos totais e *Streptococcus* mutans/ *S. sobrinus* (log_{10} UFC/mL) foram quantificadas no biofilme. O grau de desmineralização dentária foi analisado por meio da microrradiografia transversal-TMR. Os dados foram comparados estatisticamente (Kruskal-Wallis/Dunn, $p<0,05$). Na análise do lactato, os adoçantes stevia pura, aspartame puro, xilitol e controle não diferiram entre si, reduzindo em 92% a produção deste ácido. Os grupos stevia finn, aspartame finn e sacarose apresentaram maior produção de ácido lático, sendo similares entre si (0,47 ± 0,14, 0,43 ± 0,10, 0,44 ± 0,13 g/L para o esmalte; 0,69 ± 0,19, 0,65 ± 0,11, 0,67 ± 0,24 g/L para a dentina, respectivamente, $p<0,0001$). Em relação à contagem de UFCs para lactobacilos totais e *S. mutans/ S. sobrinus*, os grupos xilitol e controle não apresentaram crescimento de colônias sobre o esmalte. As espécies cresceram sob exposição a stevia finn, aspartame finn e sacarose no esmalte e dentina (5,69 ± 0,44, 5,69 ± 0,82, 5,75 ± 0,60 log_{10} UFC lactobacilos totais /mL de; 7,57 ± 0,37, 7,61 ± 0,64, 7,49 ± 0,41 log_{10} UFC *S. mutans*/mL para esmalte; 0,00 ± 3,98, 5,30 ± 5,30, 5,30 ± 0,15 log_{10} UFC lactobacilos totais/mL; 7,83 ± 0,36,

7,87 ± 0,18; 7,78 ± 0,31 log_{10} UFC *S. mutans* /mL para a dentina, respectivamente). A desmineralização do esmalte e da dentina foi significativamente reduzida para os grupos xilitol, controle, stevia pura e aspartame puro (aproximadamente 85% e 83% de redução, respectivamente) em comparação com stevia finn, aspartame finn e sacarose que, por sua vez, não diferiram entre si (ΔZ: 3084,7 ± 834,3, 3174,7 ± 603,1, 2913,7 ± 646,7 vol%.µm para o esmalte e 3945,7 ± 689,7, 3626,8 ± 617,3, 3543,3 ± 432,5 vol%.µm para a dentina, respectivamente). Em conclusão, adoçantes comerciais à base de stevia e aspartame (marca Finn) mostraram-se tão cariogênicos quanto à sacarose neste modelo experimental, o que se deve aos outros componentes destes adoçantes, uma vez que as formas puras não foram cariogênicas.

Palavras-chave: açúcar, adoçante, biofilme dentário, cárie dentária, dentina, esmalte.

ABSTRACT

Effect of sweetener containing Stevia on the development of dental caries in enamel and dentin under a microcosm biofilm model

This study compared the effect of a commercial and pure sweetener containing Stevia to that of aspartame (commercial and pure), sucrose (common sugar), xylitol (a natural sweetener known to be anti-cariogenic) on the development of dental caries in a microcosm biofilm model formed over enamel and dentin. For this, 228 bovine enamel and 228 samples of bovine root dentin samples (4 mm x 4 mm) were prepared. In 24-well plates, each enamel or dentin sample was exposed to 1.5 mL of inoculum (human saliva-glycerol + McBain saliva, 1:50), for 8 h. After the initial 8 h, the inoculum was removed, the samples were washed with PBS (5 s), received 1.5 mL of fresh medium (McBain artificial saliva) for 16 h, completing the initial 24 h. From the 2nd to the 5th day of biofilm cultivation, the samples were exposed daily to McBain saliva supplemented with 0.2% of the respective sweeteners /sugar: Stevia, aspartame (both in pure or commercial form, Finn brand), xylitol, sucrose and control saliva (McBain) without supplementation (n = 3/plate, biological quadruplicate). The cultivation was carried out at 5% CO_2 and 37ºC. The production of lactic acid (g/L) and colony-forming units (CFU) for total microorganisms, total lactobacilli, total streptococci and *Streptococcus mutans*/ *S. sobrinus* (log_{10} CFU/mL) were quantified in the biofilm. The degree of dental demineralization was analyzed using transverse microradiography-TMR. The data were compared statistically (Kruskal-Wallis / Dunn, $p < 0.05$). In the lactate analysis, the sweeteners pure stevia, pure aspartame, xylitol and control did not differ, reducing by 92% the production of this acid. The groups stevia finn, aspartame finn and sucrose showed higher production of lactic acid, being similar to each other (0.47 ± 0.14, 0.43 ± 0.10, 0.44 ± 0.13 g/L for enamel; 0.69 ± 0.19, 0.65 ± 0.11, 0.67 ± 0.24 g/L for dentin, respectively, $p < 0.0001$). Regarding the CFU counting for total lactobacilli and *S. mutans/S. sobrinus*, xylitol and control groups did not show colony growth on enamel. The species grew under exposure to stevia finn, aspartame finn and sucrose in enamel and dentin (5.69 ± 0.44, 5.69 ± 0.82, 5.75 ± 0.60 log_{10} CFU total lactobacilli/mL; 7.57 ± 0.37, 7.61 ± 0.64, 7.49 ± 0.41 log_{10} CFU *S. mutans* /mL; 0.00 ± 3.98, 5.30 ± 5.30, 5.30 ± 0.15 log_{10} CFU total lactobacilli/mL; 7.83 ± 0.36, 7.87 ± 0.18; 7.78 ± 0.31 log_{10} CFU *S. mutans*/mL for enamel and dentin, respectively). Enamel and dentin demineralization was

significantly reduced for the xylitol, control, pure stevia and pure aspartame groups (approximately 85% and 83% reduction, respectively) compared to stevia finn, aspartame finn and sucrose which, in turn, did not differ from each other (ΔZ: 3084.67 ± 834.26, 3174.67 ± 603.10, 2913.67 ± 646.69 vol%.µm for enamel and 3945.67 ± 689.69, 3626.79 ± 617.26, 3543.33 ± 432.50 vol%.µm for dentin). In conclusion, commercial sweeteners based on stevia and aspartame (Finn brand) proved to be as cariogenic as sucrose in this experimental model, which is due to the other components of these sweeteners, since the pure forms were not cariogenic.

Keywords: dental biofilm, dental cavity, dentin, enamel, sugar, sweetener.

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LISTA DE ABREVIATURA E SIGLAS

CCD	camera canon
CFU	colony forming units
cm	Centimeters
ΔZ	integrated mineral loss
DMFT	decayed, missing, filled teeth
EPS	extracellular polysaccharide
G	gram
kV	kilovolts
L	liter
Ld	lesion depth
mA	milliampere
Min	minutes
mL	milliliter
Nm	nanometer
PBS	phosphate-buffered saline
pH	hydrogen potential
R	average mineral loss
Ra	arithmetic roughness
S	seconds
TMR	transverse microradiography
µL	microliters
Vol%	Percentage of volume
WHO	World Health Organization
µm	micrometer

SUMÁRIO

1 INTRODUCTION

1 INTRODUCTION

Dental caries is a multifactorial oral disease highly impacting for the affected person, caused by a biofilm in disbiose, rich in acidogenic, aciduric and EPS (extracellular polysaccharide) producer microorganisms. These microorganisms are capable of metabolizing different types of sugar, mainly sucrose from the diet, forming acids that alter the biofilm pH and cause tooth demineralization (Keyes, 1960; Bowen, 2002; Marsh et al., 2011; Pitts et al., 2017; Ayoub et al., 2020).

Considering the relationship between dental caries and socioeconomic condition, it is obvious to infer that the consumption of common sugar (sucrose) is highly related to the disease, not only because it is low cost and easy access by the population, but also because bacteria can metabolize it easily. In addition, sucrose is the main substrate for the action of the glycosyltransferase enzyme for production of EPS, which is an important virulence factor for cariogenic bacteria (Paes-Leme et al., 2006; Ccahuana-Vásquez et al., 2007; Li et al., 2020). Accordingly, the World Health Organization (WHO) has recommended that the consumption of added sugar should not exceed more than 5% of the total energy consumed (around 10 kg/year), so that the number of DMFT is less than 3 in 12 years old children (Moyhinan, 2016; Moynihan & Miller, 2020).

To replace common sugar, non-caloric sweeteners have been used by 28% of the population, in order to control obesity and diabetes mellitus (Jayalath et al., 2015; Azad et al., 2016; Manios et al., 2020). Sweeteners can be from natural (stevia) or synthetic origin (aspartame, acesulfame, sodium cyclamate, sucralose). In general, they present high sweetness (greater than sucrose itself). However, there is no evidence about the beneficial effects (control of obesity and diabetes) as well as about the side effects (cancer, headache and others) induced by the frequent consume of sweeteners (Lohner et al., 2017; Nichol et al., 2018; Santos et al., 2018). With respect to dental caries, few studies have shown that non-caloric sweeteners seem to be inert or at least not as cariogenic as sucrose (Giacaman et al., 2013; Giacaman, 2018).

