

Salivary pellicle modification with polyphenol-rich teas and natural extracts to improve protection against dental erosion

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ABSTRACT

Objective: To investigate the modification of the salivary pellicle with different polyphenol-rich teas and natural extracts for the protection against dental erosion.

Methods: We performed two experiments: one with teas (Green tea, Black tea, Peppermint tea, Rosehip tea, negative control [NC]) and other with natural extracts (Grape seed, Grapefruit seed, Cranberry, Propolis, NC), where NC was deionized water. A total of 150 enamel specimens were used (n = 15/group). Both experiments followed the same design, consisting of 5 cycles of: salivary pellicle formation (30 min, 37 °C), modification with the solutions (30 min, 25 °C), further salivary pellicle formation (60 min, 37 °C) and erosive challenge (1 min, 1% citric acid, pH 3.6). Relative surface microhardness (rSMH), relative surface reflection intensity (rSRI) and amount of calcium release (CaR) were evaluated. Data were analysed with Kruskal-Wallis and Wilcoxon rank sum tests with Bonferroni correction ($\alpha = 0.05$).

Results: Regarding teas, Black and Green teas showed the best protection against dental erosion, presenting higher rSMH and lower CaR than NC. Peppermint tea was not different to NC and Rosehip tea caused erosion, showing the highest CaR and greatest loss of SMH and SRI. Regarding natural extracts, Grape seed and Grapefruit seed extracts presented the best protective effect, with significantly higher rSMH and lower CaR. Cranberry caused significantly more demineralization; and Propolis did not differ from NC.

Conclusion: Green tea, Black tea, Grape seed extract and Grapefruit seed extract were able to modify the salivary pellicle and improve its protective effect against enamel erosion, but Rosehip tea and Cranberry extract caused erosion.

Clinical relevance: Some bio-products, such as teas and natural extracts, improve the protective effect of the salivary pellicle against enamel erosion. More studies should be performed in order to test the viability of their use as active ingredients for oral care products.

1. Introduction

The acquired salivary pellicle forms instantly on tooth surfaces upon contact with saliva. It consists of a layer of proteins, peptides, lipids and other macromolecules that promotes a natural protection of the tooth surfaces, modulating the process of dental erosion [1]. Initially, there is the adsorption of a dense basal layer, comprised mainly of mucin,

histatin, statherin and acidic proline-rich proteins (PRP) [2,3]. Thereafter, a maturation process of protein-protein interactions occurs, resulting in globular and granular structures in the outer layers of the pellicle [4]. The basal layer is very resistant to dissolution by acids [5]. It contributes most to the protective effect against demineralization, with some of its components being particularly acid resistant [6]. Also, thicker pellicles offer more protection [3,7], but frequent erosive

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challenges can remove part of the salivary pellicle, allowing erosion to progress [8].

Efforts have been made to increase the protective effect of the salivary pellicle against dental erosion. Some plant extracts have shown to increase the thickness of the salivary pellicle [9]. This effect was associated to the polyphenol content of the extracts, as polyphenols can aggregate salivary proteins, which increases the protein adsorption to the enamel surface, intensifying the protective effect of the salivary pellicle against dental erosion.

Polyphenols are abundant in nature and very diverse. Many of them offer several health benefits, which includes anti-inflammatory, antioxidant and anti-bacterial effects. Their basic chemical structure includes a phenol ring, and they may contain galloyl esters or hydroxyl groups. These moieties can form complexes with salivary proteins, especially PRP [10], increasing protein adsorption to the salivary pellicle, thereby increasing its thickness [8]. Furthermore, polyphenols have shown to change the proteomic profile of the salivary pellicle [11]. All these changes could potentially increase the protective effect of the salivary pellicle against dental erosion.

A variety of polyphenols are found in teas and plant extracts. The present study was divided into two experiments, where we investigated 1) teas and 2) natural extracts, and their ability to modify the salivary pellicle, thereby improving its protection against enamel erosion. Our null hypotheses are: 1) the teas do not increase the protective properties of the salivary pellicle, and 2) the natural extracts do not increase the protective properties of the salivary pellicle.

2. Material and methods

2.1. Ethical aspects

For both teeth and saliva, the experiment was carried out in accordance with the approved guidelines and regulations of the local Ethics Committee (Kantonale Ethikkommission: KEK). The teeth and saliva had been pooled and were categorized as “irreversibly anonymized” by the ethics committee, so no previous approval from the committee was necessary.

