

Pellicle modification with natural bioproducts: Influence on tooth color under erosive conditions

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Abstract

Salivary pellicle was modified with bioproducts and we assessed the change in tooth color and the protection of enamel to erosion. Human enamel specimens were assigned to one of three solutions: grape seed extract or black tea (bioproducts), or deionized water (negative control); after which one half the specimens underwent erosive challenges. The specimens underwent 15 cycles involving salivary pellicle formation (10 min, 37°C), incubation in solution (2 min, 25°C), subsequent pellicle formation (90 min, 37°C). Half of the specimens was kept in a humid chamber and the other half was submitted to erosion (2 min, 1% citric acid). After 15 such cycles, the pellicle was removed. Tooth color and the surface reflection intensity were assessed after every five cycles and after pellicle removal. For non-eroded specimens, the exposure to bioproducts promoted significantly greater color change than the deionized water, with increases in yellow appearance. After pellicle removal, the color was similar in all non-eroded specimens. The bioproducts increased the surface reflection intensity over cycles. For the erosion-exposed specimens, erosion itself resulted in color change. Black tea and deionized water resulted in increased yellow appearance. Exposure to the bioproducts resulted in higher relative surface reflection intensity values over time, but only grape seed extract resulted in higher relative surface reflection intensity value at the time of pellicle removal. The bioproducts caused transient staining effect, which was reduced after pellicle removal. For enamel submitted to erosion, grape seed extract resulted in less color change and better protection of enamel against erosion than black tea or water.

KEYWORDS

dental erosion, enamel, polyphenols, salivary pellicle, tooth discoloration

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INTRODUCTION

The acquired enamel pellicle has a crucial protective function for the tooth surface. This organic layer is formed in two stages with an initial deposition of salivary proteins on the tooth surface, forming a tenacious basal layer [1], and afterward, a continuous deposition of biopolymers by protein-protein interactions, forming a globular and granular structure. The pellicle then reaches a considerable thickness, reducing the contact of acids with tooth surfaces [1], thus modulating dental erosion.

If the acid challenges occur repeatedly during an erosive process, the pellicle can be partially removed, and the demineralization of the tooth surface may progress. In an attempt to improve the resistance of the acquired pellicle, certain plant extracts and teas have been used to modify it [2–5]. The polyphenols present in these extracts can rapidly bind to salivary proteins such as proline-rich proteins and histidine-rich proteins (histatins) [3, 6]. The polyphenols are, therefore, incorporated into the pellicle structure, possibly increasing its thickness [3, 4] and consequently enhance its protective effect against acid attacks. On the other hand, as an undesirable effect, polyphenols have also been associated with tooth staining [7–9], which has been investigated but the exact mechanism is not completely understood.

Tooth staining is a complex multifactorial process that depends on the type of polyphenol-protein crosslink reaction [10]. Besides the fact that polyphenols have high affinity to the salivary proteins, basic proline-rich proteins seem to be directly related to staining. These proteins favor an increasing binding of anthocyanin and theaflavins to hydroxyapatite [10]. So, the polyphenol-protein crosslink reaction occurs on the pellicle/tooth interface, which is considered the main locus for tooth staining related to dietary intake. Thus, tooth staining might be an unwanted side-effect of polyphenol exposure from the dietary intake, which can concern esthetically conscious patients.

In this context, the present study verified how grape seed extract and tea can change the tooth color of pellicle-covered enamel when submitted or not submitted to erosive challenges. Our null hypotheses are: 1) the grape seed extract and tea do not promote tooth color change on enamel not submitted to erosion, 2) the grape seed extract and tea do not promote tooth color change on enamel submitted to erosion, and 3) the plant extract and tea do not promote protection against erosion.

MATERIAL AND METHODS

Specimen preparation

Sixty enamel specimens were prepared from human incisors. The teeth were obtained from a biobank and had been stored

in 2% chloramine T trihydrate solution. According to the local ethical committee, the teeth are “irreversibly anonymized,” and a previous ethical approval is not necessary. They were embedded in acrylic resin (Paladur; Heraeus Kulzer), ground flat and polished with silicon carbide grinding papers (FEPA grit P#1200, P#2500, and P#4000). Between each abrasive paper, the specimens were sonicated in deionized water for 5 min. When embedding the specimens, a spacer of 200 μm was used and consequently only 200 μm of the top layer of enamel surface was removed during grinding. When ready, the specimens were stored in a mineral solution (1.5 mM CaCl_2 , 1.0 mM KH_2PO_4 , 50 mM NaCl, pH = 7 [11]) until the beginning of the experiment. Immediately before the experiment, the specimens were polished under constant water cooling, with a 1 μm diamond paste for 1 min and then submitted to sonication with deionized water.

