GROWTH AND VIABILITY OF *STREPTOCOCCUS MUTANS* IN SUCROSE WITH DIFFERENT CONCENTRATIONS OF *STEVIA REBAUDIANA BERTONI*

Escobar E[1], DDS, Piedrahita M[1], DDS, Gregory RL[2], PhD.

[1]CES University, Medellín, Colombia.

[2]Department Biomedical and Applied Science, Indiana University School of Dentistry, Indianapolis, IN, USA.

Corresponding author: Estefani Escobar

E-mail: Estefescobar17@outlook.com

Telephone: (+57) 311-368-6728

Acknowledgments

This study was funded by CES University. Special thanks to Dr. Ru Zhang for all the support and guidance throughout the study; to the Oral Health Research Institute of Indiana University; to Luis Gonzalo Alvarez for his professional help in statistics and to Dr. Emery Alvarez for making this possible.

This is the author's manuscript of the article published in final edited form as:

Escobar, E., Piedrahita, M., & Gregory, R. L. (2020). Growth and viability of Streptococcus mutans in sucrose with different concentrations of Stevia rebaudiana Bertoni. Clinical Oral Investigations, 24(9), 3237–3242. https://doi.org/10.1007/s00784-020-03197-5

Abstract

Objective: To evaluate total absorbance, planktonic growth, biofilm formation, viability, metabolic activity and pH of *Streptococcus mutans* UA159 cultures when different dilutions of *Stevia Rebaudiana Bertoni* were applied and to determine the minimum inhibitory concentration (MIC) and the minimum biofilm inhibitory concentration (MBIC) of Stevia on *S. mutans*. **Materials and methods**: The effects of different dilutions of Stevia (0-400 mg/ml) on *S. mutans* total growth, planktonic growth, biofilm formation, viability, metabolic activity and pH during a 72-hour growth period were evaluated in this *in vitro* study. A stock solution was prepared by mixing 10 ml of tryptic soy broth (TSB) supplemented with 1% sucrose (TSBS) and 4 g of Stevia. **Results:** *S. mutans* total growth and biofilm formation decreased with reduced concentrations of Stevia. Furthermore, the MIC was 25 mg/ml and the MBIC was 6.25 mg/ml. Complete eradication of *S. mutans* was not observed with any of the Stevia concentrations. Planktonic growth of *S. mutans* was not repressed by high concentrations of Stevia and most of the Stevia concentrations generated an increased pH. **Conclusion:** Because Stevia reduces biofilm and acid production, Stevia can be considered a non-cariogenic sweetener. **Clinical relevance:** This study confirms the anticariogenic effect of Stevia, like it has been previously reported; but more studies on the most effective concentration are needed and in the present study, the minimum inhibitory concentration (MIC) and the minimum biofilm inhibitory concentration (MBIC) was determined in the presence of sucrose. Additionally, this is the first study to evaluate the effect of Stevia on *S. mutans* metabolic activity.

Key words: *Stevia Rebaudiana Bertoni*, *Streptococcus mutans,* Dental caries, Anticariogenic effect.

Introduction

Dental caries is a multifactorial disease, which results from the interaction between several host factors (saliva, dental anatomy, oral hygiene), microflora and a diet rich in fermentable carbohydrates [1-3]. For the disease to develop, bacterial colonization and biofilm formation is needed [4]. Several microorganisms are included in the pathogenesis of dental caries; *Streptococcus mutans* (SM) is considered the most cariogenic of all oral streptococci [5,6]. According to Takahashi and Nyvad, many researchers have identified SM as the major pathogen of dental caries because they are frequently isolated from cavitated carious lesions, are highly acidogenic and aciduric and produce surface antigen I/II and water insoluble glucan, which promotes bacterial adhesion to the tooth surface and to other bacteria [1,7,8]. Usually the composition of the biofilm remains relatively constant over time and in balance with the host [4]. However, this natural balance can be altered and disease can occur [4]. For example, poor oral hygiene and high concentrations of sugar favors the proliferation of cariogenic bacteria, such as SM [9,10]. These microorganisms produce organic acid such as lactic acid when they metabolize fermentable carbohydrates which causes local pH values to decrease, resulting in demineralization of tooth surfaces [2]. In developing countries, such as Colombia, dental caries remains a public health problem [11]. The last National Study of Oral Health (ENSAB IV 2014) reported that the prevalence of dental caries in Colombian children at twelve years of age was 88.49% [12].