Among the sweeteners, Stevia rebaudiana Bertoni, belonging to the Asteraceae family, is native from South America. Stevia leaves contain steviol glycosides, including stevioside, rebaudioside (A to F), steviolbioside and isosteviol, which are responsible for the sweet taste of the plant and have commercial value worldwide as a sugar substitute in food, beverages and medicines. In addition to its value as a sweetener, stevia and its glycosides have therapeutic effects against several diseases, such as cancer, diabetes mellitus, hypertension, inflammation, cystic fibrosis, obesity and tooth decay (Momtazi-Borojeni et al., 2017). Few studies have evaluated the effect of stevia on the development of cariogenic biofilm and/or of carious lesions, with inconclusive results regarding its anti-caries potential (Giacaman et al., 2013; Ma & Blanksma, 2015; Ferrazzano et al., 2015; Kishta et al., 2016; Escobar et al., 2020). There is still a lack of evidence about its safety and benefits, which enable a safer recommendation of Stevia (Samuel et al., 2018).

Considering that obesity is associated with dental caries (Tinanoff & Holt, 2017; Marro et al., 2021), due to the behavioral nature and dependency of sugar consumption for both diseases, and that the population have replaced common sugar by non-caloric sweeteners, it is necessary to know their anti-caries potential. Therefore, the aim of the work was to compare the effect of sweetener containing stevia (natural sweetener) to that with aspartame (synthetic sweetener), sucrose (common sugar) and xylitol (natural sweetener) on the development of dental caries in a microcosm biofilm model formed on enamel and dentin.

2 ARTICLE

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Article formatted according to Caries Research Guidelines.

Effect of sweetener containing Stevia on the development of dental caries in enamel and dentin under a microcosm biofilm model

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Short title: Effect of sweetener containing Stevia on dental caries

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Abstract

This study compared the effect of a commercial and pure sweetener containing stevia to that of aspartame (commercial and pure), sucrose (common sugar), xylitol (natural sweetener) on the development of dental caries in a microcosm biofilm model. For this, 228 bovine enamel and 228 root dentin (4 mm x 4 mm) were exposed to microcosm biofilm model using human saliva. From the 2nd to the 5th day of biofilm cultivation, the samples were exposed daily to McBain saliva supplemented with 0.2% of the respective sweeteners/sugar, under 5% CO_2 and 37ºC. The lactic acid (g/L) and the colony-forming units for total microorganisms, total lactobacilli, total streptococci and *Streptococcus mutans/ S. sobrinus* (log_{10} CFU/mL) were quantified. The demineralization was analyzed by TMR. The data were compared statistically (Kruskal-Wallis/ Dunn, $p<0.05$). Pure stevia, pure aspartame, xylitol and control were able to significantly reduce 92% of lactate production. Stevia finn, aspartame finn and sucrose showed similar production of lactic acid (0.47 ± 0.14, 0.43 ± 0.10, 0.44 ± 0.13 g/L; 0.69 ± 0.19, 0.65 ± 0.11, 0.67 ± 0.24 g/L, for enamel and dentin, respectively, $p <0.0001$). With respect to total lactobacilli and *S. mutans/S. sobrinus* CFU, xylitol and control groups did not show growth on enamel, while CFU numbers were found in stevia finn, aspartame finn and sucrose groups for both tissues (5.69 ± 0.44, 5.69 ± 0.82, 5.75 ± 0.60 log_{10} CFU total lactobacilli/mL; 7.57 ± 0.37, 7.61 ± 0.64, 7.49 ± 0.41 log_{10} CFU *S. mutans*/mL for enamel; 0.00 ± 3.98, 5.30 ± 5.30, 5.30 ± 0.15 log_{10} CFU total lactobacilli/mL; 7.83 ± 0.36, 7.87 ± 0.18; 7.78 ± 0.31 log_{10} CFU *S. mutans* /mL for dentin, respectively). Enamel and dentin demineralization was significantly reduced for the groups xylitol, control, pure stevia and pure aspartame (around 85% and 83% reduction, respectively) compared to stevia finn, aspartame finn and sucrose, which in turn did not differ each other (ΔZ: 3084.7 ± 834.3, 3174.7 ± 603.1, 2913.7 ± 646.7 vol%.µm for enamel e 3945.7 ± 689.7, 3626.8 ± 617.3, 3543.3 ± 432.5 vol%.µm for dentin, respectively). Commercial sweeteners containing stevia and aspartame proved to be as cariogenic as sucrose in this experimental model, which is due to the other components, since the pure forms were not cariogenic.

Introduction

Dental caries is a multifactorial oral disease highly impacting for the affected person especially when involves dentin. It is caused by a biofilm in disbiose, rich in acidogenic, aciduric and EPS (extracellular polysaccharide) producer microorganisms. These microorganisms are capable of metabolizing different types of sugar, mainly sucrose from the diet, forming acids that alter the biofilm pH and cause tooth demineralization (Keyes, 1960; Bowen, 2002; Marsh et al., 2011; Pitts et al., 2017; Ayoub et al., 2020).

Considering the relationship between dental caries and socioeconomic condition, it is obvious to infer that the consumption of common sugar (sucrose) is highly related to the disease, not only because it is low cost and easy access by the population, but also because bacteria can metabolize it easily. In addition, sucrose is the main substrate for the action of the glycosyltransferase responsible for the production of EPS, which is an important virulence factor of cariogenic bacteria (Paes-Leme et al., 2006; Ccahuana-Vásquez et al., 2007; Li et al., 2020). Accordingly, the World Health Organization (WHO) has recommended that the consumption of added sugar should not exceed more than 5% of the total energy consumed (around 10 kg/year), so that the number of DMFT is less than 3 in 12 years old children (Moyhinan, 2016; Moynihan & Miller, 2020).

To replace common sugar, non-caloric sweeteners have been used by 28% of the population, in order to control obesity and diabetes mellitus (Jayalath et al., 2015; Azad et al., 2016; Manios et al., 2020). Sweeteners can be from natural (stevia) or synthetic origin (aspartame, acesulfame, sodium cyclamate, sucralose). In general, they present high sweetness, which is greater than sucrose itself. However, there is no evidence about the beneficial effects (control of obesity and diabetes) as well as about the side effects (cancer, headache and others) induced by the frequent consume of sweeteners (Lohner et al., 2017; Nichol et al., 2018; Santos et al., 2018). With respect to dental caries, few studies have shown that non-caloric sweeteners seem to be inert or at least not as cariogenic as sucrose (Giacaman et al., 2013; Giacaman, 2018).

Among the sweeteners, *Stevia rebaudiana Bertoni*, belonging to the Asteraceae family, is native from South America. Stevia leaves contain steviol glycosides, including stevioside, rebaudioside (A to F), steviolbioside and isosteviol, which are responsible for the sweet taste of the plant and have commercial value

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Considering that obesity is associated with dental caries (Tinanoff & Holt, 2017; Marro et al., 2021), due to the behavioural nature and dependency of sugar consumption for both diseases, and that the population have replaced common sugar by non-caloric sweeteners, it is necessary to know their anti-caries potential. Therefore, the aim of the work was to compare the effect of sweetener containing stevia (natural sweetener) to that with aspartame (synthetic sweetener), sucrose (common sugar) and xylitol (natural sweetener) on the development of dental caries in a microcosm biofilm model formed on enamel and dentin. The null hypothesis was that stevia has the same cariogenic potential as the other sweeteners and common sugar.

Material and Methods

Saliva collection

This study was firstly approved by the local Ethical Committee (CEEA 12647819.5.0000.5417). After sign the informed consent, saliva was collected from 10 healthy donors, who have followed the inclusion criteria: 1) normal salivary flow (stimulated saliva flow > 1 mL/min and non-stimulated saliva flow > 0.3 mL/min), 2) with previous history of caries, but no caries active (no active white spot and/or cavitated lesions), 3) without gingivitis/periodontitis (gum bleeding or tooth mobility) and, 4) without ingestion of antibiotics 3 months prior the experiment. On the day of collection, participants did not brush their teeth for 24 h. Furthermore, they were not allowed to ingest food or drinks at least for 2 h before saliva collection. The saliva was collected under stimulation by chewing a rubber material for 10 min during the morning. After collection, saliva was diluted in glycerol (70% saliva and 30% glycerol). Aliquots of 1 mL were stored at -80°C (Pratten et al. 2003).

Tooth sample preparation and treatment groups

Two hundred and twenty-eight enamel and 228 root dentin samples (4 mm x 4 mm) were prepared from bovine teeth, using a semi-precision cutting machine (Buehler, Enfield, USA). The samples fixed in acrylic discs were polished in a metallographic polishing machine (Arotec, Cotia, Brazil) using water-cooled silicon-carbide discs (320 and 600-grade papers ANSI grit; Buehler, Enfield, USA) to remove grooves (amount of removed tissue for enamel: 0.259 ± 0.119 mm and dentin: 0.321 ± 0.113 mm). The roughness was measured using a contact profilometer and Mahr Surf XCR 20 software (Mahr, Göttingen, Germany) for the selection and randomly allocation of the samples in the experimental groups (enamel: Ra = 0.130 ± 0.032 µm; dentin: Ra = 0.287 ± 0.047 µm). Two thirds of the sample's surfaces were protected with nail polish to obtain control areas for the TMR analysis. The samples were sterilized using ethylene oxide [Gas exposure time (30% ETO/ 70% CO_2) for 4 h under a pressure of 0.5 ± 0.1 kgF/cm^2].

From the total samples, eighty-four samples were applied for lactic acid analysis, sixty for colony-forming unit (CFU) counting and eighty-four for transverse microradiography-TMR analysis. Enamel and dentin samples were randomly divided in the treatments according to the Ra means. All sugars/sweeteners were diluted in McBain saliva at 0.2% Their compositions are displayed in Table 1.

