The effect of solutions containing extracts of *Vochysia tucanorum* Mart., *Myrcia bella* Cambess., *Matricaria chamomilla* L. and *Malva sylvestris* L. on cariogenic bacterial species and enamel caries development

Aline Silva Braga[a], Fernanda Pereira de Souza Rosa de Melo[b], Luiz Leonardo Saldanha[b], Anne Lígia Dokkedal[b], Tobias Meißner[c], Maximilian Bemmann[c], Ellen Schulz-Kornas[c], Rainer Haak[c], Mohamed Mostafa Hefny Abdelbary[d], Georg Conrads[d], Ana Carolina Magalhães[a]*, Marcella Esteves-Oliveira[c,e]

[a]Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Al. Octávio Pinheiro Brisolla, 9-75, 17012-901 Bauru, Brazil.

[b]Department of Biological Sciences, School of Science, São Paulo State University (UNESP), Av. Eng. Luís Edmundo Carrijo Coube, 2085, 17033-360, Bauru, Brazil.

[c]Department of Cariology, Endodontology, and Periodontology, University of Leipzig, Liebigstr. 12, 04103, Leipzig, Germany and

[d]Division of Oral Microbiology and Immunology, Department of Operative and Preventive Dentistry and Periodontology, RWTH Aachen University Hospital, Pauwelsstrasse 30, 52074, Aachen, Germany.

[e] Department of Restorative, Preventive and Pediatric Dentistry, zmk Bern, University of Bern, Switzerland.

Short title: Effects of solutions containing natural extracts on enamel caries development

*Corresponding author:
Department of Biological Sciences
Bauru School of Dentistry - University of São Paulo
Al. Octávio Pinheiro Brisolla, 9-75
Bauru-SP, Zip code: 17012-901, Brazil
Phone/Fax. + 55 14 32358497
E-mail: acm@usp.br

Number of Tables: 3

Number of Figures: 6

Word count: 4,171

Key words: Antimicrobial; dental caries; dental hygiene; oral biofilm.

Abstract

This study evaluated the effect of experimental solutions containing plant extracts on bacterial species and on enamel caries prevention. Microcosm biofilm was produced from human saliva mixed with McBain saliva (0.2% sucrose) on bovine enamel for 5 days (3 days under anaerobiosis and 2 days under aerobiosis) at 37º C. From the 2nd day until the end, the treatments were applied (1x60s/day): *Vochysia tucanorum* (10 mg/ml); *Myrcia bella* (5 mg/ml); *Matricaria chamomilla* (80 mg/ml); *Malva sylvestris*, fluoride and xylitol (Malvatricin Plus®); 0.12% Chlorhexidine (PerioGard®) and PBS (negative control). The medium pH was measured. A qPCR was performed for *Streptococcus mutans* and *Lactobacilli* spp. Enamel demineralization was measured by SD-OCT. The data were compared using Kruskal-Wallis/Dunn, ANOVA-two-way/Bonferroni and ANOVA-Tukey ($p<0.05$) tests. The pH decreased after sucrose exposure; only CHX reestablished pH > 5.5 at the last day. CHX also eliminated *Lactobacilli* spp., while the other treatments did not differ significantly from PBS. Malvatricin Plus® and CHX eliminated *S. mutans*, while the other treatments did not differ from PBS. Similar results were seen concerning the reduction of lesion depth (µm) and reflectivity. The experimental natural extracts solutions were ineffective against cariogenic bacteria and to prevent the development of enamel caries.

Introduction

The dental biofilm is a complex layer containing different microbial species and a rich extracellular matrix, which covers the tooth surfaces. Ambiental changes (nutrients and/or atmosphere) can induce microbiological changes in dental biofilm, favoring species able to produce metabolites that damage to the tooth surface or the periodontal tissue [Takahashi, 2015]. When the dysbiosis occurs in the supragingival biofilm, often due to the exposition to sucrose, selection of bacteria with an acid-producing/acid-tolerating phenotype occurs and increases the risk of dental caries development [Marsh, 2018].

The use of different antimicrobial agents to control the biofilm in dysbiosis is desirable [Pitts et al., 2017]. Accordingly, medicinal plants have long been studied since they are a rich source of polyphenols, terpenoids and alkaloids compounds with antimicrobial, anti-biofilm, anti-glycosyltransferase and anti-caries activities. Although several studies have investigated extract of different plants on cariogenic species [Philip et al., 2019], there is no conclusive evidence that they are as effective as the conventional synthetic antimicrobials in reducing dental caries development, through the control of biofilm viability/metabolism and/or the direct effect on tooth demineralization [Verkaik et al., 2011; Ledder et al., 2014].

Brazil has one of the greatest floristic diversity in the world and encompasses two biodiversity hotspots [Myers et al., 2000] for conservation and exploitation priorities — the Brazilian savanna (Cerrado) and the Atlantic rainforest (Mata Atlântica). *Myrcia bella* Cambess. (Myrtaceae) and *Vochysia tucanorum* Mart. (Vochysiaceae) are species naturally occurring in Cerrado, with tremendous medicinal potential [Hashimoto, 1996; Carnevale Neto et al., 2011; Forzza et al., 2012]. Some species of *Vochysia* were tested against *Staphylococcus aureus* [Hess et al., 1995], while *Myrcia bella* was tested on *Escherichia coli* [Dos Santos et al., 2018].

Matricaria chamomilla L. (Asteraceae) is a well-known medicinal plant used in traditional medicine for oral treatments in Europe and Western Asia, and in contrast to the above-mentioned plants, it has already been included in oral care products. This plant has been tested as an antimicrobial agent against some bacteria species [Munir et al., 2014]. Although it has been shown ineffective as antimicrobial on microcosm biofilm when added in a dentifrice [Ledder et al., 2014], it was effective against dental caries when used as solution showing an anti-caries effect [Braga et al., 2020]. Additionally, Chamomilla solution also showed anti-inflammatory action on gingivitis [Goes et al., 2016].

Malva sylvestris L. (Malvaceae), native in Europe, North Africa and Asia, has been reported as a potent antimicrobial, anti-inflammatory, antioxidant and anticancer agent. Currently, it has been incorporated in a commercial mouthwash and showed anti-caries effects on bovine enamel specimens [Gasparetto et al., 2012; Braga et al., 2018; Braga et al., 2020].

Considering that dental caries is one of the most impacting oral disease worldwide [Kassebaum et al., 2015], there is a need to find agents able to act on dental biofilm in dysbiosis in order to reduce enamel demineralization. Therefore, the present study aimed to investigate and to compare the effect of different solutions containing natural extracts (*V. tucanorum, M. bella*, *M. chamomilla*, or *M. sylvestris* – the latter as a commercial product) on the (%) of the aciduric bacterial taxa (*Lactobacilli* spp. and *Streptococcus mutans*) using qPCR. As further parameters, pH changes above biofilm growth and the enamel demineralization (using spectral domain optical coherence tomography, SD-OCT) were measured, under a mixed microcosm biofilm model.

Material and methods

Ethical aspects and saliva collection

The Ethics Committee (CEEA 84325518.2.0000.5417) of Bauru School of Dentistry-USP (Bauru-Brazil) approved the study. The study was conducted according to the Declaration of Helsinki. Before saliva collection, the participants signed the informed consent. Ten healthy participants (23.8±3 years old, eight women and two men, with signed informed consent) took part in the study. The definition of inclusion criteria of the saliva donors as well as the procedures for saliva collection under stimulation (salivary flow > 1ml/min, for 10 min of collection) followed previously reported protocols [Braga et al., 2019]. The saliva was collected once in the morning period; in total 132 ml (pool) of saliva was collected and diluted in glycerol (70% saliva and 30% glycerol) and 1 ml aliquots were stored at -80° C [Pratten et al., 2003]. In a pilot study, it was ensured that the total number of bacterial genomes was in the magnitude of 10^7 genomes / µl DNA extract of saliva/biofilm and that cariogenic *S. mutans* and lactobacilli did grow out of the inoculum to form a biofilm after 5 days.