Collection of stimulated human saliva

Stimulated human saliva was collected from 15 volunteers in good general health, aged 20–61 years and representing both genders. Before saliva collection, the volunteers were instructed not to eat or drink, except water, for 1 h. The collection was performed in the morning, between 9 and 10 am. The volunteers chewed on a piece of paraffin film for 10 min, and all stimulated saliva was collected in cooled flasks. The saliva was then pooled, centrifuged for 15 min at 4°C at 4400 rpm, and protease inhibitors were added to avoid degradation of proteins. The protease inhibitors consisted of a mixture of 69.68 mg/ml phenylmethanesulfonyl fluoride, 50.05 mg/ml N-ethylmaleimide, and 79.29 mg/ml phenanthroline in 400 ml of methanol [12]. Then, saliva aliquots were stored at -80°C until the start of the experiment. Because the saliva was pooled, the local ethical committee considers it as “irreversibly anonymized,” and an ethical approval was not necessary.

Preparation of experimental solutions

Deionized water was used as negative control, and the solutions of grape seed extract and black tea were prepared fresh as described in a previous study [2]. The extract was prepared by dissolving the contents (powder) of the grape seed extract capsules (Fairvital B.V.) in deionized water, mixing for 30 min at room temperature and filtering. For black tea (Coop Group), the leaves were immersed in warm water (80°C) and steeped for 30 min. The leaves were then filtered. Before the solutions were used in the experiment, both were cooled to room temperature, and they kept their native pH (~ 5.2 for grape seed extract and ~ 5.0 for black tea).

Experimental design

The present study followed a prospective and parallel group design with experimental solutions in three levels (grape seed extract, black tea, and deionized water) and condition of the enamel surface in two levels (submitted to erosion or not).

The experiment comprised of 15 cycles, each consisting of pellicle formation (120 μ l, 10 min, 37°C, no agitation), followed by pellicle modification with experimental solutions (5 ml, 2 min, 25°C, 70 rpm) [2, 13, 14], and subsequent salivary pellicle formation (120 μ l, 90 min, 37°C, no agitation). After these steps, half of the specimens were kept in the humid chamber (sound enamel), while the other half (eroded enamel) were submitted to an erosive challenge (2 min, 1% citric acid, pH 3.6, 70 rpm, 35°C). After 15 cycles, the pellicle was removed from the enamel surface by immersion in 3% NaOCl solution (5 ml, 5 min, 25°C, 70 rpm). Tooth color and reflectivity measurements were performed at the following moments for both sound and eroded enamel: at baseline; and after 5, 10, and 15 cycles; and after pellicle removal.

Tooth color assessment

Tooth color was assessed by using a spectrophotometer (CD26; Konica Minolta) adjusted for small area (SAV) measurements (3 mm diameter). The D65 illuminant standard with the reflectance mode and ultraviolet light was used. The angle of observation was 10° with the specular component included. During measurements, the specimens were kept in the humid chamber to avoid dehydration. They were individually placed on a gray background and three measurements were performed and averaged. The values were obtained with the software Spectramagic NX (Konica Minolta). To assess the changes in tooth color, color difference was calculated by CIELAB (ΔE_{ab}), and CIEDE2000 (ΔE_{00}) color grading systems. CIELAB is a widely adopted method in tooth color studies and the CIEDE2000 represents the color differences perceived by human eye better [15]. Then, the color difference for both systems was calculated according to the following formulas:

$$\Delta E_{ab}^* = \left[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2 \right]^{1/2} \quad (1)$$

where ΔL^* indicates the difference in lightness between two recordings of a specimen made at two different time points, Δa^* indicates the difference in the red-green axis, and Δb^* indicates the difference in the blue-yellow axis.