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139 *Microcosm biofilm formation and treatments*

140 The human saliva was defrosted and mixed with McBain artificial saliva
141 (McBain, 2009) in a proportion of 1:50. The McBain saliva contained 2.5 g/L mucin
142 from porcine stomach (type II), 2.0 g/L bacteriological peptone, 2.0 g/L tryptone, 1.0
143 g/L yeast extract, 0.35 g/L NaCl, 0.2 g/L KCl, 0.2 g/L $CaCl_2$, 0.1 g/L cysteine
144 hydrochloride, 0.001 g/L hemin, 0.0002 g/L vitamin K1, at pH 7.0. All reagents were
145 from Sigma-Aldrich. This solution was added to each well containing an enamel or
146 dentin sample (v=1.5 mL/well) in 24-well plate, which was incubated at 5% CO_2 and
147 37˚C. After 8h, the medium was removed, the enamel/dentin samples were washed
148 using phosphate-buffered saline (PBS, 5 s) and fresh McBain saliva was added into
149 the wells (v=1.5 mL/well). The plates were incubated at 5% CO_2 and 37˚C for further
150 16h, completing the first day.

151 From the 2^{nd} to the 5^{th} day, McBain saliva supplemented with 0.2%
152 sweeteners/sugar was added to each well containing an enamel or dentin sample.
153 The plate was incubated at the same conditions described above (Zhang et al. 2013).
154 Figure 1 summarizes the experimental protocol.

155

156 *Lactic acid production analysis*

157 After the 5^{th} day, biofilm samples were incubated in buffered peptone water
158 (BPW) (Synth, Diadema, Brazil) supplemented with 0.2% of sweeteners/ sugar (v = 1
159 mL/sample) for 3 h, at 5% CO_2 and 37ºC, to allow the biofilm produces lactic acid.
160 Lactate concentrations were evidenced by enzymatic method (lactic dehydrogenase
161 method, Boehringer Mannheim, Germany) in the BPW solution according to the
162 manufacture instruction. The absorbance was measured at 340 nm using a
163 microplate reader (Fluorstar Optima - BMG Labtech, Ortenberg, Germany). The
164 values were expressed as g lactate/L (BPW).

165

166 *Colony-forming unit (CFU) counting*

167 For CFU counting, 100 µL of the bacterial suspension from microcosm biofilm
168 were then diluted to 10^{-4} and spread on petri dishes (25 µL/dish) containing four
169 different types of agar: 1) Brain Heart Infusion agar (BHI, Difco, Detroit, USA) for total
170 microorganisms and 2) Mitis Salivarius Agar (MSA, Neogen, Indaiatuba, Brazil)
171 containing 20% sucrose and 1% potassium tellurite for total streptococci (Lima et al.

2009); 3) SB-20M (Saravia et al. 2011) containing 15 g bacto-casitone (Difco, Detroit, USA), 5 g yeast extract (Kasvi, Curitiba, Brazil), 0.2 g L-Cysteine hydro-chloride (Sigma, Steinheim, Germany), 0.1 g sodium sulfite (Sigma, Steinheim, Germany), 20.0 g sodium acetate (Synth, Diadema, Brazil), 200.0 g coarse granular cane sugar, 15.0 g agar (Kasvi, Curitiba, Brazil), and 1 L distilled water. After autoclaving for 20 min at 120º C, 0.2 U/mL bacitracin (Sigma, Steinheim, Germany) was added for determination of mutans streptococci (*S. mutans and S. sobrinus*); and 4) Rogosa (Kasvi, Curitiba, Brazil) supplemented with 0.13% glacial acetic acid to assess the number of lactobacilli (Lima et al. 2009).

The plates were then incubated at 5% CO_2 and 37ºC. After 48h, the CFU numbers were counted and transformed in $\log_{10}$ CFU/mL (Cheng et al. 2012).

Transverse microradiography (TMR)

After cleaning, all enamel and dentin samples were transversally sectioned and polished to obtain slices with 80-100 µm (enamel) and 100-120 µm (dentin) of thickness. The enamel and dentin slices were fixed in a sample-holder together with an aluminium calibration step wedge with 14 steps. A microradiograph was taken using an x-ray generator (Softex, Tokyo, Japan) on the glass plate at 20 kV and 20 mA (at a distance of 42 cm) for 13 min. The glass plates were developed for 7 min, rinsed in deionized water, fixed for 7 min in a dark environment, and then rinsed in running water for 10 min and air-dried (all procedures were done at 20°C). The developed plate was analysed using a transmitted light microscope fitted with a 20x objective (Zeiss, Oberkochen, Germany), a CCD camera (Canon, Tokyo, Japan) and a computer. Two images per sample were taken using data-acquisition (version 2012) and interpreted using calculation (version 2006) softwares from Inspektor Research System bv (Amsterdam, The Netherlands). The mineral content was calculated based on the work of Angmar et al. (1963), assuming 87 vol% of mineral content for sound enamel and 50% of mineral content for sound dentin. The lesion depth (LD, µm), the integrated mineral loss (ΔZ, vol%.µm) and the average mineral loss over the lesion depth (R, vol%) were calculated.

Statistical Analysis

All experiments were performed in biological quadruplicate (n_{final} = 12). Data were statistically analysed using software Graph Pad Instat for Windows (GraphPad Software, San Diego, USA).

The normality (Kolmogorov-Smirnov's test) and homogeneity (Bartlett's test) of the data were tested. Since the data did not pass the homogeneity test, all data were compared using Kruskal-Wallis followed by Dunn test. The level of significance was set at 5%.

Results

Enamel

Pure stevia, pure aspartame, xylitol and control presented similar low lactate production compared to the commercial versions of the sweeteners and sucrose. On the other hand, stevia finn, aspartame finn and sucrose presented the highest lactic acid production, with no difference between them (Figure 2).

Sucrose induced the highest growth of total microorganism compared to all groups, except to stevia finn that did not differ from all groups. No growth of total lactobacilli, total streptococci and *S. mutans* was seen for xylitol and control groups, while stevia finn, aspartame finn and sucrose presented similar microorganisms' growth (Table 2).

The integrated mineral loss and lesion depth were reduced in enamel samples exposed to pure stevia, pure aspartame, xylitol and control (≈ 85% reduction) compared to sucrose, which in turn had a similar cariogenic effect to the tested commercial sweeteners (aspartame finn and stevia finn) (Table 3 and Figure 3). With respect to mineral loss mean, only enamel samples belonging to xylitol and control groups presented reduced values compared to sucrose.

Dentin

Pure stevia, pure aspartame, xylitol and control presented similar low lactate production compared to the commercial versions of the sweeteners and sucrose. On the other hand, stevia finn, aspartame finn and sucrose presented the highest lactic acid production, with no difference between them (Figure 4).

Stevia finn, xylitol and control reduced significantly the number of total microorganism and total lactobacilli in the biofilm. Stevia finn also reduced CFU for

total streptococci significantly, but only xylitol and control had antimicrobial effect on *S. mutans* (around 1.8 $\log_{10}$ reduction compared to the other groups) (Table 4).

The integrated mineral loss, mineral loss mean and lesion depth were reduced in dentin samples exposed to pure stevia, pure aspartame, xylitol and control (> 83% reduction) compared to sucrose, which in turn had a similar cariogenic effect to the tested commercial sweeteners (aspartame finn and stevia finn) (Table 5 and Figure 5).

Discussion

The natural sweetener stevia is considered one of the best substitutes for sucrose, as it has low calories and no report about adverse effects (Contreras, 2013; Salvador-Reyes et al., 2014). The plant, from which this sweetener is obtained, is rich in carbohydrates, proteins, crude fibber, minerals (K^+, Ca^{2+}, Na^+, Mg^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}) and essential amino acids. It has a high percentage of steviol (stevioside, steviolbioside, rebaudioside AF and dulcoside) (Escobar et al., 2020), which in turn has been responsible for its antimicrobial effect (Ferrazzano et al., 2015). However, its role in preventing caries and promoting oral health is not fully understood.

Stevioside offers several advantages over other non-caloric substitutes since it is heat-stable, resistant to acid hydrolysis and non-fermentable (Giongo et al., 2014). The data suggest that steviol glycosides are not cariogenic and may have beneficial effects in preventing dental caries (Samuel et al., 2018).

Our work has shown that a commercial sweetener containing stevia is as cariogenic as sucrose, although some antimicrobial effect has been seen in the biofilm formed on dentin. Escobar et al. (2020) demonstrated that, even with the presence of stevia, *S. mutans* managed to metabolize sucrose and to produce acids, being stevia ineffective in reducing microbial viability.

Part of stevia's cariogenic effect, seen in the present work, can be justified by the presence of lactose with an estimated concentration of 94% This sugar may have been metabolized by the biofilm bacteria and responsible for the dental demineralization. When there are two sources of energy supply (for example, stevia and lactose), bacteria can adapt to the conditions of the environment and metabolize one of the two sources of energy from which it obtains more energy, as discussed by. Therefore, we decided to test the pure form of stevia, whose data of lactic acid assay

and TMR confirmed that the sweetener is in fact not cariogenic and that the results found for the commercial product was due to the presence of lactose. We did not perform CFU counting in case of pure stevia, since the results of the other methods were enough to show their non-cariogenic potential. Based on it, the null hypothesis was rejected.

