UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

THAMYRIS DE SOUZA CARVALHO

Acquired enamel pellicle engineering for protection against dental erosion: in vivo evaluation of the protective effect of sugarcane-derived cystatin (CaneCPI-5), hemoglobin and statherin-derived peptide (StN15)

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“Saiba que suas decisões e não suas condições, que determinam o seu destino. Para ter o que você nunca teve, você precisa fazer o que nunca fez”.

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RESUMO

Este trabalho avaliou, in vivo, 1) as alterações na composição proteica da PAE após tratamento da superfície dentária com cistatina derivada da cana-de-açúcar (CaneCPI-5), hemoglobina humana (HB), peptídeo derivado da estaterina (StN15) ou a combinação das 3 proteínas (Comb) antes da formação da PAE e posterior desafio erosivo intrínseco ou extrínseco; 2) o potencial protetor desses tratamentos contra a erosão intrínseca ou extrínseca do esmalte. Dez voluntários participaram de um protocolo cruzado e triplo-cego, constituído por 10 fases. Em cada fase, após profilaxia, fizeram bochecho (1 min; 10 mL) com (1) água deionizada, (2) CaneCPI-5 0,1 mg/mL, (3) HB 1 mg/mL, (4) StN15 $1,88 \times 10^{-5}$M ou solução contendo a Comb (5). A PAE foi formada (2h) e a biópsia do esmalte foi realizada sobre o dente 21. Nesta área foi realizado desafio erosivo com ácido cítrico 1% pH 2,5 ou HCl 0,01 M pH 2 por 10s. Os íons cálcio liberados do esmalte foram analisados pelo método de Arsenazo. Sobre as superfícies dos demais dentes foram realizados os mesmos desafios erosivos. A PAE foi coletada com papeis filtro de eletrodo, embebidos em ácido cítrico 3% e as amostras foram analisadas por proteômica quantitativa livre de marcadores. Na erosão extrínseca, o tratamento com as proteínas/peptídeos, isolados ou combinados, aumentou várias proteínas ácido-resistentes na PAE, em comparação ao controle. Os maiores aumentos foram observados para PRPs (32 vezes, StN15), profilina (15 vezes, combinação), alfa-amilase (9 vezes; StN15), queratinas (8 vezes, CaneCPI-5 e HB), histatina-1 (7 vezes, StN15), imunoglobulinas (6,5 vezes, StN15), lactotransferrina (4 vezes, CaneCPI-5), cistatinas, lisozima, proteína S-100-A9 e actinas (3,5 vezes, StN15), albumina sérica (3,5 vezes, CaneCPI-5 e HB) e hemoglobina (3 vezes, StN15). Anexina, calmodulina, queratina, tubulina e cistatinas foram identificadas exclusivamente após tratamento com as proteínas/peptídeo, isolados ou combinados. Grupos 2, 3 e 4 tiveram Ca liberado do esmalte significativamente menor em comparação ao grupo 1 (Kruskal-Wallis / Dunn´s, $p < 0,05$). Assim, os tratamentos com CaneCPI-5, HB ou StN15 aumentam notavelmente as proteínas ácido-resistentes na PAE, protegendo contra a erosão. Na erosão intrínseca, os tratamentos também aumentaram várias proteínas ácido-

resistentes na PAE, em comparação ao controle. Os aumentos foram observados para piruvato quinase PKM (11 vezes, CaneCPI-5), imunoglobulinas e proteína 3B da glândula submaxilar regulada por androgênio (4 vezes, StN15) e Hb e lisozima-C (2 vezes, StN15). Várias proteínas não tipicamente descritas na PAE, mas que se ligam ao cálcio ou outras proteínas, foram identificadas exclusivamente nos grupos tratados com as proteínas/peptídeos testados, isolados ou combinados. As concentrações médias (SD, mM) de cálcio liberado do esmalte foram 3,67 ± 1,48^{a}, 3,11 ± 0,72^{a}, 1,94 ± 0,57^{b}, 2,37 ± 0,90^{a} e 2,38 ± 0,45^{a} para os grupos 1-5, respectivamente (ANOVA/Tukey, $p < 0,05$). Assim, os tratamentos com CaneCPI-5, HB ou StN15 aumentaram notavelmente as proteínas ácido-resistentes na PAE, mas apenas a HB foi capaz de proteger contra a erosão intrínseca. Em conclusão, todas as proteínas/peptídeos avaliados aumentam proteínas ácido-resistentes na PAE, independentemente do tipo de desafio erosivo, mas apenas a HB protegeu o esmalte da erosão intrínseca.

Palavras-chave: Erosão dentária, película adquirida do esmalte, estaterina, caneCPI-5, hemoglobina, proteoma.

ABSTRACT

Acquired enamel pellicle engineering for protection against dental erosion: in vivo evaluation of the protective effect of sugarcane-derived cystatin (CaneCPI-5), hemoglobin and statherin-derived peptide (StN15)

Current study examined, in vivo 1) acquired enamel pellicle (AEP) protein composition after treatment of tooth surface utilizing sugarcane-derived cystatin (CaneCPI-5), human hemoglobin (HB), statherin-derived peptide (StN15) or its combination (Comb) prior AEP formation and following intrinsic or extrinsic erosive attack; 2) preventive potential of these treatments versus intrinsic or extrinsic enamel erosive demineralization. Ten volunteers participated in a crossover and triple-blind protocol, composed of ten phases. In every phase, following prophylaxis, volunteers rinsed (1 min; 10 mL) with (1) deionized H_2O, (2) 0.1 mg/mL CaneCPI-5, (3) 1 mg/mL HB, (4) 1.88×10^{-5}M StN15 or solution containing Comb (5). Following AEP formation (2h), enamel biopsy was performed on tooth 21. In this area, an erosive attack was executed utilizing 1% citric acid pH 2.5 or with 0.01 M HCl pH 2 for 10s. Calcium ions released from enamel were analyzed by Arsenazo method. The remaining teeth endured identical erosive challenges. Further, electrode filter papers soaked in 3% citric acid was utilized to collect AEP. Specimens were assessed by quantitative label-free proteomics. In extrinsic erosion, treatment utilizing proteins/peptides, alone or in combination, boosted multiple proteins acid-resistant within AEP in contrast to control. The greatest boost occurred on PRPs (32-fold, StN15), profilin (15-fold, combination), alpha-amylase (9-fold; StN15), keratins (8-fold, CaneCPI-5 and HB), histatin-1 (7-fold, StN15), immunoglobulins (6.5-fold, StN15), lactotransferrin (4-fold, CaneCPI-5), cystatins, lysozyme, S-100-A9 protein and actins (3.5-fold, StN15), serum albumin (3.5-fold, CaneCPI-5 and HB) and hemoglobin (3-fold, StN15). However, solely after proteins/peptides treatment, alone or in combination, Annexin, calmodulin, keratin, tubulin and cystatins were detected. Groups two, three and four had expressively lower enamel Ca release in contrast to group one (Kruskal-Wallis / Dunn's, $p < 0.05$). Thus, treatments with CaneCPI-5, HB or StN15 notably increase proteins acid-resistant within AEP, preventing erosion. In intrinsic erosion, the treatments also boosted multiple proteins acid-resistant within AEP in contrast to control. Observing an increase for PKM pyruvate kinase (11-fold, CaneCPI-5), immunoglobulins and submaxillary

gland androgen-regulated protein 3B (4-fold, StN15), and Hb and lysozyme-C (2-fold, StN15). Multiple proteins not usually described within AEP, but that bind calcium or other proteins were exclusively in groups treated within tested proteins/peptides, alone or in combination. The mean concentrations (SD, mM) of calcium released from enamel were 3.67 ± 1.48^{a}, 3.11 ± 0.72^{a}, 1.94 ± 0.57^{b}, 2.37 ± 0.90^{a} and 2.38 ± 0.45^{a} for groups 1-5, respectively (ANOVA/Tukey, $p<0.05$). Thus, treatments utilizing CaneCPI-5, HB or StN15 notably increased proteins acid-resistant within AEP, but only HB was able to prevent intrinsic erosion. Concluding, all the proteins/peptide evaluated increased proteins acid-resistant within AEP, regardless type of erosive challenge, but only Hb protected enamel against intrinsic erosion.

Keywords: Dental erosion, acquired enamel pellicle, statherin, caneCPI-5, hemoglobin, proteome.

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AEP	Acquired enamel pellicle
AFM	Atomic force microscopy
CaneCPI-5	Sugarcane-derived cystatin
Comb	Combination
ETW	Erosive tooth wear
GERD	Gastroesophageal reflux disease
HB	Human hemoglobin
IADR	International Association for Dental Research
IPTG	Isopropyl-beta-D-Thiogalactosidio
MIX	Mixed
PRPs	Proline-rich proteins
StN15	Statherin-derived peptide

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1- Introduction

1 INTRODUCTION

Irreversible loss of mineralized tooth substance, caused by intrinsic or extrinsic acids of non-bacterial origins is defined as dental erosion (Schlueter et al., 2020). Erosive injury develops in stages. Initially, there is tooth surface softening, followed by an ongoing dissolution, of enamel crystals, layer by layer (Lussi et al., 2011). The etiology of erosion is multifactorial, with the lesion being the result of an intricate relation between factors related to patient and nutritional factors (Lussi at al., 2014). Alternatively, saliva provides important biological properties that counteract erosive challenges. In addition to its buffering and remineralizing capacity, saliva contributes to the development of acquired enamel pellicle (AEP), a layer consisting primarily of proteins, but also containing glycoproteins and lipids, which prevents direct contact between tooth surface and acids (Hara et al., 2006; Buzalaf et al., 2012; Vukosavljevic et al., 2014).

Development of AEP occurs shortly following contact of saliva with enamel surface, quickly increasing its thickness, remaining stabilized for up to thirty minutes (Hannig, 1999). At the beginning, there is adherence of some precursor proteins that have great attraction for hydroxyapatite, developing electrostatic interactions with dental enamel (Hay, 1973). Among them are proline-rich proteins (PRPs), statherin and histatins, according to in vitro studies (Yao et al., 2000; Vitorino et al., 2004), although in situ researches have also proved the presence of mucins, amylase, cystatins, lysozyme and lactoferrin (Vitorino et al., 2007; Siqueira et al., 2012). The first layer of proteins that adheres seems to be the one that provides the highest dental demineralization protection, since it is a highly electron dense layer. Posterior layers have a substantial relaxed structure in comparison to the basal layer (Hannig, 1999), being formulated of globular proteins, that links to the predecessor proteins. Therefore, it is predicted that changes in predecessor proteins might alter the later proteins, what do not show hydroxyapatite affinity, but binds to predecessor proteins. In spite of this, “acquired pellicle engineering” concept was recently suggested, which aims to modify AEP composition and also prevent tooth demineralization (Vukosavljevic et al., 2014).

In the literature, a plethora of researches had shown that incorporation of salivary proteins within AEP surface affects its ability to protect against erosive

tooth wear (ETW), which is described as tooth wear having erosion as the main causal factor (Schlueter et al., 2020). Patients with erosion present half of proteins amount within AEP in comparison to patients without erosion (Carpenter et al., 2014). Among the protective proteins are mucins that, when adhered to tooth enamel, alone or in combination with other proteins, inhibit enamel demineralization induced by erosive attacks (Cheaib et al., 2011). Additionally, to mucins, statherin and PRPs are able to sustain saturation levels regard to calcium and phosphate in the oral cavity, preventing precipitation at neutral pH and these ions release after acid attacks during the process of demineralization. It was lately found that AEP calcium concentration is reduced by 50% in patients with dental erosion, and statherin, a calcium-binding protein, is 35% less available compared to patients without erosion (Carpenter et al., 2014). With regard to dietary proteins, casein appears to have preventive properties against acid attack, especially when combined with mucin (Cheaib et al., 2011).

Interestingly, even after severe erosive attacks, some AEP components are not stripped from enamel surface. Our research group has recently observed, through quantitative proteomics approaches, that some proteins remain adhered to the enamel even following erosive attacks that simulate extrinsic acids (citric acid 1%, pH 2.5, for 20 seconds) (Delecrode et al., 2015) or intrinsic acids (Taira et al., 2018). It was observed that some proteins had their concentrations relatively increased after exposure to citric acid, compared to exposure to deionized water, such as cystatin-B (20.7 times), lysozyme-C (2.8 times) and PRP1/ 2 (2.5 times) (Delecrode et al., 2015).

Specifically, cystatins are reversible inhibitors of cysteine peptidases, whose mechanism of action are based on competitive inhibition, throughout blockade of proteolytic activity (Abrahamson, 1993). Human cystatin B, however, has a very high cost, so that, considering the inclusion of these proteins in dental products for erosion prevention, it would be interesting to develop alternatives with better cost-effectiveness. Phytocystatins are inhibitors of plant cysteine peptidases, whose family was first suggested by Kondo et al., in 1991 (Kondo et al., 1991). These proteins have a consensus sequence L-A-R-F-A-V-X(3)-N, exclusive to plant cystatins, which justifies their inclusion in a new family (Margis et al., 1998). The first sugarcane cystatin produced in a

heterologous expression system was named Cana cystatin (CaneCPI-1) (Soares-Costa et al., 2002). This protein demonstrated inhibitory activity contra cysteine peptidases, including human cathepsins (Oliva et al., 2004). In addition to CaneCPl-1, another 4 sugarcane cystatins were produced recombinantly and studied for their inhibitory activity (Gianotti et al., 2006; Gianotti et al., 2008; Miguel, 2014). The CaneCPI-4 protein showed significant inhibitory activity contra human cathepsins B and L (Gianotti et al., 2008). Recently, a new cystatin from sugarcane, CaneCPI-5, was identified and characterized, which showed activities very similar to those of CaneCPI-4, being able to effectively inhibit a significant amount of cysteine peptidases, including cathepsin B, which is resistant to inhibition by cystatins. On the other hand, it proved to be much more soluble when produced in a bacterial expression system, what promotes its formulation and purification, in addition to presenting a strong adherence to quartz cuvettes, suggesting that it could bind more strongly to tooth enamel (Santiago et al., 2017). Thus, by accumulating these desirable characteristics, CaneCPI-5 was one of the proteins tested in the current research, in attempt to increase acid resistance within AEP. Due to the existing homology between plant and animal cystatins (Margis et al., 1998) and the low cost of production of plant cystatins, they can be excellent alternatives for insertion in dental products aimed at preventing caries and dental erosion. A publication by our research group revealed, by atomic force microscopy (AFM), that CaneCPI-5 has a high binding strength to enamel (6 times higher than the control). Topographic images of enamel samples coated with mucin 2.7 mg/mL, casein 10 mg/mL and CaneCPI-5 0.086 mg/mL were also taken before and after incubation with citric acid (0.65%, pH 3.4 for 1 min). Solely CaneCPI-5 prevented citric acid-induced damage to the enamel. In addition, using an in vitro early erosion model (Cheaib et al., 2011), we rattested that treating the enamel surface with CaneCPI-5 protects enamel against early erosion, with the best concentration being 0.1 mg/mL (Santiago et al., 2017), with no additional benefits utilizing higher concentrations.

Results make clear the great potential of CaneCPI-5 in preventing tooth erosion due to its aptitude to interrelate with tooth enamel. It is also a protein of low production cost and high thermal stability, which makes it ideal for inclusion

in dental products. However, before new products are developed for insertion in the market, further studies are needed, using models closer to clinical reality.

More recently, we searched for proteins resistant to disposition by intrinsic acids (Taira et al., 2018). This issue is extremely important, since gastric acids pH is lower and its buffering capability is higher than dietary acids, which steers to a generally more serious destruction of tooth tissue (Moazzez et al., 2014). In addition, patients with eating disorders have been reported to be at increased erosion risk (OR = 12.4), which even increases after self-induced vomiting (OR = 19.6) (Hermont et al., 2014). In a recent study (Taira et al., 2018), we observed that statherin remained in the AEP in vivo after challenges with HCl 0.01 M (pH 2) and HCl 0.1M (pH1), even in cases of AEPs formed in the short-term (for only 3 min). Statherin is a phosphorylated salivary protein, with 43 amino acid residues with a primary sequence alike osteopontin and casein, capable of binding calcium. Its negative charge density (due to phosphorylation of serines two and three) and helical conformation in the N-terminal region are major for the interaction with hydroxyapatite (Raj et al., 1992), which has been confirmed in experiments involving nuclear magnetic resonance solid state (Naganagowda et al., 1998). In addition, an in vitro research described that at least 15 N-terminal residues or farther are required in statherin-derived peptides to decrease enamel demineralization (Shah et al., 2011). These data together indicate that statherin is an AEP protein resistant to displacement by intrinsic acids and, therefore, with great potential to be integrated into ETW preventive dental products such as mouthwash solutions. Epidemiological data corroborate these findings, since in patients with dental erosion, the concentration of statherin within AEP is reduced by 35% (Carpenter et al., 2014). Reduction in statherin concentration was also observed within AEP collected in vivo from regions with erosion, when compared to regions without erosion within the same patient (Mutahar et al., 2017). Thus, statherin-derived peptides with at least 15 N-terminal residues seem to be excellent candidates to protect against erosion when adsorbed to the enamel surface, which was investigated in the current research.

Another interesting study from our group compared differences in the AEP protein profile, collected from the vestibular surface, in volunteers with gastroesophageal reflux disease (GERD) without dental demineralization, in

comparison to those with GERD with dental demineralization and control patients (without GERD and without tooth erosion). Among the proteins with differential expression, hemoglobin called our attention, as several subunits were found to be expressed more than 3 times higher in the group of patients with GERD and without erosion, in comparison to patients with the same disease, but with erosive lesions (Martini et al., 2019). Hemoglobins are not usually incorporated amidst AEP protein components. The first research related its presence in the pellicle was lately conducted by our group, and this protein was identified exclusively in the posterior dental arches area (Ventura et al., 2017). This could be the cause why this protein had not been identified within AEP in prior proteomics researches, since they gathered pellicle only from the anterior teeth (Siqueira et al., 2007; Lee et al., 2013; Delecrode et al., 2015; Zimmermann et al., 2019). The hemoglobin affinity for hydroxyapatite has been well known, since hydroxyapatite columns present an excellent performance for the purification of hemoglobin (Kawasaki et al., 1985). Due to its ability to adsorb hemoglobin, hydroxyapatite microspheres (Qi et al., 2013) or polyhedral (Yu et al., 2017) have been elaborated for controlled delivery of this protein. Interesting, adsorption of hemoglobin to hydroxyapatite elevates as the pH reduces, which can be clarified by electrostatic interactions among hemoglobin molecules and hydroxyapatite, which occur through van der Waals forces, hydrophobic or electrostatic interactions. Hemoglobin isoelectric point is around 6.8-7.0, making this protein positively charged when the pH is under 6.8 (Yu et al., 2017). Patients with GERD usually show a lower pH in the oral cavity than healthy patients, and a linkage has been found between pH < 4 in the distal esophagus and pH <5.5 in the oral cavity (Bartlett et al., 1996).