Recently, the use of sweeteners as sugar substitutes for the prevention of dental caries has been implemented [13]. *Stevia Rebaudiana Bertoni* is a perennial shrub of the Asteraceae family, native of Paraguay and Brazil and it is currently cultivated in other countries like China, Malaysia, Singapore, South Korea, Taiwan, Thailand, United States, Canada and in Europe [13,14]. Stevia is considered one of the best sugar substitutes because it is 300 times sweeter than sucrose, it is low in calories and no adverse effects have been reported [15,16]. The plant is rich in carbohydrates, protein, crude fiber, minerals (K, Ca, Na, Mg, Cu, Mn, Fe, Zn), essential amino acids and has a high percentage of steviol glycosides (stevioside, steviolbioside, rebaudioside A-F and dulcoside). The leaves also contain 80 to 85% water [15,17].

Stevia Rebaudiana Bertoni possesses multiple medicinal properties; anti-hyperglycemic, anti-hypertensive, anti-oxidant, anti-tumor, anti-diarrheal, diuretic, gastro and renal protective, anti-viral and immunomodulatory actions have been reported [5,15-17]. Numerous *in vivo* and *in vitro* studies have demonstrated an anticariogenic effect, *Stevia* inhibits growth of *Streptococcus* and *Lactobacillus* [5], reduces extracellular polysaccharide and biofilm formation [18,19], it is bactericidal [16,18] and does not significantly affect pH values, attributing it to causing a low acidogenic potential of the bacteria [13,15]. Due to the anticariogenic property, anti-inflammatory and cicatrizant capacity, Stevia is also of great importance in oral health [13,15,17].

There are several studies that provide evidence for the use of Stevia in the prevention of dental caries, but more studies on its mechanism of action and most effective concentration are needed [5,15,18,20]. The aim of this *in vitro* study was to

evaluate total absorbance, biofilm formation, planktonic growth, viability, metabolic activity and pH of *S. mutans* when different dilutions of *Stevia Rebaudiana Bertoni* are applied and to determine the minimum inhibitory concentration (MIC) and the minimum biofilm inhibitory concentration (MBIC).

Materials and Methods

Bacterial strain, media and solution preparation

S. mutans UA159 (American Type Culture Collection ATCC 700611) was initially cultivated on a Mitis Salivarius Bacitracin agar plate and inoculated into 5 ml of tryptic soy broth (TSB) and incubated overnight in 5% CO_2 at 37ºC. Pure *Stevia Rebaudiana Bertoni* leaf extract (Mood & Mind LLC, Orlando, FL, USA) was used. A stock solution was prepared by mixing 10 ml of TSB supplemented with 1% sucrose (TSBS) and 4 g of Stevia and filter-sterilizing through a 0.45 µm filter.

Inhibition of *S. mutans* total absorbance, planktonic growth and biofilm formation

Serial dilutions of Stevia (0, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25, 50, 100, 200, 400 mg/ml) in TSBS were prepared, 190 µL of each Stevia concentration was added to wells of sterile 96-well flat bottom microtiter plates and inoculated with 10 µL of *S. mutans* from the overnight TSB culture and incubated for 24 hours at 37ºC in 5% CO_2. The total absorbance of the wells indicating the relative total amount of biofilm and planktonic cells, was measured at 595 nm in a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA). For planktonic growth, 120 µL of the bacterial cultures from each well was transferred to a new 96-well plate and the

optical density at 595 nm was measured. In order to determine the effect of Stevia on biofilm formation, the remaining planktonic culture fluid from the first 96-well plate was gently shaken out, biofilms were fixed with 200 µL of 10% formaldehyde for 30 minutes, washed three times with sterile deionized water and stained with 200 µL of 0.3% crystal violet for 30 minutes. After washing the biofilms three times, crystal violet was extracted from the biofilm cells by incubation for 60 minutes with 200 µL of 2-propanol. The absorbance was read at 490 nm.