A similar result was seen for the commercial sweetener containing aspartame (and also lactose) in our work, which was as cariogenic as stevia and sucrose. Aspartame is a dipeptide ester (aspartic acid is attached to the N-terminal portion of phenylalanine), presented as a white crystalline powder with a refreshing aroma and characteristic of a sweet flavour. In an aqueous solution, aspartame is about 160 times sweeter than sucrose (Matsukubo & Takazoe, 2006).

According to Giacaman et al. (2013), using a monospecie biofilm (*S. mutans*), aspartame reduced the biofilm pH around 5.4 after 80h, value higher than that induced by sucrose, but very close to the critical pH for apatite. In the cited study, the biofilm exposed to stevia during this period had a pH of around 6.0, value closed with what was found for Giongo et al. (2014) using 7% stevioside and 93% lactose. Regarding the biofilm biomass, all the sugars and sweeteners mentioned showed a high value compared to the control group (NaCl). All sugars and sweeteners showed cariogenic potential, but sucrose was the most cariogenic one. After 5 days of biofilm cultivation, enamel demineralization induced by fructose (30% loss of microhardness), aspartame (approximately 23%) and stevia (approximately 18%) was significantly lower when compared to the sucrose (approximately 43%). In our study, the cariogenic potential of the commercial aspartame was due to the presence of lactose (94%), which was proved by the TMR images of pure aspartame. Differently what was shown above, we found similarity between the commercial sweeteners and sucrose, which may be due to the experimental model (continuous exposure of microcosm biofilm to sweeteners/sugar for 5 days) and the response variable (TMR).

Artificial sweeteners are usually hundreds of times sweeter than sucrose. In our study, we tested all groups at the same concentration, but in the real situation the amount of sweetener applied is usually much lower than sucrose. Products containing sweeteners, for this reason, cannot be sold in their pure form. For example, a tablet of a commercial product advertised as sucralose usually contains only about 10% sucralose, with higher proportions of other carbohydrates, usually

lactose, starch or starch hydrolysates (Giacaman et al., 2013). Although lactose is considered one of the least cariogenic common sugars, evidence points out that repeated exposures to this carbohydrate can lead microorganisms (*S. mutans*) to adapt and to produce acids from lactose, more quickly over time (Hamilton & Letbag, 1979; Birkhed et al., 1993; Zeng et al., 2010).

As expected, xylitol (anti-cariogenic) and control (no supplementation) groups significantly reduced the number of microorganisms in biofilm. Xylitol (sugar alcohol of 5 C) is not easily metabolized by cariogenic microorganisms. The decrease in the pH of the biofilm caused by this sugar is not enough to cause enamel demineralization (Marghalani et al., 2017). *Streptococcus mutans*, gram-positive bacteria identified as being primarily responsible for the caries process, do not metabolize xylitol for obtaining energy. Xylitol is transported into cells by the induced action of a fructose transporter, however, it causes a futile cycle because it is not going through to glycolytic reactions or, when accumulated, induces cell damage (Ly et al., 2008; Masoud et al., 2015; Riley et al., 2015). Xylitol is also known to improve remineralization of artificial caries lesions *in vitro* and *in situ* (Cardoso et al., 2014; Cardoso et al., 2016).

Future studies can be designed in order to understand the cariogenic potential of different pure and commercial sweeteners using models closer to the *in vivo* condition such as *in situ* models. With more scientific evidence, the population shall be advised about the different cariogenic potential among the sweeteners brand names.

In conclusion, commercial sweeteners containing stevia and aspartame (Finn trademark) demonstrated to be as cariogenic as sucrose in this experimental model, due to the presence of other components, since the pure forms of the sweeteners are not cariogenic.

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Statement of Ethics

This study was approved by the local Ethics Committee (Number: 12647819.5.0000.5417) and Ethics committee on animal research (CEUA, Number: 004/2019).

Disclosure Statement

The authors declare no potential conflict of interest.

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Author Contributions

Nascimento CA, Kim RR and Ferrari CR performed the experiments. Nascimento CA, Braga AS, Souza BM and Magalhães AC designed the project. Magalhães AC supervised all the experiments. Nascimento CA and Magalhães AC analyzed the data and wrote the manuscript. All authors revised and approved the paper.

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Table 1. Composition of sweeteners

Product's name	Brand / City-Country	Composition
Finn 100% Stevia®	Hypera Pharma/São Paulo–Brazil	Lactose, steviol glycoside sweetener and silicon dioxide anti-humectant.
Pure Stevia	Merck	Stevioside; Rebaudiosides A, B, C and D; Dulcoside A; Rubusoside; Steviolbioside; Stevioside.
Finnaspartame®	Hypera Pharma/São Paulo–Brazil	Lactose, aspartame sweetener and anti-wetting silicon dioxide.
Pure Aspartame	Merck	Aspartame
Sucrose	Merck	Sucrose
Xilitol (Finesweet)®	Airon Ind. E Com. de Prod. Alimt. LTDA/ Ribeirão Preto-Brazil	Xylitol

* The pure sweeteners were tested in lactate assay and TMR analysis.

Table 2. CFU counting log_{10}/mL [median (interquartile range)] for Total MØ, total lactobacilli, total streptococci and *S. mutans/S. sobrinus* in the microcosm biofilm formed on the enamel and exposed to different sugars / sweeteners.

Groups	Total MØ	total lactobacilli	total streptococci	*S. mutans/ S. sobrinus*
Stevia Finn	6.54 (0.41)^{AB}	5.69 (0.44)^{A}	7.27 (0.53)^{A}	7.57 (0.37)^{A}
Aspartame Finn	6.84 (1.31)^{A}	5.69 (0.82)^{A}	7.42 (0.32)^{A}	7.61 (0.64)^{A}
Xylitol	5.89 (0.45)^{A}	5.30 (0.00)**	5.60 (0.23)**	5.60 (0.63)**
Sucrose	7.15 (0.36)^{B}	5.75 (0.60)^{A}	7.09 (0.61)^{A}	7.49 (0.41)^{A}
Control (no supplementation)	6.16 (0.89)^{A}	6.26 (0.66)**	5.30 (0.23)**	5.78 (1.30)**

**Groups excluded from statistical analysis because they did not show considerable colonies growth. With respect to total lactobacilli, total streptococci and *S. mutans*, Xylitol group presented 80%, 53.4% and 46.7% of samples without growth, respectively, while the Control group presented 86.7%, 66.7% and 60%, respectively.

Kruskal-Wallis/Dunn test ($p < 0.0001$, $p = 0.809$, $p = 0.169$ and $p = 0.9579$ for total Microorganisms, total lactobacilli, total streptococci and *S. mutans /S. sobrinus*, respectively). n = 12. Different letters represent groups with statistical differences.

Table 3. TMR analysis of the enamel samples

Groups	ΔZ (vol%.μm)	LD (μm)	R (vol%)
Stevia Finn	3084.67 (834.26)^{A}	89.78 (27.61)^{A}	31.59 (5.77)^{A}
Pure Stevia	668.46 (302.45)^{B}	25.39 (15.93)^{B}	27.82 (10.17)^{ABC}
Aspartame Finn	3174.67 (603.10)^{A}	86.87 (25.56)^{A}	33.95 (5.36)^{A}
Pure Aspartame	410.83 (209.93)^{B}	23.19 (18.08)^{B}	22.62 (8.96)^{BC}
Sucrose	2913.67 (646.69)^{A}	78.05 (19.47)^{A}	31.14 (4.06)^{AB}
Xylitol	422.00 (196.48)^{B}	21.23 (7.55)^{B}	19.91 (5.19)^{C}
Control (no supplementation)	326.00 (173.24)^{B}	15.25 (7.36)^{B}	20.16 (3.87)^{C}

Kruskal-Wallis / Dunn [median (interquartile range), ΔZ- $p < 0.0001$; P- $p < 0.0001$; R- $p < 0.0001$]. n=12. Different letters in the same column represent groups with significant differences.

Table 4. CFU counting log_{10}/mL [median (interquartile range)] for Total MØ, total lactobacilli, total streptococci and *S. mutans/S. sobrinus* in the microcosm biofilm formed on the dentin and exposed to different sugars /sweeteners

Grupos	MØ Totais	total lactobacilli	total streptococci	*S. mutans/ S. sobrinus*
Stevia Finn	6.42 (0.44)^{A}	0.00 (3.98)^{AB}	5.80 (0.40)^{A}	7.83 (0.36)^{A}
Aspartame Finn	7.09 (0.67)^{BC}	5.30 (5.30)^{BC}	6.31 (0.45)^{AB}	7.87 (0.18)^{A}
Xylitol	6.72 (0.27)^{AB}	0.00 (0.00)^{A}	6.60 (0.34)^{B}	6.15 (0.37)^{B}
Sucrose	7.24 (0.38)^{C}	5.30 (0.15)^{C}	6.45 (0.25)^{B}	7.78 (0.31)^{A}
Control (no supplementation)	6.82 (0.36)^{AB}	0.00 (0.00)**	6.55 (0.54)^{B}	5.78 (1.21)^{B}

**Groups excluded from statistical analysis because they did not show considerable colonies growth. For control group, there was no growth of total lactobacilli CFU.

Kruskal-Wallis/Dunn test ($p < 0.0001$ for all species).

n= 12. Different letters represent groups with statistical differences.