2.2. Specimen preparation

One hundred and fifty enamel specimens were obtained from human molars, which were selected from a pool of extracted teeth that had been stored in 2% chloramine T trihydrate solution. They were embedded in acrylic resin (Paladur, Heraeus Kulzer GmbH, Hanau, Germany) and serially ground flat and polished under constant cooling (LabPol 21, Struers, Ballerup, Denmark) with decreasing grain size (18.3 μm , 10 μm , 5 μm and 3 μm). Between each step, the specimens were sonicated in deionized water for 1 min. Afterwards, they were stored in a mineral solution (1.5 mM CaCl_2 , 1.0 mM KH_2PO_4 , 50 mM NaCl, pH = 7.0 [12]) until the start of the experiments, when they were further polished, under constant cooling, with a 1 μm diamond paste for 1 min and then sonicated in deionized water. This procedure allows the removal of mineral deposits that might have formed during storage in the mineral solution.

2.3. Collection of stimulated human saliva

Stimulated human saliva was obtained from healthy donors, aged 20–30 years and from both genders. They were informed not to eat or drink (except water) for 2 h before the saliva collection, performed in the morning. The donors chewed on a paraffin wax for 10 min, and all the stimulated saliva was collected in chilled vials. After the collection, the saliva was pooled and centrifuged for 20 min at 4 °C (4000 g). The supernatant was divided in small aliquots and stored at -80 °C until use.

2.4. Experimental groups

The present study comprises 2 independent experiments, one testing teas and the other natural extracts. For the teas, they were steeped at a 50:1 (v:w) water to tea ratio, in warm water (80 °C) for 30 min. This should yield a maximum extraction of tea polyphenols without degradation of the leaves [13]. The leaves were then filtered and the tea allowed to cool to 25 °C before use. For the natural extracts, we used commercially available extracts sold as food supplements (Table 1). A previous study testing the reactivity of proanthocyanidins to salivary proteins used a concentration of 1 g/l [14], so solutions with this same concentration of polyphenols were prepared for the present study (according to the manufacturers' declaration of polyphenol content). For the Propolis extract, no information on the polyphenol content was available. Values from 5 to 50% have been reported for Propolis, therefore we prepared a solution containing 2 g/l of Propolis, assuming the polyphenol concentration would then be in a similar range as for the other extracts. The solutions were prepared by dissolving the contents (powder) of the extract capsules in deionized water, mixing for 30 min at room temperature and later filtering. All solutions were prepared fresh daily and, to avoid chemical alterations to the polyphenol molecules, they were used in their native (non-adjusted) pH. The list of experimental products and their pH are presented in Table 1.

2.5. Experimental design

Both experiments followed the same design: 5 cycles of salivary pellicle formation (30 min, 37 °C, no agitation), followed by pellicle modification with the experimental solutions (30 min, 25 °C, 70 rpm, travel path 50 mm), subsequent salivary pellicle formation (60 min, 37 °C, no agitation); the specimens were then submitted to an erosive challenge (1 min, 1% citric acid, pH 3.6, 70 rpm, travel path 50 mm), and kept in a humid chamber until the next cycle. After each procedure, the specimens were washed with deionized water and dried with oil-free air. The citric acid used for erosion was stored for calcium analyses.

The response variables evaluated were: relative surface microhardness (rSMH), relative surface reflection intensity (rSRI) and amount of calcium released to the citric acid (CaR).

2.6. Surface microhardness measurement

Surface microhardness was measured with a Knoop diamond, with 10 g load and dwell time of 10 s (UHL VMHT Microhardness Tester, UHL technische Mikroskopie GmbH & Co. KG, Asslar, Germany). For each SMH measurement, six indentations were performed with 25 μm distance from each other. The average value from the six indentations was

Table 1
Experimental groups and pH of the solutions used in experiments 1 and 2.

Experiment 1	
Teas *	pH
Negative control (NC; deionized water)	~6.2
Green tea	5.4
Black tea	5.0
Peppermint tea	6.3
Rosehip tea (containing hibiscus)	2.9
Experiment 2	
Natural extracts	pH
Negative control (NC; deionized water)	~6.2
Grape seed extract †	5.8
Grapefruit seed extract ‡	7.2
Propolis extract ‡	7.6
Cranberry extract §	3.2

* All teas were from Coop Qualité & Prix, Switzerland.

† Fairvital B.V., Germany.

‡ Dr. Ehrenberger Synthese GmbH, Austria.