Effect on bacterial viability

After preparing 3 ml of each Stevia concentration in TSB (0-400 mg/ml) in sterile 5 ml test tubes, 30 µL of an overnight culture of *S. mutans* was added, vortexed and incubated for 24 hours at 37ºC in 5% CO_2. Additionally, a control group was used; 3 ml of TSB only, inoculated with *S. mutans* and incubated as previously described. 1:100 dilutions of each treated culture were made using sterile saline (0.9% NaCL) and spiral plated on blood agar plates. The plates were incubated for 24 hours in 5% CO_2 at 37ºC. The number of colony forming units (CFU) for each concentration of Stevia was determined using an automated colony counter (Synbiosis, Inc., Frederick, MD, USA) and compared to the values from the TSB control without Stevia.

Effect on biofilm metabolic activity

The metabolic activity of *S. mutans* biofilm treated with Stevia was measured using a method described by Pierce *et al* [21] for *Candida albicans* and adapted for *S. mutans* by Huang et al [23], based on the reduction of 2,3 - bis (2-methoxy-4-nitro-5-

sulfophenyl) - 2H-tetrazolium-5-carboxanilide (XTT) by biofilm cells to a water-soluble orange compound [21]. Established biofilms were grown in sterile 96-well flat bottom microtiter plates by adding 10 µL of an overnight culture of *S. mutans* to 190 µL of TSBS and incubating in 5% CO_2 at 37ºC overnight. The culture medium was gently shaken out, 200 µL of the Stevia dilutions in TSBS were added to designated wells and incubated at 37ºC for 24 hours in 5% CO_2. After incubation, the media was removed with a micropipette. The treated biofilms were washed three times with sterile saline. Fresh XTT/menadione solution was prepared and 200 µL of the XTT/ menadione reagent was added to each well. The plates were wrapped with aluminum foil, incubated for two hours at 37ºC and the absorbance read at 490 nm in a spectrophotometer.

pH measurements

3 ml of each dilution of Stevia in TSBS (0-400 mg/ml) was inoculated with 100 µL of *S. mutans* overnight culture in sterile 12-well flat bottom tissue culture plates and incubated overnight in 5% CO_2 at 37ºC. The pH of the culture medium was measured inside each well with a pH meter (Fisher Scientific, accumet AB15 pH meter, San Diego, CA, USA) at 0, 4, 8, 24, 28, 32, 48, 52 and 56 h after inoculation. Before each test session the pH electrode was calibrated using a buffer solution at pH 7. Three ml of sterile TSBS, was used as a control group and the uninoculated control group measurements were performed at the three different time points (0, 4 and 8 hours) during the first day of the experiment only.

Statistical analysis

The IMB-S PSS program was used for the statistical analysis of the data. The quantitative variables (total absorbance, biofilm formation, planktonic growth, bacterial viability, XTT and pH) were evaluated using Shapiro-Wilk's test to verify that the set of data has a normal distribution. It was found that the variables total absorbance, biofilm formation, bacterial viability and pH did not have a normal distribution and the variable XTT did have a normal distribution. For those who did not have a normal distribution, Kruskal-Wallis test was used to compare if there were differences between the different concentrations of Stevia while for the variable XTT the ANOVA test was used. Stevia concentrations were compared for differences in total absorbance, biofilm formation, planktonic growth, bacterial viability and XTT using one-way ANOVA. Experiments were repeated 3 times. When the Kruskal-Wallis ANOVA test showed statistically significant differences with respect to the different Stevia concentrations, the Tukey's post-hoc test was used. Additionally, the effects of Stevia concentration and time on pH were analyzed using Two-way ANOVA. A 5% significance level was used for all tests.