Table 5. TMR analysis of the dentin samples

Groups	ΔZ (vol%.μm)	LD (μm)	R (vol%)
Stevia Finn	3945.67 (689.69)^{A}	115.23 (18.25)^{A}	30.06 (3.74)^{A}
Pure Stevia	557.14 (249.35)^{B}	54.64 (36.34)^{B}	11.65 (7.51)^{B}
Aspartame Finn	3626.79 (617.26)^{A}	120.87 (13.68)^{A}	30.95 (4.78)^{A}
Pure Aspartame	520.71 (181.89)^{B}	42.24 (21.81)^{B}	14.26 (5.57)^{B}
Sucrose	3543.33 (432,50)^{A}	117.83 (9.33)^{A}	31.42 (3.12)^{A}
Xylitol	720.00 (156.32)^{B}	41.33 (12.83)^{B}	14.00 (2.89)^{B}
Control (no supplementation)	673.57 (176.61)^{B}	47.49 (15.39)^{B}	14.41 (2.66)^{B}

Kruskal-Wallis / Dunn [median (interquartile range), ΔZ- $p < 0.0001$; P- $p < 0.0001$; R- $p < 0.0001$]. n=12. Different letters in the same column represent groups with significant differences.

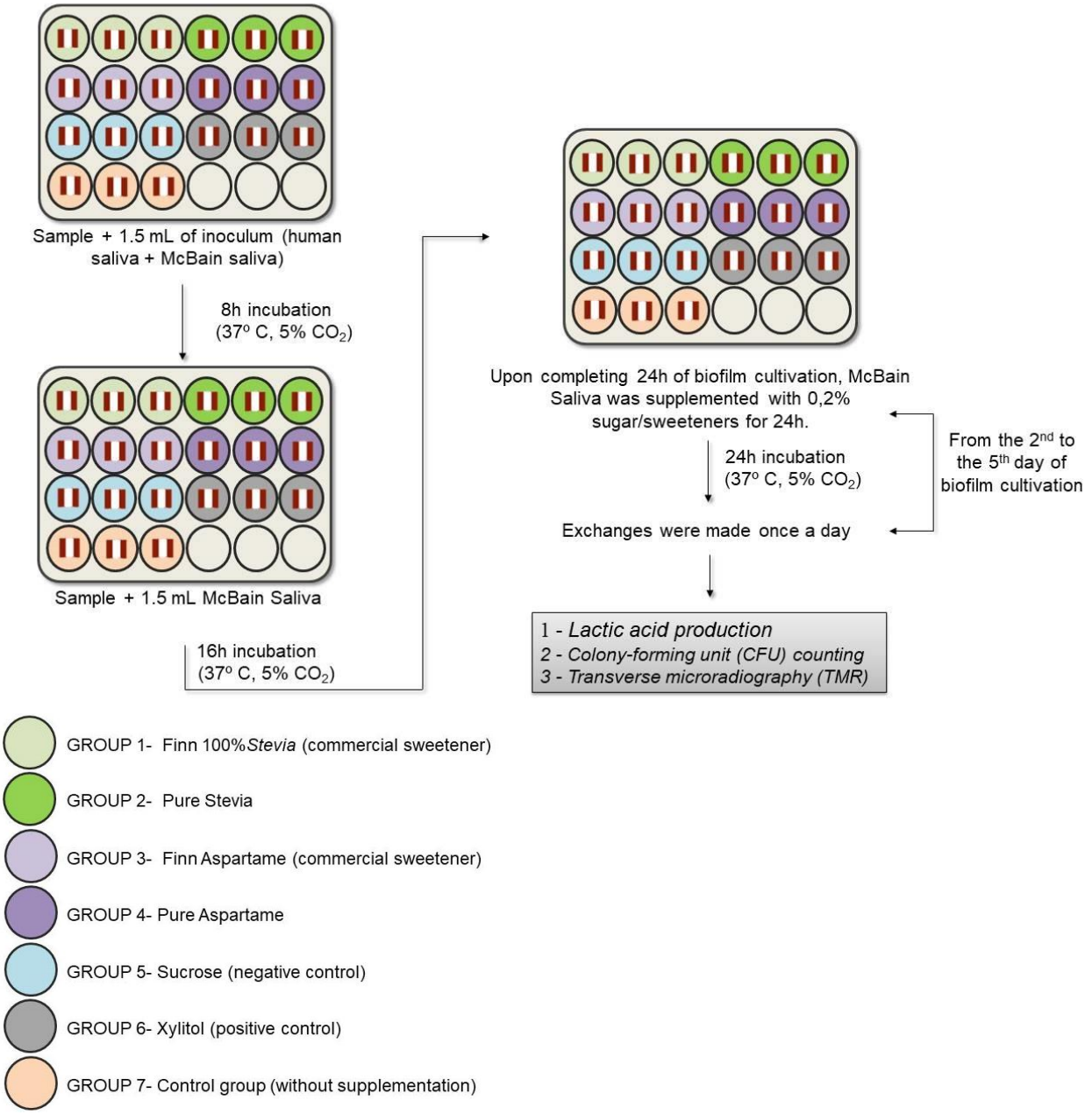

Figure 1. Representative images of the experimental protocol.

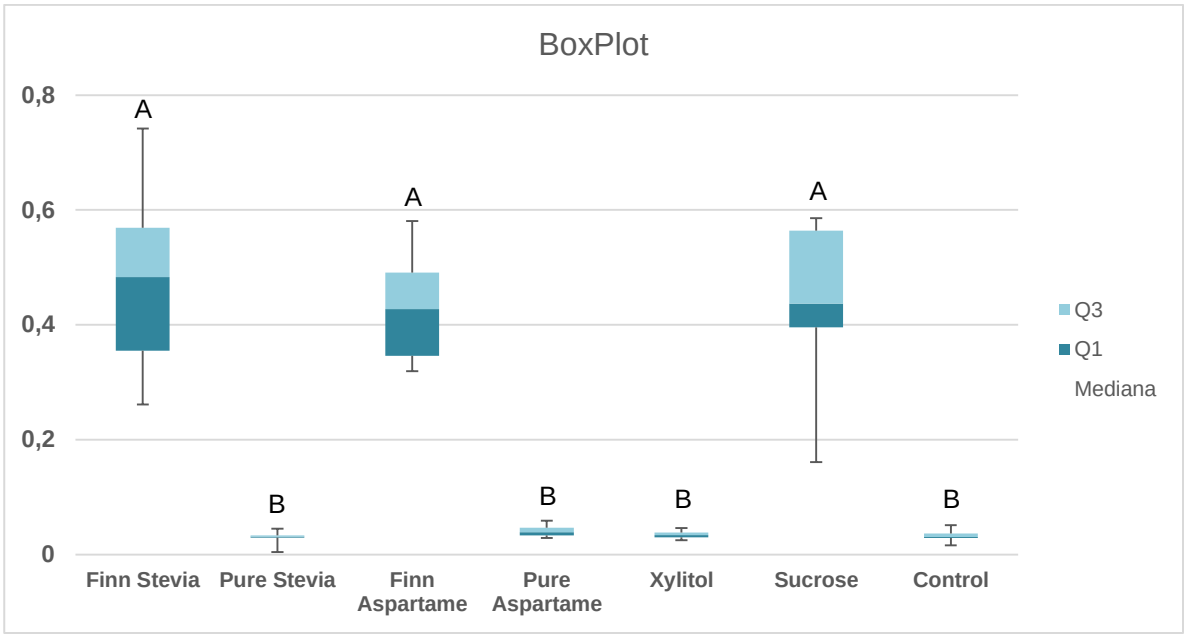

Figure 2. Box plot of lactic acid production analysis of the microcosm biofilm on enamel samples (g/L). Kruskal-Wallis / Dunn ($p < 0.0001$). n=12. Different letters in the same column represent groups with significant differences