Therefore, the lower pH in the oral cavity of patients with GERD may enhance hemoglobin adsorption on tooth surfaces, since it grants this protein a positive charge. Since, higher levels of hemoglobin is noticed in patients with GERD and without erosion suggests that this protein may have intrinsic erosion preventive role. Recently, our research group evaluated the differential protein expression in the saliva of these patients, having observed an increase of more than 20 times in the alpha subunit of hemoglobin in patients with GERD without erosion. A proof-of-concept study was also carried out, in which it was verified that treating the enamel surface utilizing hemoglobin, in concentrations ranging

between 1 and 4 mg/mL, prevents enamel initial intrinsic erosion in vitro (Martini et al., 2020).

The elaboration of this research was grounded on previous studies by our research team started approximately 7 years ago which, using proteomic strategies that allowed us to identify candidate proteins or enamel protection against ETW (Delecrode et al., 2015; Taira et al., 2018; Martini et al., 2019). Additionally, recent studies by our group, involving in vitro (Santiago et al., 2017; Taira et al., 2018; Martini et al., 2020; Reis et al., 2023) in situ (Pela et al., 2021) and in vivo (Carvalho et al., 2020; Pela et al., 2023) protocols, demonstrated good performance of sugarcane-derived cystatin (CaneCPI-5), statherin-derived peptide (StN15) and human hemoglobin (HB) in reducing enamel erosive demineralization.

Using this knowledge, in this study, focused on acquired pellicle engineering, we evaluated, in vivo, 1) AEP protein composition following tooth surface treatment utilizing CaneCPI-5, HB, StN15 or its mix (Comb) prior of AEP formation and subsequent intrinsic or extrinsic erosive attack; 2) the protective potential of these treatments against intrinsic or extrinsic enamel erosive demineralization. The thesis is organized in the form of two articles. In the first one, already published, an extrinsic erosive challenge was employed, while in the second, which is under final review for publication, the effect of an intrinsic erosive challenge, under the same conditions, was evaluated.

2- Articles

Article 1

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Acquired pellicle protein-based engineering protects against erosive demineralization

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ABSTRACT

Objectives: To evaluate, *in vivo*: 1) proteomic alterations in the acquired enamel pellicle (AEP) after treatment with sugarcane-derived cystatin (CaneCPI-5), hemoglobin (HB), statherin-derived peptide (StN15) or their combination before the formation of the AEP and subsequent erosive challenge; 2) the protection of these treatments against erosive demineralization.

Materials and methods: In 5 crossover phases, after prophylaxis, 10 volunteers rinsed (10 mL, 1 min) with: deionized water-1, 0.1 mg/mL CaneCPI-5-2, 1.0 mg/mL HB-3, 1.88×10^{-3} M StN15-4 or their combination-5. AEP was formed (2 h) and enamel biopsy (10 µL, 1%citric acid, pH 2.5, 10 s) was performed on one incisor for calcium analysis. The same acid was applied on the vestibular surfaces of the remaining teeth. The acid-resistant proteins within the remaining AEP were collected. Samples were quantitatively analyzed by label-free proteomics.

Results: Treatment with the proteins/peptide, isolated or combined, increased several acid-resistant proteins in the AEP, compared with control. The highest increases were seen for PRPs (32-fold, StN15), profilin (15-fold, combination), alpha-amylase (9-fold; StN15), keratins (8-fold, CaneCPI-5 and HB), Histatin-1 (7-fold, StN15), immunoglobulins (6.5-fold, StN15), lactotransferrin (4-fold, CaneCPI-5), cystatins, lysozyme, protein S-100-A9 and actins (3.5-fold, StN15), serum albumin (3.5-fold, CaneCPI-5 and HB) and hemoglobin (3-fold, StN15). Annexin, calmodulin, keratin, tubulin and cystatins were identified exclusively upon treatment with the proteins/peptide, alone or combined. Groups 2, 3 and 4 had significantly lower Ca released from enamel compared to group 1 (Kruskal-Wallis/Dunn's, $p < 0.05$).

Conclusions: Treatment with CaneCPI-5, HB or StN15 remarkably increases acid-resistant proteins in the AEP, protecting against erosion.

Clinical significance: Our results show, for the first time, that treatment with proteins/peptide remarkably increases acid-resistant proteins in the AEP, protecting against erosive demineralization. These findings open an avenue for a new preventive approach for erosive demineralization, employing acquired pellicle engineering procedures that may in the future be incorporated into dental products.

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1. Introduction

One of the most important preventive factors against erosive demineralization is the acquired enamel pellicle (AEP), an organic, bacteria-free film that covers enamel, composed mainly by proteins and glycoproteins [1]. AEP prevent direct contact between acids and dental surface, acting as a mechanical barrier against acid attack [2–4].

The development of the AEP takes place shortly after contact of the enamel surface with saliva, with a rapid increase in its thickness, which will be stabilized for up to 30 minutes [5]. In the beginning, some precursor proteins with strong affinity for hydroxyapatite develop electrostatic interactions with the enamel, being adsorbed onto the enamel surface [6]. This first layer of proteins seems to provide the greatest protection against dental demineralization, since it is a very electron dense layer. The subsequent layers have a much looser structure when compared to the basal layer, being composed of globular proteins, which bind to precursor proteins [5]. Thus, it is expected that the change in the binding of precursor proteins can alter the binding of subsequent proteins, which have no affinity for hydroxyapatite, but adhere to the precursor proteins. With this in mind, the concept "acquired pellicle engineering" was proposed, intending to modify the composition of the AEP, further preventing dental demineralization [4].

Interestingly, some components of the AEP, especially those constituting its basal layer, are not removed from the tooth surface, even after severe erosive challenges [7]. This prompted our group to investigate, using quantitative proteomic approaches, which proteins within the AEP are resistant to removal by erosive acids. Among them, cystatin [8], statherin [9] and hemoglobin [10] were identified. Due to their high affinity for hydroxyapatite, these proteins are potential candidates to be included in dental products for procedures of "acquired pellicle engineering", aiming to protect the teeth against erosive demineralization. It was hypothesized that upon treatment of the enamel surface with these proteins, alone or in combination, there would be the formation of a reinforced basal layer that would alter the proteome of the subsequently formed AEP. This stronger AEP would then make the tooth surface more resistant to erosive demineralization.

To investigated this, the present proof-of-concept study evaluated, *in vivo*: 1) alterations in the proteome of the AEP after treatment with sugarcane-derived cystatin (CaneCPI-5), human hemoglobin (HB), statherin-derived peptide (StN15) or their combination before the formation of the AEP and subsequent erosive challenge; and 2) the protective potential of these treatments against erosive demineralization. The null hypotheses evaluated were: 1) treatment CaneCPI-5, HB, statherin-derived peptide (StN15) or their combination before the formation of the AEP and subsequent erosive challenge does not change the proteome of the remaining AEP; and 2) does not protect enamel against erosive demineralization.

2. Materials and methods

2.1. Ethical aspects and subjects

The protocol of this study was approved by the Ethics Committee of Bauru School of Dentistry, University of São Paulo, (#CAAE 99709318.1.0000.5417). All volunteers were made aware of the importance of the research and participated after signing the Informed Consent Form. The sample size was calculated with MSstats [11] using data from our previous experiment [9], considering $\alpha = 0.05$ and $1\text{-}\beta = 0.8$. The effect size (difference in protein abundance) was considered as 1.5. The estimated number of samples was 3/group. Considering the low amount of proteins typically recovered from the AEP in vivo, we decided to include 10 volunteers, in order to constitute 3 pools (biological triplicates).

Volunteers from both genders (6 female, 4 male; 18–35 years of age) participated in this *in vivo* triple-blind study (volunteers, researchers and analysts). They were non-smokers, had good general and oral health (without active caries lesions, gingivitis, periodontitis or any other oral condition affecting the composition of the oral fluids), were not using medications that would reduce salivary flow and did not have restorations on the buccal surfaces of the upper and lower teeth. The stimulated and unstimulated salivary flow was greater than 1 and 0.25 mL/min, respectively. Volunteers who presented risk factors for erosion, such as excessive consumption of carbonated drinks, fruit juices or acidic fruits, swimmers or patients with gastric disorders, such as bulimia and gastroesophageal reflux, were excluded. Clinical examinations and salivary flow exams were conducted by a dentist in a dental clinic with volunteers in the supine position using artificial light, air compressor, suction, clinical mirror and probe.

2.2. In vivo experiment

The factor under study was the type of protein solution used in the form of mouthwash, at 5 levels. It was a crossover and triple-blind study, consisting of 5 parallel phases, with all volunteers participating in all study groups. To avoid the circadian effects on the composition of the AEP, its collection occurred in the morning.

In each phase, after prophylaxis, volunteers rinsed with 10 mL (1 min) of the following solutions: deionized water (1), 0.1 mg/mL CaneCPI-5 (2), 1.0 mg/mL HB (3), 1.88×10^{-5}M StN15 (4) or combination of the 3 proteins/peptide (5). The concentrations of CaneCPI-5 [12], HB [13] and StN15 [14] were based in previous in vitro studies from our group. The pH of the protein solutions was native. The volunteers were deprived of eating and drinking for 2 hours and the AEP was allowed to form. After 120 min, an adhesive tape containing an orifice (4.92 mm^2) was attached to the left central incisor and 10 µL of 1% citric acid (pH 2.5) were applied on the exposed dental surface for 10 s. The drop was collected after 10 s with a pipette for calcium analysis, using the Arsenazo III method [15].

On the vestibular surfaces of the other upper and lower teeth, 200 µL of the same acid was applied for 10 s with a pipette. After washing with deionized water for acid removal, the acid-resistant proteins within the AEP were collected using electrode filter papers, soaked in 3 % citric acid [16]. In order to avoid contamination of the gingival margin, only two thirds of the buccal coronal surfaces were rubbed, where 3 % citric acid papers were used for each hemiarch. Thus, 4 papers were obtained from each volunteer, and a pool was made with the papers of 3-4 volunteers for each treatment. Therefore, the proteomic analysis of AEP was performed in biological triplicate.

2.3. Proteomic analysis and statistical analysis

After protein extraction and quantification, which occurred exactly as described by Ventura et al. [17], the samples were submitted to nano reverse phase liquid chromatography coupled to mass spectrometry (nLC-ESI-MS/MS), using pre-established parameters [17]. Samples were run in triplicates. Label-free proteomic quantification was performed using Protein Lynx Global Service (PLGS) software. Difference in expression among the groups was calculated using Monte-Carlo algorithm embedded in the software and expressed as $p < 0.05$ for proteins present in lower abundance and $1\text{-}p > 0.95$ for proteins present in higher abundance.

The data related to the Ca concentration released from the enamel were analyzed using the GraphPad InStat software (version 3.0 for Windows). Data did present normal distribution (Kolmogorov-Smirnov test) and were analyzed by Kruskal-Wallis and Dunn's test ($p < 0.05$).

3. Results

3.1. Effect of rinsing with proteins/peptide increases in the protein profile of the AEP

The mean ± SD amounts of proteins recovered from the AEP were

8.2 ± 2.5, 21.7 ± 5.2, 17.5 ± 3.9, 16.2 ± 3.7 and 19.6 ± 4.3 μg, respectively, for groups 1-5. Treatment with the tested proteins/peptide, isolated or combined, increased several acid-resistant proteins in the AEP, compared with control. The highest increases were seen for PRPs (up to 32-fold, StN15), profilin (up to 15-fold, combination), alpha-amylase (up to 9-fold; StN15), keratin (up to 8-fold, CaneCPI-5 and HB), histatin-1 (up to 7-fold, StN15), immunoglobulins (up to 6.5-fold, StN15), lactotransferrin (up to 4-fold, CaneCPI-5), cystatins, lysozyme, protein S-100-A9 and actins (up to 3.5-fold, StN15), serum albumin (up to 3.5-fold, caneCPI-5 and HB) and hemoglobin (up to 3-fold, StN15). Interestingly, several isoforms of 14-3-3 protein, histone H2B, annexin, calmodulin, keratin, tubulin and cystatins, as well as myeloperoxidase were identified exclusively upon treatment with the proteins/peptide, alone or in combination (Tables 1–4).

3.2. Effect of rinsing with proteins/peptide on enamel erosive demineralization

In order to check if the alterations in the proteomic profile of the AEP after rinsing with the proteins/peptide alone or in combination would affect the resistance of enamel to erosive demineralization we analyzed the concentration of calcium (Ca) released from the enamel. There was a significant difference among the groups ($KW = 25.452$, $p < 0.0001$). The groups treated with StN15, CaneCPI-5 and HB had significantly lower Ca release compared to the negative control (deionized water). The combination of the 3 proteins, however, did not significantly reduce the Ca release when compared to deionized water. In addition, StN15 significantly reduced Ca release compared to the combination (Fig. 1).

4. Discussion

The present study was designed to test the concept that acquired pellicle engineering by the enrichment of the enamel surface with potentially protective proteins/peptide changes the proteome of the AEP, thus increasing the protection against erosive demineralization. The proteins/peptide evaluated were chosen based on previous studies that revealed their potential as acid-resistant molecules in the AEP submitted to erosive challenges [8–10]. All the proteins/peptide evaluated provoked extensive changes in the protein composition of the remaining AEP and significantly reduced enamel erosive demineralization. Thus, both null hypotheses were rejected.

CaneCPI-5 is a sugarcane derived cystatin that was recently cloned by our group and shown to have strong affinity to hydroxyapatite. At the concentration employed in the present study (0.1 mg/mL), it significantly reduced initial erosive demineralization *in vitro* [12]. Regarding statherin, it has been shown that it is not necessary to employ the whole molecule to achieve protection against demineralization. Statherin-derived peptides containing at least 15 N-terminal residues have been shown to provide protection [18]. In the present study, we evaluated StN15, a peptide representing the first 15 N-terminal residues of statherin, with phosphorylated serine residues in positions 2 and 3. The density of negative charges in the N-terminus of the statherin-derived peptides is important for the interaction with hydroxyapatite [19], since the negative charges of the phosphate residues are attracted by the calcium residues within the hydroxyapatite. Regarding the concentration of StN15 employed (1.88×10^{-5} M), it corresponds to the mean range of statherin concentrations found in saliva [20] and was shown to provide protection against initial erosion in vitro [14]. HB was evaluated in the present study because it was shown to be increased in the AEP [10] and saliva [13] of gastroesophageal reflux disease (GERD) patients without erosive tooth wear, when compared with GERD patients with erosive tooth wear. Moreover, HB has strong affinity for hydroxyapatite and hydroxyapatite columns are used to purify this protein [21]. The HB concentration chosen was based on our recent *in vitro* study, which showed that 1.0 mg/mL provides the same protection as CaneCPI-5 at 0.1 mg/mL against initial enamel

Table 1
Proteins with significantly altered expression that remained in the acquired enamel pellicle after rinse with 0.1 mg/mL CaneCPI-5 for 1 minute in comparison with water (H_2O), followed by formation of the acquired enamel pellicle for 2 hours and subsequent challenge with 1% citric acid pH 2.5 for 10 seconds.

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* CANE: H_2O
P13647	**Keratin_ type II cytoskeletal 5**	**83**	**8.08**
P04259	**Keratin_ type II cytoskeletal 6B**	**230**	**7.32**
P02538	**Keratin_ type II cytoskeletal 6A**	**246**	**7.10**
P48668	**Keratin_ type II cytoskeletal 6C**	**246**	**6.89**
P01859	**Immunoglobulin heavy constant gamma 2**	**149**	**5.16**
P01861	**Immunoglobulin heavy constant gamma 4**	**176**	**4.57**
P01857	**Immunoglobulin heavy constant gamma 1**	**486**	**4.44**
Q562R1	**Beta-actin-like protein 2**	**1478**	**4.14**
P02814	**Submaxillary gland androgen-regulated protein 3B**	**3876**	**3.97**
P01860	**Immunoglobulin heavy constant gamma 3**	**179**	**3.94**
P02788	**Lactotransferrin**	**227**	**3.94**
Q9BYX7	**Putative beta-actin-like protein 3**	**1141**	**3.94**
P02810	**Salivary acidic proline-rich phosphoprotein 1/2**	**389**	**3.86**
P25311	**Zinc-alpha-2-glycoprotein**	**237**	**3.86**
Q9UJZ1	**Stomatin-like protein 2_ mitochondrial**	**65**	**3.63**
P60709	**Actin_ cytoplasmic 1**	**5974**	**3.49**
P63261	**Actin_ cytoplasmic 2**	**5974**	**3.49**
P04746	**Pancreatic alpha-amylase**	**2963**	**3.46**
Q9UGM3	**Deleted in malignant brain tumors 1 protein**	**72**	**3.39**
P04745	**Alpha-amylase 1**	**4579**	**3.35**
P19961	**Alpha-amylase 2B**	**3771**	**3.29**
P68133	**Actin_ alpha skeletal muscle**	**4504**	**3.22**
Q6S8J3	**POTE ankyrin domain family member E**	**2559**	**3.22**
P62736	**Actin_ aortic smooth muscle**	**4504**	**3.19**
P63267	**Actin_ gamma-enteric smooth muscle**	**4504**	**3.19**
P01876	**Immunoglobulin heavy constant alpha 1**	**997**	**3.16**
P68032	**Actin_ alpha cardiac muscle 1**	**4504**	**3.13**
P02768	**Serum albumin**	**5056**	**3.06**
A0M8Q6	**Immunoglobulin lambda constant 7**	**171**	**2.97**
P01877	**Immunoglobulin heavy constant alpha 2**	**565**	**2.94**
A5A3E0	**POTE ankyrin domain family member F**	**2500**	**2.94**
P0CF74	**Immunoglobulin lambda constant 6**	**226**	**2.92**
P13646	**Keratin_ type I cytoskeletal 13**	**153**	**2.77**
P00738	**Haptoglobin**	**281**	**2.61**
P01834	**Immunoglobulin kappa constant**	**627**	**2.53**
P04792	**Heat shock protein beta-1**	**1774**	**2.44**
Q96DA0	**Zymogen granule protein 16 homolog B**	**744**	**2.34**
P0DOY3	**Immunoglobulin lambda constant 3**	**226**	**2.20**
P0CG38	**POTE ankyrin domain family member I**	**1359**	**2.18**
P06702	**Protein S100-A9**	**9379**	**2.18**
P0CG04	**Immunoglobulin lambda constant 1**	**249**	**2.16**
P0DOY2	**Immunoglobulin lambda constant 2**	**226**	**2.16**
B9A064	**Immunoglobulin lambda-like polypeptide 5**	**226**	**2.08**
P05109	**Protein S100-A8**	**3832**	**2.08**
P12273	Prolactin-inducible protein	1127	1.72
P04083	Annexin A1	2548	1.38
P59666	Neutrophil defensin 3	2682	0.69
P59665	Neutrophil defensin 1	2682	0.68
P15516	Histatin-3	3388	0.08
P31946	14-3-3 protein beta/alpha	245	CANE*
P62258	14-3-3 protein epsilon	273	CANE*
Q04917	14-3-3 protein eta	245	CANE*
P61981	14-3-3 protein gamma	245	CANE*
P31947	14-3-3 protein sigma	329	CANE*