§ BioProphyl® GmbH (Urocyon), Germany.

computed as the SMH of each specimen. The SMH was analyzed at baseline (SMH_{initial}) and after the final cycle (SMH_{final}). The relative SMH (rSMH) was calculated using the following formula: rSMH =

$$\left(\frac{\text{SMH}_{\text{final}}}{\text{SMH}_{\text{initial}}} \right) \times 100.$$

2.7. Surface reflection intensity measurement

The surface reflection intensity (SRI) was measured with a table-top reflectometer [15,16], with a specific software to register the value of highest reflection intensity (SRI value). The measurements were performed at baseline (SRI_{initial}) and after the final cycle (SRI_{final}). Prior final SRI measurement, after the last experimental cycle, the specimens were immersed in 3% NaOCl (5 min, 25 °C, 70 rpm, travel path 50 mm) in order to remove remnants the salivary pellicle. The relative SRI (rSRI) was calculated according to the subsequent formula: rSRI =

$$\left(\frac{\text{SRI}_{\text{final}}}{\text{SRI}_{\text{initial}}} \right) \times 100.$$

2.8. Amount of calcium released in the citric acid

The amount of calcium released to the citric acid (CaR) for each specimen, after each erosive challenge, was determined using an atomic absorption spectrometer (AAnalyst 400, Perkin Elmer Analytical Instruments, Waltham, MA, USA). To eliminate the interference of other ions, lanthanum nitrate (0.5 %, lanthanum nitrate hexahydrate: La [NO₃]₃.6H₂O) was added to the citric acid [17]. The calcium concentrations were then normalized to the surface areas of the enamel specimens. The surface area of each specimen was measured with a light microscope (Leica, M420) connected to a camera (Leica, DFC495). Under 16 x magnification and using the software program IM500, the contour of the exposed enamel area was traced and the enamel area calculate. The cumulative amount of calcium released was considered

for the statistical analyses, which represents the total amount of calcium released by each specimen after all cycles, and is expressed in nmol of Ca²⁺ per mm² of enamel.

2.9. Statistical analysis

Data of rSMH, rSRI and CaR were analyzed separately for each experiment. Firstly, Shapiro-Wilk test was performed to analyze data distribution. The final SMH for Rosehip tea could not be measured because it was below the detection limit, so it was not included in the statistical analysis, but are presented in the results. Since for some groups data did not meet the assumptions of normality, Kruskal-Wallis tests were performed. Post-hoc pairwise comparisons were performed with Wilcoxon rank sum test with Bonferroni correction for multiple tests. Analyses were performed with R 3.5.3 and significance level of $\alpha = 0.05$. Additionally, we analyzed Pearson's correlation between rSMH and rSRI (n = 135 data points, since values of rSMH for Rosehip tea were not included in the analysis).

3. Results

3.1. Experiment 1

Green tea and Black tea showed significantly higher rSMH than Peppermint tea and the NC group, which in turn did not differ from each other (Fig. 1). Green tea and Black tea also presented the lowest values of CaR, without differences between each other, but only Black tea was significantly different to the NC. Rosehip tea was very erosive and the final SMH could not be measured as it was below the detection limit, so it was not included in the statistical analysis. Accordingly, this group presented very low rSRI and the highest CaR, significantly differing from all other groups (Figs. 2 and 3).

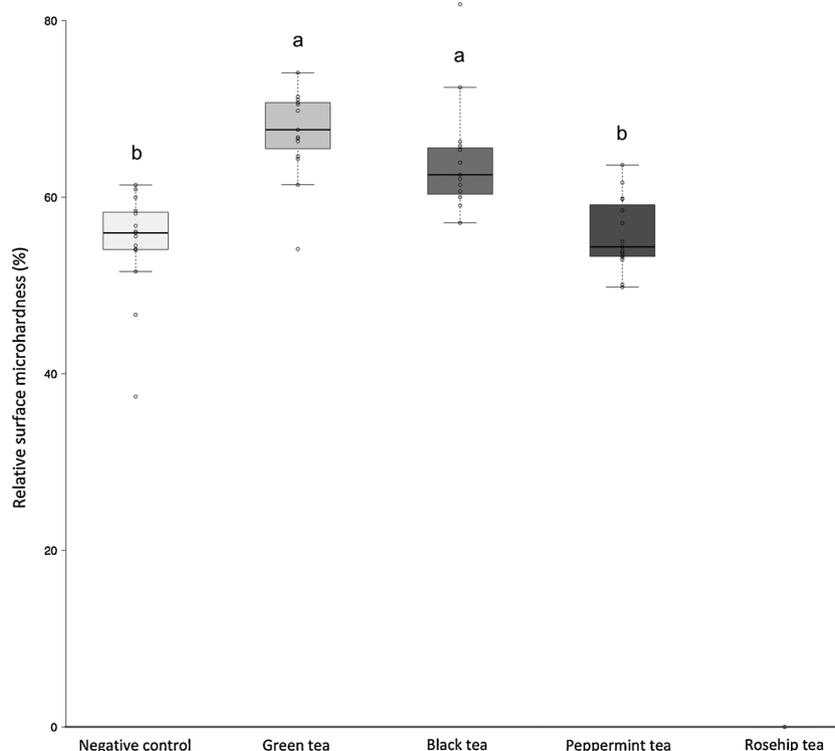


Fig. 1. Relative surface microhardness (%) for the teas. Different letters indicate significant differences ($p < 0.05$). Rosehip tea was not included in the statistical analysis, because it presented final SMH below the detection limit.