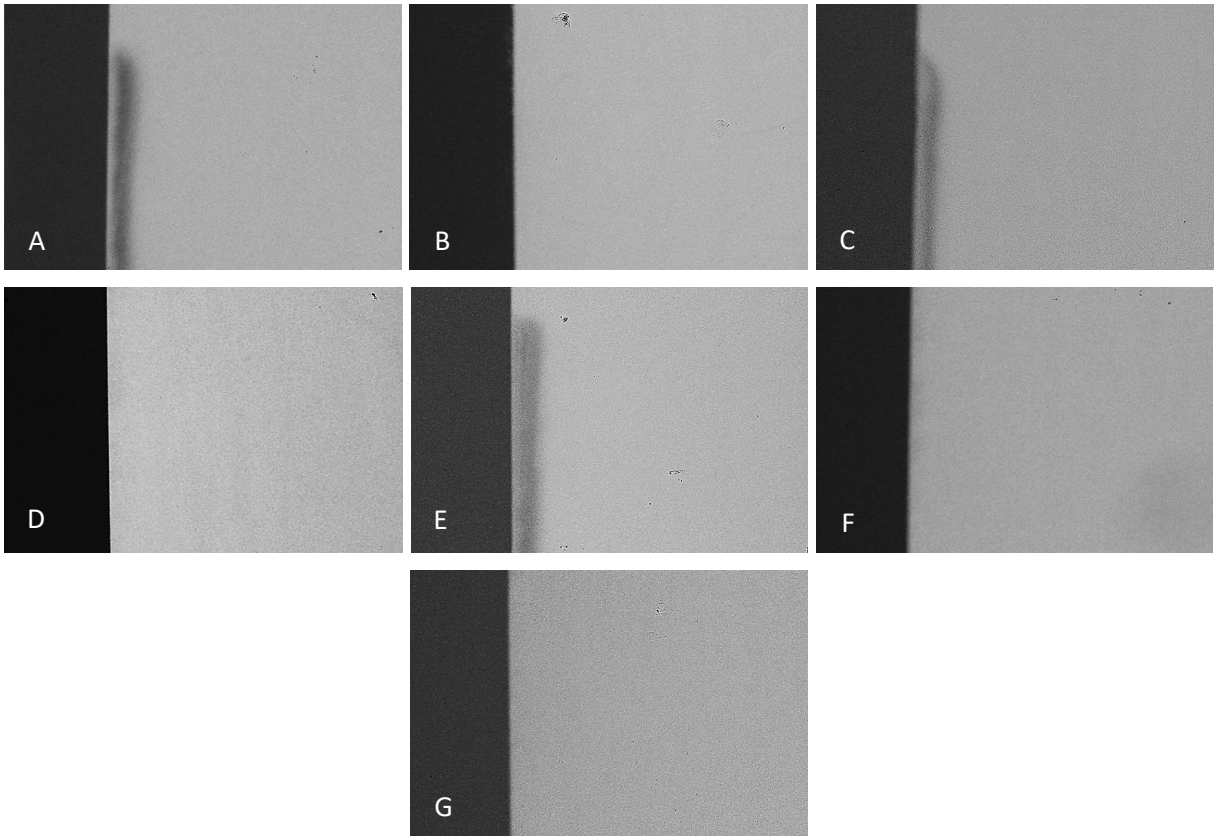

Figure 3. Representative images of TMR for enamel: A – stevia finn, B – pure stevia, C – aspartame finn, D – pure aspartame, E – sucrose, F – xylitol, G – control.

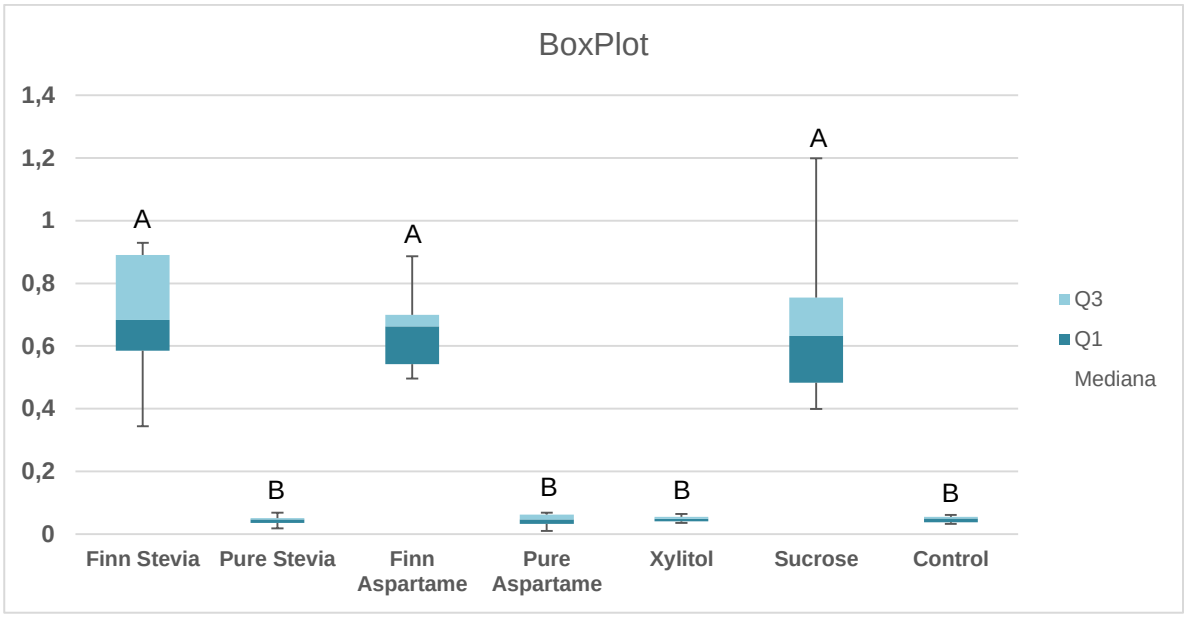

Figure 4. Box plot of lactic acid production analysis of the microcosm biofilm on dentin samples (g/L). Kruskal-Wallis / Dunn ($p < 0.0001$). n= 12. Different letters in the same column represent groups with significant differences.

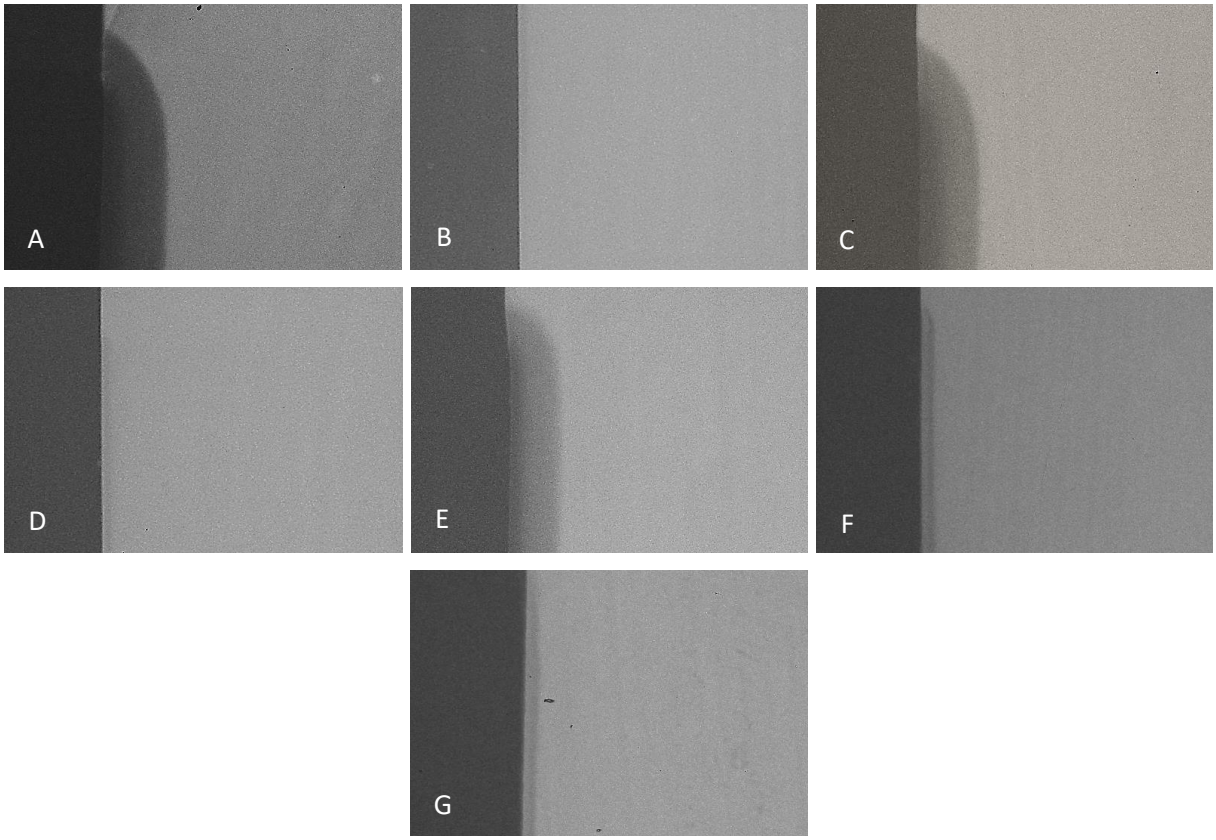

Figure 5. Representative images of TMR for dentin: A – stevia finn, B – pure stevia, C – aspartame finn, D – pure aspartame, E – sucrose, F – xylitol, G – control.

3 DISCUSSION

3 DISCUSSION

The natural sweetener Stevia is considered one of the best substitutes for sucrose, as it is sweet, has low calories and no report of adverse effect (Contreras, 2013; Salvador-Reyes et al., 2014). The plant, from which this sweetener is obtained, is rich in carbohydrates, proteins, crude fiber, minerals (K^+, Ca^{2+}, Na^+, Mg^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}) and essential amino acids. It has a high percentage of steviol (stevioside, steviolbioside, rebaudioside AF and dulcoside) (Escobar et al., 2020), which in turn has been responsible for its antimicrobial effect (Ferrazzano et al., 2015). However, its role in preventing caries and promoting oral health is not fully understood.

Steviosides offer several advantages over other non-caloric substitutes such as heat-stable, resistant to acid hydrolysis and they are non-fermentable (Giongo et al., 2014). The data suggest that steviol glycosides are not cariogenic and may have beneficial effects in preventing dental caries (Samuel et al., 2018).

Our work has shown that a commercial sweetener containing stevia is as cariogenic as sucrose, although some antimicrobial effect has been seen in the biofilm formed on dentin. Escobar et al. (2020) demonstrated that, even with the presence of Stevia, *S. mutans* managed to metabolize sucrose and produce acids, being stevia ineffective in reducing microbial viability under sucrose exposure.

Part of Stevia's cariogenic effect, seen in our work, can be justified by the presence of lactose; being its concentration estimated in 94% This sugar may have been metabolized by the biofilm bacteria and caused caries. When there are two sources of energy supply (for example, stevia and lactose), bacteria can adapt to the conditions of the environment and metabolize one of the two sources of energy from which it obtains more energy (Aragão et al., 2019). Therefore, we decided to test the pure form of Stevia, whose results of lactic acid assay and TMR confirmed that the sweetener is not cariogenic and that the results found for the commercial product was due to the presence of lactose. We did not perform CFU counting in case of pure stevia and pure aspartame, since the results of the other methods were enough to show their non-cariogenic potential.