$$\Delta E_{00} = \left[\left(\frac{\Delta L'}{K_L S_L} \right)^2 + \left(\frac{\Delta C'}{K_C S_C} \right)^2 + \left(\frac{\Delta H'}{K_H S_H} \right)^2 + R_T \left(\frac{\Delta C'}{K_C S_C} \right) \left(\frac{\Delta H'}{K_H S_H} \right) \right]^{1/2} \quad (2)$$

where $\Delta L'$ indicates the difference in lightness, $\Delta C'$ indicates the difference in chroma, and $\Delta H'$ indicates the difference in hue. R_T represents the rotation function. K_L , K_C , K_H are parametric factors for lightness (L), chroma (C), and hue (H), respectively. S_L , S_C , S_H are weighting functions for lightness (L), chroma (C), and hue (H), respectively.

To calculate the differences (Δ) in color (CIELAB or CIEDE2000) and in CIELAB coordinates after 5, 10, and 15 cycles and after pellicle removal, values measured at these time points were subtracted from the baseline values. The interpretation of the results was based on the 50:50% perceptibility threshold ($\Delta E_{ab} = 1.2$ and $\Delta E_{00} = 0.8$) and on the 50:50% acceptability threshold ($\Delta E_{ab} = 2.7$ and $\Delta E_{00} = 1.8$) [16].

Surface reflection intensity assessment

Aiming to evaluate the effect of the erosive challenges on the enamel surface, the surface reflection intensity was assessed with a table-top reflectometer connected to a computer with the specific software [17, 18]. The specimen was placed in a standardized position on the platform of the device under a laser beam (635 nm; oeMarket). The platform was moved aiming to adjust the laser beam on the enamel surface to register the highest value of reflection intensity. As the specimens were polished, they exhibited the highest values of surface reflection intensity at the baseline. This condition corresponds to non-eroded enamel surface. During the experiment, the values of this measurement may be decreased, and lower values signify higher erosive demineralization. To analyze the data, the relative surface reflection intensity was calculated, according to the following formula:

$$rSRI = \frac{SRI_i}{SRI_{baseline}} \times 100 \quad (3)$$

where $SRI_{baseline}$ is the baseline surface reflection intensity value, and SRI_i is the surface reflection intensity measurement after 5, 10, or 15 cycles, and after pellicle removal.

Statistical analysis

The data were submitted to the normality test (Shapiro Wilk test) and some groups did not meet a normal distribution. For both tooth color and relative surface reflection intensity, we used Kruskal-Wallis and post-hoc Dunn's tests to compare specimens according to erosion or not and across each evaluated time point (5, 10, or 15 cycles, and after pellicle removal). To evaluate the effect of erosion on tooth color, we considered the values of differences in color (ΔE_{ab} , ΔE_{00} , ΔL^* , Δa^* , and Δb^*) of enamel submitted to erosion in after exposure to demineralized water. To compare the effect of evaluated time

within the same group, we used Friedman's tests with post-hoc Wilcoxon Matched Pairs tests. A significance level of 5% was used, and Bonferroni corrections were made for multiple tests (three independent tests were performed).

RESULTS

Tooth color

Table 1 depicts the progression of tooth color alteration throughout the study for all experimental groups in both conditions tested. Until five cycles, the bioproducts caused a significant color change verified by the median values of color difference of both color systems used. This was observed for the specimens not submitted to erosion. When the specimens were submitted to erosive challenge, significantly higher ΔE_{ab} and ΔE_{00} values in the negative control group was found in comparison to the bioproducts. This characteristic was observed in other evaluated times. Then, the erosive demineralization process itself caused a significant color change. Throughout the 15 cycles, all groups not submitted to erosion maintained the color difference values, whereas all the groups submitted to the erosion exhibited an increase in both ΔE_{ab} and ΔE_{00} values. Even so, exposure to black tea and deionized water resulted in greater color change in comparison to grape seed extract. After pellicle removal, for the no erosion condition, no significant differences were detected between the solutions ($p < 0.001$), and for the specimens submitted to erosion, significantly higher color change was observed for the negative control group.