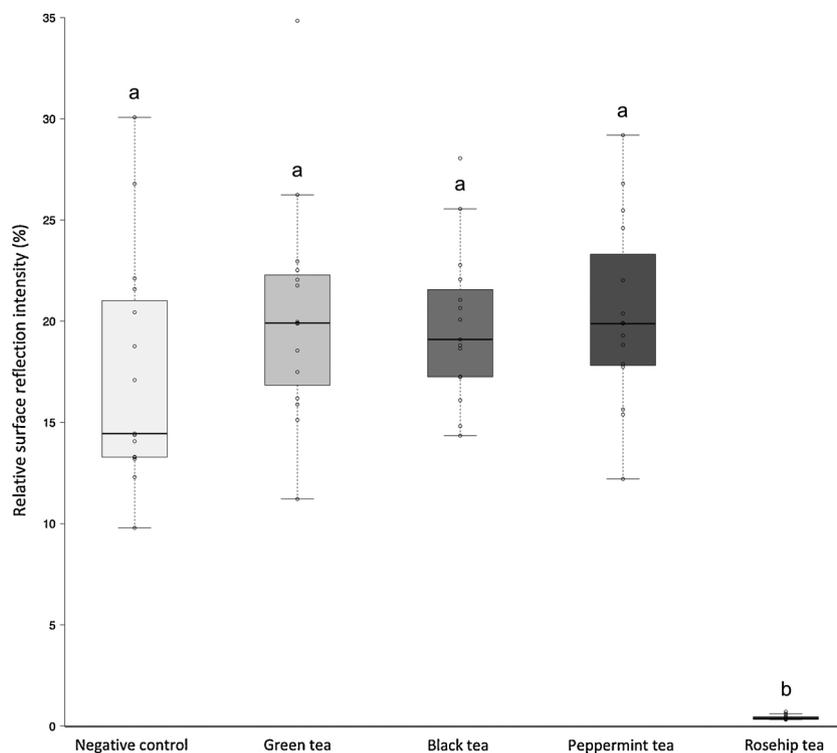


Fig. 2. Relative surface reflection intensity (%) for the teas. Different letters indicate significant differences ($p < 0.05$).

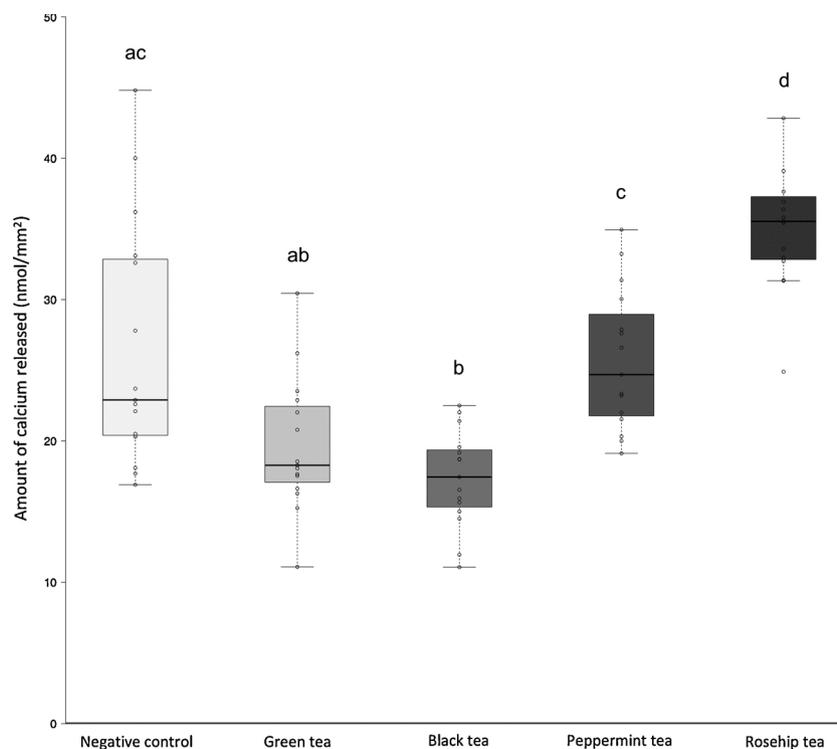


Fig. 3. Total amount of calcium released to the citric acid for the teas. Different letters indicate significant differences ($p < 0.05$).