Treatments

Vochysia tucanorum Mart. and *Myrcia bella* Cambess. leaf samples were collected in March 2017 at the Jardim Botânico Municipal de Bauru (S 22°20´ 30´´ W 49° 00´ 30´´), São Paulo, Brazil. Voucher specimens were prepared, identified and deposited at the Herbarium of the UNESP – UNBA under code number 5508 (*M. bella*) and 5141 (*V. tucanorum*). The access and shipment of component of genetic heritage

were issued under authorization No. 010468/2014-51 of Genetic Heritage Management Council (CGEN). Leaves' extracts (EtOH:H_2O 7:3, v/v) of *M. bella* and *V. tucanorum* were prepared according to previous studies [Saldanha et al., 2013; Machado et al., 2016]. Briefly, the powdered hot air dried (45^o C) were extracted through percolation using 70% EtOH as a solvent at room temperature and lyophilized. This process afforded the hydroethanolic extracts with 28% and 17% of yields for *M. bella* and *V. tucanorum*, respectively. The *Matricaria chamomilla* L. (flower and stalk) extract was purchased (*Quimer Insumos vegetais,* São Paulo, Brazil).

Table 1 shows the concentrations of the tested experimental solutions prepared with water, the commercial mouthwashes and the negative control (PBS). The concentrations of the experimental solutions were determined according to the results of minimum bactericide concentration (MBC) previously tested on *S. mutans* strain ATCC25175 as a reference, under aerobic conditions (37° C, 5% CO_2) [Pires et al., 2018]. The commercial products were tested without dilution. The company did not provide the concentration of the active components in the commercial mouthrinses.

Tooth specimen preparation

The bovine specimens were donated by cattle slaughtered in the food manufacturing industry (Frigol S.A, Lençóis Paulista-SP, Brazil). The study was approved by Ethics Committee on Animal Research (CEUA, Number: 002/2018, Bauru School of Dentistry, University of São Paulo, Bauru, Brazil) following the guidelines of the CONCEA (National Council for Control of Animal Experimentation). No animals were harm in order to conduct this study. Thirty-six bovine enamel specimens (4 mm x 4 mm) were polished and evaluated with respect to an average roughness (*Ra*) (contact profilometer Mahr, Göttingen, Germany) [Braga et al., 2019] to standardize the enamel surface for biofilm growth. Two parts of each 1/3 of the enamel surface were covered with red nail polish (Estreia-Colorama®, Rio de Janeiro, Brazil) in order to protect it from the biofilm and to create two reference areas (sound enamel), enabling later appropriate analysis of dental enamel demineralization through optical coherence tomography (SD-OCT). Thereafter, the specimens were sterilized using ethylene oxide [Gas exposure time (30% ETO/70%CO_2) for 4 h under 0.5 ± 0.1 kgF/cm^2 pressure]. Enamel specimens were

158 randomly distributed into six groups (n=6, Table 1), by using their mean *Ra* as criteria
159 (*Ra*: 0.155±0.03 μm).
160
161 *Preparation of artificial saliva*
162 McBain artificial saliva, containing 2.5 g/l mucin from porcine stomach (type II),
163 2.0 g/l tryptone, 2.0 g/l bacteriological peptone, 1.0 g/l yeast extract, 0.35 g/l NaCl, 0.2
164 g/l $CaCl_2$, 0.2 g/l KCl, 0.1 g/l cysteine hydrochloride, 0.001 g/l hemin, and 0.0002 g/l
165 vitamin K1, was prepared. Some components were sterilized using an autoclave.
166 However, mucin was sterilized by pasteurization to protect molecular structure and
167 function, whereas cysteine hydrochloride, hemin and vitamin K1 were sterilized using a
168 syringe filter.
169
170 *Microcosm biofilm formation*
171 Human saliva was mixed with McBain saliva [McBain, 2009] at a proportion of
172 1:50. Microcosm biofilm was formed as described by previous work [Braga et al., 2019],
173 however, under anaerobiosis for three days (to allow growth of anaerobic oral species)
174 and changing to aerobiosis (7% CO_2) for the last two days (Figure 1). Therefore, we
175 called this model as mixed microcosm biofilm.
176 Each enamel specimen was fixed in a 24-well microtiter plate, using liquid silicon
177 in the bottom of each well. Human saliva/McBain saliva solution was added (v=1.5
178 ml/well) and the plates were incubated anaerobically in GasPak (BD, Sparks, USA) at
179 37˚C, for the first 8 h. Thereafter, the specimens were washed with PBS and exposed to
180 fresh McBain saliva containing 0.2% sucrose and incubated at the same conditions until
181 completing the 1^{st} day of the experiment.
182 From the 2^{nd} to the 5^{th} day, the specimens were treated with the experimental
183 solutions (Table 1) at room temperature, once a day (1 ml/well, 1 minute). The specimens
184 were washed using PBS, and fresh McBain saliva containing 0.2% sucrose was added.
185 Then, the microplates were incubated under strictly anaerobic conditions at 37°C for
186 additional 2 days, and at the last 2 days, under 7% CO_2 and 37º C [Conrads et al., 2019].
187 The experiments were performed in duplicate (n=3/replicate), as shown in Figure 1.
188
189

pH Monitoring

The medium pH (McBain artificial saliva) values (in the layer right above biofilm) were monitored every day, starting after the first 8h and 24h of biofilm formation. From day 2 to 5, the biofilm pH was measured before performing the treatments once a day, by using a MiniTrode (Hamilton, Bonaduz, Switzerland) at room temperature as previously reported [Walther et al., 2019].

Molecular analyses (genome count determination) – qPCR

After 5 days of biofilm formation, the samples were removed from 24-well-plates and transferred into Eppendorf tubes containing 250 µl of 0.9% saline solution with 3 glass spheres (3 mm in diameter) and stored in the freezer at -70°C. At the next day, the microtubes were defrosted and samples vortexed for 10 s to provoke complete removal of the biofilm from the samples surface; this solution was transferred to a new microtube, where 100 µl was used to DNA extraction using the QIAamp® DNA Mini Kit (QIAGEN, Düsseldorf, Germany) according to the manufacturer protocol. The DNA samples were stored at -20°C. The cleaned enamel specimens were stored with sterilized water in a domestic refrigerator to measure the demineralization by SD-OCT analysis.

The quantity of microorganisms was analyzed by quantitative polymerase chain reaction (qPCR) using the software QuantStudio version 3 (Applied Biosystems by Thermo Fisher Scientific, Waltham, USA), as previously described by Henne et al. [2015; 2016]. Shortly, specific primers were applied to measure *Streptococcus mutans* and *Lactobacilli* spp. (Table 2). A combination of 0.1 µl Pr.F (Primer Forward), 0.1 µl Pr.R (Primer Reverse), 10 µl Rastem/Master Mix solution and 8.8 µl water were pipetted into 96-well plates containing respective primers; after that, 0.1 µl of DNA samples were pipetted in each well. As a calibration curve, known concentrations of the tested microorganisms were applied (positive control) and water functioned as negative control. The data were obtained by QuantStudio Design & Analysis software v.1.4.3, and exported to Excel Microsoft, in biological triplicate [Walther et al., 2019].