Considering the CIELAB coordinates (Table 2), the experimental solutions presented similar values of lightness (ΔL) after 5 cycles with no significant alteration in these values for the specimens not submitted to erosion. However, for specimens submitted to erosion the negative control presented considerably greater lightness (ΔL) in comparison to the bioproducts. Throughout the 15 cycles, black tea exposure led to a significant decrease in values, exhibiting a darker enamel surface for the specimens not submitted to erosion. Interestingly, the same behavior of this experimental group was observed for the specimens submitted to erosion. After pellicle removal, the ΔL value for black tea decreased for the no erosion condition, while it increased for grape seed extract and deionized water, leading to a significant difference in ΔL values between the latter and the other two bioproduct solutions ($p < 0.001$). In contrast, the ΔL values for black tea exposed eroded specimens increased, but remained darker than the negative control, with significantly lower values ($p < 0.001$).

Regarding Δa , no significant differences were found between the experimental solutions until five cycles for the no erosion condition. In contrast, the bioproducts exhibited

TABLE 1 ΔE_{ab} and ΔE_{00} median values [1° quartile (25%); 3° quartile (75%)] for enamel submitted or not to erosive challenges according to the evaluated times

Condition	Color difference	Experimental solution	5 cycles	10 cycles	15 cycles	After pellicle removal
No erosion	ΔE_{ab}	Grape seed extract	1.84 [1.09; 2.31]	Aa 1.84 [1.39; 3.63]	Aa 2.24 [1.63; 2.75]	Aa 2.18 [1.11; 3.27]
		Black tea	2.49 [1.63; 4.37]	Aa 3.76 [2.50; 6.01]	Aa 4.87 [3.88; 5.97]	Ab 1.47 [1.07; 2.65]
		Deionized water	0.71 [0.43; 0.91]	Ba 0.74 [0.70; 0.89]	Ba 0.89 [0.65; 1.14]	Ab 2.84 [1.93; 3.44]
	ΔE_{00}	Grape seed extract	1.64 [0.99; 2.09]	Aa 1.98 [1.56; 3.24]	Aa 2.27 [1.81; 2.66]	Aa 1.94 [0.96; 2.79]
		Black tea	1.81 [1.28; 3.49]	Aa 3.09 [1.97; 4.51]	Aa 3.71 [3.00; 4.43]	Aa 1.20 [0.87; 2.13]
		Deionized water	0.70 [0.39; 0.84]	Ba 0.70 [0.60; 0.87]	Ba 0.84 [0.59; 1.08]	Ab 2.49 [1.77; 2.83]
Erosion	ΔE_{ab}	Grape seed extract	2.13 [1.52; 3.12]	Aab 1.67 [1.26; 2.73]	Aa 3.85 [2.29; 4.59]	Ab 3.44 [1.44; 3.55]
		Black tea	2.64 [2.16; 2.97]	Aa 3.43 [2.37; 4.11]	ABa 5.17 [4.17; 6.34]	Aa 2.27 [1.71; 2.87]
		Deionized water	5.68 [4.19; 6.94]	Bab 4.39 [3.85; 5.06]	Ba 5.79 [4.39; 7.28]	Bb 5.41 [4.33; 6.91]
	ΔE_{00}	Grape seed extract	1.94 [1.31; 2.59]	Aab 1.53 [1.02; 2.11]	Aa 3.50 [2.40; 3.93]	Aa 2.84 [1.21; 3.27]
		Black tea	2.09 [1.78; 2.30]	Aa 2.56 [1.91; 3.55]	ABa 4.07 [3.55; 4.49]	Bb 1.89 [1.36; 2.37]
		Deionized water	4.86 [3.63; 6.09]	Bab 3.73 [3.31; 4.49]	Ba 4.85 [3.77; 6.38]	Bab 4.40 [3.80; 5.99]

Different upper case letters indicate significant differences between groups within evaluated time for each color difference assessment (Kruskal-Wallis and post hoc Dunn's test); different small case letters indicate significant differences between the evaluated times in the same experimental solution for each color difference assessment (Friedman and post hoc Wilcoxon Matched Pairs Test). No erosion and erosion conditions were analyzed separately.