3.2. Experiment 2

Grape seed extract showed the highest rSMH values, followed by Grapefruit seed extract, with significant difference between them and the NC group. They also presented the highest values of rSRI. Propolis extract was the only group that did not show rSMH significantly

different from the NC ($p = 0.555$). Cranberry extract was very erosive and presented the lowest rSMH and rSRI values, significantly differing from all other groups (Figs. 4 and 5, respectively). Grape seed, Grapefruit seed and Propolis extracts showed significantly lower amount of CaR when compared to the NC, without difference between them (Fig. 6). Cranberry extract was significant different only to Grape seed

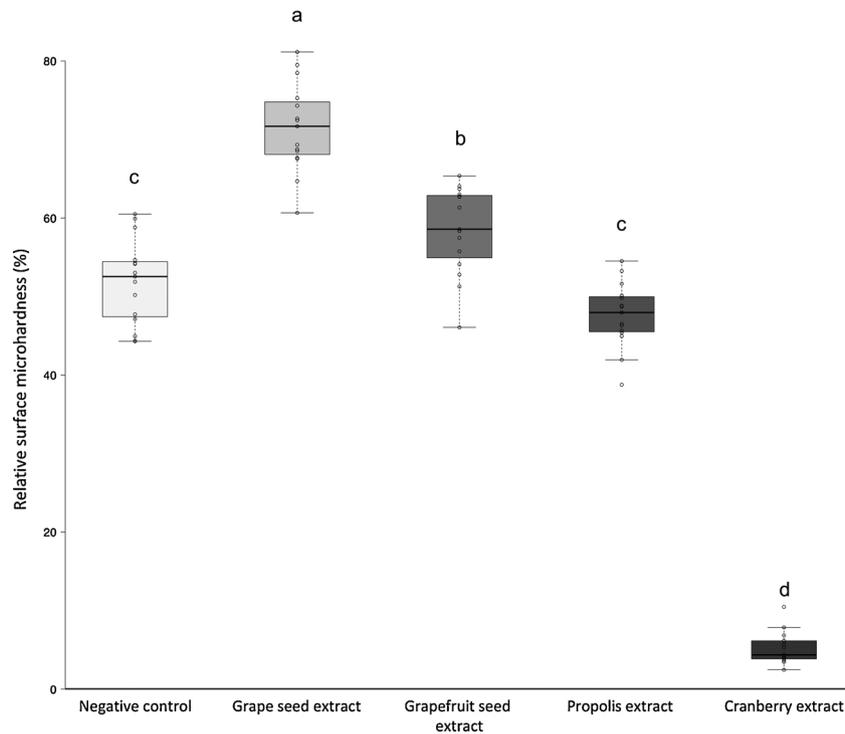


Fig. 4. Relative surface microhardness (%) for the plant extracts. Different letters indicate significant differences ($p < 0.05$).

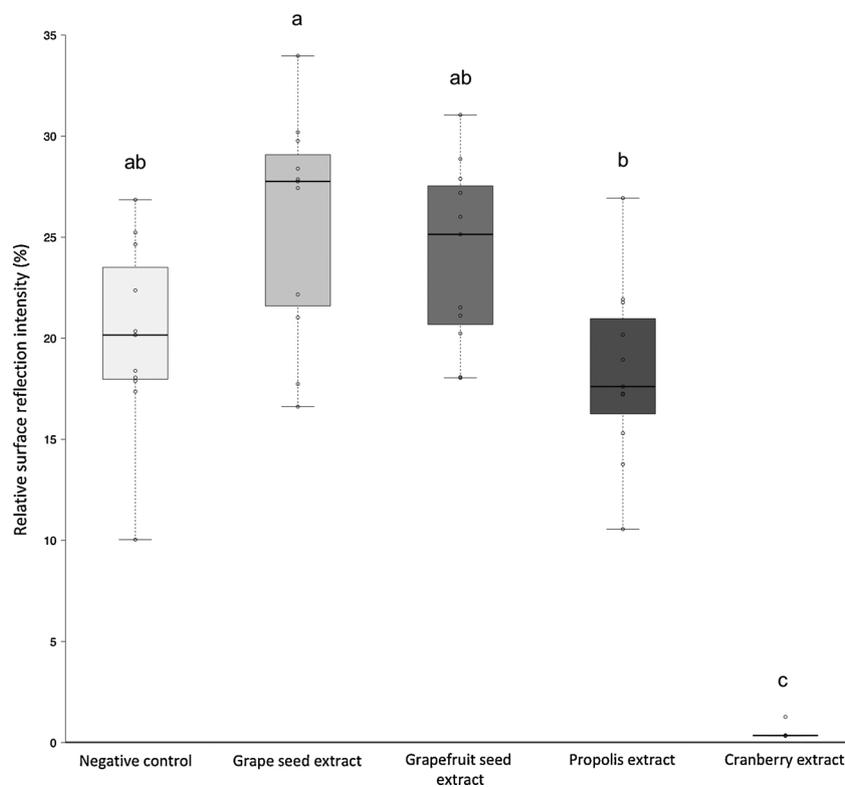


Fig. 5. Relative surface reflection intensity (%) for the plant extracts. Different letters indicate significant differences ($p < 0.05$).

extract, presenting higher amount of CaR.