SD-OCT imaging and analysis

Spectral domain optical coherence tomography (SD-OCT, Telesto II; Thorlabs GmbH, Dachau, Germany) was used to quantify artificial induced lesions three-

dimensionally following the protocol of Schneider et al. [2017]. The Telesto II SP2 probe head was equipped with a wideband laser source of center wavelength at 1310 nm (±120 nm) with values of the axial or lateral resolution of < 7.5 (air) or 15 µm. Before using the SD-OCT, the nail polish covering the sample surface was removed in order to obtain images from sound and demineralized enamel surfaces. A maximum field of view of 5.5 mm x 5.5 mm x 3.5 mm (δx=10.00, δy=3.54 µm, δz=20 µm), imaging speed 48 kHz, sensitivity ≤ 85 dB and A-scan average of five were used. An image stack of the middle part of the surface area (width = 1.5 mm) of the specimen was recorded, to get a representative area independent of edge effects, and including both sound and demineralized enamel. Per image stack 25 OCT-B-scans, from image 101 to 176 with a distance between the images of δz=20 µm, were generated.

The images were exported from the SD-OCT software ThorImage version 4.4 using ImageJ version v.1.4.5, and lower boundary values of 20 dB and an upper boundary of 80 dB were set. We developed the open-source software Caries Lesion Quantification (CarLQuant Software version 1; the code of the open-source software is accessible through the open-source data repository DOI: XXX will be given upon acceptance of the manuscript) to quantify semi-automatically depth and area of the artificially induced-caries lesions. The software was set to detect the sound and demineralized areas and to calculate the mean values of lesion depth (pixel), the reflectivity of the sound area (gray values), and the reflectivity of the lesion area (gray values). The raw values of lesion depth were given in pixels and re-calculated into µm. A detailed description of the detection procedure is given in Figure 2.

Micro-Computer Tomography (Micro-CT) imaging

One representative specimen from each group was scanned by X-ray microtomography using Skyscan 1172 (Bruker Micro-CT, Kontich, Antwerp, Belgium) at 100 kV and 100 µA. A filter (Al+Cu) was used to reduce the beam-hardening artifacts. Six specimens were scanned at 180° rotation with a rotation step of 0.20° with a pixel size of 3.95 mm, averaging five readings and a random movement of 10 in oversize with three segments, during 5 h of scanning for each segment. The images were reconstructed using NRecon version 1.7.4.2 (BrukerMicro-CT), applying a beam-hardening correction

of 50% and a ring artifacts reduction of 30%. The images were calibrated and rotated in Data Viewer version 1.5.6.2 (Bruker Micro-CT).

Statistical Analysis

Data were statistically compared using Graph Pad Instat and Graph Pad Prism software (GraphPad Software, San Diego, USA). The distribution and homogeneity were tested using Kolmogorov and Smirnov's and Bartlett's tests, respectively. With respect to the medium pH, the data were compared with two-way ANOVA/Bonferroni's test (factors: treatment and periods of analysis: 8h, 24h, 2, 3, 4 and 5 days). *Lactobacilli* spp. and *S. mutans* levels and lesion depth (µm) were analyzed using Kruskal-Wallis and Dunn's multiple comparisons test. The values of the reflectivity area (gray values) were compared using ANOVA/ Tukey's Multiple Comparison test. The level of significance was set at 5%.

Results

pH changes and risk for demineralization

Table 3 shows the pH values during the microcosm biofilm growth. The pH values were reduced for all treatments after 24h of biofilm growth. From 24h to 120h, there were no further significant differences in pH values between the periods, for all treatments. However, there was significant difference among the treatments, where CHX (positive control) significantly showed increased pH values compared to PBS (negative control) and all other groups (except Malvatricin Plus®). In case of CHX, the restored pH value was finally (at 120h) higher than the critical pH (> 5.5) for hydroxyapatite demineralization. No significant interaction between the analyzed factors was found.

qPCR and changes in numbers of aciduric bacteria

Figure 3 shows the genome counts for *Lactobacilli* spp. and *Streptococcus mutans* recovered from the microcosm biofilm formed over the specimen surfaces. Complete elimination (reduction under cut-off which is usually <10 cells) of lactobacilli was caused by chlorhexidine included in the product PerioGard® only, while all the other treatments were similar to PBS (negative control). With respect to *Streptococcus mutans*, the commercial mouthwashes Malvatricin Plus® and PerioGard® containing *Malva sylvestris*

and chlorhexidine, respectively, were both able to reduce this species under the detection limit.

SD-OCT

The commercial mouthwashes containing *Malva sylvestris* and chlorhexidine were able to significantly reduce the lesion depth (µm) compared to PBS (negative control). The experimental solution containing *Matricaria chamomilla* was statistically equal compared to *Malva sylvestris*, but also similar to PBS (negative control) (Figure 4.a). The difference between sound and lesion area (grayscale/reflectivity) were significantly lower for both commercial mouthwashes (Malvatricin Plus® and PerioGard®) compared to PBS (negative control) (Figure 4.b). The experimental solutions were unable to reduce enamel demineralization (the difference in grayscale). Figure 5 shows representative SD-OCT images from specimens containing demineralized and sound areas. The images show the demineralized middle area (slight gray) and at both edges, the control sound areas.

Micro-Computer Tomography (Micro-CT) imaging

Representative images from micro-CT give a visual impression of enamel with sound and lesion area (Figure 6). The lesion presented by PBS (the negative control) appears darker and deeper compared to the lesions presented by the other groups. Specimens treated by *Malva sylvestris* and 0.12% CHX showed shallow lesions with minor enamel alterations compared to the other groups (Figure 6).

Discussion

The number of oral care products containing natural agents on the market is steadily increasing. However, most products have not been even tested for the prevention of oral diseases or with respect to side effects [Cheng et al., 2015]. There is an urgent need to validate their positive and negative effects in a more consistent matter. Few studies done so far in this field have tested the plant extracts and their main compounds applying different models, concentrations and vehicles [Verkaik et al., 2011; Philip et al., 2019] making a comparison of the results difficult.

Microcosm biofilm used in the present study retains most of the cultivable microorganisms found in the oral cavity [Filoche et al., 2007]. The present protocol was modified compared to a previous study [Braga et al., 2018], since it presented two different atmospheres: i) anaerobic, at the beginning to allow the growth of (obligate as well as facultative) anaerobes from the saliva donors and to mimic the deep biofilm layer; ii) aerobic atmosphere after 3 days to allow reproduction of the biofilm surface and complete consumption of sugar by organisms with a respiratory chain. This is the first time that a mixed microcosm biofilm model was applied to induce enamel demineralization, which has been measured by SD-OCT.

It is already known that sugars are metabolized by supragingival saccharolytic bacteria such as *Streptococcus mutans* and related species [Takahashi, 2015]. During the caries process, *Streptococcus mutans* create a retentive and acidic niche for the secondary colonization of lactobacilli, which are also known as acidogenic and aciduric bacteria involved in the lesion progression [Caufield et al., 2015]. Therefore, both *Streptococcus mutans* and lactobacilli (*Lactobacilli* spp.) were quantified by qPCR, since this method has a very good accuracy compared to CFU counting [Mira, 2018].

In addition, we analyzed enamel demineralization by SD-OCT, a new non-invasive imaging method able to detect and quantify dental caries lesions [Schneider et al., 2017], taking advantage of measuring demineralization without the need of cutting the specimen. It is also a straightforward technique that can be used to visualize and quantify the differences between sound and lesion areas in real-time and to generate a three-dimensional model of the lesion [Schneider et al., 2017; Sahyoun et al., 2020].

To validate the information provided by SD-OCT, a representative image of each group was obtained by micro-CT, which is a sensitive method to detect early lesions produced *in vitro* [Hamba et al., 2012], being applied as a reference standard for the analysis of proximal enamel surfaces [Oliveira et al., 2019]. The selected micro-CT images confirm that a demineralized surface area is detectable. We refrained from further interpretation since the micro-CT data was conducted with a very small sample (n=1 per treatment), due to the long period of scanning.