Results

Visible bacterial growth was observed with all Stevia concentrations (Fig. 1). However, statistically significant decreases in total growth were detected with 25 ($p = 0.0328$), 50 ($p = 0.0012$), 100 ($p < .0001$), 200 ($p < .0001$) and 400 ($p < .0001$) mg/ml of Stevia compared to the 0 Stevia control. The remaining dilutions were not statistically different. The minimum inhibitory concentration (MIC), which is defined as the lowest concentration of an agent that inhibits the visible growth of a

microorganism, was 25 mg/ml. In general, the planktonic growth of *S. mutans* was not repressed by high concentrations of Stevia (results not shown).

When comparing biofilm formation to the control group (Fig. 2), all the concentrations higher than 3.13 mg/ml exhibited significant decreases. Overall, biofilm growth tended to decline as the Stevia concentrations increased. The minimum biofilm inhibitory concentration (MBIC), considered as the lowest concentration of an agent that inhibits the visible biofilm formation of a microorganism, was 6.25 mg/ml.

When the bacterial viability was analyzed, complete eradication of *S. mutans* was not observed with any of the Stevia concentrations (Fig. 3). The differences between the control group and any of the Stevia dilutions were not statistically significant. The inhibitory effect of Stevia was also examined by an agar diffusion test using the highest Stevia concentrations (100, 200 and 400 mg/ml; results not shown). No inhibition zones were identified. Therefore, we can conclude that Stevia does not kill *S. mutans*.

In general, biofilm metabolic activity increased with increasing Stevia concentrations up to 100 mg/ml and specifically started to increase at approximately 1.56 mg/ml of Stevia (Fig. 4; $p = 0.0232$). The maximum metabolic activity was noted at 25 mg/ml ($p = 0.0007$). Significantly lower metabolic activity was observed at 400 mg/ml of Stevia compared to the 0 control ($p = 0.0002$).

Increasing the length of incubation caused significant pH changes during 72 hours of incubation with the different Stevia dilutions (Fig. 5). Overall, at each Stevia concentration the pH of the culture decreased over the 72-incubation period. With

increasing Stevia concentrations, there was a correponding increased pH compared to the 0 control, although these were not all statistically significant. The highest Stevia concentration (400 mg/ml) provided the highest pH (approximately 4.2) compared to the 0 control at 3.9.

Discussion

There is a definite relationship between the dietary consumption of sucrose and the incidence of dental caries. Therefore, a reduction in sugar intake and/or its replacement with non-fermentable sweeteners are important ways to contribute to the prevention of dental caries [13,17]. The use of Stevia is becoming quite popular and many studies have reported that this natural sweetener is not cariogenic [13,15,18,19]. In 1992, Das *et al* fed sixty *S. sobrinus*–colonized albino Sprague-Dawley rats with 30% sucrose; 0.5% stevioside or 0.5% rebaudioside A (the two main Stevia components) for 5 weeks and they concluded that neither stevioside nor rebaudioside A were cariogenic [22].

The present study was aimed to evaluate total growth, planktonic growth, biofilm formation, viability, metabolic activity and pH of *S. mutans* when different dilutions of *Stevia Rebaudiana Bertoni* were applied and to determine the minimum inhibitory concentration (MIC) and the minimum biofilm inhibitory concentration (MBIC). The results demonstrate that *S. mutans* total growth and biofilm formation decreased with Stevia. The MIC and MBIC were 25 and 6.25 mg/ml, respectively.