A similar finding was seen for the commercial sweetener containing aspartame (and also lactose) in our work, which was as cariogenic as stevia and sucrose. Aspartame is a dipeptide ester (aspartic acid is attached to the N-terminal portion of phenylalanine), presented as a white crystalline powder with a refreshing aroma and characteristic of a sweet flavor. In an aqueous solution, aspartame is about 160 times sweeter than sucrose (Matsukubo & Takazoe, 2006).

According to Giacaman et al. (2013) using a monospecie biofilm (*S. mutans*), aspartame reduced the biofilm pH around 5.4 after 80h, value higher than that induced by sucrose, but very close to the critical pH for apatite. In the cited study, the biofilm exposed to stevia during this period had a pH of around 6.0, value closed with what was found for Giongo et al. (2014) using 7% stevioside and 93% lactose. Regarding the biomass of the biofilm, all the sugars and sweeteners mentioned showed a high value compared to the control group (NaCl). All sugars and sweeteners showed cariogenic potential, but lower than the sucrose. After 5 days of biofilm cultivation, enamel demineralization induced by fructose (30% loss of microhardness), aspartame (approximately 23%) and stevia (approximately 18%) was significantly lower when compared to the positive control group (sucrose, approximately 43%). In our study, the cariogenic potential of the commercial aspartame was due to the presence of lactose (94%), which was proved by the lactic acid assay and TMR images of samples exposed to pure aspartame. Differently what was shown above, we found similarity between the commercial sweeteners and sucrose, which may be due to the experimental model (microcosm biofilm and continuous sweetener exposure for 5 days) and the response variable (TMR) applied in the present study.

Artificial sweeteners are usually hundreds of times sweeter than sucrose. In our study, we tested all groups at the same concentration, but in the real situation the amount of sweetener applied is much lower than sucrose. Products containing sweeteners, for this reason, cannot be sold in their pure form. For example, a tablet of a commercial product advertised as sucralose usually contains only about 10% sucralose with higher proportions of other carbohydrates, usually lactose, starch or starch hydrolysates (Giacaman et al., 2013). Although lactose is considered one of the least cariogenic sugars, evidence points out that repeated exposures to this carbohydrate can lead microorganisms (*S. mutans*) to adapt and to produce acids

from lactose, more quickly over time (Hamilton & Letbag, 1979; Birkhed et al., 1993; Zeng et al., 2010; Aragão et al., 2019).

As expected, xylitol (anti-cariogenic) and control (no supplementation) groups significantly reduced the number of microorganisms in biofilm. Xylitol (sugar alcohol of 5 C) is not easily metabolized by cariogenic microorganisms. The decrease in the biofilm pH caused by this sugar is not enough to induce enamel demineralization (Marghalani et al., 2017). *Streptococcus mutans*, a gram-positive bacteria identified as being primarily responsible for the caries process, do not metabolize xylitol for energy. Xylitol is transported into cells by the induced action of a fructose transporter, however, it causes a futile cycle because it is not going through to glycolytic reactions or, when accumulated, induces cell damage (Ly et al., 2008; Masoud et al., 2015; Riley et al., 2015). Xylitol is also known to improve remineralization of artificial caries lesions *in vitro* and *in situ* (Cardoso et al., 2014; Cardoso et al., 2016).

Future studies can be designed in order to understand the cariogenic potential of different pure and commercial sweeteners using models closer to the *in vivo* condition such as *in situ* models. With more scientific evidence, the population shall be advised about the different cariogenic potential among the sweeteners brand names.

In conclusion, commercial sweeteners containing stevia and aspartame (Finn trademark) demonstrated to be as cariogenic as sucrose in this experimental model, due to the presence of other components, since the pure forms of the sweeteners are not cariogenic.

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APÊNDICE

APÊNDICE A- **TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**

Caro aluno de pós-graduação do Laboratório de Bioquímica da Faculdade de Odontologia de Bauru, através deste termo, lhe convidamos para participar da pesquisa intitulada “Efeito do adoçante Stevia sobre o desenvolvimento da cárie dentária em esmalte e dentina sob um modelo de biofilme microcosmo”. A nossa pesquisa tem como objetivo comparar o efeito do Stevia (adoçante natural) a do aspartame (adoçante sintético), da sacarose e do xilitol sobre o desenvolvimento da cárie dentária em um modelo de biofilme microcosmo em esmalte e dentina, o qual será conduzido no laboratório. Para isso, gostaríamos de solicitar a doação de sua saliva, para que possamos ter as bactérias bucais necessárias para a formação do biofilme no laboratório. Esta pesquisa será feita por mim (Ana Carolina Magalhães) com colaboração dos pesquisadores Luiz Ricardo Pero Vecchia, Aline Silva, Carolina Ruis Ferrari, Beatriz Martines de Souza, e Rafaela Ricci Kim.

É importante destacar que de acordo com o item IV.6.b da resolução 466/12 você terá garantida a liberdade do consentimento para a participação ou não na pesquisa, sem qualquer represália. Portanto, garantimos que não será coagido e nem sofrerá restrições de suas atividades usuais na FOB/USP.

Anteriormente à coleta de saliva, você será submetido a uma avaliação da sua condição bucal e a algumas perguntas para verificarmos se você se enquadra dentro dos critérios de inclusão da pesquisa: ter histórico de lesões cariosas mas não ser cárie ativo (apresentar lesões de mancha branca ativa ou lesões cavitadas), não apresentar sinais de gengivite/periodontite (presença de sangramento e mobilidade dentária) e não ter ingerido antibióticos nos últimos 3 meses. Lactantes, gestantes, fumantes e indivíduos com doenças crônicas serão automaticamente excluídos da amostra.

Uma vez selecionado, ficará combinado que você deverá não escovar os dentes por 24 h assim como ficar em jejum (comida e água) 2 h anteriores à coleta da saliva. A saliva será coletada no período da manhã, por volta das 9-10h, em uma data a ser agendada. A saliva será coletada sob estimulo por mastigação de uma parafina plástica- Parafilm (que é uma película plástica, sem cheiro, sem cor, resistente à água) durante 10 minutos. Durante este período, você cuspirá toda a saliva em um recipiente plástico. O objetivo desta estimulação de saliva é aumentar a quantidade de saliva produzida para termos volume de amostra suficiente.

Aproveitaremos a oportunidade e mensuraremos o seu fluxo salivar, o que também está dentro do critério de inclusão da pesquisa, e lhe informaremos se ele está adequado ou não.

A sua participação neste trabalho acarretará em risco mínimo, que acontecerá no caso de você ter alergia ao plástico utilizado para mastigação ou se você apresentar enjoos na hora da coleta. Nestes casos, você deverá comunicar o responsável pela pesquisa, que estará presente no momento da coleta, o qual o(a) liberará da participação na pesquisa, sem penalização alguma. Ainda ressaltamos que o fato de ficar 24 h sem escovar os dentes não acarretará prejuízos a sua saúde bucal.

Os gastos que forem gerados por este trabalho ficarão a cargo da responsável pelo projeto. Importante ressaltar que não está sendo considerado nenhum pagamento ou recompensa material pela sua participação neste estudo. Você terá garantido o direito à indenização compensatória caso fique comprovado que a sua participação acarretou algum problema a você. É importante destacar que como benefício direto, logo após a coleta, você terá direito a uma profilaxia profissional e a um lanche.

Você não precisará passar por nenhum outro tipo de procedimento adicional. Isso quer dizer que utilizaremos apenas a saliva. Queremos deixar claro que não existe a menor obrigação de você doar saliva para a pesquisa. Isso é totalmente voluntário. A saliva doada que não for usada na pesquisa será descartada no lixo contaminado do laboratório de Bioquímica.

Esta pesquisa gerará como benefício indireto um maior conhecimento sobre o efeito do adoçante Stevia no desenvolvimento da cárie dentária em esmalte e dentina, o que poderá em longo prazo gerar novos produtos que poderão auxiliar na prevenção desta doença bucal.

Você pode recusar-se em assinar este termo para a não participação na pesquisa e mesmo após assinar este termo, caso mude de idéia e queira retirar seu consentimento em qualquer fase da pesquisa, poderá fazê-lo sem nenhuma represália. Todo o trabalho será feito sem a sua identificação, preservando completamente sua identidade. Ao concordar em participar desta pesquisa, você receberá uma via igualmente válida deste termo. O direito à indenização lhe será permitido, caso ocorra algum dano decorrente da sua participação nesta pesquisa. Caso necessite de ajuda financeira para participar desta pesquisa (no caso,

transporte da sua residência à FOB e vice-versa, para a coleta de saliva) ela poderá ser ressarcida pelo pesquisador.