TABLE 2 CIELAB coordinates median values [1st quartile (25%); 3rd quartile (75%)] for enamel submitted or not to erosive challenges according to the evaluated times

Condition	Coordinate	Experimental solution	5 cycles	10 cycles	15 cycles	After pellicle removal				
No erosion	ΔL	Grape seed extract	0.59 [-0.84; 1.31]	Aa	0.41 [-1.75; 0.98]	Aa	-0.31 [-1.55; 0.81]	ABa	-1.15 [-2.07; 0.15]	Aa
		Black tea	-0.05 [-2.28; 1.03]	Aa	-2.29 [-4.45; -0.88]	Bb	-2.39 [-3.72; -0.17]	Ab	-0.84 [-2.54; -0.04]	Aa
		Deionized water	0.04 [-0.09; 0.32]	Aa	0.44 [0.16; 0.58]	Ab	0.54 [0.13; 0.88]	Bb	2.23 [1.80; 2.80]	Bc
	Δa	Grape seed extract	0.06 [0.01; 0.14]	Aa	0.88 [0.44; 1.06]	Ab	0.49 [0.28; 1.14]	Ab	-0.06 [-0.14; 0.19]	Aa
		Black tea	0.14 [0.02; 0.26]	Aa	0.53 [0.47; 0.72]	Ab	0.62 [0.33; 0.79]	Ab	-0.19 [-0.36; 0.02]	Aa
		Deionized water	0.01 [-0.30; 0.10]	Aa	0.07 [-0.06; 0.33]	Ba	0.09 [-0.14; 0.35]	Ba	-0.08 [-0.25; 0.15]	Aa
	Δb	Grape seed extract	-0.21 [-0.81; 0.17]	Aa	0.56 [0.28; 0.96]	Aab	0.62 [0.17; 1.85]	Ab	0.80 [-0.02; 1.57]	Aab
		Black tea	0.71 [0.03; 1.68]	Aa	2.18 [-0.29; 3.32]	Ba	4.55 [2.43; 4.76]	Bb	-0.16 [-1.03; 0.46]	Ac
		Deionized water	-0.03 [-0.32; 0.18]	Aa	-0.20 [-0.38; 0.36]	Aa	0.26 [-0.31; 0.39]	Aa	-0.86 [-2.13; -0.46]	Ba
Erosion	ΔL	Grape seed extract	0.18 [-1.33; 0.88]	Aa	0.78 [-0.88; 1.20]	Aa	1.51 [0.72; 2.68]	Ab	3.40 [0.52; 3.45]	ABb
		Black tea	-0.35 [-1.01; 0.02]	Aa	-1.22 [-2.71; -0.62]	Aa	-1.74 [-2.17; -1.10]	Ba	1.58 [0.20; 2.68]	Ab
		Deionized water	5.26 [3.89; 6.58]	Ba	4.14 [3.77; 4.80]	Ba	4.94 [3.69; 7.15]	Aa	4.96 [4.03; 6.29]	Ba
	Δa	Grape seed extract	0.48 [0.27; 0.67]	Aa	0.33 [0.24; 0.47]	ABa	1.27 [0.98; 1.56]	Ab	-0.04 [-0.27; 0.01]	Ac
		Black tea	0.25 [0.17; 0.34]	Aa	0.40 [0.29; 0.54]	Aa	1.25 [1.14; 1.36]	Ab	-0.31 [-0.36; -0.12]	Ab
		Deionized water	-0.22 [-0.35; -0.08]	Ba	0.05 [-0.28; 0.15]	Ba	-0.07 [-0.44; 0.00]	Ba	-0.03 [-0.36; 0.07]	Aa
	Δb	Grape seed extract	-0.28 [-1.14; 1.64]	ABa	-0.13 [-0.80; 0.93]	Aa	3.14 [0.99; 3.68]	Ab	0.41 [-0.26; 1.12]	Aa
		Black tea	2.53 [2.05; 2.91]	Ba	2.83 [1.51; 3.19]	Ba	4.76 [3.39; 6.01]	Ab	0.05 [-0.85; 0.54]	Ac
		Deionized water	-1.52 [-2.21; -1.17]	Aa	-1.02 [-1.58; -0.14]	Aa	-2.16 [-2.34; -1.07]	Bb	-1.31 [-1.70; -0.66]	Ab

Different upper case letters indicate significant differences between groups within evaluated time for each CIELAB coordinate (Kruskal-Wallis and post hoc Dunn's test); different small case letters indicate significant differences between the evaluated times in the same experimental solution (Friedman and post hoc Wilcoxon Matched Pairs Test). No erosion and erosion conditions were analyzed separately.

higher Δa values in comparison to deionized water. After 10 and 15 cycles, black tea and grape seed extract exposure resulted in significantly higher values than deionized water for specimens not submitted to erosion ($p < 0.050$), inducing a more reddish appearance of the enamel surface. The same pattern was verified with the specimens submitted to erosion, in which a stronger shade of red (higher Δa values) at T15 in the specimens treated with grape seed extract or black tea ($p < 0.001$) was found. After pellicle removal, no differences were observed between the groups for no erosion condition, with a decreased in Δa values. For specimens submitted to erosion, the red color diminished, and the Δa values reduced in the bioproduct groups.