Considering that the medium pH values changed over time, our study showed that the commercial mouthwashes containing *Malva sylvestris* and chlorhexidine presented a pH recovery, which may be related to the reduction of *S. mutans* level in the biofilm and

the lesion depth (Figure 6). Interesting was that - despite the fact that chlorhexidine has eliminated (dramatically reduced under cut-off) the cariogenic species according to qPCR results - it did not completely protect enamel against demineralization. This observation highlights the involvement of other species on the development of dental caries, which is in accordance with previous findings and the currently widely accepted extended ecological plaque hypothesis for the etiology of dental caries [Simón-Soro and Mira, 2015; Conrads and About, 2018].

It is important to mention that even though the commercial agent containing chlorhexidine showed a high reduction of both caries-associated bacteria (*S. mutans* and lactobacilli) as well as a significant decreased enamel demineralization, this agent is not currently recommended for clinical caries prevention. Chlorhexidine is a broad-spectrum antiseptic causing elimination of not only pathogenic bacteria but also of commensal species. According to the current ecological plaque hypothesis, commensal bacterial are needed to main the equilibrium in the oral microbiome and, thus, the oral health. Besides that, recent studies have shown that the use of broad-spectrum antimicrobial agents do not revert dysbiosis, since the susceptible surfaces of the teeth normally become repopulated with a similar microbiome, as soon as the therapy is stopped [Burne, 2018]. Finally, chlorhexidine also causes some important side-effects when used for long-term [Cieplik et at., 2019]. Therefore, it is currently believed that new strategies, such as cariogenic virulence inhibiting natural agents, to modulate rather than eliminate the microbiome and with low side-effects might be the most promising [Philip et al., 2020]. However, this aspect is still to be further proven.

With respect to antimicrobial effect of natural agents, the only mouthwash containing a natural agent with effect on *S. mutans* was *M. sylvestris* (Malvatricin Plus®– Daudt). This finding is different from what was recently shown by our group [Braga et al., 2020] using the standard microcosm biofilm model (aerobic growth). Braga et al. [2020] found an antimicrobial effect of *M. sylvestris* only on lactobacilli, but not on *S. mutans*. The contrasting results may be due to the differences in atmosphere for biofilm growth between both studies. The anaerobic conditions can have favored the growth of lactobacilli species (indeed qualified as mostly anaerobic) reducing the effect of antimicrobial agents on these microorganisms [Caufield et al., 2015]. On the other hand, *S. mutans* seems to be more susceptible to the treatments under anaerobic conditions.

Another possible explanation might be the different microbial quantification method applied by Braga et al. [2020], which performed the counting of colony-forming unit (CFU counting).

With respect to enamel demineralization, only *M. sylvestris* had a protective effect comparable to the chlorhexidine-product, which is in agreement with the results of our previous study [Braga et al., 2018]. In addition, Braga et al. [2020] showed an anti-caries effect not only for *M. sylvestris*, but also for *M. chamomilla*, which might have been due to the atmospheric conditions and the method applied to measure demineralization (transverse microradiography – TMR) in their study. With respect to the methods, lesion depth measured by OCT and TMR has shown good agreement for enamel caries lesions found in extracted teeth [Staninec et al., 2011].

The idea that the environment has influence on biofilm quality has been previously discussed. Variations in nutrient supply, pH, temperature and atmosphere may select microorganisms in biofilm more adapted to dynamic growth conditions [Kreth et al., 2008]. Despite the microbiological differences between the studies of Braga et al. [2018, 2020] and the present work, both models (aerobic and mixed) produced enamel lesions with a similar depth. However, probably the role of the microorganisms on the caries development was different between them, which deserves to be further studied.

According to our results and from previous studies, only *M. sylvestris containing* solution (the commercial mouthwash) has some potential effect to be further tested. *M. sylvestris* contains malvone, aromatic compounds, monoterpenes and tetrahydroxylated acyclic diterpes [Veshkurova et al., 2006]. Malvone has been responsible by the antimicrobial effect of *M. sylvestris* [Razavi et al., 2011], since it has shown to inhibit strains of *S. mutans* and *Lactobacillus casei* [Da Silva et al., 2012]. We also have to consider that the tested commercial product contains fluoride (able to inhibit bacterial sugar uptake and glycolysis and to hard enamel by formation of fluorohydroxyapatite) and xylitol (sucrose substitute), which also play important role making enamel more resistant to the effect of the acids, decreasing demineralization and accelerating the remineralization [Buzalaf et al., 2011; Cardoso et al., 2016]. It also contains a few other antimicrobial substances such as menthol and triclosan albeit in minor concentrations. Therefore, we cannot affirm that the beneficial effect of the commercial mouthwash is due to the presence of the natural agent malvone only.

Further studies using microbiome and metabolomics approaches are desirable to better understand the effect of the atmosphere in the biofilm development and the dynamic of dysbiosis under the influence of plant extracts. The new methods applied to measure enamel demineralization shall be further explored and compared to the gold-standard method (TMR).

In comparison to the commercial products PerioGard® (based on chlorhexidine) and Malvatricin Plus® (based on *M. sylvestris* extract), the tested new experimental solutions, containing several less investigated natural plant extracts, were ineffective in reducing *Lactobacillus* species and *S. mutans* as well as enamel demineralization under the study conditions chosen.

Acknowledgments

This study was conducted as part of the doctoral thesis of Braga AS. We wish to thank Mrs. Beate Melzer-Krick (RWTH Aachen University Hospital) and Mrs. Claudia Rüger (University of Leipzig, Department of Cariology, Endodontology and Periodontology) for technical support. We would like to thank the anonymous saliva donors.

Statement of Ethics

This study was approved by the local Ethics Committee (Number: 84325518.2.0000.5417) and Ethics committee on animal research (CEUA, Number: 002/2018).

Disclosure Statement

The authors declare no potential conflict of interest.

Funding Sources

This work was supported by the São Paulo Research Foundation (FAPESP 2017/00556-0 and 2017/17249-2, 2018/26506-1).

Author Contributions

Braga AS, Meißner T, Bemmann M and Abdelbary MMHA performed the experiments. de Melo FPSR, Saldanha LL and Dokkedal AL collected the plants and prepared the extracts. Schulz-Kornas E, Haak R and Conrads G designed the project, Magalhães AC and Esteves-Oliveira M designed the project and supervised all the experiments. Braga AS and Magalhães AC analyzed the data and wrote the manuscript. All authors revised and approved the paper.

References

Braga AS, de Melo Simas LL, Pires JG, Souza BM, de Souza Rosa de Melo FP, Saldanha LL et al. Antibiofilm and anti-caries effects of an experimental mouth rinse containing *Matricaria chamomilla* L. extract under microcosm biofilm on enamel. J Dent. 2020;99:103415.

Braga AS, Girotti LD, de Melo Simas LL, Pires JG, Pelá VT, Buzalaf MAR et al. Effect of commercial herbal toothpastes and mouth rinses on the prevention of enamel demineralization using a microcosm biofilm model. Biofouling. 2019;35(7):796–804.

Braga AS, Pires JG, Magalhães AC. Effect of a mouthrinse containing *Malva sylvestris* on the viability and activity of microcosm biofilm and on enamel demineralization compared to known antimicrobials mouthrinses. Biofouling. 2018;34(3):252–261.

Burne RA. Getting to know "the known unknowns": Heterogeneity in the oral microbiome. Adv. Dent. Res. 2018;29(1):66-70.

Buzalaf M, Pessan JP, Honório HM, Ten Cate JM. Mechanisms of action of fluoride for caries control. Monogr Oral Sci. 2011;22:97–114.

Cardoso CA, Cassiano LP, Costa EN, Souza-E-Silva CM, Magalhães AC, Grizzo LT et al. Effect of xylitol varnishes on remineralization of artificial enamel caries lesions in situ. J Dent. 2016;50:74–78.

Carnevale Neto F, Pilon AC, Silva DHS, Bolzani VS, Castro-Gamboa I. Vochysiaceae: secondary metabolites, ethnopharmacology and pharmacological potential. Phytochem Rev. 2011;10:413–429.