3.3. Correlation between rSMH and rSRI

There was a positive medium to high correlation ($r = 0.73$; $p < 0.01$) between rSMH and rSRI, which is depicted in Fig. 7.

4. Discussion

The protocol we used in the present study was based on the hypothesis that polyphenols from teas and natural extracts would bind to proteins from the salivary pellicle, which in turn would attract more proteins from the saliva. Therefore, we performed the treatments (either

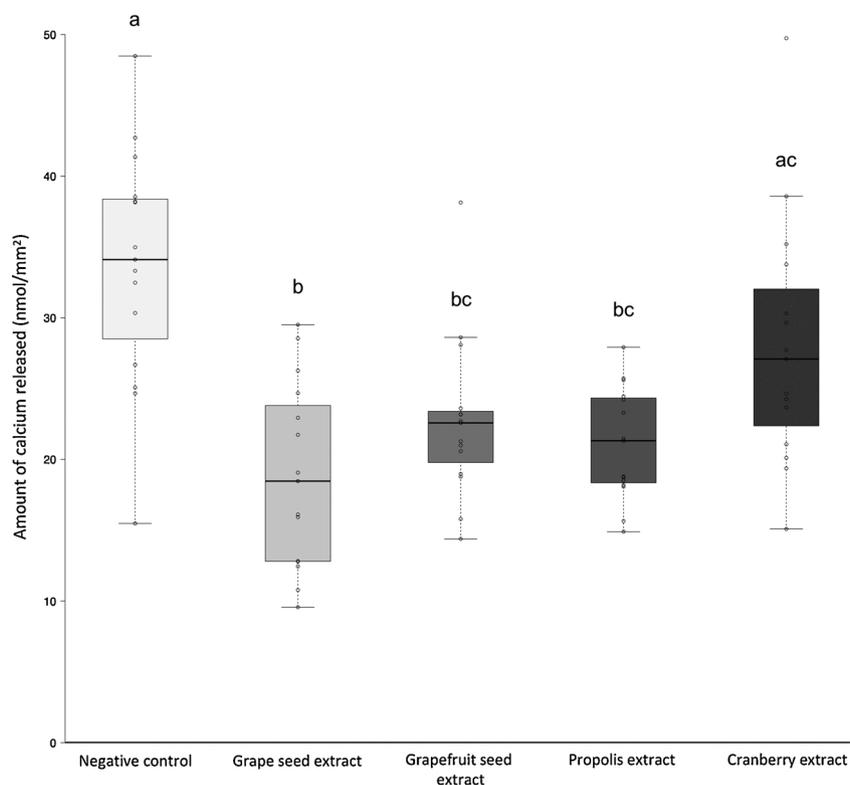


Fig. 6. Total amount of calcium released to the citric acid for the plant extracts. Different letters indicate significant differences ($p < 0.05$).

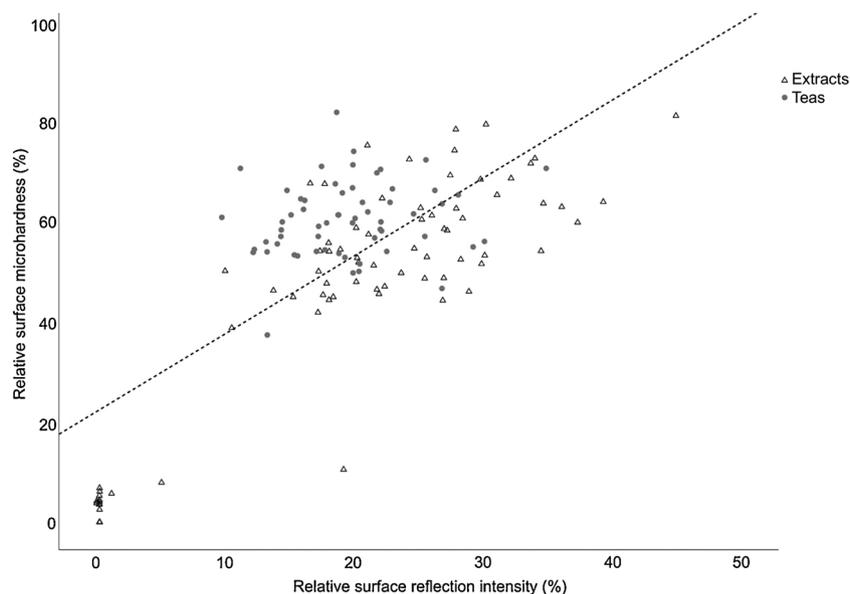


Fig. 7. Scatter plot of relative surface microhardness and relative surface reflection intensity for all specimens (Experiments 1 and 2, except for Rosehip tea, which rSMH values were not included in the analysis). NOTE: The dotted line depicts the regression line ($y = 1.6x + 22$).