(*continued on next page*)

Table 1 (*continued*)

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* CANE: H_2O
P27348	14-3-3 protein theta	245	CANE*
P63104	14-3-3 protein zeta/delta	277	CANE*
Q86SQ4	Adhesion G-protein coupled receptor G6	132	CANE*
P02763	Alpha-1-acid glycoprotein 1	154	CANE*
P01009	Alpha-1-antitrypsin	87	CANE*
Q9P2G1	Ankyrin repeat and IBR domain-containing protein 1	279	CANE*
Q01484	Ankyrin-2	106	CANE*
P12429	Annexin A3	172	CANE*
P08133	Annexin A6	97	CANE*
P04280	Basic salivary proline-rich protein 1	486	CANE*
O43670	BUB3-interacting and GLEBS motif-containing protein ZNF207	280	CANE*
P27482	Calmodulin-like protein 3	352	CANE*
P23280	Carbonic anhydrase 6	1310	CANE*
P23528	Cofilin-1	345	CANE*
Q8NEU8	DCC-interacting protein 13-beta	498	CANE*
P53602	Diphosphomevalonate decarboxylase	138	CANE*
Q9BY07	Electrogenic sodium bicarbonate cotransporter 4	149	CANE*
Q5JZY3	Ephrin type-A receptor 10	543	CANE*
Q04637	Eukaryotic translation initiation factor 4 gamma 1	304	CANE*
P78344	Eukaryotic translation initiation factor 4 gamma 2	155	CANE*
Q7Z6M2	F-box only protein 33	117	CANE*
P21333	Filamin-A	63	CANE*
P14136	Glial fibrillary acidic protein	150	CANE*
Q13439	Golgin subfamily A member 4	144	CANE*
Q96A08	Histone H2B type 1-A	425	CANE*
P33778	Histone H2B type 1-B	637	CANE*
P62807	Histone H2B type 1-C/E/F/G/I	637	CANE*
P58876	Histone H2B type 1-D	637	CANE*
Q93079	Histone H2B type 1-H	637	CANE*
P06899	Histone H2B type 1-J	637	CANE*
O60814	Histone H2B type 1-K	637	CANE*
Q99880	Histone H2B type 1-L	637	CANE*
Q99879	Histone H2B type 1-M	637	CANE*
Q99877	Histone H2B type 1-N	637	CANE*
P23527	Histone H2B type 1-O	637	CANE*
Q16778	Histone H2B type 2-E	637	CANE*
Q5QNW6	Histone H2B type 2-F	637	CANE*
Q8N257	Histone H2B type 3-B	637	CANE*
P57053	Histone H2B type F-S	637	CANE*
O96028	Histone-lysine N-methyltransferase NSD2	181	CANE*
Q15811	Intersectin-1	165	CANE*
Q9NZM3	Intersectin-2	163	CANE*
P13645	Keratin_ type I cytoskeletal 10	96	CANE*
P19012	Keratin_ type I cytoskeletal 15	86	CANE*
P35908	Keratin_ type II cytoskeletal 2 epidermal	424	CANE*
P19013	Keratin_ type II cytoskeletal 4	164	CANE*
Q86Y46	Keratin_ type II cytoskeletal 73	290	CANE*
O95678	Keratin_ type II cytoskeletal 75	420	CANE*
Q5XKE5	Keratin_ type II cytoskeletal 79	420	CANE*
Q8IV03	Leucine rich adaptor protein 1-like	168	CANE*
Q9Y608	Leucine-rich repeat flightless-interacting protein 2	256	CANE*
P33121	Long-chain-fatty-acid–CoA ligase 1	219	CANE*
Q8NCY6	Myb/SANT-like DNA-binding domain-containing protein 4	114	CANE*
P05164	Myeloperoxidase	216	CANE*
P60660	Myosin light polypeptide 6	291	CANE*
Q7Z406	Myosin-14	455	CANE*
Q14CX7	N-alpha-acetyltransferase 25_ NatB auxiliary subunit	251	CANE*
Q8NGW1	Olfactory receptor 6B3	109	CANE*
Q6ZVD8	PH domain leucine-rich repeat-containing protein phosphatase 2	150	CANE*
Q6VY07	Phosphofurin acidic cluster sorting protein 1	86	CANE*
Q15102	Platelet-activating factor acetylhydrolase IB subunit gamma	402	CANE*
O15460	Prolyl 4-hydroxylase subunit alpha-2	488	CANE*

Table 1 (*continued*)

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* CANE: H_2O
Q9NY61	Protein AATF	553	CANE*
Q8IY45	Protein AMN1 homolog	113	CANE*
Q8NCA5	Protein FAM98A	90	CANE*
Q9Y5E8	Protocadherin beta-15	94	CANE*
Q8IXJ9	Putative Polycomb group protein ASXL1	143	CANE*
Q7Z2F6	Putative protein ZNF720	165	CANE*
Q96QB1	Rho GTPase-activating protein 7	195	CANE*
Q92622	Run domain Beclin-1-interacting and cysteine-rich domain-containing protein	129	CANE*
O75494	Serine/arginine-rich splicing factor 10	241	CANE*
P02787	Serotransferrin	168	CANE*
P42229	Signal transducer and activator of transcription 5A	101	CANE*
P51692	Signal transducer and activator of transcription 5B	101	CANE*
Q9Y448	Small kinetochore-associated protein	123	CANE*
Q5MJ70	Speedy protein A	106	CANE*
Q9C093	Sperm flagellar protein 2	113	CANE*
Q86VE3	Spermidine/spermine N(1)-acetyltransferase-like protein 1	76	CANE*
Q15772	Striated muscle preferentially expressed protein kinase	93	CANE*
Q9Y6N5	Sulfide:quinone oxidoreductase_ mitochondrial	101	CANE*
C9J3V5	Testis-expressed protein 22	170	CANE*
Q8N584	Tetratricopeptide repeat protein 39C	141	CANE*
P07202	Thyroid peroxidase	104	CANE*
Q9NX61	Transmembrane protein 161A	127	CANE*
B1AL88	Transmembrane protein FAM155A	122	CANE*
Q9H972	Uncharacterized protein C14orf93	209	CANE*
Q9NRW7	Vacuolar protein sorting-associated protein 45	143	CANE*
Q9P202	Whirlin	83	CANE*
Q8TBZ8	Zinc finger protein 564	87	CANE*
Q96MU6	Zinc finger protein 778	667	CANE*
Q9C0K1	Zinc transporter ZIP8	90	CANE*
P00330	Alcohol dehydrogenase 1	1227	H2O*
Q8N7 × 0	Androglobin	70	H_2O*
Q8N9B4	Ankyrin repeat domain-containing protein 42	82	H_2O*
P03973	Antileukoproteinase	313	H_2O*
P13942	Collagen alpha-2(XI) chain	122	H_2O*
Q8WXI2	Connector enhancer of kinase suppressor of ras 2	94	H_2O*
Q53SF7	Cordon-bleu protein-like 1	128	H_2O*
Q9UBG3	Cornulin	142	H_2O*
Q86T13	C-type lectin domain family 14 member A	66	H_2O*
O14976	Cyclin-G-associated kinase	75	H_2O*
Q96RT1	Erbin	56	H_2O*
Q5T1M5	FK506-binding protein 15	42	H_2O*
Q14687	Genetic suppressor element 1	89	H_2O*
P47897	Glutamine–tRNA ligase	79	H_2O*
Q8IWJ2	GRIP and coiled-coil domain-containing protein 2	73	H_2O*
P0DMV8	Heat shock 70 kDa protein 1A	32	H_2O*
P17066	Heat shock 70 kDa protein 6	33	H_2O*
P69905	Hemoglobin subunit alpha	540	H_2O*
P68871	Hemoglobin subunit beta	1520	H_2O*
P02042	Hemoglobin subunit delta	257	H_2O*
P02100	Hemoglobin subunit epsilon	257	H_2O*
P69891	Hemoglobin subunit gamma-1	257	H_2O*
P69892	Hemoglobin subunit gamma-2	257	H_2O*
P56524	Histone deacetylase 4	87	H_2O*
P02533	Keratin_ type I cytoskeletal 14	28	H_2O*
Q04695	Keratin_ type I cytoskeletal 17	28	H_2O*
P08493	Matrix Gla protein	690	H_2O*
A0JLT2	Mediator of RNA polymerase II transcription subunit 19	372	H_2O*
Q9P1T7	MyoD family inhibitor domain-containing protein	69	H_2O*
P07737	Profilin-1	97	H_2O*
Q9P2K9	Protein dispatched homolog 3	37	H_2O*
Q08188		50	H_2O*

(*continued on next page*)

Table 1 (*continued*)

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* CANE: H_2O
	Protein-glutamine gamma-glutamyltransferase E		
Q9HBR0	Putative sodium-coupled neutral amino acid transporter 10	73	H_2O*
Q9H1J1	Regulator of nonsense transcripts 3A	60	H_2O*
Q9P2K3	REST corepressor 3	61	H_2O*
Q9NRY4	Rho GTPase-activating protein 35	53	H_2O*
Q9P0V9	Septin-10	249	H_2O*
A1 × 283	SH3 and PX domain-containing protein 2B	54	H_2O*
P12757	Ski-like protein	44	H_2O*
Q9Y6J9	TAF6-like RNA polymerase II p300/CBP-associated factor-associated factor 65 kDa subunit 6 L	91	H_2O*
Q9Y4G6	Talin-2	76	H_2O*
A0A1B0GUA7	Testis-expressed protein 51	72	H_2O*
Q13144	Translation initiation factor eIF-2B subunit epsilon	76	H_2O*
Q8IXQ3	Uncharacterized protein C9orf40	143	H_2O*
Q7L1V2	Vacuolar fusion protein MON1 homologB	206	H_2O*
Q7Z2W4	Zinc finger CCCH-type antiviral protein 1	42	H_2O*
Q86XU0	Zinc finger protein 677	94	H_2O*

Proteins highlighted in bold are increased or decreased more than 2-fold.

[a] Identification is based on proteins ID from UniProt protein database, reviewed only (http://www.uniprot.org/).

[b] Proteins with expression significantly altered are organized according to the ratio.

* Indicates unique proteins in alphabetical order.

erosion [13].

The protocol carried out for proteomic analysis followed a methodology that was recently developed by our research group, which increases the protein identification of the samples [17]. Our results showed that the treatment of the dental surface *in vivo* with proteins that have affinity for hydroxyapatite considerably increased the amount of proteins immobilized within the AEP after the erosive challenge (8.2 µg for the control group *versus* 16.2-21.7 µg for the experimental groups). Moreover, it was observed a remarkable increase in several proteins in the AEP that are potentially resistant to removal by acids (Tables 1–4), protecting against erosive demineralization (Fig. 1). Interestingly, the families of proteins with changes in expression as well as the ratios found were distinct for the different proteins evaluated (Tables 1–4). However, considering the main outcome of the experiment (protection against erosive demineralization), these differences do not seem to be of importance, since all the isolated proteins/peptide provided similar protection. The combination of the 3 proteins, however, was not able to provide protection in comparison with the control. This might be because the concentrations of the 3 proteins in combination were similar to the ones used when the proteins were isolated. Considering that all of them have affinity for hydroxyapatite, they might have competed for binding sites onto this mineral. Further evaluation of the effect of the combination of these proteins at lower concentrations is interesting, since different proteins alter the composition of the AEP in a distinct manner (Tables 1–3).

One important finding of the present study is that despite some proteins with increased expression or exclusively found upon treatment with the proteins/peptide are those secreted by the salivary glands that are typically found in the AEP (e.g. PRPs, cystatins, amylase, lactotransferrin, histatins), most of them are intracellular proteins (e.g. keratins, histones, actin and profilin, tubulins, 14-3-3 proteins). It is known that the precursor proteins found in the basal layer of the AEP are the most protective ones against erosive demineralization [7]. Thus, increasing the amount of proteins with strong affinity for hydroxyapatite

Table 2
Proteins with significantly altered expression that remained in the acquired enamel pellicle after rinse with 1.0 mg/mL Hemoglobin (HB) for 1 minute in comparison with water (H_2O), followed by formation of the acquired enamel pellicle for 2 hours and subsequent challenge with 1% citric acid pH 2.5 for 10 seconds.

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* HB:H_2O
P13647	**Keratin, type II cytoskeletal 5**	**83**	**7.92**
P07737	**Profilin-1**	**97**	**6.55**
P04259	**Keratin, type II cytoskeletal 6B**	**230**	**6.30**
P48668	**Keratin, type II cytoskeletal 6C**	**246**	**6.11**
P02538	**Keratin, type II cytoskeletal 6A**	**246**	**6.05**
Q53SF7	**Cordon-bleu protein-like 1**	**128**	**4.90**
P01861	**Immunoglobulin heavy constant gamma 4**	**176**	**4.81**
P01860	**Immunoglobulin heavy constant gamma 3**	**179**	**4.26**
P02814	**Submaxillary gland androgen-regulated protein 3B**	**3876**	**4.18**
P01857	**Immunoglobulin heavy constant gamma 1**	**486**	**3.94**
P02768	**Serum albumin**	**5056**	**3.63**
P01859	**Immunoglobulin heavy constant gamma 2**	**149**	**3.49**
P13646	**Keratin, type I cytoskeletal 13**	**153**	**3.46**
P00738	**Haptoglobin**	**281**	**3.32**
Q9UBG3	**Cornulin**	**142**	**3.16**
P0CF74	**Immunoglobulin lambda constant 6**	**226**	**3.13**
P04792	**Heat shock protein beta-1**	**1774**	**3.03**
P06702	**Protein S100-A9**	**9379**	**3.00**
Q562R1	**Beta-actin-like protein 2**	**1478**	**2.89**
P01876	**Immunoglobulin heavy constant alpha 1**	**997**	**2.89**
P05109	**Protein S100-A8**	**3832**	**2.89**
P0DOY3	**Immunoglobulin lambda constant 3**	**226**	**2.80**
P63261	**Actin, cytoplasmic 2**	**5974**	**2.72**
P0DOY2	**Immunoglobulin lambda constant 2**	**226**	**2.72**
P02788	**Lactotransferrin**	**227**	**2.69**
A0M8Q6	**Immunoglobulin lambda constant 7**	**171**	**2.69**
P60709	**Actin, cytoplasmic 1**	**5974**	**2.66**
P01877	**Immunoglobulin heavy constant alpha 2**	**565**	**2.66**
P68032	**Actin, alpha cardiac muscle 1**	**4504**	**2.56**
P63267	**Actin, gamma-enteric smooth muscle**	**4504**	**2.53**
Q9BZW7	**Testis-specific gene 10 protein**	**93**	**2.53**
P68133	**Actin, alpha skeletal muscle**	**4504**	**2.51**
P62736	**Actin, aortic smooth muscle**	**4504**	**2.51**
P04080	**Cystatin-B**	**1982**	**2.46**
Q9BYX7	**Putative beta-actin-like protein 3**	**1141**	**2.46**
P0CG04	**Immunoglobulin lambda constant 1**	**249**	**2.44**
Q6S8J3	**POTE ankyrin domain family member E**	**2559**	**2.39**
A5A3E0	**POTE ankyrin domain family member F**	**2500**	**2.32**
P0CG39	**POTE ankyrin domain family member J**	**657**	**2.20**
Q9UGM3	**Deleted in malignant brain tumors 1 protein**	**72**	**2.18**
P0CG38	**POTE ankyrin domain family member I**	**1359**	**2.18**
P01037	**Cystatin-SN**	**2445**	**2.12**
P04745	**Alpha-amylase 1**	**4579**	**2.08**
P04083	**Annexin A1**	**2548**	**2.05**
P12273	**Prolactin-inducible protein**	**1127**	**2.05**
P19961	**Alpha-amylase 2B**	**3771**	**2.01**
P04746	**Pancreatic alpha-amylase**	**2963**	**2.01**
Q96DA0	Zymogen granule protein 16 homolog B	744	1.92
P01036	Cystatin-S	2529	1.57
P69892	Hemoglobin subunit gamma-2	257	1.48
P59665	Neutrophil defensin 1	2682	1.32
P59666	Neutrophil defensin 3	2682	1.31
B9A064	Immunoglobulin lambda-like polypeptide 5	226	0.07
P31946	14-3-3 protein beta/alpha	1424	HB*
P62258	14-3-3 protein epsilon	1424	HB*
Q04917	14-3-3 protein eta	1424	HB*
P61981	14-3-3 protein gamma	1424	HB*
P31947	14-3-3 protein sigma	1615	HB*
P27348	14-3-3 protein theta	1424	HB*
P63104	14-3-3 protein zeta/delta	1801	HB*

(*continued on next page*)

Table 2 (*continued*)