Caufield PW, Schön CN, Saraithong P, Li Y, Argimón S. Oral Lactobacilli and dental caries: A model for niche adaptation in humans. J. Dent. Res. 2015;94(9 Suppl):110S–118S.

476 Cheng L, Li J, He L, Zhou X. Natural products and caries prevention. Caries Res.
477 2015;49(1):38–45.
478 Cieplik F, Jakubovics NS, Buchalla W, Maisch T, Hellwig E, Al-Ahmad A. Resistance
479 toward chlorhexidine in oral bacteria-is there cause for concern? Front. Microbiol.
480 2019;22;10:587.
481 Conrads G, About I. Pathophysiology of dental caries. Monogr Oral Sci. 2018;27:1–10.
482 Conrads G, Wendt LK, Hetrodt F, Deng ZL, Pieper D, Abdelbary M et al. Deep
483 sequencing of biofilm microbiomes on dental composite materials. J. Oral Microbiol.
484 2019;11(1):1617013.
485 Da Silva NB, Alexandria AK, De Lima AL, Claudino LV, De Oliveira Carneiro TF, Da
486 Costa AC et al. In vitro antimicrobial activity of mouth washes and herbal products
487 against dental biofilm-forming bacteria. Contemp. Clin. Dent. 2012;3(3):302–305.
488 Dos Santos C, Galaverna RS, Angolini C, Nunes V, de Almeida L, Ruiz A et al.
489 Antioxidative, antiproliferative and antimicrobial activities of phenolic compounds from
490 three *Myrcia* species. Molecules. 2018;23(5):986.
491 Filoche SK, Soma KJ, Sissons CH. Caries-related plaque microcosm biofilms developed
492 in microplates. Oral Microbiol. Immunol. 2007;22(2):73–79.
493 Forzza RC, Baumgratz JF, Bicudo CE, Canhos DA, Carvalho AAJr, Coelho MN et al.
494 New Brazilian floristic list highlights conservation challenges. BioScience.
495 2012;62(1):39–45.
496 Gasparetto JC, Martins CA, Hayashi SS, Otuky MF, Pontarolo R. Ethnobotanical and
497 scientific aspects of *Malva sylvestris* L.: a millennial herbal medicine. J. Pharm.
498 Pharmacol. 2012;64(2):172–189.
499 Goes P, Dutra CS, Lisboa MR, Gondim DV, Leitão R, Brito GA et al. Clinical efficacy
500 of a 1% *Matricaria chamomile* L. mouthwash and 0.12% chlorhexidine for gingivitis
501 control in patients undergoing orthodontic treatment with fixed appliances. J. Oral Sci.
502 2016;58(4):569–574.
503 Hamba H, Nikaido T, Sadr A, Nakashima S, Tagami J. Enamel lesion parameter
504 correlations between polychromatic micro-CT and TMR. J. Dent. Res. 2012;91(6):586–
505 591.
506 Hashimoto G. Illustrated Cyclopedia of Brazilian Medicinal Plants. Aboc-Sha,
507 Kamakura, Japan, 1996.

508 Heilig HG, Zoetendal EG, Vaughan EE, Marteau P, Akkermans AD, de Vos WM.
509 Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human
510 intestine as determined by specific amplification of 16S ribosomal DNA. Appl. Environ.
511 Microbiol. 2002;68(1):114–123.
512 Henne K, Gunesch AP, Walther C, Meyer-Lueckel H, Conrads G, Esteves-Oliveira M.
513 Analysis of bacterial activity in sound and cariogenic biofilm: A pilot in vivo study.
514 Caries Res. 2016;50(5):480-488.
515 Henne K, Rheinberg A, Melzer-Krick B, Conrads G. Aciduric microbial taxa including
516 *Scardovia wiggsiae* and *Bifidobacterium* spp. in caries and caries free subjects. Anaerobe.
517 2015;35(Pt A):60–65.
518 Hess SC, Brum RL, Honda NK, Cruz AB, Moretto E, Cruz RB et al. Antibacterial activity
519 and phytochemical analysis of *Vochysia divergens* (Vochysiaceae). J. Ethnopharmacol.
520 1995;47(2):97–100.
521 Kassebaum NJ, Bernabé E, Dahiya M, Bhandari B, Murray CJ, Marcenes W. Global
522 burden of untreated caries: a systematic review and metaregression. J. Dent. Res.
523 2015;94(5):650–658.
524 Kreth J, Zhang Y, Herzberg MC. Streptococcal antagonism in oral biofilms:
525 *Streptococcus sanguinis* and *Streptococcus gordonii* interference with *Streptococcus*
526 *mutans*. J. Bacteriol. 2008;190(13):4632–4640.
527 Ledder RG, Latimer J, Humphreys GJ, Sreenivasan PK, McBain AJ. Bacteriological
528 effects of dentifrices with and without active ingredients of natural origin. Appl. Environ.
529 Microbiol. 2014;80(20):6490–6498.
530 Machado AC, Souza LP, Saldanha LL, Pieroni LG, Matos AA, Oliveira FA et al.
531 "Aroeira" (*Myracrodruon urundeuva*) methanol extract: the relationship between
532 chemical compounds and cellular effects. Pharm. Biol. 2016;54(11):2737–2741.
533 Marsh PD. In sickness and in health-what does the oral microbiome mean to us? An
534 ecological perspective. Adv. Dent. Res. 2018;29(1):60–65.
535 McBain AJ. Chapter 4: In vitro biofilm models: an overview. Adv. Appl. Microbiol.
536 2009;69:99–132.
537 Mira A. Oral microbiome studies: potential diagnostic and therapeutic implications. Adv.
538 Dent. Res. 2018;29(1):71–77.

Munir N, Iqbal AS, Altaf I, Bashir R, Sharif N, Saleem F et al. Evaluation of antioxidant and antimicrobial potential of two endangered plant species *Atropa belladonna* and *Matricaria chamomilla*. Afr. J. Tradit. Complement. Altern. Med. 2014;11(5):111–117.

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GA, Kent J. Biodiversity hotspots for conservation priorities. Nature. 2000;403(6772):853–858.

Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology (Reading). 2002;148(Pt 1):257–266.

Oliveira LB, Massignan C, Oenning AC, Rovaris K, Bolan M, Porporatti AL et al. Validity of micro-CT for in vitro caries detection: a systematic review and meta-analysis. Dentomaxillofac Radiol. 2019;49(7):20190347.

Philip N, Leishman S, Walsh L. Potential role for natural products in dental caries control. Oral Health Prev. Dent. 2019;17(5):479–485.

Philip N, Leishman SJ, Bandara HMHN, Healey DL, Walsh LJ. Randomized controlled study to evaluate microbial ecological effects of CPP-ACP and Cranberry on dental plaque. JDR Clin. Trans. Res. 2020;5(2):118-126.

Pires JG, Zabini SS, Braga AS, de Cássia Fabris R, de Andrade FB, de Oliveira RC et al. Hydroalcoholic extracts of *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart. leaves on *Streptococcus mutans* biofilm and tooth demineralization. Arch. Oral Biol. 2018;91:17–22.

Pitts NB, Zero DT, Marsh PD, Ekstrand K, Weintraub JA, Ramos-Gomez F et al. Dental caries. Nat. Rev. Dis. Primers. 2017;3:17030.

Pratten J, Wilson M, Spratt DA. Characterization of in vitro oral bacterial biofilms by traditional and molecular methods. Oral Microbiol. Immunol. 2003;18(1):45–49.

Razavi SM, Zarrini G, Molavi G, Ghasemi G. Bioactivity of *Malva sylvestris* L., a medicinal plant from iran. Iran J. Basic Med. Sci. 2011;14(6):574–579.

Sahyoun CC, Subhash HM, Peru D, Ellwood RP, Pierce MC. An experimental review of optical coherence tomography systems for noninvasive assessment of hard dental tissues. Caries Res. 2020;54(1):43–54.