with teas or natural extracts) between two moments of exposure to saliva. This would thus modify more effectively the salivary pellicle, increasing its protective effect [8,18]. Indeed, some of the teas and natural extracts were able to improve the protection of the salivary pellicle against erosion, but others did not. Actually, some even caused more enamel demineralization. So, we can reject the both null hypotheses.

Considering the teas, Green tea and Black tea were able to increase the protective effect of the salivary pellicle. It was expected for them to behave similarly, since both are made from the same plant leaves

(*Camellia sinensis*). However, due to differences in the method of processing and oxidation levels, they have important chemical differences, especially in their polyphenolic contents. In Green tea, the most abundant polyphenols are catechins, such as epigallocatechin-3-gallate (EGCG), while in Black tea, theaflavins predominate [19–21]. Theaflavins are produced during fermentation, by the enzymatic co-oxidation of pairs of catechins [21]. Despite this difference, most of the other polyphenols in their composition are similar [22] and this chemical difference does not seem to have a large impact on their interaction with the salivary pellicle. In fact, modification of the salivary

pellicle has already been shown with EGCG. The pellicle becomes thicker and more electron-dense [23,24], with an impact on its proteomic profile, where greater amounts of acid-resistant proteins, such as statherin, are integrated into its structure [11]. Additionally, Black tea is able to adsorb onto the salivary pellicle, increasing the persistence of the pellicle to surfactants [18,25]. So, in our experiment, the positive results from Green tea and Black tea are probably due to the EGCG and theaflavins, which interacted with the salivary pellicle, increasing its anti-erosive potential. These teas are also known to naturally contain fluoride. Although it is a high concentration for consumption (< 2 ppm F^-), which can have a positive effect on caries, the concentration is too low to have any meaningful effect against dental erosion.

Rosehip tea resulted in excessive demineralization because of its low pH. Pure Rosehip tea has a high pH (~ 6.3) [26] and a high polyphenol content [27,28]. However, the tea used in this experiment also contained hibiscus [26], and this is probably the reason for the low pH (Table 1). This, in turn, counteracted any possible positive effect that the polyphenols from Rosehip could have had. Further studies of pure Rosehip are still warranted. Peppermint tea was generally not different from the control group, so we can assume that it did not have any influence on the salivary pellicle. Despite the presence of polyphenols in Peppermint infusions, mainly flavones and flavanones [29], the concentration is much lower than that found in Green and Black teas [29]. So, the effect was not as evident, because the polyphenolic concentration plays an important role [23,24].

Noteworthy, all teas were prepared with a 30 min steep time, which is thought to yield the maximum extraction of polyphenols [13]. Still, different leaves/teas might release different amounts of polyphenols within this time, leading to different concentrations, thereby to different polyphenol/protein interactions, since the latter is influenced by ratio, structure and concentration of both polyphenols and proteins [30]. This can explain the different results observed in the present study, since the teas contain different types and concentrations of polyphenols. The fundamental purpose of the present study was to analyze different teas and natural extracts, and not compare polyphenols, so measuring polyphenolic concentrations in the different solutions was not within our scope. Further studies could be performed on testing pure polyphenols at the same concentrations. Moreover, teas are normally steeped for shorter times, so lower polyphenols content are expected. This imposes a difficulty for the modification of the salivary pellicle only through the ingestion of teas. They remain, however, a good option to be tested as anti-erosion agents in oral care products.

Regarding the natural extracts, Grape seed and Grapefruit seed extracts presented the most promising results. The Grape seed extract used in the present study contains mainly purified oligomeric proanthocyanidin (OPC), which include catechin and epicatechin units with partial galloylation [31], and can be considered the responsible for the best results observed for this group. OPCs can form hydrophobic interactions or hydrogen bonds with proteins, or even oxidation followed by covalent condensations [10,32,33]. Since polyphenols interact initially with proline-rich proteins and statherin [34], which are present in the basal layer of the pellicle, we assume that OPC is interacting with this basal layer, attracting even more proteins to the pellicle, and ultimately changing its structure and improving its protective effect [23,24]. Since the basal layer of the pellicle is formed very quickly, one can speculate that even a much shorter initial incubation period in saliva than the one used here (30 min) might be effective in providing the necessary base for the interaction with the polyphenols. Concerning Grapefruit seed extract, it contains flavonoids, specifically flavanones, and mainly naringenin [35]. Although the exact mechanism of interaction between these molecules and salivary proteins is not yet known, a binding similar to OPCs can be assumed. Moreover, Grapefruit seed extract also contains calcium that might also have helped to protect the enamel against demineralization [36]. The results observed in the present study are promising and both extracts are viable options to be added in oral care products.