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* HB:H_2O
P07108	Acyl-CoA-binding protein	482	HB*
P02763	Alpha-1-acid glycoprotein 1	632	HB*
P01009	Alpha-1-antitrypsin	120	HB*
P01023	Alpha-2-macroglobulin	189	HB*
P30533	Alpha-2-macroglobulin receptor-associated protein	134	HB*
P12429	Annexin A3	500	HB*
P02647	Apolipoprotein A-I	298	HB*
P06576	ATP synthase subunit beta_ mitochondrial	190	HB*
Q8NHQ9	ATP-dependent RNA helicase DDX55	107	HB*
O95429	BAG family molecular chaperone regulator 4	94	HB*
P04280	Basic salivary proline-rich protein 1	2560	HB*
Q9H159	Cadherin-19	131	HB*
P0DP23	Calmodulin-1	2272	HB*
P0DP24	Calmodulin-2	2272	HB*
P0DP25	Calmodulin-3	2272	HB*
P27482	Calmodulin-like protein 3	2665	HB*
P23280	Carbonic anhydrase 6	423	HB*
P16070	CD44 antigen	227	HB*
P51788	Chloride channel protein 2	267	HB*
P23528	Cofilin-1	267	HB*
P01024	Complement C3	69	HB*
Q9NWV4	CXXC motif containing zinc binding protein	243	HB*
P24903	Cytochrome P450 2F1	444	HB*
Q5TAQ9	DDB1- and CUL4-associated factor 8	246	HB*
Q8N1I0	Dedicator of cytokinesis protein 4	56	HB*
P13716	Delta-aminolevulinic acid dehydratase	116	HB*
Q02487	Desmocollin-2	215	HB*
Q9P1A6	Disks large-associated protein 2	201	HB*
Q9UBX2	Double homeobox protein 4	103	HB*
P0CJ85	Double homeobox protein 4-like protein 2	103	HB*
P0CJ86	Double homeobox protein 4-like protein 3	103	HB*
P0CJ88	Double homeobox protein 4-like protein 5	103	HB*
P0CJ89	Double homeobox protein 4-like protein 6	103	HB*
P0CJ90	Double homeobox protein 4-like protein 7	103	HB*
O95071	E3 ubiquitin-protein ligase UBR5	90	HB*
Q5MY95	Ectonucleoside triphosphate diphosphohydrolase 8	82	HB*
Q01469	Fatty acid-binding protein 5	1115	HB*
P02679	Fibrinogen gamma chain	111	HB*
P21333	Filamin-A	276	HB*
Q12950	Forkhead box protein D4	92	HB*
P04075	Fructose-bisphosphate aldolase A	708	HB*
Q96I76	G patch domain-containing protein 3	251	HB*
P47929	Galectin-7	573	HB*
P19447	General transcription and DNA repair factor IIH helicase subunit XPB	188	HB*
P04406	Glyceraldehyde-3-phosphate dehydrogenase	566	HB*
P00739	Haptoglobin-related protein	197	HB*
O94927	HAUS augmin-like complex subunit 5	176	HB*
Q96A08	Histone H2B type 1-A	153	HB*
P33778	Histone H2B type 1-B	641	HB*
P62807	Histone H2B type 1-C/E/F/G/I	641	HB*
P58876	Histone H2B type 1-D	641	HB*
Q93079	Histone H2B type 1-H	641	HB*
P06899	Histone H2B type 1-J	641	HB*
O60814	Histone H2B type 1-K	641	HB*
Q99880	Histone H2B type 1-L	641	HB*
Q99879	Histone H2B type 1-M	641	HB*
Q99877	Histone H2B type 1-N	641	HB*
P23527	Histone H2B type 1-O	641	HB*
Q16778	Histone H2B type 2-E	641	HB*
Q5QNW6	Histone H2B type 2-F	641	HB*
Q8N257	Histone H2B type 3-B	641	HB*
P57053	Histone H2B type F-S	641	HB*
O00291	Huntingtin-interacting protein 1	118	HB*
P01871	Immunoglobulin heavy constant mu	276	HB*
P01591	Immunoglobulin J chain	804	HB*
O00458	Interferon-related developmental regulator 1	832	HB*
Q8TAC2	Josephin-2	178	HB*
O60259	Kallikrein-8	134	HB*

Table 2 (*continued*)

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* HB:H_2O
Q15323	Keratin_ type I cuticular Ha1	396	HB*
Q14532	Keratin_ type I cuticular Ha2	239	HB*
O76009	Keratin_ type I cuticular Ha3-I	175	HB*
Q14525	Keratin_ type I cuticular Ha3-II	468	HB*
O76011	Keratin_ type I cuticular Ha4	205	HB*
Q92764	Keratin_ type I cuticular Ha5	277	HB*
O76013	Keratin_ type I cuticular Ha6	248	HB*
O76014	Keratin_ type I cuticular Ha7	221	HB*
O76015	Keratin_ type I cuticular Ha8	221	HB*
P13645	Keratin_ type I cytoskeletal 10	552	HB*
Q99456	Keratin_ type I cytoskeletal 12	270	HB*
P19012	Keratin_ type I cytoskeletal 15	676	HB*
P08779	Keratin_ type I cytoskeletal 16	578	HB*
P05783	Keratin_ type I cytoskeletal 18	287	HB*
P08727	Keratin_ type I cytoskeletal 19	252	HB*
P35900	Keratin_ type I cytoskeletal 20	197	HB*
Q9C075	Keratin_ type I cytoskeletal 23	175	HB*
Q2M2I5	Keratin_ type I cytoskeletal 24	248	HB*
Q7Z3Y7	Keratin_ type I cytoskeletal 28	244	HB*
Q6A162	Keratin_ type I cytoskeletal 40	297	HB*
P04264	Keratin_ type II cytoskeletal 1	225	HB*
P35908	Keratin_ type II cytoskeletal 2 epidermal	162	HB*
P19013	Keratin_ type II cytoskeletal 4	499	HB*
Q86Y46	Keratin_ type II cytoskeletal 73	302	HB*
O95678	Keratin_ type II cytoskeletal 75	672	HB*
Q5XKE5	Keratin_ type II cytoskeletal 79	672	HB*
Q5T3J3	Ligand-dependent nuclear receptor-interacting factor 1	125	HB*
Q9Y2H9	Microtubule-associated serine/threonine-protein kinase 1	906	HB*
P05164	Myeloperoxidase	1593	HB*
P14649	Myosin light chain 6B	355	HB*
P60660	Myosin light polypeptide 6	544	HB*
O43847	Nardilysin	87	HB*
P14543	Nidogen-1	75	HB*
Q13287	N-myc-interactor	129	HB*
O75376	Nuclear receptor corepressor 1	164	HB*
Q9UQ90	Paraplegin	275	HB*
Q6VY07	Phosphofurin acidic cluster sorting protein 1	80	HB*
P01833	Polymeric immunoglobulin receptor	170	HB*
Q9UL51	Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2	88	HB*
P20648	Potassium-transporting ATPase alpha chain 1	713	HB*
Q9HAT1	Protein ERGIC-53-like	199	HB*
Q9BS92	Protein NipSnap homolog 3B	169	HB*
Q8WZA1	Protein O-linked-mannose beta-1_2-N-acetylglucosaminyltransferase 1	210	HB*
Q9H0W8	Protein SMG9	437	HB*
Q99497	Protein/nucleic acid deglycase DJ-1	591	HB*
Q14289	Protein-tyrosine kinase 2-beta	302	HB*
A8MUU1	Putative fatty acid-binding protein 5-like protein 3	383	HB*
Q86U02	Putative uncharacterized protein encoded by LINC00596	175	HB*
P14618	Pyruvate kinase PKM	706	HB*
Q9Y2J0	Rabphilin-3A	279	HB*
O95267	RAS guanyl-releasing protein 1	162	HB*
P52566	Rho GDP-dissociation inhibitor 2	123	HB*
Q6P5S7	Ribonuclease kappa	220	HB*
Q9Y6N7	Roundabout homolog 1	125	HB*
Q15468	SCL-interrupting locus protein	226	HB*
P02787	Serotransferrin	2016	HB*
Q9NRF2	SH2B adapter protein 1	332	HB*
Q9H299	SH3 domain-binding glutamic acid-rich-like protein 3	153	HB*
Q86VE3	Spermidine/spermine N(1)-acetyltransferase-like protein 1	509	HB*
Q15772	Striated muscle preferentially expressed protein kinase	120	HB*
O15400	Syntaxin-7	180	HB*
P10599	Thioredoxin	202	HB*
Q86UQ8	Transcription factor NF-E4	109	HB*

(*continued on next page*)

Table 2 (*continued*)

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* HB:H_2O
Q9C040	Tripartite motif-containing protein 2	270	HB*
P17752	Tryptophan 5-hydroxylase 1	122	HB*
P07332	Tyrosine-protein kinase Fes/Fps	572	HB*
Q9H9J4	Ubiquitin carboxyl-terminal hydrolase 42	350	HB*
P08670	Vimentin	433	HB*
Q96N77	Zinc finger protein 641	300	HB*
Q96MU6	Zinc finger protein 778	205	HB*
Q8N7 × 0	Androglobin	70	H_2O*
Q8N9B4	Ankyrin repeat domain-containing protein 42	82	H_2O*
Q12955	Ankyrin-3	79	H_2O*
P13942	Collagen alpha-2(XI) chain	122	H_2O*
Q8WXI2	Connector enhancer of kinase suppressor of ras 2	94	H_2O*
Q86T13	C-type lectin domain family 14 member A	66	H_2O*
O14976	Cyclin-G-associated kinase	75	H_2O*
Q96RT1	Erbin	56	H_2O*
Q5T1M5	FK506-binding protein 15	42	H_2O*
Q14687	Genetic suppressor element 1	89	H_2O*
P47897	Glutamine–tRNA ligase	79	H_2O*
P0DMV8	Heat shock 70 kDa protein 1A	32	H_2O*
P0DMV9	Heat shock 70 kDa protein 1B	32	H_2O*
P17066	Heat shock 70 kDa protein 6	33	H_2O*
P56524	Histone deacetylase 4	87	H_2O*
Q8N1A0	Keratin-like protein KRT222	104	H_2O*
A0JLT2	Mediator of RNA polymerase II transcription subunit 19	372	H_2O*
Q9P1T7	MyoD family inhibitor domain-containing protein	69	H_2O*
Q9P2K9	Protein dispatched homolog 3	37	H_2O*
Q08188	Protein-glutamine gamma-glutamyltransferase E	50	H_2O*
Q9HBR0	Putative sodium-coupled neutral amino acid transporter 10	73	H_2O*
Q9H1J1	Regulator of nonsense transcripts 3A	60	H_2O*
Q9P2K3	REST corepressor 3	61	H_2O*
Q9NRY4	Rho GTPase-activating protein 35	53	H_2O*
Q9P0V9	Septin-10	249	H_2O*
A1 × 283	SH3 and PX domain-containing protein 2B	54	H_2O*
P12757	Ski-like protein	44	H_2O*
Q9Y6J9	TAF6-like RNA polymerase II p300/CBP-associated factor-associated factor 65 kDa subunit 6 L	91	H_2O*
Q9Y4G6	Talin-2	76	H_2O*
A0A1B0GUA7	Testis-expressed protein 51	72	H_2O*
Q13144	Translation initiation factor eIF-2B subunit epsilon	76	H_2O*
Q8IXQ3	Uncharacterized protein C9orf40	143	H_2O*
Q7Z2W4	Zinc finger CCCH-type antiviral protein 1	42	H_2O*
Q86XU0	Zinc finger protein 677	94	H_2O*

Proteins highlighted in bold are increased or decreased more than 2-fold.

[a] Identification is based on proteins ID from UniProt protein database, reviewed only (http://www.uniprot.org/).

[b] Proteins with expression significantly altered are organized according to the ratio.

* Indicates unique proteins in alphabetical order.

in the basal layer, as tested in the present study, may also increase the amount of other proteins that do not bind to hydroxyapatite, but bind to the precursor proteins, which could have been the case of the intracellular proteins. One example of this is the increase of actin, found upon treatment with HB and StN15 and in protein combination. In the same study groups, profilin (an actin-binding protein) was also increased. Thus, actin and profilin might be part of the protein aggregates that are taken up in the second stage of formation of the AEP [5]. It is noteworthy that treatment with the proteins/peptide increased several proteins with antimicrobial properties in the AEP, such as several isoforms of immunoglobulins, cystatins, histatins and lysozyme. Therefore, it is possible that they also provide protection against dental caries, which should be evaluated in further studies.

One limitation of the present study was the lack of a positive control

Table 3
Proteins with significantly altered expression that remained in the acquired enamel pellicle after rinse with StN15 - StN15pS2pS3 (1.88×10^{-5}M) for 1 minute in comparison with water (H_2O), followed by formation of the acquired enamel pellicle for 2 hours and subsequent challenge with 1% citric acid pH 2.5 for 10 seconds.

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* StN15: H_2O
P02814	**Submaxillary gland androgen-regulated protein 3B**	**3876**	**32.46**
P07737	**Profilin-1**	**97**	**11.70**
P19961	**Alpha-amylase 2B**	**3771**	**9.12**
P02812	**Basic salivary proline-rich protein 2**	**273**	**8.67**
P04746	**Pancreatic alpha-amylase**	**2963**	**8.67**
P04745	**Alpha-amylase 1**	**4579**	**7.39**
P15515	**Histatin-1**	**1504**	**7.10**
P01834	**Immunoglobulin kappa constant**	**627**	**6.49**
P02810	**Salivary acidic proline-rich phosphoprotein 1/2**	**389**	**6.49**
P25311	**Zinc-alpha-2-glycoprotein**	**237**	**6.30**
P13647	**Keratin_ type II cytoskeletal 5**	**83**	**6.17**
A0M8Q6	**Immunoglobulin lambda constant 7**	**171**	**5.10**
P04259	**Keratin_ type II cytoskeletal 6B**	**230**	**4.81**
P48668	**Keratin_ type II cytoskeletal 6C**	**246**	**4.81**
P00738	**Haptoglobin**	**281**	**4.76**
P02538	**Keratin_ type II cytoskeletal 6A**	**246**	**4.71**
P01876	**Immunoglobulin heavy constant alpha 1**	**997**	**4.48**
P0CF74	**Immunoglobulin lambda constant 6**	**226**	**4.48**
Q9UGM3	**Deleted in malignant brain tumors 1 protein**	**72**	**4.35**
P12273	**Prolactin-inducible protein**	**1127**	**4.26**
P01877	**Immunoglobulin heavy constant alpha 2**	**565**	**4.01**
Q96DA0	**Zymogen granule protein 16 homolog B**	**744**	**4.01**
P01861	**Immunoglobulin heavy constant gamma 4**	**176**	**3.97**
Q9UBG3	**Cornulin**	**142**	**3.74**
P01857	**Immunoglobulin heavy constant gamma 1**	**486**	**3.74**
P01037	**Cystatin-SN**	**2445**	**3.56**
P61626	**Lysozyme C**	**1523**	**3.56**
P0CG04	**Immunoglobulin lambda constant 1**	**249**	**3.49**
P0DOY2	**Immunoglobulin lambda constant 2**	**226**	**3.46**
B9A064	**Immunoglobulin lambda-like polypeptide 5**	**226**	**3.46**
P06702	**Protein S100-A9**	**9379**	**3.46**
P0DOY3	**Immunoglobulin lambda constant 3**	**226**	**3.35**
Q562R1	**Beta-actin-like protein 2**	**1478**	**3.32**
P01036	**Cystatin-S**	**2529**	**3.22**
P02100	**Hemoglobin subunit epsilon**	**257**	**2.97**
P69892	**Hemoglobin subunit gamma-2**	**257**	**2.97**
P02042	**Hemoglobin subunit delta**	**257**	**2.89**
P01859	**Immunoglobulin heavy constant gamma 2**	**149**	**2.89**
P02788	**Lactotransferrin**	**227**	**2.89**
P69891	**Hemoglobin subunit gamma-1**	**257**	**2.86**
P01860	**Immunoglobulin heavy constant gamma 3**	**179**	**2.86**
P02768	**Serum albumin**	**5056**	**2.66**
P69905	**Hemoglobin subunit alpha**	**540**	**2.66**
P04792	**Heat shock protein beta-1**	**1774**	**2.56**
Q9BYX7	**Putative beta-actin-like protein 3**	**1141**	**2.56**
Q8IWJ2	**GRIP and coiled-coil domain-containing protein 2**	**73**	**2.51**
P68871	**Hemoglobin subunit beta**	**1520**	**2.48**
P63261	**Actin_ cytoplasmic 2**	**5974**	**2.41**
P62736	**Actin_ aortic smooth muscle**	**4504**	**2.32**
Q9UJZ1	**Stomatin-like protein 2_ mitochondrial**	**65**	**2.32**
P63267	**Actin_ gamma-enteric smooth muscle**	**4504**	**2.29**
P60709	**Actin_ cytoplasmic 1**	**5974**	**2.27**
P68032	**Actin_ alpha cardiac muscle 1**	**4504**	**2.25**
Q6S8J3	**POTE ankyrin domain family member E**	**2559**	**2.23**

(*continued on next page*)

Table 3 (*continued*)

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* StN15: H_2O
P68133	**Actin_ alpha skeletal muscle**	**4504**	**2.16**
P04080	**Cystatin-B**	**1982**	**2.12**
P09228	**Cystatin-SA**	**1029**	**2.05**
A5A3E0	**POTE ankyrin domain family member F**	**2500**	**2.05**
P59666	Neutrophil defensin 3	2682	1.95
P05109	Protein S100-A8	3832	1.93
Q8TAX7	Mucin-7	874	1.68
P59665	Neutrophil defensin 1	2682	1.60
P0CG38	POTE ankyrin domain family member I	1359	1.52
P04083	Annexin A1	2548	1.28
P31946	14-3-3 protein beta/alpha	184	StN15*
P62258	14-3-3 protein epsilon	184	StN15*
Q04917	14-3-3 protein eta	184	StN15*
P61981	14-3-3 protein gamma	184	StN15*
P31947	14-3-3 protein sigma	479	StN15*
P27348	14-3-3 protein theta	184	StN15*
P63104	14-3-3 protein zeta/delta	586	StN15*
O15528	25-hydroxyvitamin D-1 alpha hydroxylase_ mitochondrial	351	StN15*
Q9BTT0	Acidic leucine-rich nuclear phosphoprotein 32 family member E	213	StN15*
P01009	Alpha-1-antitrypsin	143	StN15*
P48728	Aminomethyltransferase_ mitochondrial	312	StN15*
Q01432	AMP deaminase 3	292	StN15*
Q6UB99	Ankyrin repeat domain-containing protein 11	189	StN15*
P07355	Annexin A2	915	StN15*
P02647	Apolipoprotein A-I	628	StN15*
P05090	Apolipoprotein D	300	StN15*
P04280	Basic salivary proline-rich protein 1	432	StN15*
Q7Z5Y6	Bone morphogenetic protein 8A	120	StN15*
Q8TDL5	BPI fold-containing family B member 1	156	StN15*
Q9UBW5	Bridging integrator 2	202	StN15*
Q9H251	Cadherin-23	319	StN15*
Q13554	Calcium/calmodulin-dependent protein kinase type II subunit beta	168	StN15*
P27482	Calmodulin-like protein 3	257	StN15*
P23280	Carbonic anhydrase 6	2105	StN15*
O75390	Citrate synthase_ mitochondrial	494	StN15*
P23528	Cofilin-1	1132	StN15*
Q5T9S5	Coiled-coil domain-containing protein 18	77	StN15*
Q5W186	Cystatin-9	285	StN15*
P01034	Cystatin-C	571	StN15*
Q5VZ89	DENN domain-containing protein 4C	123	StN15*
Q14919	Dr1-associated corepressor	138	StN15*
Q9HAT8	E3 ubiquitin-protein ligase pellino homolog 2	515	StN15*
Q8IY85	EF-hand calcium-binding domain-containing protein 13	1589	StN15*
Q9BY07	Electrogenic sodium bicarbonate cotransporter 4	240	StN15*
P01588	Erythropoietin	169	StN15*
Q7L2H7	Eukaryotic translation initiation factor 3 subunit M	410	StN15*
O15360	Fanconi anemia group A protein	176	StN15*
Q01469	Fatty acid-binding protein 5	456	StN15*
P04075	Fructose-bisphosphate aldolase A	155	StN15*
Q9BY60	Gamma-aminobutyric acid receptor-associated protein-like 3	238	StN15*
A6NK44	Glyoxalase domain-containing protein 5	163	StN15*
Q96A08	Histone H2B type 1-A	334	StN15*
P33778	Histone H2B type 1-B	447	StN15*
P62807	Histone H2B type 1-C/E/F/G/I	447	StN15*
P58876	Histone H2B type 1-D	447	StN15*
Q93079	Histone H2B type 1-H	447	StN15*
P06899	Histone H2B type 1-J	447	StN15
O60814	Histone H2B type 1-K	447	StN15*
Q99880	Histone H2B type 1-L	447	StN15*
Q99879	Histone H2B type 1-M	447	StN15*
Q99877	Histone H2B type 1-N	447	StN15*
P23527	Histone H2B type 1-O	447	StN15*
Q16778	Histone H2B type 2-E	447	StN15*