Saldanha LL, Vilegas W, Dokkedal AL. Characterization of flavonoids and phenolic acids in *Myrcia bella* Cambess. using FIA-ESI-IT-MS(n) and HPLC-PAD-ESI-IT-MS combined with NMR. Molecules (Basel, Switzerland). 2013;18(7):8402–8416.

571 Schneider H, Park KJ, Rueger C, Ziebolz D, Krause F, Haak R. Imaging resin infiltration
572 into non-cavitated carious lesions by optical coherence tomography. J. Dent. 2017;60:94–
573 98.
574 Simón-Soro A, Mira A. Solving the etiology of dental caries. Trends Microbiol.
575 2015;23(2):76–82.
576 Staninec M, Douglas SM, Darling CL, Chan K, Kang H, Lee RC et al. Non-destructive
577 clinical assessment of occlusal caries lesions using near-IR imaging methods. Lasers
578 Surg. Med. 2011;43(10):951–959.
579 Takahashi N. Oral microbiome metabolism: from "who are they?" to "what are they
580 doing?" J. Dent. Res. 2015;94(12):1628–1637.
581 Verkaik MJ, Busscher HJ, Jager D, Slomp AM, Abbas F, van der Mei HC. Efficacy of
582 natural antimicrobials in toothpaste formulations against oral biofilms in vitro. J. Dent.
583 2011;39(3):218–224.
584 Veshkurova O, Golubenko Z, Pshenichnov E, Arzanova I, Uzbekov V, Sultanova E et al.
585 Malvone A, a phytoalexin found in *Malva sylvestris* (family Malvaceae). Phytochemistry.
586 2006;67(21):2376–2379.
587 Walther C, Meyer-Lueckel H, Conrads G, Esteves-Oliveira M, Henne K. Correlation
588 between relative bacterial activity and lactate dehydrogenase gene expression of co-
589 cultures in vitro. Clin. Oral Investig. 2019;23(3):1225–1235.

Figure Legends

Fig. 1. Experimental biofilm protocol and the response variables.

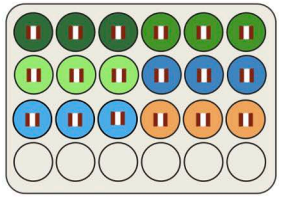
Fig. 2. a. SD-OCT-B scan (Automatic mode), the right side shows the legend for different lines used to the analysis with CarLQuant software. **b.** Grayscale showing the specimen reflectivity. **c.** Graphic showing the grayscale of the specimen surface, the enamel and dentin areas.

Fig. 3. Boxplot of genome counts of caries-associated bacteria *Lactobacilli* spp. and *S. mutans* present in microcosm biofilm after treatments. Genomes per microliter of samples were determined for each bacterial taxon by qPCR. **A.** *Lactobacilli* spp. ($p<0.0009$); **b.** *S. mutans* ($p=0.0215$). Different letters show significant differences among the groups (Kruskal-Wallis/ Dunn' multiple comparison test).

Fig. 4. a. Boxplot of lesion depth (µm) (Kruskal-Wallis/Dunn' multiple comparison test, $p<0.0004$). **b.** Mean ±SD of reflectivity difference between sound and lesion areas (gray values, ANOVA/Tukey's multiple comparisons test, $p<0.0001$). Different letters show significant differences among the groups. High value on grayscale means higher difference between sound and demineralized enamel area (higher demineralization). Low value means smaller difference between sound and demineralized enamel areas (lower demineralization).

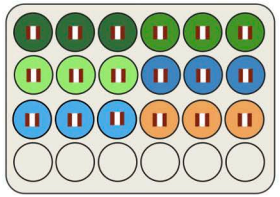
Fig. 5. Representative SD-OCT images of a specimen from each group. The white arrow shows the lesion area in white color.

Fig. 6. Representative µ-CT images of a specimen from each group. The black arrow shows the lesion area in dark color.



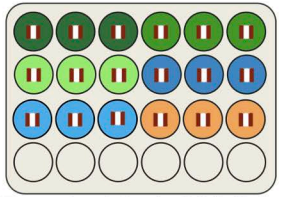
Enamel + 1.5 ml of inoculum plus McBain saliva

8h incubation
(37° C, under anaerobic conditions)



Enamel + 1.5 ml of McBain saliva with sucrose

16h incubation
(37° C, under anaerobic conditions)

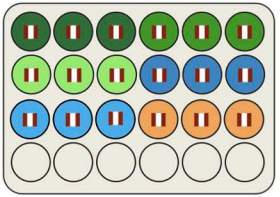


Enamel + 1.5 ml of McBain saliva with sucrose

Changed every 24h

Treatment with the commercial or experimental mouth rinses (1x/1min)

From day 2 to 3: anaerobic conditions
From day 4 to 5: aerobic condictions 7% CO_2