Regarding Δb , no significant alteration was observed in the groups not submitted to erosion. Differently, for the specimens submitted to erosion, black tea caused a significant increase in yellow appearance of enamel already after five cycles. Throughout the 15 cycles, higher Δb values for black tea in comparison to other groups was found in both conditions (submitted or not to erosion), except after 15 cycles for specimens submitted to erosion, in which grape seed extract caused a significant increase in yellowish appearance and no differences were found between the bioproducts. After pellicle removal, the values again decreased considerably for both conditions ($p < 0.050$).

Surface reflection intensity

Until five cycles, the specimens not submitted to erosion exhibited higher values of surface reflection intensity for all groups exposed to experimental solutions. On the other hand, for the specimens submitted to erosion, grape seed extract presented a higher surface reflection intensity value than the other two groups. The black tea showed a significant decrease in surface reflection intensity when submitted to erosion than did the non-eroded specimens. Throughout the cycles, in general, the surface reflection intensity values were maintained for the specimens not submitted to erosion. In contrast, the experimental groups exhibited a significant decrease in surface reflection intensity during the erosive challenges. However, the values for both grape seed extract and black tea exposed specimens decreased, and the median values for grape seed extract exposed specimens were still significantly greater than seen for the water exposed specimens ($p < 0.001$). After pellicle removal, black tea exposed specimens presented significantly higher surface reflection intensity values than the other exposures when the specimens were not submitted to erosion. However, grape seed extract exposure promoted the highest surface reflection intensity among erosively challenged specimens ($p < 0.001$) (Table 3).

TABLE 3 Relative surface reflection intensity (%) median values [1° quartile (25%); 3° quartile (75%)] for enamel submitted or not to erosive challenges according to the evaluated times

Condition	Experimental solution	5 cycles	10 cycles	15 cycles	After pellicle removal
No erosion	Grape seed extract	82.07 [77.80; 86.48]	Aa 75.28 [74.35; 80.32]	Aa 92.77 [82.76; 96.44]	Ab 86.26 [77.85; 94.54]
	Black tea	91.67 [88.45; 93.76]	ABa 75.97 [74.97; 77.89]	Ab 81.79 [74.59; 85.13]	Bb 104.36 [97.94; 106.82]
	Deionized water	94.75 [88.06; 97.61]	Ba 93.49 [85.79; 96.67]	Ba 92.68 [87.25; 95.73]	Aa 91.81 [87.01; 97.37]
Erosion	Grape seed extract	93.04 [90.42; 101.19]	Aa 109.58 [103.85; 115.63]	Ab 60.91 [59.25; 67.14]	Ac 32.41 [31.11; 38.46]
	Black tea	74.08 [69.53; 75.60]	Ba 96.03 [89.76; 98.83]	Ab 52.36 [48.89; 57.81]	ABc 16.31 [14.88; 17.03]
	Deionized water	34.62 [32.17; 40.36]	Ca 43.32 [37.29; 44.70]	Ba 48.50 [43.79; 49.36]	Bb 23.54 [19.88; 28.11]

Different upper case letters indicate significant differences between groups within evaluated time (Kruskal-Wallis and post hoc Dunn's test); different small case letters indicate significant differences between the evaluated times in the same experimental solution (Friedman and post hoc Wilcoxon Matched Pairs Test). No erosion and erosion conditions were analyzed separately.

DISCUSSION

There is an ongoing increase in the use of natural supplements in dentistry where polyphenol-rich plant extracts can be used to modulate the caries and erosion processes [2–5, 19, 20]. The polyphenols can adsorb onto acquired enamel pellicle, due to their strong interaction with salivary proteins, leading to reduced bacterial adherence, increased thickness of the pellicle, and consequently, inhibition of the direct contact of acids with tooth surface [3, 20, 21]. However, polyphenols can also be responsible for tooth staining.