Table 3 (*continued*)

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* StN15: H_2O
Q5QNW6	Histone H2B type 2-F	447	StN15*
Q8N257	Histone H2B type 3-B	447	StN15*
P57053	Histone H2B type F-S	447	StN15*
P62805	Histone H4	887	StN15*
O96028	Histone-lysine N-methyltransferase NSD2	114	StN15*
P01871	Immunoglobulin heavy constant mu	302	StN15*
A0A0J9YX35	Immunoglobulin heavy variable 3-64D	128	StN15*
P01591	Immunoglobulin J chain	4960	StN15*
Q96SY0	Integrator complex subunit 14	239	StN15*
Q9NWB7	Intraflagellar transport protein 57 homolog	217	StN15*
P06870	Kallikrein-1	970	StN15*
Q14145	Kelch-like ECH-associated protein 1	95	StN15*
P19012	Keratin_ type I cytoskeletal 15	147	StN15*
P08779	Keratin_ type I cytoskeletal 16	139	StN15*
P08727	Keratin_ type I cytoskeletal 19	246	StN15*
P19013	Keratin_ type II cytoskeletal 4	371	StN15*
Q86Y46	Keratin_ type II cytoskeletal 73	137	StN15*
O95678	Keratin_ type II cytoskeletal 75	98	StN15*
Q8NBT2	Kinetochore protein Spc24	209	StN15*
Q96S06	Lipase maturation factor 1	249	StN15*
O60244	Mediator of RNA polymerase II transcription subunit 14	136	StN15*
Q9BQA1	Methylosome protein 50	133	StN15*
Q96J65	Multidrug resistance-associated protein 9	144	StN15*
P05164	Myeloperoxidase	176	StN15*
P14649	Myosin light chain 6B	116	StN15*
P60660	Myosin light polypeptide 6	890	StN15*
O00308	NEDD4-like E3 ubiquitin-protein ligase WWP2	296	StN15*
O00533	Neural cell adhesion molecule L1-like protein	154	StN15*
P49321	Nuclear autoantigenic sperm protein	54	StN15*
Q7Z3K3	Pogo transposable element with ZNF domain	684	StN15*
P01833	Polymeric immunoglobulin receptor	482	StN15*
P25789	Proteasome subunit alpha type-4	191	StN15*
Q9HAT1	Protein ERGIC-53-like	686	StN15*
Q5W0V3	Protein FAM160B1	632	StN15*
P31949	Protein S100-A11	522	StN15*
O95785	Protein Wiz	220	StN15*
Q99497	Protein/nucleic acid deglycase DJ-1	204	StN15*
A6NMY6	Putative annexin A2-like protein	915	StN15*
Q9Y2K5	R3H domain-containing protein 2	106	StN15*
Q12967	Ral guanine nucleotide dissociation stimulator	588	StN15*
P46940	Ras GTPase-activating-like protein IQGAP1	173	StN15*
Q86 × 27	Ras-specific guanine nucleotide-releasing factor RalGPS2	137	StN15*
Q86V86	Serine/threonine-protein kinase pim-3	112	StN15*
P02787	Serotransferrin	1875	StN15*
P36952	Serpin B5	221	StN15*
Q8TCY0	Small integral membrane protein 11B	309	StN15*
Q8NHG7	Small VCP/p97-interacting protein	237	StN15*
Q9Y345	Sodium- and chloride-dependent glycine transporter 2	227	StN15*
Q9BXS9	Solute carrier family 26 member 6	178	StN15*
Q9C093	Sperm flagellar protein 2	229	StN15*
Q658L1	Stabilizer of axonemal microtubules 2	154	StN15*
Q15772	Striated muscle preferentially expressed protein kinase	140	StN15*
Q92537	Sushi domain-containing protein 6	271	StN15*
O15061	Synemin	146	StN15*
Q8IYX1	TBC1 domain family member 21	115	StN15*
Q16650	T-box brain protein 1	149	StN15*
Q5UIP0	Telomere-associated protein RIF1	44	StN15*
O14530	Thioredoxin domain-containing protein9	180	StN15*
Q6ZWK6	Transmembrane protease serine 11 F	112	StN15*
P45379	Troponin T_ cardiac muscle	287	StN15*
P07437	Tubulin beta chain	453	StN15*
Q13885	Tubulin beta-2A chain	453	StN15*

(*continued on next page*)

Table 3 (*continued*)

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* StN15: H_2O
Q9BVA1	Tubulin beta-2B chain	453	StN15*
Q13509	Tubulin beta-3 chain	292	StN15*
P04350	Tubulin beta-4A chain	172	StN15*
P68371	Tubulin beta-4B chain	172	StN15*
Q9BUF5	Tubulin beta-6 chain	172	StN15*
Q3ZCM7	Tubulin beta-8 chain	172	StN15*
P35236	Tyrosine-protein phosphatase non-receptor type 7	153	StN15*
Q93009	Ubiquitin carboxyl-terminal hydrolase 7	259	StN15*
Q9H972	Uncharacterized protein C14orf93	373	StN15*
Q502W6	von Willebrand factor A domain-containing protein 3B	162	StN15*
Q8N9V3	WD repeat_ SAM and U-box domain-containing protein 1	75	StN15*
Q6P2D8	X-ray radiation resistance-associated protein 1	138	StN15*
Q96NG8	Zinc finger protein 582	326	StN15*
Q8N7 × 0	Androglobin	70	H_2O*
Q8N9B4	Ankyrin repeat domain-containing protein 42	82	H_2O*
P13942	Collagen alpha-2(XI) chain	122	H_2O*
Q8WXI2	Connector enhancer of kinase suppressor of ras 2	94	H_2O*
Q53SF7	Cordon-bleu protein-like 1	128	H_2O*
Q86T13	C-type lectin domain family 14 member A	66	H_2O*
O14976	Cyclin-G-associated kinase	75	H_2O*
Q96RT1	Erbin	56	H_2O*
Q5T1M5	FK506-binding protein 15	42	H_2O*
Q14687	Genetic suppressor element 1	89	H_2O*
P47897	Glutamine–tRNA ligase	79	H_2O*
P17066	Heat shock 70 kDa protein 6	33	H_2O*
P56524	Histone deacetylase 4	87	H_2O*
P02533	Keratin_ type I cytoskeletal 14	28	H_2O*
Q04695	Keratin_ type I cytoskeletal 17	28	H_2O*
Q8N1A0	Keratin-like protein KRT222	104	H_2O*
P08493	Matrix Gla protein	690	H_2O*
A0JLT2	Mediator of RNA polymerase II transcription subunit 19	372	H_2O*
Q9P1T7	MyoD family inhibitor domain-containing protein	69	H_2O*
Q9P2K9	Protein dispatched homolog 3	37	H_2O*
Q08188	Protein-glutamine gamma-glutamyltransferase E	50	H_2O*
Q9HBR0	Putative sodium-coupled neutral amino acid transporter 10	73	H_2O*
Q9H1J1	Regulator of nonsense transcripts 3A	60	H_2O*
Q9P2K3	REST corepressor 3	61	H_2O*
Q9NRY4	Rho GTPase-activating protein 35	53	H_2O*
Q9P0V9	Septin-10	249	H_2O*
A1 × 283	SH3 and PX domain-containing protein 2B	54	H_2O*
P12757	Ski-like protein	44	H_2O*
Q9Y6J9	TAF6-like RNA polymerase II p300/CBP-associated factor-associated factor 65 kDa subunit 6 L	91	H_2O*
Q9Y4G6	Talin-2	76	H_2O*
A0A1B0GUA7	Testis-expressed protein 51	72	H_2O*
Q9BZW7	Testis-specific gene 10 protein	93	H_2O*
Q13144	Translation initiation factor eIF-2B subunit epsilon	76	H_2O*
Q8IXQ3	Uncharacterized protein C9orf40	143	H_2O*
Q7L1V2	Vacuolar fusion protein MON1 homolog B	206	H_2O*
Q7Z2W4	Zinc finger CCCH-type antiviral protein 1	42	H_2O*
Q9C0A1	Zinc finger homeobox protein 2	176	H_2O*
Q86XU0	Zinc finger protein 677	94	H_2O*

Proteins highlighted in bold are increased or decreased more than 2-fold.

[a] Identification is based on proteins ID from UniProt protein database, reviewed only (http://www.uniprot.org/).

[b] Proteins with expression significantly altered are organized according to the ratio.

* Indicates unique proteins in alphabetical order.

Table 4

Proteins with significantly altered expression that remained in the acquired enamel pellicle after rinse with Combination (COMB) of the 3 proteins (Cane, StN15 and HB) at the concentrations already described for 1 minute in comparison with water (H_2O), followed by formation of the acquired enamel pellicle for 2 hours and subsequent challenge with 1% citric acid pH 2.5 for 10 seconds.

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* COMB: H_2O
P07737	**Profilin-1**	**97**	**15.18**
P01861	**Immunoglobulin heavy constant gamma 4**	**176**	**5.37**
P01860	**Immunoglobulin heavy constant gamma 3**	**179**	**5.21**
P01859	**Immunoglobulin heavy constant gamma 2**	**149**	**4.31**
Q7L1V2	**Vacuolar fusion protein MON1 homolog B**	**206**	**4.10**
P01877	**Immunoglobulin heavy constant alpha 2**	**565**	**3.86**
P01876	**Immunoglobulin heavy constant alpha 1**	**997**	**3.78**
P01857	**Immunoglobulin heavy constant gamma 1**	**486**	**3.53**
P15515	**Histatin-1**	**1504**	**3.49**
P01834	**Immunoglobulin kappa constant**	**627**	**3.35**
P13646	**Keratin_ type I cytoskeletal 13**	**153**	**3.10**
A0M8Q6	**Immunoglobulin lambda constant 7**	**171**	**3.03**
Q9UBG3	**Cornulin**	**142**	**2.97**
P02810	**Salivary acidic proline-rich phosphoprotein 1/2**	**389**	**2.86**
P02788	**Lactotransferrin**	**227**	**2.83**
Q9UGM3	**Deleted in malignant brain tumors 1 protein**	**72**	**2.59**
P0CF74	**Immunoglobulin lambda constant 6**	**226**	**2.59**
P63261	**Actin_ cytoplasmic 2**	**5974**	**2.51**
P04792	**Heat shock protein beta-1**	**1774**	**2.51**
P60709	**Actin_ cytoplasmic 1**	**5974**	**2.46**
P06702	**Protein S100-A9**	**9379**	**2.46**
P04746	**Pancreatic alpha-amylase**	**2963**	**2.36**
P02768	**Serum albumin**	**5056**	**2.29**
P12273	**Prolactin-inducible protein**	**1127**	**2.27**
P04745	**Alpha-amylase 1**	**4579**	**2.25**
P19961	**Alpha-amylase 2B**	**3771**	**2.25**
P05109	**Protein S100-A8**	**3832**	**2.25**
P04080	**Cystatin-B**	**1982**	**2.08**
Q562R1	**Beta-actin-like protein 2**	**1478**	**2.05**
P02814	Submaxillary gland androgen-regulated protein 3B	3876	1.97
P68032	Actin_ alpha cardiac muscle 1	4504	1.95
P68133	Actin_ alpha skeletal muscle	4504	1.95
P62736	Actin_ aortic smooth muscle	4504	1.90
P63267	Actin_ gamma-enteric smooth muscle	4504	1.88
P01037	Cystatin-SN	2445	1.73
Q8TAX7	Mucin-7	874	1.55
Q12955	Ankyrin-3	79	1.54
Q9BYX7	Putative beta-actin-like protein 3	1141	1.49
P09228	Cystatin-SA	1029	1.46
Q6S8J3	POTE ankyrin domain family member E	2559	1.42
P01036	Cystatin-S	2529	1.39
A5A3E0	POTE ankyrin domain family member F	2500	1.35
P04083	Annexin A1	2548	1.30
P59665	Neutrophil defensin 1	2682	0.40
P15516	Histatin-3	3388	0.28
P31947	14-3-3 protein sigma	214	COMB*
Q05823	2-5A-dependent ribonuclease	116	COMB*
Q16515	Acid-sensing ion channel 2	83	COMB*
Q9UJY5	ADP-ribosylation factor-binding protein GGA1	137	COMB*
P12429	Annexin A3	292	COMB*
Q9NUQ8	ATP-binding cassette sub-family F member 3	56	COMB*
P04280	Basic salivary proline-rich protein 1	178	COMB*
Q07817	Bcl-2-like protein 1	352	COMB*
P27482	Calmodulin-like protein 3	309	COMB*
P23280	Carbonic anhydrase 6	273	COMB*
Q9BXL7		220	COMB*

(*continued on next page*)

Table 4 (*continued*)

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* COMB: H_2O
	Caspase recruitment domain-containing protein 11		
O75175	CCR4-NOT transcription complex subunit 3	207	COMB*
Q8N137	Centrobin	206	COMB*
P12110	Collagen alpha-2(VI) chain	132	COMB*
Q2VPA4	Complement component receptor 1-like protein	79	COMB*
P17927	Complement receptor type 1	57	COMB*
P28325	Cystatin-D	187	COMB*
Q9BY07	Electrogenic sodium bicarbonate cotransporter 4	337	COMB*
O43556	Epsilon-sarcoglycan	505	COMB*
P14324	Farnesyl pyrophosphate synthase	124	COMB*
O00358	Forkhead box protein E1	58	COMB*
Q9NXC5	GATOR complex protein MIOS	66	COMB*
P04406	Glyceraldehyde-3-phosphate dehydrogenase	352	COMB*
Q96A08	Histone H2B type 1-A	121	COMB*
P33778	Histone H2B type 1-B	181	COMB*
P62807	Histone H2B type 1-C/E/F/G/I	181	COMB*
P58876	Histone H2B type 1-D	181	COMB*
Q93079	Histone H2B type 1-H	181	COMB*
P06899	Histone H2B type 1-J	181	COMB*
O60814	Histone H2B type 1-K	181	COMB*
Q99880	Histone H2B type 1-L	181	COMB*
Q99879	Histone H2B type 1-M	181	COMB*
Q99877	Histone H2B type 1-N	181	COMB*
P23527	Histone H2B type 1-O	181	COMB*
Q16778	Histone H2B type 2-E	181	COMB*
Q5QNW6	Histone H2B type 2-F	181	COMB*
Q8N257	Histone H2B type 3-B	181	COMB*
P57053	Histone H2B type F-S	181	COMB*
P01591	Immunoglobulin J chain	566	COMB*
Q9NZM3	Intersectin-2	49	COMB*
P04264	Keratin_ type II cytoskeletal 1	342	COMB*
Q07866	Kinesin light chain 1	190	COMB*
Q9HAQ2	Kinesin-like protein KIF9	193	COMB*
Q96II8	Leucine-rich repeat and calponin homology domain-containing protein 3	336	COMB*
P05164	Myeloperoxidase	325	COMB*
P59047	NACHT_ LRR and PYD domains-containing protein 5	169	COMB*
Q7RTR0	NACHT_ LRR and PYD domains-containing protein 9	65	COMB*
Q9H7 × 0	N-alpha-acetyltransferase 60	90	COMB*
P18615	Negative elongation factor E	231	COMB*
A6NDB9	Paralemmin-3	61	COMB*
O60664	Perilipin-3	372	COMB*
Q53H76	Phospholipase A1 member A	214	COMB*
P01833	Polymeric immunoglobulin receptor	171	COMB*
P28290	Protein ITPRID2	151	COMB*
Q6P5S2	Protein LEG1 homolog	208	COMB*
Q9UKK3	Protein mono-ADP-ribosyltransferase PARP4	71	COMB*
Q6GMV3	Putative peptidyl-tRNA hydrolase PTRHD1	343	COMB*
P23468	Receptor-type tyrosine-protein phosphatase delta	164	COMB*
Q9UKL0	REST corepressor 1	292	COMB*
P52566	Rho GDP-dissociation inhibitor 2	336	COMB*
P21817	Ryanodine receptor 1	120	COMB*
Q7Z7L1	Schlafen family member 11	316	COMB*
P02787	Serotransferrin	488	COMB*
Q8TF17	SH3 domain and tetratricopeptide repeat-containing protein 2	164	COMB*
Q99250	Sodium channel protein type 2 subunit alpha	225	COMB*
P78383	Solute carrier family 35 member B1	158	COMB*
O43581	Synaptotagmin-7	272	COMB*
Q8N584	Tetratricopeptide repeat protein 39C	105	COMB*
P30048	Thioredoxin-dependent peroxide reductase_ mitochondrial	154	COMB*
Q9NQE7	Thymus-specific serine protease	122	COMB*

Table 4 (*continued*)

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* COMB: H_2O
Q9C035	Tripartite motif-containing protein 5	634	COMB*
P07437	Tubulin beta chain	262	COMB*
Q13885	Tubulin beta-2A chain	262	COMB*
Q9BVA1	Tubulin beta-2B chain	262	COMB*
P04350	Tubulin beta-4A chain	262	COMB*
P68371	Tubulin beta-4B chain	262	COMB*
Q9NYH9	U3 small nucleolar RNA-associated protein 6 homolog	139	COMB*
Q9H972	Uncharacterized protein C14orf93	180	COMB*
Q99437	V-type proton ATPase 21 kDa proteolipid subunit	197	COMB*
Q641Q2	WASH complex subunit 2A	129	COMB*
Q9Y4E1	WASH complex subunit 2C	129	COMB*
O14628	Zinc finger protein 195	96	COMB*
Q3KQV3	Zinc finger protein 792	68	COMB*
P06733	Alpha-enolase	384	H_2O*
Q8N7 × 0	Androglobin	70	H_2O*
Q8N9B4	Ankyrin repeat domain-containing protein 42	82	H_2O*
P13929	Beta-enolase	384	H_2O*
P13942	Collagen alpha-2(XI) chain	122	H_2O*
Q8WXI2	Connector enhancer of kinase suppressor of ras 2	94	H_2O*
Q53SF7	Cordon-bleu protein-like 1	128	H_2O*
Q86T13	C-type lectin domain family 14 member A	66	H_2O*
O14976	Cyclin-G-associated kinase	75	H_2O*
Q96RT1	Erbin	56	H_2O*
Q5T1M5	FK506-binding protein 15	42	H_2O*
P09104	Gamma-enolase	384	H_2O*
Q14687	Genetic suppressor element 1	89	H_2O*
P47897	Glutamine–tRNA ligase	79	H_2O*
Q8IWJ2	GRIP and coiled-coil domain-containing protein 2	73	H_2O*
P0DMV8	Heat shock 70 kDa protein 1A	32	H_2O*
P0DMV9	Heat shock 70 kDa protein 1B	32	H_2O*
P17066	Heat shock 70 kDa protein 6	33	H_2O*
P69905	Hemoglobin subunit alpha	540	H_2O*
P56524	Histone deacetylase 4	87	H_2O*
P02533	Keratin_ type I cytoskeletal 14	28	H_2O*
Q04695	Keratin_ type I cytoskeletal 17	28	H_2O*
P13647	Keratin_ type II cytoskeletal 5	83	H_2O*
P02538	Keratin_ type II cytoskeletal 6A	246	H_2O*
P04259	Keratin_ type II cytoskeletal 6B	230	H_2O*
P48668	Keratin_ type II cytoskeletal 6C	246	H_2O*
Q8N1A0	Keratin-like protein KRT222	104	H_2O*
P08493	Matrix Gla protein	690	H_2O*
A0JLT2	Mediator of RNA polymerase II transcription subunit 19	372	H_2O*
Q9P1T7	MyoD family inhibitor domain-containing protein	69	H_2O*
Q9P2K9	Protein dispatched homolog 3	37	H_2O*
Q08188	Protein-glutamine gamma-glutamyltransferase E	50	H_2O*
Q9HBR0	Putative sodium-coupled neutral amino acid transporter 10	73	H_2O*
Q9H1J1	Regulator of nonsense transcripts 3A	60	H_2O*
Q9NRY4	Rho GTPase-activating protein 35	53	H_2O*
Q9P0V9	Septin-10	249	H_2O*
A1 × 283	SH3 and PX domain-containing protein 2B	54	H_2O*
P12757	Ski-like protein	44	H_2O*
Q9UJZ1	Stomatin-like protein 2_ mitochondrial	65	H_2O*
Q9Y6J9	TAF6-like RNA polymerase II p300/CBP-associated factor-associated factor 65 kDa subunit 6 L	91	H_2O*
Q9Y4G6	Talin-2	76	H_2O*
A0A1B0GUA7	Testis-expressed protein 51	72	H_2O*
Q9BZW7	Testis-specific gene 10 protein	93	H_2O*
Q13144	Translation initiation factor eIF-2B subunit epsilon	76	H_2O*
Q8IXQ3	Uncharacterized protein C9orf40	143	H_2O*
Q7Z2W4	Zinc finger CCCH-type antiviral protein 1	42	H_2O*

(*continued on next page*)

Table 4 (*continued*)

[a]Acession number	Protein name	PLGS Score	[b]*Ratio* COMB: H_2O
Q9C0A1	Zinc finger homeobox protein 2	176	H_2O*
Q86XU0	Zinc finger protein 677	94	H_2O*

Proteins highlighted in bold are increased or decreased more than 2-fold.