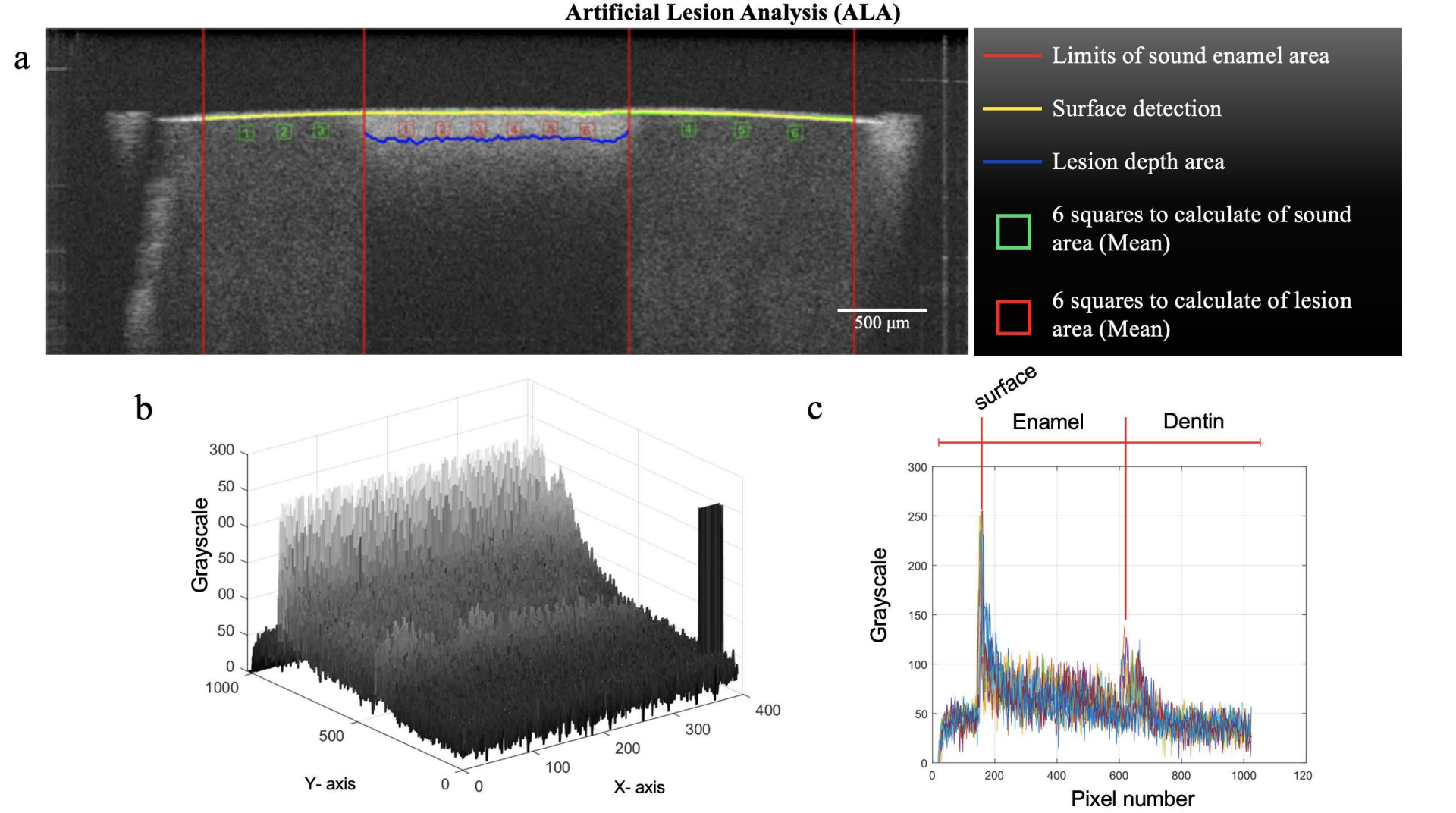


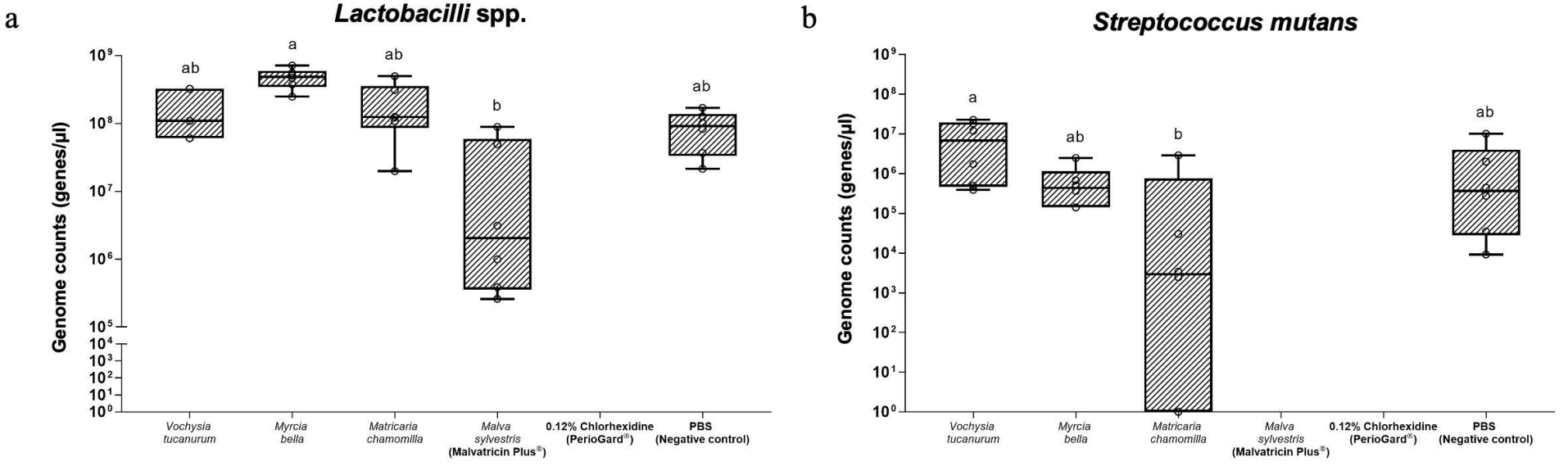
Enamel + 1.5 ml of McBain saliva with sucrose

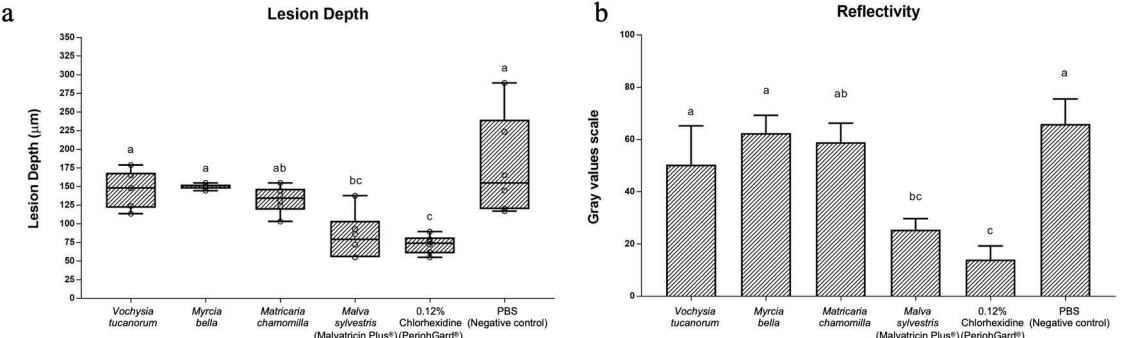
After 5 days

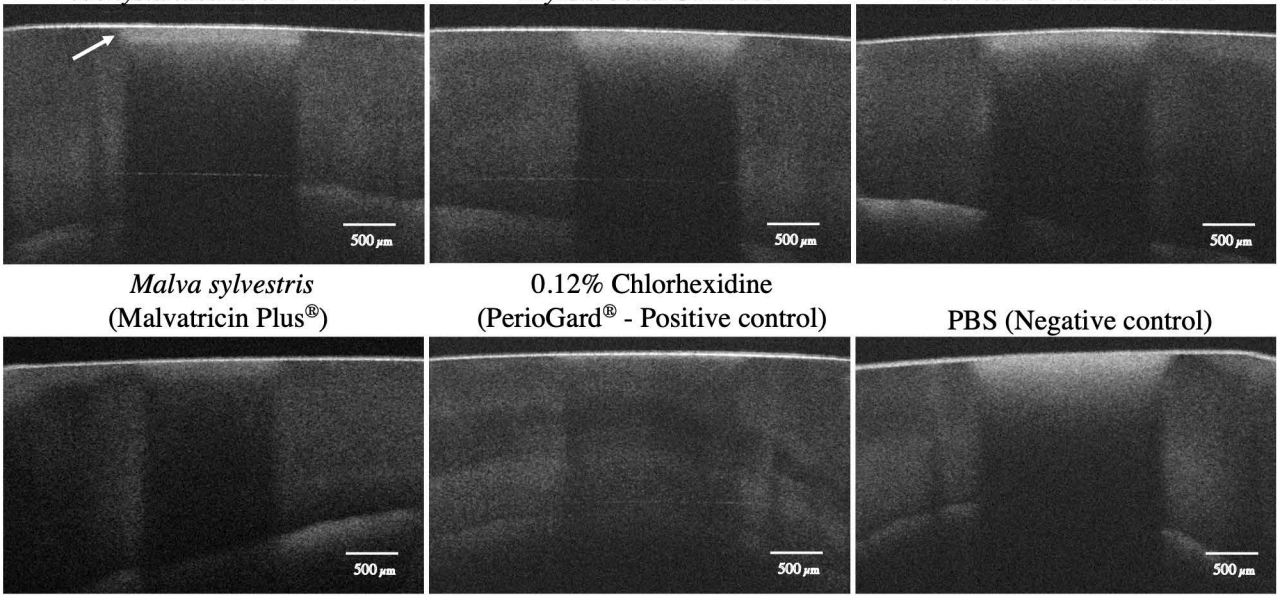
qPCR

SD-OCT

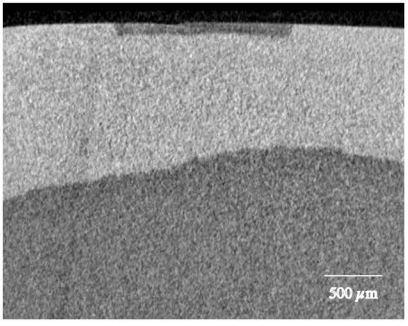

Artificial Lesion Analysis (ALA)
a
500 µm
Limits of sound enamel area
Surface detection
Lesion depth area
6 squares to calculate of sound area (Mean)
6 squares to calculate of lesion area (Mean)
b
Grayscale
Y- axis
X- axis
c
surface
Enamel
Dentin
Grayscale
Pixel number


a
Lactobacilli spp.
Genome counts (genes/µl)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
ab
a
ab
b
ab
Vochysia tucanurum
Myrcia bella
Matricaria chamomilla
Malva sylvestris (Malvatricin Plus®)
0.12% Chlorhexidine (PerioGard®)
PBS (Negative control)
b
Streptococcus mutans
Genome counts (genes/µl)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
a
ab
b
ab
Vochysia tucanurum
Myrcia bella
Matricaria chamomilla
Malva sylvestris (Malvatricin Plus®)
0.12% Chlorhexidine (PerioGard®)
PBS (Negative control)