Tooth staining is a complex physical and chemical interaction between the tooth surface and a colored material (chromogen) [9]. In this study, we used polyphenols from grape seed extract and black tea; the first contains oligomeric proanthocyanidin (OPC) or condensed tannins [22–24], and the second contains theaflavins, thearubigins, and other catechin polymeric structures such as epigallocatechin gallate [25–28]. All these polyphenols exhibit high affinity to salivary proteins [3, 4] and, thus, adhere to the salivary pellicle, contributing to the staining process. However, the exact mechanism of action of this interaction is still not fully elucidated.

Here, we have reported on color in both the CIELAB scale and the CIEDE2000. Although CIEDE2000 better represents the color differences perceived by the human eye [15, 16], several studies still use the CIELAB system to compare treatment efficacy as well as degree of staining. So, to maintain comparability to other studies, we included both values. Our results show that both natural bioproducts promoted color change, so we can reject the first null hypothesis.

The tooth color difference observed in enamel not submitted to erosion but exposed to grape seed extract or black tea was higher than the perceptibility threshold for both CIELAB ($\Delta E_{ab} = 1.2$) and CIEDE2000 ($\Delta E_{00} = 0.8$) systems [16] at all evaluated times. Clinically, this could mean that patients using products containing such polyphenols might perceive a change in the color of their teeth. However, although staining might be perceptible, the color change may be acceptable until 15 cycles for the grape seed extract and, until five cycles for black tea, considering the acceptability threshold of $\Delta E_{ab} = 2.7$ for the CIELAB system. This means that in a clinical situation, the patient might accept the mismatch between the stained and non-stained regions. It must be emphasized that such values should be interpreted with caution, since the original study compared the color of teeth with that of restorative materials [16].

When enamel was submitted to successive erosive challenges, all experimental solutions presented color difference values above the perceptible threshold. Particularly, the negative control group presented values significantly higher than the bioproducts until 10 cycles, which corroborates the observation that the erosion process itself changes the color of

specimens [29]. The use of bioproducts on eroded specimens also induced a color difference higher than the perceptibility threshold, leading to an unacceptable outcome for the human eye. For black tea, the color difference was moderately unacceptable already after 10 cycles. Differently, the grape seed extract presented numerically lower values, albeit not statistically significantly so. Interestingly, the negative control (showing the effect of the erosion process itself) exhibited clearly unacceptable color differences already after five cycles, which was characterized by increased lightness (whiter) and negative values in the b axis (increased blueness). In contrast, both bioproducts induced redness of the enamel specimens and, in general, they also promoted yellowness, especially after 15 cycles. This suggests that the polyphenol-based solutions interacted with the salivary pellicle, leading to a significant color change (shown in the Δa^* and Δb^* values), and significantly protected the enamel surface against acid attacks [2, 4, 5], which is reflected with lower ΔL^* values and the lack of change in b values. Then, since we generally observed a change to a lighter color in the specimens, we also rejected the second null hypothesis. The Δa^* values show that both the grape seed extract and the black tea induced a reddish appearance that increased after 10 cycles, which can be explained by the dark orange and red color provided by their polyphenols [26]. As the theaflavins are the result of catechin oxidation, they are responsible for the orange or orange-red color in the tea while the thearubigins are red-brown or dark-brown pigments [30–32]. On the other hand, as the grape seed extract contains anthocyanin-based polyphenols, they exhibit dark purple to violet and reddish colors, which also explains the Δa^* and Δb^* values for this group [33, 34]. In addition, grape seed extract did not influence the yellow appearance of enamel not submitted to erosion, since the Δb^* values were similar to those of the negative control. In contrast, black tea exhibited a yellowing effect on sound enamel after 10 cycles. The staining potential can be explained by the intense color provided by the polyphenols themselves that built up in the acquired pellicle throughout the cycles. Remarkably, despite the significant color change promoted by these bioproducts, we found no color differences after pellicle removal. This is interesting because in the clinical situation, any color change occurring from the application of bioproducts will probably be transient. Therefore, once the salivary pellicle is partially removed during tooth brushing, the teeth should go back to their baseline color.