Brambilla *et al* investigated the effect of Stevia extracts on *in vitro S. mutans* biofilm formation and the *in vivo* pH of plaque. Similar to this study, a reduction in biofilm

formation was confirmed. They used solutions containing 1% stevioside or rebaudioside A and there was an approximately 36-40% reduction in biofilm formation with stevioside and rebaudioside A compared with sucrose. They also analyzed the plaque pH of twenty volunteers after rinsing with 10% solutions of stevioside, rebaudioside A and sucrose for one minute. The sucrose rinse produced a significantly lower pH value compared to the Stevia extracts which ranged from pH 6.92 to 7.3. Therefore, they concluded that Stevia can also be considered non-acidogenic [13]. This high pH was not noted in the current *in vitro* study, with approximately pH 4.2 being the highest pH observed at a concentration of 400 mg/ml of Stevia. However, the methodology in both studies was very different [13]. In the study conducted by Brambilla the measurements were performed directly in the mouth in 3 proximal dental sites at 0, 5, 10, 15, 30, 45 and 60 minutes, while in this study the pH of the culture medium was measured during a longer period of time (72 hours) and therefore there was a greater opportunity for acid production.

Another study assessed the cariogenic potential of several commercial sweeteners including Stevia in an artificial caries model [18]. *S. mutans* biofilms were cultured on bovine enamel slabs and exposed to Stevia for 5 days. 10% sucrose and 0.9% NaCl were used as caries positive and caries negative controls, respectively. In this earlier study, Stevia treatment resulted in a smaller amount of biomass and reduced the number of viable cells when compared with sucrose, with similar counts to the negative control. Although the data exhibited a decrease in bacterial counts, Stevia still allowed some bacterial growth. This was also observed in the present study; complete eradication of *S. mutans* did not result with any of the Stevia concentrations

possibly due to the increased biofilm observed with sucrose. The current study suggests that Stevia is not a bactericidal agent, but does inhibit *S. mutans* biofilm growth.

Gamboa and Chaves [5] prepared powdered Stevia from the plant leaves in hexane, methanol, ethanol, ethyl acetate, and chloroform and evaluated the antibacterial capability of the five solvent extracts against 16 bacterial strains of the genera *Streptococcus* and Lactobacillus using an agar diffusion method. The inhibition zones were measured and the minimum inhibitory concentrations were determined (MIC: lowest concentration of the extract that produces an inhibition zone of at least 6 mm). The results indicated that the MICs for all the extracts ranged between 30 and 120 mg/ml. They also found inhibition zones for the streptococcal strains between 8.6 and 13.3 mm, however, the inhibition zones were significantly higher for the lactobacilli strains. In the present study, no inhibition zones were observed even with the highest Stevia concentrations and the MIC, determined by a microplate method, was lower at 25 mg/ml.

In conclusion, this is the first study to evaluate the effect of Stevia on *S. mutans* metabolic activity. The maximum metabolic activity was noted at 25 mg/ml and the lowest at 400 mg/ml of Stevia. It is important to highlight that the results of this study were obtained using an *in vitro* approach in the presence of sucrose and that oral biofilm comprises a metabolically active and organized consortium of hundreds of bacterial species not just *S. mutans*. Further *in vivo* studies must be conducted to test the effect of Stevia on the main microorganisms associated with caries such as

Streptococcus sobrinus, *Streptococcus gordonii*, and different *Lactobacillus* and *Actinomyces* species [5].

Conclusions

1. The results indicate that Stevia inhibits *S. mutans* total growth and biofilm formation. The minimum inhibitory concentration was 25 mg/ml and the minimum biofilm inhibitory concentration was 6.25 mg/ml.
2. Complete eradication of *S. mutans* was not observed with any of the Stevia concentrations. Therefore, Stevia does not have a bactericidal effect.
3. In general, planktonic growth of *S. mutans* was not repressed by high concentrations of Stevia.
4. Most of the Stevia concentrations generated an increase in pH compared to the 0 mg/ml Stevia control. A pH of approximately 4.1 was the highest pH observed with the largest Stevia concentration examined.
5. Based on our in vitro observations, Stevia can be considered a non-cariogenic sweetener.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Funding: This investigation was fully funded by CES University. The funding source had no involvement in data collection, analysis interpretation or in the decision to submit the article for publication.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Not required.