[a] Identification is based on proteins ID from UniProt protein database, reviewed only (http://www.uniprot.org/).

[b] Proteins with expression significantly altered are organized according to the ratio.

* Indicates unique proteins in alphabetical order.

recognized for its ability to reduce erosive demineralization, such as SnF_2 rinse [22]. Despite the mechanism of action of SnF_2 to reduce erosion does not involve acquired pellicle engineering, it would have been interesting to compare the performance of this agent with that of the proteins/peptides tested in the present study.

5. Conclusion

Our results show, for the first time, that treatment with CaneCPI-5, HB or StN15 remarkably increases acid-resistant proteins in the AEP, which protects against erosive demineralization. These findings open an avenue for a new preventive approach for erosive demineralization, employing acquired pellicle engineering procedures that may in the future be incorporated into dental products.

CRediT authorship contribution statement

Thamyris Souza Carvalho: Validation, Formal analysis, Data curation, Investigation, Visualization, Writing - review & editing. **Tamara Teodoro Araújo:** Formal analysis, Investigation, Writing - review & editing. **Talita Mendes Oliveira Ventura:** Formal analysis, Investigation, Writing - review & editing. **Aline Dionizio:** Formal analysis, Investigation, Writing - review & editing. **João Victor Frazão Câmara:** Investigation. **Samanta Mascarenhas Moraes:** Investigation. **Vinicius Taioqui Pelá:** Investigation. **Tatiana Martini:** Investigation. **Julia Chaparro Leme:** Investigation. **Ana Luiza Bogaz Derbotolli:** Investigation. **Larissa Tercilia Grizzo:** Investigation. **Edson Crusca:** Resources, Writing - review & editing. **Priscila Yumi Tanaka Shibao:** Resources, Writing - review & editing. **Reinaldo Marchetto:** Resources, Writing - review & editing. **Flavio Henrique-Silva:** Resources, Writing - review & editing. **Juliano Pelim Pessan:** Resources, Writing - review & editing. **Marília Afonso Rabelo Buzalaf:** Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Supervision, Project administration.

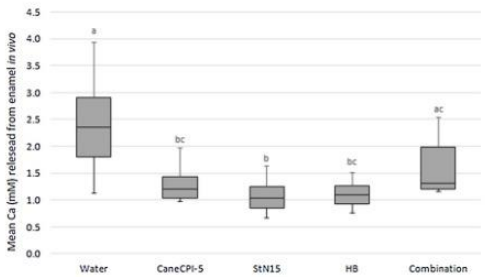

Fig. 1. Calcium released after challenge with 10 µL of 1% citric acid (pH 2.5), for enamel treated in vivo after rinsing for 1 min with 10 mL of deionized water, 0.1 mg/mL CaneCPI-5, 1.0 mg/mL hemoglobin (HB), 1.88×10^{-5} M statherin-derived peptide (StN15) or combination of the 3 proteins/peptide and subsequent acquired enamel pellicle formation for 2 h (Kruskal-Wallis and Dunn's test, $p < 0.05$, $n = 10$).

Declaration of Competing Interest

The authors have declared no conflict of interest.

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Declaration of exclusive use of the article (**ANNEX C**)

Proof of final review (**ANNEX D**)

Hemoglobin protects enamel against intrinsic enamel erosive demineralization

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Short title: Hemoglobin reduces intrinsic erosion

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Abstract

Introduction: This study investigated the changes in the acquired enamel pellicle (AEP) proteome when this integument is formed in vivo after treatment with sugarcane-derived cystatin (CaneCPI-5), hemoglobin (HB), and a statherin-derived peptide (StN15), or their combination and then exposed to an intrinsic acid challenge. The effectiveness of these treatments in preventing intrinsic erosion was also evaluated. **Methods:** Ten volunteers, after prophylaxis, in 5 crossover phases, rinsed with the following solutions (10mL, 1min): control (deionized water-H_2O)-group 1, 0.1mg/mL CaneCPI-5- group 2, 1.0mg/mL HB- group 3, $1.88x10^{-5}$M StN15- group 4 or a blend of these- group 5. Following this, AEP formation occurred (2h) and an enamel biopsy (10µL, 0.01M HCl, pH 2.0, 10s) was conducted on one incisor. The biopsy acid was then analyzed for calcium (Arsenazo method). The vestibular surfaces of the other teeth were treated with the same acid. Acid-resistant proteins in the residual AEP were then collected and analyzed quantitatively via proteomics. **Results:** Compared to control, treatment with the proteins/peptide, mixed or isolated, markedly enhanced acid-resistant proteins in the AEP. Notable increases occurred in pyruvate kinase PKM (11-fold, CaneCPI-5), immunoglobulins and submaxillary gland androgen-regulated protein 3B (4-fold, StN15), Hb and lysozyme-C (2-fold, StN15). Additionally, a range of proteins not commonly identified in the AEP, but known to bind calcium or other proteins were in groups treated with the tested proteins/peptide either in isolation or as a mixture. The mean (SD, mM) calcium concentrations released from enamel were 3.67 ± 1.48^{a}, 3.11 ± 0.72^{a}, 1.94 ± 0.57^{b}, 2.37 ± 0.90^{a} and 2.38 ± 0.45^{a} for groups 1-5, respectively (RM-ANOVA/Tukey, $p<0.05$). **Conclusions:** Our findings demonstrate that all treatments, whether using a combination of proteins/peptides or in isolation, enhanced acid-resistant proteins in the AEP. However, only HB showed effectiveness in protecting against intrinsic erosive demineralization. These results pave the way for innovative preventive methods against intrinsic erosion, using "acquired pellicle engineering" techniques.

1 INTRODUCTION

The organic bacteria-free layer covering the enamel, known as acquired enamel pellicle (AEP), is primarily composed of proteins and glycoproteins, but also includes lipids [1, 2]. It serves as a crucial biological factor to prevent erosive demineralization, acting as a physical protection against acid dissolution [3, 4, 5].

The AEP forms in distinct stages [6]. In the initial stage, leading proteins such as proline-rich proteins (PRPs), histatins, statherin and cystatins, which have a strong affinity for hydroxyapatite and form electrostatic connections with dental enamel, make up the basal layer of the AEP [7]. This layer is extremely electron-dense and appears to provide the majority of the defense against erosive demineralization. Conversely, the later layers exhibit a more relaxed arrangement compared to the initial coating, being composed of globular proteins, which attach to proteins of the basal layer [6].

Interestingly, some proteins forming the foundation of the AEP, even after extensive and severe acid challenges, remain attached to the enamel surface [8, 9]. This prompted our research group to explore, through quantitative proteomic analysis, which proteins within the AEP are stable and not displaced by acids. Several proteins were identified, but cystatin [10], statherin [11] and hemoglobin (HB) [12] were emphasized. These proteins exhibit a high affinity for hydroxyapatite, and it it is proposed, through "acquired pellicle engineering" approaches, that these proteins are ideal candidates to reinforce the AEP, thereby enhancing the enamel surface’s resistance to demineralization by erosive acids. These proteins, whether used alone or in mixture, pave the way for an innovative erosion preventive strategy and are potential candidates for incorporation into dental products.

In a preceding proof-of-concept *in vivo* investigation, we showed that protein treatment significantly increases proteins that are more resistant to removal by citric acid, mimicking extrinsic erosive challenges [13]. However, researching protection against intrinsic erosive demineralization is crucial because the pH of gastric acids is lower, and their buffering ability is stronger when compared with the dietary acids, resulting in more severe damage to dental hard tissues [14]. Therefore, the aim of this study was to assess *in vivo*: 1) the changes in the proteome AEP following treatments with sugarcane-derived cystatin (CaneCPI-5), HB, statherin-derived peptide (StN15) or their mixture prior to AEP formation and subsequent intrinsic acid challenge; 2) the potential of these treatments for protection against intrinsic erosion. The tested null hypotheses were: 1) treatment with CaneCPI-5, HB, StN15 or their mixture prior to

AEP formation and subsequent acid challenge does not modify the proteome of the remaining AEP; and 2) does not protect against intrinsic enamel erosion.

2 Materials and methods

2.1 Ethical aspects and subjects

The Human Ethics Committee (No. 99709318.1.0000.5417) approved the protocol for this study. Prior to the commencement of the study, written informed consent was obtained from all volunteers. For the proteomic analysis, the sample size (n=10) was determined using MSstats [14], based on findings from a previous study [13], with $\alpha = 0.05$ and $1-\beta = 0.8$ considered. The effect size (difference in protein quantity) was set at as 1.5. The anticipated number of volunteers was 3/group. Due to the limited quantity of proteins extracted from the AEP *in vivo*, we recruited 10 participants, enabling the realization the experimental design of 5 groups, while forming 3 pools (biological triplicates). Sample size was also calculated for calcium released from enamel, based on our previous study [13]. With a minimum detectable difference in calcium release of 1.31 mM and standard deviation (SD) of 0.82 mM, an α error of 5% and a β error of 20%, the required sample size was n=7. We decided to include 10 volunteers in the present study, which was sufficient for both response variables (proteomics and calcium released from enamel).

Ten volunteers (4 males and 6 females, age range 18–35) participated in this *in vivo* study. The volunteers met the following inclusion criteria: good health, both oral and general (could not present gingivitis, dental caries, periodontal disease, nor any oral cavity changes that might alter oral fluids' composition), absence of restorations on the buccal surfaces of all upper and lower teeth, non-smokers and not on medication that might decrease saliva flow. Mean (SD) unstimulated and stimulated salivary flows were 0.37(0.07) and 1.29(0.39) mL/min, respectively. Individuals with risk factors for dental erosion, such as excessive consumption of acidic fruits, soft drinks, those with gastric disorders such as gastroesophageal reflux and bulimia, or swimmers, were excluded from participating in this study.

2.2 Types of mouthwash treatments

This research protocol adopted an in vivo, triple-blind (researchers, participants and analysts) and crossover (5 treatment phases) design. All participants underwent each

of the 5, with two participants randomly assigned to one of the 5 treatment groups (selected by computerized random numbers). The rinse solutions used were deionized water (control) (1), 0.1 mg/mL CaneCPI-5 (2), 1.0 mg/mL HB (3), $1.88x10^{-5}$M StN15 (4) or a combination of all proteins/peptide (5).

2.3 In vivo experiment

To mitigate circadian effects in the composition of the AEP, sampling was performed in the morning. In each stage, after prophylaxis, participants rinsed with 10 mL (1 min) of the compounds outlined in section 2.2. Participants were instructed to abstain from drinking and eating for a period of 2h so the AEP was allowed to form. Following, an adhesive tape with an area of 4.92 mm^2 was placed on the left central incisor. Then the exposed surface was treated with 10 µL of 0.01 M hydrochloric acid, (pH 2.0) for 10s and the entire volume was collected with a pipette for calcium analysis performed using the Arsenazo III method [15]. For this purpose, a calibration curve was established with varying calcium concentrations (0.25 to 8.0 mM), and absorbance was measured at 650 nm and 25 ◦C, using a Biotek spectrophotometer. Analyses were conducted in duplicate.

The vestibular surfaces of the remaining lower and upper teeth were randomly treated with 200 µL of the same acid for a duration of 10 seconds, applied using a pipette. Each quadrant of the mouth was rinsed with deionized water to remove the acid, dried gently with compressed air and isolated with cotton rolls to prevent lip contamination. Acid-resistant proteins within AEP were harvested using electrode filter papers, saturated in 3% citric acid [16]. To prevent gingival margin contamination, only 2/3 of the buccal coronal surfaces were treated, where 3% citric acid papers were used for each quadrant. Thus, four papers were obtained from each participant, and a pool was formed by combining papers from 3-4 participants for each treatment. This uniform pooling approach, involving the same participants, aided the proteomic analysis of the AEP in biological triplicate.

2.4 Statistical and proteomic analysis

Protein extraction and quantification were carried out as reported by Ventura et al., 2017. Samples underwent nano reverse phase liquid chromatography coupled to mass spectrometry (nLC-ESI-MS/MS), using the system nanoAcquity UPLC-Xevo QTof MS (Waters, Manchester, UK). All parameters employed were described

elsewhere [16]. Samples were assessed in technical triplicates. Nine MS raw files from every pooled group were submitted to label-free proteomic quantification using Protein Lynx Global Service (PLGS) software (Version 3.0, Waters, Manchester, UK), as reported elsewhere [16]. Variation in expression among groups was verified by *t* test ($p<0.05$), using PLGS software.

2.5 Calcium analysis and statistical analysis

Concentrations of Ca ions released from dental enamel were evaluated using GraphPad InStat software (GraphPad software Inc. La Jolla, CA, USA; version 3.0 for Windows). After checking normality with the test Kolmogorov-Smirnov and homogeneity with Bartlett´s test, data were analyzed by repeated-measures ANOVA and Tukey´s ($p<0.05$).

2.6 Bioinformatics and statistical analysis

Analyses were performed using the Bioinformatics & Evolutionary Genomics software. To create the graph, the IDs of all proteins molecules showing significant changes in the AEP after with HB compared to control, followed by formation of AEP for two hours and further HCl challenge were included. These protein IDs used in the bioinformatics analysis are listed in the complementary table 2. Enrichment-Bonferroni's test was applied.

3 Results

3.1 Rinsing with proteins/peptide increases acid-resistant proteins in the AEP

The average amounts of proteins recovered from the AEP were 6.4 ± 1.5, 8.6 ± 2.2, 9.1 ± 3.9, 13.6 ± 2.7 e 11.5 ± 3.3 µg, treatments 1-5 respectively. The groups treated with proteins/peptide, whether alone or in combination, showed significant increases in many acid-resistant proteins within AEP, compared to the water group. The most notable increases were observed in pyruvate kinase PKM (11-fold, CaneCPI-5), immunoglobulins and submaxillary gland androgen-regulated protein 3B (4-fold, StN15), as well as Hb and lysozyme-C (2-fold, StN15). Furthermore, several proteins not typically found within the AEP, but that bind calcium or other proteins were uniquely identified in the groups treated with the proteins/peptide tested, either alone or in combination (Complementary Tables 1-4).

3.2 Rinsing with Hemoglobin reduces intrinsic enamel erosion and affects biological/molecular processes related to demineralization

After a 10-second challenge with HCl 0.01 M at pH 2, the mean concentrations (± SD) of released Ca were 3.67 ± 1.48, 3.11 ± 0.72, 1.94 ± 0.57, 2 .37 ± 0.90 and 2.38 ± 0.45 mM for the groups where volunteers rinsed with deionized H_2O, CaneCPI-5, HB, StN15 or a mixture of the three proteins, respectively. RM-ANOVA revealed a significant variation among the groups ($F = 2.807$, $p = 0.043$). Tukey´s test only found a significant difference only between the hemoglobin group and the control ($p<0.05$) (Figure 1).

Due to the differences in Ca results, the HB group underwent further analysis regarding biological and molecular processes. The biological and/or molecular processes identified in the comparison of the HB *vs*. H_2O groups were: humoral immune response (37%), keratinocyte differentiation (22%), antimicrobial peptide-mediated antimicrobial humoral immune response (13%), focal adhesion cluster (11%), alpha hemoglobin binding (8%), regulation of epidermal development (6%), and alpha-amylase activity (3%) (Figure 2).

4 Discussion

Acquired pellicle engineering with acid-resistant proteins has emerged as an innovative and promising tool to prevent dental caries [17] and ETW [13, 18, 19]. Various studies, utilizing *in vitro* [20, 21, 22, 23]*, in situ* [18] *and in vivo* [13, 19, 24] protocols, have demonstrated the potential of CaneCPI-5, StN15 and HB in reducing dental demineralization. However, before dental products containing these proteins/peptide can be widely employed to manage dental caries and/or ETW, a deeper understanding of their mechanism of action and additional evidence on their efficacy, particularly from studies that closely mimic clinical conditions, are needed. The *in vivo* protocol used in this study fulfills this requirement. It was initially developed (and proven effective) for evaluating the capability of these proteins/peptide to alter the AEP's proteome and to lessen initial extrinsic dental enamel erosion (induced by citric acid) [13, 19]. In this study, we focused on an intrinsic erosive challenge, induced by an enamel biopsy of a central incisors with 0.01M HCl, pH 2.0, for 10 s. It appears that in acquired pellicle engineering with proteins/peptides, there is an acid-specific protective effect offered by the proteins. For instance, cystatin B, was found to increase 20- and 13-fold in AEP challenged with citric and lactic acids, respectively [10]. Conversely, statherin levels increased after exposure to HCl [11], and hemoglobin (HB)

showed a threefold increase in AEP in GERD patients without ETW compared to GERD patients with ETW [12]. This variation might be attributed to the different pH levels of these acids and the distinct pK values of the involved proteins and peptides. In this study, after challenge with HCl, the HB treatment notably decreased Ca released from enamel compared to the water group, leading to the rejection of our second null hypothesis. However, this was not observed for CaneCPI-5, Stn15 or their mixture, suggesting that among the evaluated treatments, HB is likely the most effective in preventing demineralization from intrinsic erosive challenges (Figure 1).