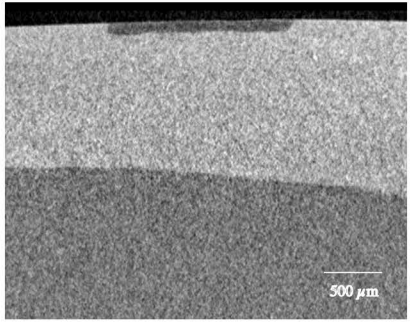

a
Lesion Depth
Lesion Depth (µm)
350
325
300
275
250
225
200
175
150
125
100
75
50
25
0
a
a
ab
bc
c
a
Vochysia tucanorum
Myrcia bella
Matricaria chamomilla
Malva sylvestris (Malvatricin Plus®)
0.12% Chlorhexidine (PeriohGard®)
PBS (Negative control)
b
Reflectivity
Gray values scale
100
80
60
40
20
0
a
a
ab
bc
c
a
Vochysia tucanorum
Myrcia bella
Matricaria chamomilla
Malva sylvestris (Malvatricin Plus®)
0.12% Chlorhexidine (PeriohGard®)
PBS (Negative control)


Vochysia tucanorum Mart.
Myrcia bella Cambess.
Matricaria chamomilla L.
500 µm
500 µm
500 µm
Malva sylvestris
(Malvatricin Plus®)
0.12% Chlorhexidine
(PerioGard® - Positive control)
PBS (Negative control)
500 µm
500 µm
500 µm

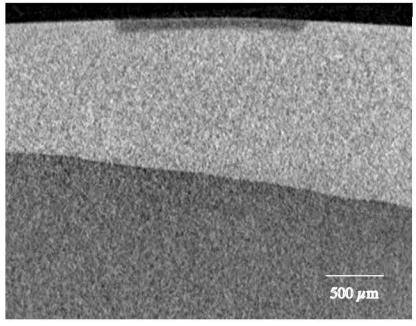
Vochysia tucanorum Mart.



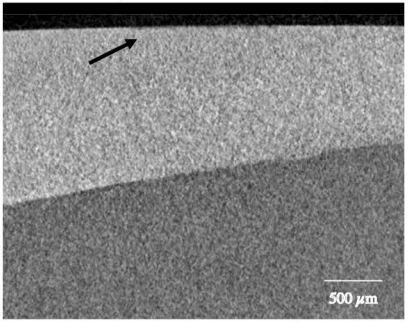
Myrcia bella Cambess.



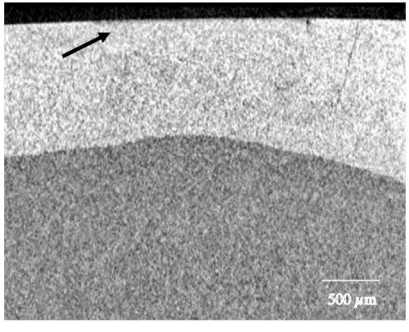
Matricaria chamomilla L.



Malva sylvestris
(Malvatricin Plus®)



0.12% Chlorhexidine
(PerioGard® - Positive control)



PBS (Negative control)

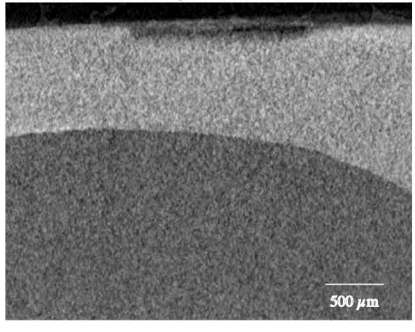


Table 1. Experimental solutions and commercial mouthwashes.

Treatments	Company/City-Country	Composition/ Extract concentration
Vochysia tucanorum	_	10 mg/ml (water)
Myrcia bella	_	20 mg/ml (water)
Matricaria chamomilla	_	80 mg/ml (water)
Malva sylvestris (Malvatricin Plus®)	Daudt/Rio de Janeiro-Brazil	**Active component:** *Malva sylvestris* extract (Mallow), menthol, sorbitol, triclosan, xylitol, zinc chloride and sodium fluoride (225 ppm of fluoride).
0.12% chlorhexidine (PerioGard® - positive control of experimental model) (CHX)	Colgate-Palmolive/ São Paulo-Brazil	**Active component:** 0.12% chlorhexidine gluconate, sorbitol and cetylpyridinium chloride.
PBS (negative control)	-	-

Table 2. Oligonucleotides used for amplification of 16S rRNA gene.

Name	Sequence (5´→3´)	Organism, target, fragment size [bp]	Temperature profile	Reference
Nadkarni-F	TCCTACGGGAGGCAGCAGT	All bacteria, 16S, 466	95 °C, 10 min 95 °C, 10 s 60 °C, 10 s 72 °C, 25 s 40 cycles	Nadkarni et al. [2002]. *
Nadkarni-R	GGACTACCAGGGTATCTAATCCTGTT			
Smut16S-81-F	CTTGCACACCGTGTTTTCT	*S. mutans*, 16S, 519	95 °C, 10 min 95 °C, 10 s 55 °C, 10 s 72 °C, 25 s 50 cycles	Henne et al. [2015]
Smut16S-600-R	TTTTACTCCAGACTTTCCTG			
SD-Lab-158a	GGAAACAGRTGCTAATACCG	*Lactobacilli* spp., 16S, 549	95 °C, 10 min 95 °C, 10 s 55 °C, 10 s 72 °C, 25 s 50 cycles	Heilig et al. [2002]
SD-Lab-Re	CACCGCTACACATGGAG			

Sequence of microorganisms 16S-rRNA (gene) with fragment size (base pairs) and temperature profile of qPCR cycles. * Nadkarni-Primers were used to calculate the total microorganisms for the pilot study.

Table 3. The medium pH values (Mean±Standard Deviation) during the microcosm biofilm growth

Treatment	Values pH/Time (hours)				
	8h^{A}	**24h^{B}**	**72h^{B}**	**96h^{B}**	**120h^{B}**
Vochysia tucanorum [a]	5.52±0.01	4.20±0.01	4.17±0.11	4.22±0.17	4.38±0.26
Myrcia bella [a]	5.53±0.03	4.10±0.12	4.19±0.01	4.16±0.08	4.01±0.05
Matricaria chamomilla [a]	5.57±0.03	4.19±0.01	4.10±0.03	4.22±0.08	4.23±0.24
Malva sylvestris (Malvatricin Plus®) [ab]	5.58±0.02	4.16±0.05	4.49±0.09	4.88±0.07	**5.44±0.31**
0.12% Chlorhexidine (PerioGard® - positive control) [b]	5.55±0.02	4.17±0.01	4.81±0.63	**5.35±0.36**	**5.65±0.04**
PBS (negative control) [a]	5.61±0.03	4.16±0.01	4.08±0.03	4.23±0.06	4.37±0.07

Different letters (lower case) show significant differences among the treatments and different letters (upper case) show significant differences among the periods of analysis for all groups. Two-way ANOVA/Bonferroni's multiple comparison test. Treatment $p=0.0003$; time $p<0.0001$ and interaction $p=0.0812$.