References

1. Ojeada Garcés J, Oviedo García E, Salas L (2013) *Streptococcus mutans* and dental caries. Rev. CES Odont 26(1):44-56. http://www.scielo.org.co/pdf/ceso/v26n1/v26n1a05.pdf
2. Selwitz R, Ismail A, Pitts N (2007) Dental caries. Lancet 369:51-59. https://doi.org/10.1016/S0140-6736(07)60031-2
3. Gupta P, Gupta N, Singh H (2014) Prevalence of Dental Caries in relation to Body Mass Index, Daily Sugar Intake and Oral Hygiene Status in 12-Year-

Old School Children in Mathura City: A Pilot Study. International Journal of Pediatrics Volume 2014.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3945027/

9. Do T, Devine D, Marsh P (2013) Oral biofilms: molecular analysis, challenges and future prospects in dental diagnostics. Clinical, Cosmetic and Investigational Dentistry 5:11-19.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3652372/

10. Gamboa F, Chaves M (2012) Antimicrobial potential of extracts from *Stevia Rebaudiana* leaves against bacteria of importance in dental caries. Acta Odontol Latinoam 25(2):171-175.
http://www.scielo.org.ar/pdf/aol/v25n2/v25n2a03.pdf

11. Jiang S, Gao X, Jin L, Lo E (2016) Salivary Microbiome Diversity in Caries Free and Caries Affected Children. International Journal of Molecular Sciences 17(12).
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5187778/pdf/ijms-17-01978.pdf

7. Takahashi N, Nyvad B (2011) The Role of Bacteria in the Caries Process: Ecological Perspectives. J Dent Res 90(3):294-303. https://journals.sagepub.com/doi/abs/10.1177/0022034510379602?rfr_dat=cr_pub%3Dpubmed&url_ver=Z39.882003&rfr_id=ori%3Arid%3Acrossref.org&journalCode=jdrb

8. Pedro Núñez D, García Bacallao L (2010) Biochemistry of dental caries. Revista Habanera de Ciencias Médicas 9(2):156-166.

http://scielo.sld.cu/pdf/rhcm/v9n2/rhcm04210.pdf

9. Kidd E (2011) The implications of the new paradigm of dental caries. Journal of Dentistry 39 Suppl 2: S3-8. https://www.sciencedirect.com/science/article/pii/S0300571211002739?via%3Dihub
10. Huang R, Li M, Gregory R (2011) Bacterial interactions in dental biofilm Virulence 2(5):435-444. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3322631/pdf/viru0205_0435.pdf
11. Martignon S, Gomez J, Tellez M, Ruiz J, Marin L, Rangel M (2013) Current Cariology Education in Dental Schools in Spanish Speaking Latin American Countries. Journal of Dental Education 77(10):1330-7. http://www.jdentaled.org/content/jde/77/10/1330.full.pdf
12. Minsalud (2012) Cuarto Estudio Nacional de Salud Bucal (ENSAB IV). https://www.minsalud.gov.co/sites/rid/Lists/BibliotecaDigital/RIDE/VS/PP/ENSAB-IV-Metodologia.pdf
13. Brambilla E, Cagetti M, Lonescu A, Campus G, Lingstrom P (2014), An in vitro and in vivo Comparison of the Effect of *Stevia Rebaudiana* Extracts on Different Caries Related Variables: A Randomized Controlled Trial Pilot Study. Caries Research 48(1):19-23. https://www.karger.com/Article/Abstract/351650

14. Thomas J, Glade M (2010) Stevia: It's Not Just About Calories. The Open Obesity Journal 2:101-109. https://pdfs.semanticscholar.org/fd0b/5df46a776069b89244000885d65f0277318b.pdf