In this present study, the concentrations of proteins/peptide were based on prior research. CaneCPI-5 at 0.1 mg/mL has been effective against extrinsic erosion *in vitro* [20]*, in situ* [18] and *in vivo* [13], but no studies have shown its effectiveness against intrinsic erosion, aligning with our findings. Typically, gastric acids have a lower pH and higher buffering capacity than dietary acids [25], making intrinsic erosion control more challenging. In our previous extrinsic erosion *in vivo* study, using a similar protocol where CaneCPI-5 protected against extrinsic erosion, 1% citric acid pH 2.5 was [13], while in this study, to simulate intrinsic erosion, we used 0.01 M HCl at pH 2.0.

The statherin-derived peptide in this study contains the 15 N-terminal statherin residues, with phosphorylated serine residues at second and third positions. The entire structure of statherin is not necessary for protective action against erosive challenges [26]. The presence of negative charge density at the N-terminus of statherin-derived peptides facilitates the interaction with hydroxyapatite [27], as the negative charges of the phosphate residues are attractedto the calcium ions of hydroxyapatite. The concentration of StN15 used was 1.88×10^{-5}M, similar to the average concentrations of statherin in saliva [28].

Hemoglobin was evaluated in the present study because recent by our group showed that this protein increased significantly in both AEP [12] and saliva [21] of GERD patients with without ETW compared to those with GERD but with ETW. Additionally, HB is known to have high affinity for hydroxyapatite and is often purified using hydroxyapatite columns [29]. The concentration of HB used was based on an *in vitro* study showing that 1.0 mg/mL HB provides similar protection as 0.1 mg/mL CaneCPI-5 against early intrinsic enamel demineralization [21].

Proteomic analysis was conducted using a protocol developed by our research group, which enhanced protein identification in the samples [30]. Our results revealed that treatment of the tooth surface *in vivo* with proteins having high affinity for

hydroxyapatite increased the number of proteins immobilized in the AEP after the acid challenge (6.4 µg for the control-water group against 8.6-13.6 µg for the experimental groups). Additionally, an increase in many acid-resistant proteins in the AEP was observed (Complementary Tables 1-4), leading to the rejection of the first null hypothesis. Interestingly, protein families with alterations in expression were different among the distinct proteins analyzed (Complementary Tables 1-4). A significant finding of this study is that several proteins not typically associated with the AEP, but that bind calcium or other proteins, were detected exclusively in the groups treated with the tested proteins/peptide, either isolated or in combination. In addition, many proteins with enhanced expression or observed only in the groups that rinsed with the proteins/peptide solutions are produced by the salivary glands and are commonly found in the AEP (e.g., PRPs, cystatin, lysozyme-C, submaxillary gland androgen-regulated protein 3B). These were identified in the groups treated with the rinse solutions containing Cane-CPI-5 and StN15. However, the mixture of the 3 proteins was not as effective in providing protection compared to the water group. The reason behind this finding might be related to the fact that the concentration of each protein in the mixture was equivalent to the concentration of each protein when it was used alone. Considering that they all have great affinity for hydroxyapatite, they may have competed for binding sites on this mineral. Further examination of the effect of mixing these 3 proteins at lower concentrations is necessary, as distinct proteins change AEP structure in different ways (Complementary Tables 1-4).

Among the treatments tested, several intracellular proteins (for example, keratins, histones, and actins) were identified. It is known that proteins in the basal layer of AEP provide the strongest protection against erosive demineralization [8]. Thus, increasing the number of proteins with high affinity for hydroxyapatite in the basal layer, as explored in the present work, may also increase the number of distinct proteins that do not bind to hydroxyapatite, but bind to these key proteins, which may be the case of intracellular proteins. This is consistent with the results found in a recent work by our research group, in which these proteins were highly expressed in the group treated with HB in the AEP formed for 3 min [31]. In the Cane-CPI-5 treatment group, pyruvate kinase (a cadherin-binding protein, which belongs to the calcium-dependent transmembrane glycoprotein family) showed the greatest increase among the identified proteins. Thus, pyruvate kinase could be part of the aggregates of proteins formed in the second phase of AEP formation [6]. It is noteworthy that protein/peptide

treatment increased numerous proteins with antimicrobial properties within the AEP, such as multiple isoforms of immunoglobulins, cystatins, histatins, and lysozyme. Therefore, it is possible that it also provides dental caries prevention, which should be investigated in future studies.

In the current study, where a more intense erosive challenge was administered using 0.01 M HCl pH 2.0, only HB was effective in protecting enamel against erosive demineralization, unlike our previous study, which used an extrinsic erosive demineralization (1% citric acid pH 2.5) [13]. In another recent *in vivo* study by our group, a marked increase (more than 18-fold) in various HB subunits was detected in the 3-min AEP, after 1-min rinse with human HB [31]. These findings collectively support the great potential of HB to enhance the basal layer of the AEP and protect against ETW. Based on this, we conducted bioinformatics analysis to determine which biological and/or molecular processes were most affected by rinsing with HB, compared to the negative control. Most of the affected processes (Figure 2) involved antimicrobial defense, such as humoral immune response (37%) and antimicrobial peptide-mediated antimicrobial humoral immune response (13%). These processes might be significant for anticaries activity, which should be further investigated in future studies.

Among biological processes affected in the comparison between the HB and water groups, we found alpha-amylase activity (3%) and binding to alpha hemoglobin (8%), underscoring the protective effect of the treatment with the HB-containing solution. The alpha-amylase protein is typically present in the basal layer of AEP [32], and two subunits of alpha-amylases decreased upon treatment with HB (Complementary Table 2), suggesting that HB, owing to its strong affinity for hydroxyapatite, likely bound preferentially to this mineral, leaving fewer binding sites for other proteins of the basal layer. HB is a tetramer comprising 2 pairs of globins, each linked to a heme group. In adult humans, HBs consist of 96.5-98.5% HbA1 (α2β2 dimer) and 1.5-3.5% HbA2 (α2δ2 dimer), while Hb ε replaces the β chain in embryos. The various Hb subunits have distinct amino acid sequences [33]. Among them, the α chain contains the highest proportion of serine, threonine, and tyrosine residues that could be phosphorylated, imparting a negative charge to the protein, thereby enhancing its capacity to bind to hydroxyapatite [29]. Thus, it is likely that the α chain of Hb binds to Ca residues of hydroxyapatite.

Another noteworthy observation was that 11% of the processes related to focal adhesion assembly. These structures are multiprotein complexes comprising integrins, forming mechanical linkages between intracellular actin bundles and the extracellular substrate in multiple cell types. Intracellular proteins were also prominently featured in the proteomic analysis of previous studies [13, 24]. They appear to be integral to some important structural arrangements within the AEP. The fact that 22% of biological processes pertain to the differentiation of keratinocytes and 6% to the regulation of epidermal development may be associated with proteins from desquamated epithelial cells, which are also components of the AEP [32, 24].

Despite the present study being conducted *in vivo*, both the treatment and the acidic challenge were administered only once. In addition, an abrasive challenge, commonly associated with erosive conditions in the clinical setting, was not included. Thus, future studies should focus on protocols involving prolonged erosive challenges combined with abrasion by toothbrushing. Solutions [18] and chitosan gels [34] containing CaneCPI-5 have been shown to be more effective than placebo and as effective as commercial stannous mouthwash or NaF gel in preventing prolonged erosion (citric acid) combined with abrasion *in situ*. Similar results were observed for StN15 for erosive challenges conducted with HCl (unpublished data). However, HB has yet to be evaluated under similar protocols. Another limitation of the study was the requirement for the application of the mouthwashes on pellicle-free teeth, necessitating professional prophylaxis and limiting their practical use. Recently, we observed that CaneCPI-5 and vitamin-E solutions have a synergistic effect against enamel erosion, being more effective than commercial stannous mouthwash *in vitro*. Additionally, this combination was effective regardless of the presence of the pellicle during its application [35]. These findings should be validated using protocols that more closely mimic clinical conditions.

In conclusion, our results indicate that the *in vivo* enamel proteins/peptide treatment, either independently or combined, can increase acid-resistant proteins within the AEP, but only HB prevents intrinsic initial erosion. These findings lay the groundwork for an innovative approach for intrinsic erosive demineralization prevention, using engineering of the acquired pellicle techniques that could be incorporated into future dental products.

Statement of Ethics: *Hemoglobin protects enamel against intrinsic enamel erosive demineralization* was conducted under the World Medical Association Declaration of Helsink, and approved by the Human Ethics Committee of Bauru School of Dentistry, University of São Paulo (protocol No. 99709318.1.0000.5417). Participants signed a written informed consent.

Conflict of interest statement: The authors have no conflicts of interest to declare that are pertinent to the content of this study.

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Author contributions: Conceived and designed the experiments: T.S.C, R.M., F.H.-S. and M.A.R.B; Acquired data: T. S. C, T. T. A, T. M. O. V, A. D, J. V. F. C, S. M. M, J. C. L, L. T. G, E. C, P. Y. T. S, R. M, F. Henrique-Silva and J. P. P; Analyzed and interpreted data: T. S. C, T. T. A, A. D and M. A. R. B; Drafted the manuscript: T. S. C, T. T. A, A. D and M. A. R. B. Critically revised the manuscript: T. S. C and M. A. R. B; Approved the final version to be submitted: All the authors.

Data Availability Statement: All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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Figures Legends

Fig 1. Calcium released from dental enamel after rinsing (1 min) with 10 mL of water (control), 0.1 mg/mL sugarcane-derived cystatin (CaneCPI-5), 1.0 mg/mL hemoglobin (HB), $1.88x10^{-5}$ M statherin-derived peptide (StN15) or mixture of the 3 proteins/peptide, followed by formation of the AEP (2 h) and acid challenge (0.01 M hydrochloric acid pH 2) for 10 s (RM-ANOVA and Tukey´s, $p<0.05$, n=10).

Fig 2. Graph representing the biological and/or molecular processes present in the comparison 1.0 mg/mL hemoglobin (HB) *vs.* water (control).

Figure 1- Calcium released from enamel

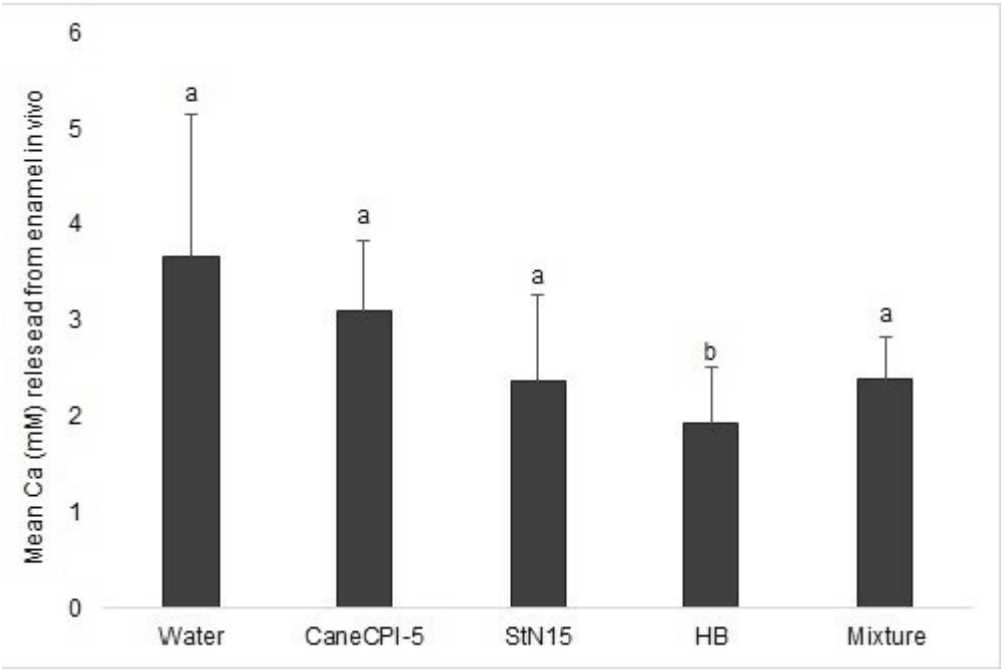

Fig 1. Calcium released from dental enamel after rinsing (1 min) with 10 mL of water (control), 0.1 mg/mL sugarcane-derived cystatin (CaneCPI-5), 1.0 mg/mL hemoglobin (HB), $1.88x10^{-5}$ M statherin-derived peptide (StN15) or mixture of the 3 proteins/peptide, followed by formation of the AEP (2 h) and acid challenge (0.01 M hydrochloric acid pH 2) for 10 s (RM-ANOVA and Tukey´s, $p<0.05$, n=10).

Figure 2- Hemoglobin *vs* control

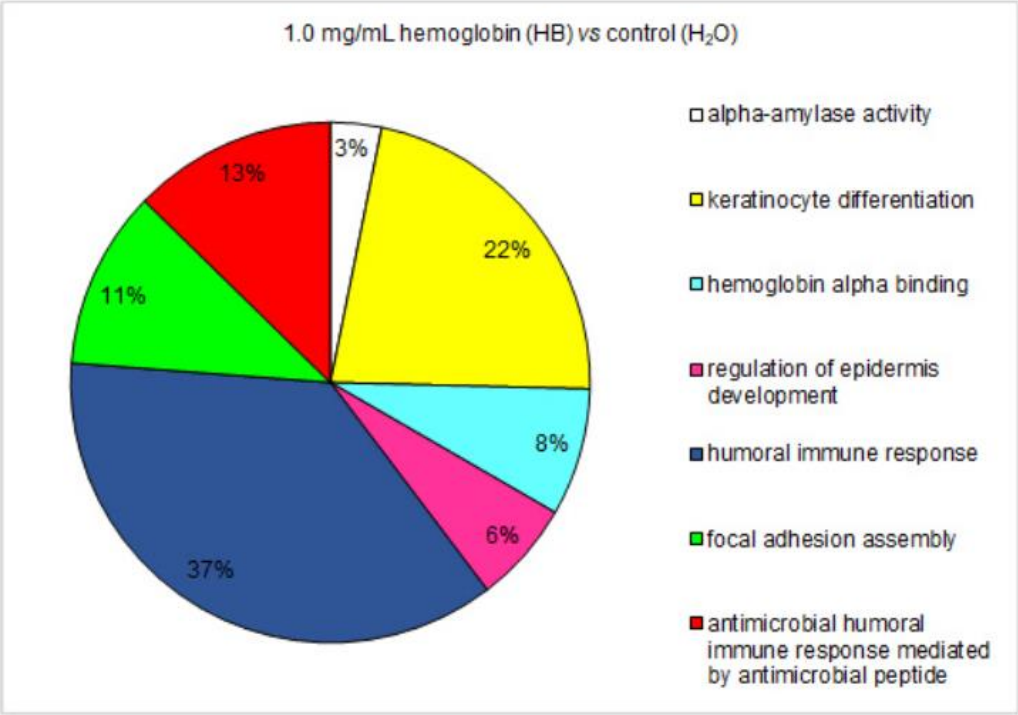

Fig 2. Graph representing the biological and/or molecular processes present in the comparison 1.0 mg/mL hemoglobin (HB) *vs.* water (control).

Complementary Tables

Table 1. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with 0.1 mg/mL sugarcane-derived cystatin (CaneCPI-5), for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* CANE:water
P14618	**Pyruvate kinase PKM**	**383**	**11,13**
P01037	**Cystatin-SN**	**467**	**2,25**
P02814	**Submaxillary gland androgen-regulated protein 3B**	**1492**	**2,23**
P02768	Serum albumin	1688	1,19
P04083	Annexin A1	183	0,73
P01876	**Immunoglobulin heavy constant alpha 1**	**213**	**0,47**
P04745	**Alpha-amylase 1**	**1445**	**0,41**
P01877	**Immunoglobulin heavy constant alpha 2**	**132**	**0,39**
P19961	**Alpha-amylase 2B**	**1394**	**0,37**
P27482	Calmodulin-like protein 3	126	CANE*
Q99795	Cell surface A33 antigen	111	CANE*
Q8N2C3	DEP domain-containing protein 4	366	CANE*
Q8TDM6	Disks large homolog 5	107	CANE*
O00423	Echinoderm microtubule-associated protein-like 1	74	CANE*
Q6PIW4	Fidgetin-like protein 1	244	CANE*
P01834	Immunoglobulin kappa constant	437	CANE*
Q13099	Intraflagellar transport protein 88 homolog	76	CANE*
Q9NYL2	Mitogen-activated protein kinase kinase kinase 20	172	CANE*
Q9GZN6	Orphan sodium- and chloride-dependent neurotransmitter transporter NTT5	152	CANE*
O43660	Pleiotropic regulator 1	128	CANE*
O15037	Protein KHNYN	114	CANE*
Q99497	Protein/nucleic acid deglycase DJ-1	200	CANE*
Q15287	RNA-binding protein with serine-rich domain 1	123	CANE*
Q9NQU5	Serine/threonine-protein kinase PAK 6	335	CANE*
Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1	95	CANE*

Table 1. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with 0.1 mg/mL sugarcane-derived cystatin (CaneCPI-5), for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(continued)

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* CANE:water
Q9BZW7	Testis-specific gene 10 protein	56	CANE*
O00268	Transcription initiation factor TFIID subunit 4	133	CANE*
Q9C035	Tripartite motif-containing protein 5	301	CANE*
Q96JP2	Unconventional myosin-XVB	104	CANE*
P02708	Acetylcholine receptor subunit alpha	77	Water*
Q9UJX3	Anaphase-promoting complex subunit 7	149	Water*
Q5T2E6	Armadillo-like helical domain-containing protein 3	96	Water*
Q6PL18	ATPase family AAA domain-containing protein 2	64	Water*
O75185	Calcium-transporting ATPase type 2C member 2	66	Water*
P27797	Calreticulin	163	Water*
P0DO97	Coiled-coil domain-containing protein 192	98	Water*
Q96A83	Collagen alpha-1(XXVI) chain	111	Water*
Q02246	Contactin-2	172	Water*
Q9UBG3	Cornulin	105	Water*
Q86XP0	Cytosolic phospholipase A2 delta	68	Water*
Q8TEA8	D-aminoacyl-tRNA deacylase 1	152	Water*
Q8N1I0	Dedicator of cytokinesis protein 4	397	Water*
Q9UGM3	Deleted in malignant brain tumors 1 protein	75	Water*
Q9Y485	DmX-like protein 1	83	Water*
Q9UNA4	DNA polymerase iota	537	Water*
Q8N136	Dynein assembly factor with WDR repeat domains 1	86	Water*
Q8NHG8	E3 ubiquitin-protein ligase ZNRF2	113	Water*
Q5MNV8	F-box only protein 47	156	Water*
P14136	Glial fibrillary acidic protein	87	Water*
P68871	Hemoglobin subunit beta	310	Water*
P02042	Hemoglobin subunit delta	310	Water*
P02100	Hemoglobin subunit epsilon	310	Water*
P69891	Hemoglobin subunit gamma-1	310	Water*