Surprisingly, Cranberry extract acted similar to Rosehip tea. Due to its low pH, it also caused more demineralization. Like Grape seed extract, Cranberry is rich in proanthocyanidins, especially polymeric proanthocyanidins [37], so it was initially expected that it would cause some modification of the salivary pellicle. However, its low pH counteracted any possible positive effect that the polyphenols could have had. Our results are, however, congruent to a previous study, where Cranberry extract was not as effective in protecting against enamel erosion [38]. In the study by Boteon, Kato, Buzalaf, Prakki, Wang, Rios and Honorio [39], however, Cranberry extract was able to reduce erosion in dentine.

Propolis extract was not able to increase the protective effect of the salivary pellicle. This is in agreement with a previous study by our group that did not observe a positive result on the protection against dental erosion [40]. Propolis has a high polyphenol content, mainly from the class of flavonoids, but the chemical composition of propolis varies according to the conditions of its production, including location and bee species [41]. Thus, one should not exclude the possibility of an increased protective effect with the use of other types of propolis extracts. Hence, future studies should investigate the effect of different types of propolis extracts on the salivary pellicle. The exact polyphenol composition and content of the used Propolis is not known, but the concentration was estimated based on values found in the literature.

It is important to point out that we used three different methods, all of which mirror the amount of demineralization caused by the erosive challenges. Regarding rSMH and rSRI, higher values represent less demineralization – or more protection from the pellicle. Differences between the groups are better observed in the rSMH than in the rSRI results, for the latter has a greater variation. This is probably due to the effect of the pellicle on the enamel surface. Although we incubated the specimens in NaOCl to remove the pellicle, we cannot discard the fact that remnant of the pellicle still persists on the enamel surface, thus influencing the results [42]. As discussed above, when polyphenols adsorb onto the salivary pellicle, it can persist even after exposure to surfactants [18,25]. In any case, these methods present a positive medium to high correlation, which shows that both methods follow, in general, the same pattern, as observed previously [16], where rSRI was shown to be a suitable and highly sensitive method for initial erosion. The method, however, is less influenced by non-modified pellicle, but it may still be influenced by a modified pellicle.

Another important point is that the experimental solutions contained different pHs. On the one hand, this can hinder direct comparisons between the solutions, but on the other hand, pH can considerably influence the effect of the polyphenols. If we had adjusted the pH for all teas/extracts, we would possibly obtain different results. So, we decided to use the natural pH for all tested products. A downside to this was that the natural pH of the Rosehip tea and Cranberry extract were as low as (or even lower than) the citric acid. This negatively impacted enamel, especially in view of the long period of time of immersion in these solutions (30 min). These solutions probably caused erosion, where calcium was also released to these solutions. So even the calcium results for these two products does not fully reflect the demineralization occurring on the enamel surface. It would be interesting to test these solutions, especially the Cranberry extract, due to its polymeric proanthocyanidin content, at a higher pH. Moreover, the time of immersion in the teas and plant extracts used is too long for clinical application. Future studies should consider shorter application times.

In summary, polyphenols are a large family of compounds, so it is difficult to pinpoint exactly which polyphenolic compound is more effective. But considering the products with best results, we can speculate that the compounds with best interaction with the salivary pellicle are EGCG (from Green tea), theaflavins (from Black tea), OPC (from Grape seed extract) and flavanones and naringenin (from Grapefruit seed extract). Nevertheless, we cannot exclude the fact that many other polyphenols are present in these products, and the interaction of different polyphenols might influence their mechanism of action. All

things considered, we conclude that Green tea, Black tea, Grape seed extract and Grapefruit seed extract can effectively modify the salivary pellicle and improve its protective effect against enamel erosion.

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CRediT authorship contribution statement

Samira Helena Niemeyer: Formal analysis, Writing - original draft. **Tommy Baumann:** Conceptualization, Methodology, Formal analysis, Writing - review & editing. **Adrian Lussi:** Resources, Writing - review & editing. **Hendrik Meyer-Lueckel:** Resources, Writing - review & editing. **Tais Scaramucci:** Writing - review & editing. **Thiago Saads Carvalho:** Conceptualization, Methodology, Data curation, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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