15. Contreras S (2013) Anticariogenic properties and effects on periodontal structures of *Stevia rebaudiana Bertoni*. Narrative review. J Oral Res 2(3): 158-166. https://dialnet.unirioja.es/servlet/articulo?codigo=4995308

16. Salvador Reyes R, Sotelo Herrera M, Paucar Menacho L (2014) Study of Stevia (*Stevia rebaudiana Bertoni*) as a natural sweetener and its use in benefit of the health. Scientia Agropecuaria 5:157-163. https://es.scribd.com/document/362512646/stevia-pdf

17. Fambrizio Ferrazzano G, Cantile T, Alcidi B, Coda M, Ingenito A, Zarrelli A, Di Fabio G, Pollio A (2016) Is *Stevia rebaudiana Bertoni* a Non-Cariogenic Sweetener? A Review. Molecules 21(1):E38. https://www.mdpi.com/1420-3049/21/1/38

18. Giacaman R, Campos P, Muñoz Sandoval C, Castro R (2013) Cariogenic potential of commercial sweeteners in an experimental biofilm caries model on enamel. Archives of Oral Biology 58(9):1116-1122. https://www.sciencedirect.com/science/article/pii/S0003996913000782?via%3Dihub

19. Blauth de Slavutzky S (2010) Stevia and sucrose effect on plaque formation. J FÜR VERBRAUCHERSCHUTZ Leb 5(2):213-216.

https://link.springer.com/article/10.1007%2Fs00003-010-0555-5

20. Grembecka M (2015) Natural Sweeteners in a human diet. Rocz Panstw Zakl Hig 66(3) 195-202. https://www.ncbi.nlm.nih.gov/pubmed/26400114

21. Pierce CG, Uppuluri P, Tristan AR, Wormley FL, Mowat E, Ramage G, Lopez Ribot JL (2008) A simple and reproducible 96-well plate based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat Protoc 3(9):1494-500. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2741160/pdf/nihms127871.pdf

22. Das S, Das AK, Murphy RA, Punwani IC, Nasution MP, Kinghorn AD (1992) Evaluation of the cariogenic potential of the intense natural sweeteners stevioside and rebaudioside A. Caries Res 26(5):363-6. https://www.karger.com/Article/Abstract/261469

23. Huang R, M Li, R.L. Gregory (2012) Effect of nicotine on growth and metabolism of *Streptococcus mutans*. Eur. J. Oral Sciences 120(4):319-325. https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1600-0722.2012.00971.x

Description of the graphs

Fig. 1. Effect of Stevia on *S. mutans* total growth. The mean, and SE of the optical density (OD) at 595 nm of *S. mutans* cells treated with different concentrations of Stevia is shown. The experiment was repeated three times and asterisks indicate statistically significant differences compared to the 0 mg/ml Stevia control (p <0.05

Fig. 2. Effect of Stevia on *S. mutans* biofilm formation. The absorbance at 490 nm of crystal violet-stained *S. mutans* biofilm with different Stevia concentrations is shown with mean, and SE. The experiment was repeated three times and asterisks indicate statistically significant differences compared to the 0 mg/ml Stevia control ($p < 0.05$).

Fig. 3. Effect of Stevia on *S. mutans* viability. The number of colony forming units (CFU) with different Stevia concentrations was determined. The mean, and SE are shown and the experiment was repeated three times.

Fig. 4. Effect of Stevia on *S. mutans* metabolic activity. The absorbance at 490 nm, reflecting the metabolic activity of *S. mutans* biofilm cells treated with different Stevia concentrations is shown with mean, and SE. The experiment was repeated three times and asterisks indicate statistically significant differences compared to the 0 mg/ml Stevia control ($p < 0.05$).

Fig. 5. Effect of different Stevia concentrations on pH of *S. mutans* culture over 72 hours of growth. The experiment was repeated three times.