Qualquer dúvida ou maiores esclarecimentos você poderá recorrer a qualquer um dos membros da equipe do projeto (Laboratório de Bioquímica 14-3235-8247) ou a pesquisadora responsável Ana Carolina Magalhães (telefone 14 / 98118-9106, e-mail acm@usp.br). Caso possua preocupações quanto aos seus direitos como participante deste estudo, ou queira fazer denúncias quanto à condução do mesmo, sinta-se a vontade para procurar o Comitê de Ética em Pesquisa, da Faculdade de Odontologia de Bauru/USP, Alameda Dr. Octávio Pinheiro Brisolla, 9-75, telefone (14)3235-8356 ou e-mail: cep@fob.usp.br e a forma de contato com CONEP – Endereço: Esplanada dos Ministérios, Bloco G, Anexo B. Sala 104B, telefone: (61) 3315-5878, e-mail: cns@saude.gov.br.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Pelo presente instrumento que atende às exigências legais, o Sr. (a) _____ _____, portador da cédula de identidade _____, após leitura minuciosa das informações constantes neste TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO, devidamente explicada pelos profissionais em seus mínimos detalhes, ciente dos serviços e procedimentos aos quais será submetido, não restando quaisquer dúvidas a respeito do lido e explicado, DECLARA e FIRMA seu CONSENTIMENTO LIVRE E ESCLARECIDO concordando em participar da pesquisa proposta. Fica claro que o participante da pesquisa, pode a qualquer momento retirar seu CONSENTIMENTO LIVRE E ESCLARECIDO e deixar de participar desta pesquisa e ciente de que todas as informações prestadas tornar-se-ão confidenciais e guardadas por força de sigilo profissional (Art. 9º do Código de Ética Odontológica).

Por fim, como pesquisador(a) responsável pela pesquisa, DECLARO o cumprimento do disposto na Resolução CNS nº 466 de 2012, contidos nos itens IV.3, item IV.5.a e na íntegra com a resolução CNS nº 466 de dezembro de 2012.

Por estarmos de acordo com o presente termo o firmamos em duas vias igualmente válidas (uma via para o participante da pesquisa e outra para o pesquisador) que serão rubricadas em todas as suas páginas e assinadas ao seu término, conforme o disposto pela Resolução CNS nº 466 de 2012, itens IV.3.f e IV.5.d.

Bauru, SP, _____ de _____ de _____.

_____ _____

Assinatura Participante da Pesquisa Nome/Assinatura do Pesquisador(a)

Ana Carolina Magalhães

O Comitê de Ética em Pesquisa – CEP, organizado e criado pela FOB-USP, em 29/06/98 (Portaria GD/0698/FOB), previsto no item VII da Resolução nº 466/12 do Conselho Nacional de Saúde do Ministério da Saúde (publicada no DOU de 13/06/2013), é um Colegiado interdisciplinar e independente, de relevância pública, de caráter consultivo, deliberativo e educativo, criado para defender os interesses dos participantes da pesquisa em sua integridade e dignidade e para contribuir no desenvolvimento da pesquisa dentro de padrões éticos.

Qualquer denúncia e/ou reclamação sobre sua participação na pesquisa poderá ser reportada a este CEP:

Horário e local de funcionamento:

Comitê de Ética em Pesquisa

Faculdade de Odontologia de Bauru-USP - Prédio da Pós-Graduação (bloco E - pavimento superior), de segunda à sexta-feira, no horário das 14hs às 17 horas, em dias úteis.

Alameda Dr. Octávio Pinheiro Brisolla, 9-75

Vila Universitária – Bauru – SP – CEP 17012-901

Telefone/FAX(14)3235-8356

e-mail: cep@fob.usp.br

ANEXOS

ANEXO A – Parecer Consubstanciado do CEP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito do adoçante Stevia sobre o desenvolvimento da cárie dentária
Pesquisador: Ana Carolina Magalhães
Área Temática:
Versão: 1
CAAE: 12647819.5.0000.5417
Instituição Proponente: Universidade de Sao Paulo
Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 3.325.154

Apresentação do Projeto:
É um estudo de análise do efeito do adoçante Stevia sobre o desenvolvimento da cárie dentária em esmalte e dentina sob um modelo de biofilme microcosmo.

Objetivo da Pesquisa:
O objetivo do projeto será comparar o efeito do Stevia (adoçante natural) ao do aspartame (adoçante sintético), da sacarose e do xilitol sobre o desenvolvimento da cárie dentária em um modelo de biofilme microcosmo em esmalte e dentina.

Avaliação dos Riscos e Benefícios:
Risco mínimo, relacionado a coleta de saliva dos participantes.
Os benefícios são relacionados à melhor compreensão dos efeitos da Stevia em relação às condições de cariogenicidade na cavidade bucal.

Comentários e Considerações sobre a Pesquisa:
Pesquisa relevante de alto potencial de contribuição para o avanço do conhecimento na área.

Considerações sobre os Termos de apresentação obrigatória:
Todos os termos foram apresentados em conformidade com as normas éticas.

Recomendações:
Não há

Conclusões ou Pendências e Lista de Inadequações:

Aprovado sem pendências.

Considerações Finais a critério do CEP:

Esse projeto foi considerado APROVADO na reunião ordinária do CEP de 08/05/2019, com base nas normas éticas da Resolução CNS 466/12. Ao término da pesquisa o CEP-FOB/USP exige a apresentação de relatório final. Os relatórios parciais deverão estar de acordo com o cronograma e/ou parecer emitido pelo CEP. Alterações na metodologia, título, inclusão ou exclusão de autores, cronograma e quaisquer outras mudanças que sejam significativas deverão ser previamente comunicadas a este CEP sob risco de não aprovação do relatório final. Quando da apresentação deste, deverão ser incluídos todos os TCLEs e/ou termos de doação assinados e rubricados, se pertinentes.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1321923.pdf	18/04/2019 11:25:47		Aceito
Outros	termo_de_aquiescencia_departamento.pdf	18/04/2019 11:19:51	LUIZ RICARDO PERO VECCHIA	Aceito
Projeto Detalhado / Brochura Investigador	projeto_de_pesquisa.docx	17/04/2019 19:59:21	LUIZ RICARDO PERO VECCHIA	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.doc	17/04/2019 19:19:36	LUIZ RICARDO PERO VECCHIA	Aceito
Declaração do Patrocinador	termo_de_outorga_fapesp.pdf	17/04/2019 18:44:23	LUIZ RICARDO PERO VECCHIA	Aceito
Declaração de Pesquisadores	DECLARACAO_DE_COMPROMISSO_DO_PESQUISADOR_COM_RESULTADOS.pdf	17/04/2019 18:39:44	LUIZ RICARDO PERO VECCHIA	Aceito
Outros	CEP_ANIMAL.pdf	17/04/2019 18:37:28	LUIZ RICARDO PERO VECCHIA	Aceito
Outros	termo_de_AquiescenciaCIP.pdf	17/04/2019 18:36:35	LUIZ RICARDO PERO VECCHIA	Aceito
Outros	termo_de_Aquiescencia_posgraduacao.pdf	17/04/2019 18:34:59	LUIZ RICARDO PERO VECCHIA	Aceito
Outros	QUESTIONARIO_TECNICO_PDF.pdf	17/04/2019 17:59:20	LUIZ RICARDO PERO VECCHIA	Aceito
Folha de Rosto	Untitled_20190416_140833.pdf	17/04/2019	LUIZ RICARDO	Aceito

USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

Continuação do Parecer: 3.325.154

Folha de Rosto	Untitled_20190416_140833.pdf	16:24:19	PERO VECCHIA	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BAURU, 14 de Maio de 2019

Assinado por:
Ana Lúcia Pompéia Fraga de Almeida
(Coordenador(a))

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Uso de Animais

Uso exclusivo da CEUA/FOB/USP
Reg. Nº **004/2019**
Recebido em: 16/04/19
Maristela Petenuci Ferrari
Secretária da CEUA – SRTE 53052

PROTOCOLO DE RECEBIMENTO DO FORMULÁRIO PARA REGISTRO DE PROTOCOLOS EFETUADOS COM CADÁVERES, OU PARTE DELES, EM ENSINO E/OU PESQUISA

Finalidade:	Pesquisa
Período:	Mar/2019a Fev/2019
Título da pesquisa:	Efeito do adoçante Stevia sobre o desenvolvimento da cárie dentária em esmalte e dentina sob um modelo de biofilme microcosmo
Pesquisador Responsável:	Profa. Dra. Ana Carolina Magalhães
Pesquisador Executor:	Luiz Ricardo Pero Vecchia
Colaboradores:	Aline Silva Braga, Beatriz Martines de Souza, Carolina Ruis Ferrari e Rafela Ricci Kim
Nota Fiscal/Termo de Doação (incluido possíveis perdas)	Termo de Doação-Frigol **Total adquirido:** 300 dentes bovinos
Nº Lote / Data do Abate	5 / 02/04/2019
Nº utilizados / Nº de grupos:	5 grupos - total utilizado 216

ANEXO B – E-mail de submissão

Fwd: CRE-2021-5-14 Manuscript submission confirmation

AM **Ana Carolina Magalhães <acm@fob.usp.br>**
09:13

Para: Caren Augustinho

---------- Forwarded message ---------
De: **Caries Research** <cre@manuscriptmanager.net>
Date: sex., 28 de mai. de 2021 às 09:12
Subject: CRE-2021-5-14 Manuscript submission confirmation
To: <acm@fob.usp.br>

Submission: CRE-2021-5-14 - Effect of sweetener containing stevia on the development of dental caries in enamel and dentin under a microcosm biofilm model

Submitting author: Prof Ana Magalhães

Attention: Prof Magalhães

Thank you very much for submitting the above manuscript. Please use the manuscript number as listed above on all correspondence about the manuscript.

The manuscript will now be forwarded to our reviewers and we shall inform you as soon as a decision has been made by the editorial board.

The progress of your manuscript can be followed from the progress report accessed from your account overview.

Kind regards,

Editorial Office