Studies evaluating the influence of erosion on tooth color are scarce. Clinically, as the erosive process progresses, the enamel layer is worn away, becoming thinner and the underlying darker (decreased values in lightness – L coordinate), yellower (increased values in yellow-blue axis – b coordinate) color of dentin shows through. However, it is still difficult to correlate the thickness of remaining enamel layer (after enamel loss) with tooth color change [35]. Furthermore, in

the present study, we only performed erosion (acid challenges), but not abrasion, which would increase the enamel loss. In addition, the erosion model was mild, with only a total of 15 min acid challenges, which probably only led to a more porous and rougher enamel surface, with very minimal surface loss. Therefore, it is reasonable to speculate that the differences in tooth color observed in our results arise from the surface porosity and roughness, rather than a thinner enamel layer. Then, as the enamel presents an irregular surface, the light scattering and reflection patterns are changed, influencing the color perception and measurements [36, 37].

In the present experiment, we removed the pellicle from the enamel surface with sodium hypochlorite, a usual method used in pellicle experiments [38, 39]. However, this solution contains a chlorine compound with bleaching characteristics. Sodium hypochlorite releases chlorine dioxide that is dissociated into chlorite, chlorate and oxygen by a disproportionation reaction [40]. Then, the oxygen oxidizes the organic molecules, removing the pigments absorbed in acquired pellicle and the pellicle itself. In our results, we observed values of color difference also in the negative control group after pellicle removal. It could be that the sodium hypochlorite might have not only removed the salivary pellicle, but also could have caused bleaching on the surface of the enamel. This effect must be investigated further using other methods of pellicle removal.

The pellicle protection of the enamel against tooth erosion [2, 4] was also observed in the surface reflection intensity results. The analysis of enamel erosion by surface reflection intensity has already been validated comparing to other analytical methods, such as surface hardness, calcium release and scanning electron microscopy of enamel surface [17, 18]. The reflectometer is capable of detecting erosion, and can be recommended for the quantification of the early softening phase of dental erosion, since the optical analysis provides the best sensitivity among the tested methods [18]. Additionally, this optical method presents several important advantages such as simplicity, fast performance, noncontact, nondestructive analysis of dental tissue, and cost-effective assembly. Then, in the present study, we recorded the highest values of relative surface reflection intensity in the grape seed extract group, followed by black tea after five cycles. The values of relative surface reflection intensity increased until 10 cycles, and then, we speculate that the acid attacks are only able to remove part of the salivary pellicle, and its basal layer remains on the tooth surface. As this remnant is kept for the following cycles, additional pellicle formation, as well as more pellicle modifications will cause more polyphenols to interact with the proteins, leading to a greater increase in pellicle thickness [38]. The presence of this pellicle on the enamel surface is related to the higher rSRI values, as observed in our results [41, 42], suggesting more protection against erosion [43]. Moreover, the higher relative

surface reflection intensity values for the grape seed extract after pellicle removal further corroborates the fact that this bioproduct significantly protects enamel against erosion [2].

Grape seed extract exhibited good results in the protection against erosion, suggesting that it is a favorable option for oral care products. Despite its staining potential for enamel that was not submitted to erosion, any color change seems to be temporary, diminishing after pellicle removal. Additionally, grape seed extract did not induce a significant yellow appearance over the time period evaluated. However, these positive results should be further studied in association with tooth brushing. On one hand, tooth brushing will partly remove the pellicle, allowing the tooth to retrieve its baseline color, but on the other hand, it intensifies surface loss under erosive conditions [44, 45] leading to a thinner enamel layer and, in turn, a change in tooth color. We can still conclude that natural bioproducts (grape seed extract and black tea) caused a significant, albeit transient, staining effect on enamel, which was reversible after pellicle removal. Of the two bioproducts, grape seed extract resulted in less color change compared with black tea and improved the protection against erosion when the enamel was submitted to the erosive challenges.

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CONFLICT OF INTEREST

No conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: Mariane Mailart, Thiago Carvalho; **Methodology:** Mariane Mailart, Burak Yilmaz, Thiago Carvalho; **Formal analysis:** Mariane Mailart; **Investigation:** Mariane Mailart, Pavel Berli. **Data curation:** Thiago Carvalho; **Writing – original draft:** Mariane Mailart; **Writing – review & editing:** Pavel Berli, Alessandra Borges, Burak Yilmaz, Tommy Baumann, Thiago Carvalho. **Supervision:** Thiago Carvalho.

DATA AVAILABILITY STATEMENT

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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