Table 1. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with 0.1 mg/mL sugarcane-derived cystatin (CaneCPI-5), for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(continued)

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* CANE:water
P69892	Hemoglobin subunit gamma-2	310	Water*
A0A2R8Y619	Histone H2B	106	Water*
Q96A08	Histone H2B type 1-A	106	Water*
P33778	Histone H2B type 1-B	292	Water*
P62807	Histone H2B type 1-C/E/F/G/I	292	Water*
P58876	Histone H2B type 1-D	292	Water*
Q93079	Histone H2B type 1-H	292	Water*
P06899	Histone H2B type 1-J	292	Water*
O60814	Histone H2B type 1-K	292	Water*
Q99880	Histone H2B type 1-L	292	Water*
Q99879	Histone H2B type 1-M	292	Water*
Q99877	Histone H2B type 1-N	292	Water*
P23527	Histone H2B type 1-O	292	Water*
Q16778	Histone H2B type 2-E	292	Water*
Q5QNW6	Histone H2B type 2-F	292	Water*
Q8N257	Histone H2B type 3-B	292	Water*
P57053	Histone H2B type F-S	292	Water*
Q4G0P3	Hydrocephalus-inducing protein homolog	192	Water*
P01857	Immunoglobulin heavy constant gamma 1	90	Water*
P01859	Immunoglobulin heavy constant gamma 2	33	Water*
P01860	Immunoglobulin heavy constant gamma 3	38	Water*
P01861	Immunoglobulin heavy constant gamma 4	38	Water*
P0CG04	Immunoglobulin lambda constant 1	191	Water*
P0DOY2	Immunoglobulin lambda constant 2	191	Water*
P0DOY3	Immunoglobulin lambda constant 3	191	Water*
P0CF74	Immunoglobulin lambda constant 6	98	Water*
A0M8Q6	Immunoglobulin lambda constant 7	98	Water*
B9A064	Immunoglobulin lambda-like polypeptide 5	191	Water*
Q9NVH2	Integrator complex subunit 7	229	Water*
Q99456	Keratin_ type I cytoskeletal 12	75	Water*
P19013	Keratin_ type II cytoskeletal 4	336	Water*

Table 1. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with 0.1 mg/mL sugarcane-derived cystatin (CaneCPI-5), for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(continued)

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* CANE:water
P13647	Keratin_ type II cytoskeletal 5	101	Water*
P02538	Keratin_ type II cytoskeletal 6A	147	Water*
P04259	Keratin_ type II cytoskeletal 6B	147	Water*
P48668	Keratin_ type II cytoskeletal 6C	147	Water*
P08729	Keratin_ type II cytoskeletal 7	87	Water*
Q86Y46	Keratin_ type II cytoskeletal 73	229	Water*
P05787	Keratin_ type II cytoskeletal 8	87	Water*
Q6KB66	Keratin_ type II cytoskeletal 80	87	Water*
Q86W92	Liprin-beta-1	202	Water*
Q9BY66	Lysine-specific demethylase 5D	77	Water*
Q8TAX7	Mucin-7	257	Water*
Q99972	Myocilin	96	Water*
Q06710	Paired box protein Pax-8	168	Water*
P0CG38	POTE ankyrin domain family member I	98	Water*
P0CG39	POTE ankyrin domain family member J	98	Water*
Q9UKI3	Pre-B lymphocyte protein 3	355	Water*
Q9HCU5	Prolactin regulatory element-binding protein	148	Water*
Q9H714	Protein associated with UVRAG as autophagy enhancer	58	Water*
Q9HAT1	Protein ERGIC-53-like	400	Water*
Q9Y5H0	Protocadherin gamma-A3	140	Water*
Q9BRP9	Putative uncharacterized protein MGC13053	148	Water*
Q86TS7	Putative UPF0730 protein encoded by LINC00643	411	Water*
A5PLK6	Regulator of G-protein signaling protein-like	139	Water*
Q8N122	Regulatory-associated protein of mTOR	102	Water*
Q9Y2P8	RNA 3'-terminal phosphate cyclase-like protein	184	Water*
Q96PQ1	Sialic acid-binding Ig-like lectin 12	242	Water*
Q9BSK2	Solute carrier family 25 member 33	122	Water*
Q15772	Striated muscle preferentially expressed protein kinase	327	Water*

Table 1. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with 0.1 mg/mL sugarcane-derived cystatin (CaneCPI-5), for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(conclusion)

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* CANE:water
Q9NXG6	Transmembrane prolyl 4-hydroxylase	166	Water*
Q7L1V2	Vacuolar fusion protein MON1 homolog B	511	Water*
O43895	Xaa-Pro aminopeptidase 2	188	Water*
Q8WYQ9	Zinc finger CCHC domain-containing protein 14	55	Water*

[a]Identification is based on proteins ID from UniProt protein database, reviewed only (http://www.uniprot.org/).
[b]Proteins with expression significantly modified are organized according to the ratio.
*Indicates unique proteins in alphabetical order.
Proteins highlighted in bold are enhanced or decreased more than 2-fold.

Table 2. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with 1.0 mg/mL Hemoglobin (HB) for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

[a]Number of acession	Proteins	PLGS Score	[b]*Ratio* HB:water
P01036	Cystatin-S	693	0,66
P02808	Statherin	10611	0,64
P06702	Protein S100-A9	3052	0,61
P02814	Submaxillary gland androgen-regulated protein 3B	1492	0,54
P02768	Serum albumin	1688	0,53
P60709	Actin_ cytoplasmic 1	1073	0,52
P63261	Actin_ cytoplasmic 2	1073	0,51
P01857	**Immunoglobulin heavy constant gamma 1**	**90**	**0,49**
P05109	**Protein S100-A8**	**972**	**0,48**
P04792	**Heat shock protein beta-1**	**2077**	**0,48**
P19013	**Keratin_ type II cytoskeletal 4**	**336**	**0,47**
P04745	**Alpha-amylase 1**	**1445**	**0,45**
P19961	**Alpha-amylase 2B**	**1394**	**0,42**
P02788	**Lactotransferrin**	**122**	**0,42**
P31947	**14-3-3 protein sigma**	**269**	**0,40**
Q6S8J3	**POTE ankyrin domain family member E**	**290**	**0,39**
P25311	**Zinc-alpha-2-glycoprotein**	**914**	**0,39**

Table 2. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with 1.0 mg/mL Hemoglobin (HB) for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(continued)

[a]Number of acession	Proteins	PLGS Score	[b]*Ratio* HB:water
P68032	**Actin_ alpha cardiac muscle 1**	**860**	**0,38**
P01876	**Immunoglobulin heavy constant alpha 1**	**213**	**0,38**
Q562R1	**Beta-actin-like protein 2**	**377**	**0,38**
P68133	**Actin_ alpha skeletal muscle**	**860**	**0,36**
P62736	**Actin_ aortic smooth muscle**	**860**	**0,36**
P63267	**Actin_ gamma-enteric smooth muscle**	**860**	**0,36**
P01877	**Immunoglobulin heavy constant alpha 2**	**132**	**0,35**
P04746	**Pancreatic alpha-amylase**	**836**	**0,35**
P04083	**Annexin A1**	**183**	**0,32**
A5A3E0	**POTE ankyrin domain family member F**	**290**	**0,32**
Q9BYX7	**Putative beta-actin-like protein 3**	**62**	**0,32**
P0CG38	**POTE ankyrin domain family member I**	**98**	**0,29**
P59665	**Neutrophil defensin 1**	**1578**	**0,24**
P59666	**Neutrophil defensin 3**	**1578**	**0,23**
P04264	**Keratin_ type II cytoskeletal 1**	**140**	**0,18**
Q8TEA8	**D-aminoacyl-tRNA deacylase 1**	**152**	**0,15**
P12814	Alpha-actinin-1	388	HB*
P35609	Alpha-actinin-2	383	HB*
Q08043	Alpha-actinin-3	383	HB*
O43707	Alpha-actinin-4	388	HB*
P27482	Calmodulin-like protein 3	232	HB*
P23280	Carbonic anhydrase 6	310	HB*
Q8WXI2	Connector enhancer of kinase suppressor of ras 2	75	HB*
P04080	Cystatin-B	341	HB*
P09228	Cystatin-SA	385	HB*
Q8WTX7	Cytosolic arginine sensor for mTORC1 subunit 1	88	HB*
Q9Y620	DNA repair and recombination protein RAD54B	272	HB*
Q09019	Dystrophia myotonica WD repeat-containing protein	79	HB*

Table 2. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with 1.0 mg/mL Hemoglobin (HB) for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(continued)

[a]Number of acession	Proteins	PLGS Score	[b]*Ratio* HB:water
Q969U6	F-box/WD repeat-containing protein 5	218	HB*
P04075	Fructose-bisphosphate aldolase A	132	HB*
P62805	Histone H4	153	HB*
P01834	Immunoglobulin kappa constant	1398	HB*
P56192	Methionine--tRNA ligase_ cytoplasmic	140	HB*
Q9UKY7	Protein CDV3 homolog	218	HB*
Q13464	Rho-associated protein kinase 1	83	HB*
O75116	Rho-associated protein kinase 2	83	HB*
Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1	92	HB*
Q03169	Tumor necrosis factor alpha-induced protein 2	360	HB*
Q96DA0	Zymogen granule protein 16 homolog B	481	HB*
P02708	Acetylcholine receptor subunit alpha	77	water*
Q9UJX3	Anaphase-promoting complex subunit 7	149	water*
Q01484	Ankyrin-2	189	water*
Q5T2E6	Armadillo-like helical domain-containing protein 3	96	water*
Q6PL18	ATPase family AAA domain-containing protein 2	64	water*
O75185	Calcium-transporting ATPase type 2C member 2	66	water*
P27797	Calreticulin	163	water*
P0DO97	Coiled-coil domain-containing protein 192	98	water*
Q96A83	Collagen alpha-1(XXVI) chain	111	water*
Q02246	Contactin-2	172	water*
Q9UBG3	Cornulin	105	water*
Q86XP0	Cytosolic phospholipase A2 delta	68	water*
Q8N1I0	Dedicator of cytokinesis protein 4	397	water*
Q9UGM3	Deleted in malignant brain tumors 1 protein	75	water*
Q9Y485	DmX-like protein 1	83	water*

Table 2. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with 1.0 mg/mL Hemoglobin (HB) for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(continued)

[a]Number of acession	Proteins	PLGS Score	[b]*Ratio* HB:water
Q9UNA4	DNA polymerase iota	537	water*
Q8N136	Dynein assembly factor with WDR repeat domains 1	86	water*
Q8NHG8	E3 ubiquitin-protein ligase ZNRF2	113	water*
Q5MNV8	F-box only protein 47	156	water*
P14136	Glial fibrillary acidic protein	87	water*
P68871	Hemoglobin subunit beta	310	water*
P02042	Hemoglobin subunit delta	310	water*
P02100	Hemoglobin subunit epsilon	310	water*
P69891	Hemoglobin subunit gamma-1	310	water*
P69892	Hemoglobin subunit gamma-2	310	water*
A0A2R8Y619	Histone H2B	106	water*
Q96A08	Histone H2B type 1-A	106	water*
P33778	Histone H2B type 1-B	292	water*
P62807	Histone H2B type 1-C/E/F/G/I	292	water*
P58876	Histone H2B type 1-D	292	water*
Q93079	Histone H2B type 1-H	292	water*
P06899	Histone H2B type 1-J	292	water*
O60814	Histone H2B type 1-K	292	water*
Q99880	Histone H2B type 1-L	292	water*
Q99879	Histone H2B type 1-M	292	water*
Q99877	Histone H2B type 1-N	292	water*
P23527	Histone H2B type 1-O	292	water*
Q16778	Histone H2B type 2-E	292	water*
Q5QNW6	Histone H2B type 2-F	292	water*
Q8N257	Histone H2B type 3-B	292	water*
P57053	Histone H2B type F-S	292	water*
P01859	Immunoglobulin heavy constant gamma 2	33	water*
P01860	Immunoglobulin heavy constant gamma 3	38	water*
P0CG04	Immunoglobulin lambda constant 1	191	water*
P0DOY2	Immunoglobulin lambda constant 2	191	water*
P0DOY3	Immunoglobulin lambda constant 3	191	water*
P0CF74	Immunoglobulin lambda constant 6	98	water*

Table 2. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with 1.0 mg/mL Hemoglobin (HB) for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(continued)

[a]Number of acession	Proteins	PLGS Score	[b]*Ratio* HB:water
A0M8Q6	Immunoglobulin lambda constant 7	98	water*
B9A064	Immunoglobulin lambda-like polypeptide 5	191	water*
Q9NVH2	Integrator complex subunit 7	229	water*
P13645	Keratin_ type I cytoskeletal 10	140	water*
P13647	Keratin_ type II cytoskeletal 5	101	water*
P02538	Keratin_ type II cytoskeletal 6A	147	water*
P04259	Keratin_ type II cytoskeletal 6B	147	water*
P48668	Keratin_ type II cytoskeletal 6C	147	water*
P08729	Keratin_ type II cytoskeletal 7	87	water*
P05787	Keratin_ type II cytoskeletal 8	87	water*
Q6KB66	Keratin_ type II cytoskeletal 80	87	water*
Q86W92	Liprin-beta-1	202	water*
Q9BY66	Lysine-specific demethylase 5D	77	water*
P61626	Lysozyme C	519	water*
Q8TAX7	Mucin-7	257	water*
P05164	Myeloperoxidase	133	water*
Q99972	Myocilin	96	water*
P60660	Myosin light polypeptide 6	498	water*
Q06710	Paired box protein Pax-8	168	water*
P0CG39	POTE ankyrin domain family member J	98	water*
Q9UKI3	Pre-B lymphocyte protein 3	355	water*
P07737	Profilin-1	134	water*
Q9HCU5	Prolactin regulatory element-binding protein	148	water*
Q9H714	Protein associated with UVRAG as autophagy enhancer	58	water*
Q9HAT1	Protein ERGIC-53-like	400	water*
Q9Y5H0	Protocadherin gamma-A3	140	water*
Q9BRP9	Putative uncharacterized protein MGC13053	148	water*
Q86TS7	Putative UPF0730 protein encoded by LINC00643	411	water*
A5PLK6	Regulator of G-protein signaling protein-like	139	water*
Q8N122	Regulatory-associated protein of mTOR	102	water*

Table 2. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with 1.0 mg/mL Hemoglobin (HB) for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(conclusion)

[a]Number of acession	Proteins	PLGS Score	[b]*Ratio* HB:water
Q9Y2P8	RNA 3'-terminal phosphate cyclase-like protein	184	water*
Q96PQ1	Sialic acid-binding Ig-like lectin 12	242	water*
Q9BSK2	Solute carrier family 25 member 33	122	water*
Q9NXG6	Transmembrane prolyl 4-hydroxylase	166	water*
Q7L1V2	Vacuolar fusion protein MON1 homolog B	511	water*
O43895	Xaa-Pro aminopeptidase 2	188	water*
Q8WYQ9	Zinc finger CCHC domain-containing protein 14	55	water*

[a]Identification is based on proteins ID from UniProt protein database, reviewed only (http://www.uniprot.org/).
[b]Proteins with expression significantly modified are organized according to the ratio.
*Indicates unique proteins in alphabetical order.
Proteins highlighted in bold are enhanced or decreased more than 2-fold.

Table 3. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with statherin-derived peptide (StN15) (1.88 x 10^{-5}M) for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* StN15:water
P01859	**Immunoglobulin heavy constant gamma 2**	**33**	**4,62**
P02814	**Submaxillary gland androgen-regulated protein 3B**	**1492**	**4,10**
P61626	Lysozyme C	519	1,86
P02042	Hemoglobin subunit delta	310	1,68
P69891	Hemoglobin subunit gamma-1	310	1,68
P02100	Hemoglobin subunit epsilon	310	1,67
P69892	Hemoglobin subunit gamma-2	310	1,67
P68871	Hemoglobin subunit beta	310	1,65
P0CG38	POTE ankyrin domain family member I	98	1,26
P01857	Immunoglobulin heavy constant gamma 1	90	0,87

Table 3. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with statherin-derived peptide (StN15) (1.88 x 10^{-5}M) for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(continued)

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* StN15:water
P05109	Protein S100-A8	972	0,84
P04792	Heat shock protein beta-1	2077	0,84
P06702	Protein S100-A9	3052	0,76
Q9BYX7	Putative beta-actin-like protein 3	62	0,76
P05164	Myeloperoxidase	133	0,75
P01860	Immunoglobulin heavy constant gamma 3	38	0,75
P13646	Keratin_ type I cytoskeletal 13	146	0,71
P25311	Zinc-alpha-2-glycoprotein	914	0,63
P13645	Keratin_ type I cytoskeletal 10	140	0,63
P01861	Immunoglobulin heavy constant gamma 4	38	0,61
P01861	Immunoglobulin heavy constant gamma 4	38	0,61
P01861	Immunoglobulin heavy constant gamma 4	38	0,61
P06899	Histone H2B type 1-J	292	0,60
P59666	Neutrophil defensin 3	1578	0,59
Q99879	Histone H2B type 1-M	292	0,59
P33778	Histone H2B type 1-B	292	0,58
P62807	Histone H2B type 1-C/E/F/G/I	292	0,58
Q8N257	Histone H2B type 3-B	292	0,58
P57053	Histone H2B type F-S	292	0,57
P58876	Histone H2B type 1-D	292	0,57
Q93079	Histone H2B type 1-H	292	0,57
O60814	Histone H2B type 1-K	292	0,57
Q99880	Histone H2B type 1-L	292	0,57
Q99877	Histone H2B type 1-N	292	0,57
P23527	Histone H2B type 1-O	292	0,57
Q16778	Histone H2B type 2-E	292	0,57
Q5QNW6	Histone H2B type 2-F	292	0,57
P59665	Neutrophil defensin 1	1578	0,55
A0A2R8Y619	Histone H2B	106	0,54
P01876	Immunoglobulin heavy constant alpha 1	213	0,54
P01877	Immunoglobulin heavy constant alpha 2	132	0,54
Q96A08	Histone H2B type 1-A	106	0,53
P02768	**Serum albumin**	**1688**	**0,45**

Table 3. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with statherin-derived peptide (StN15) (1.88 x 10^{-5}M) for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(continued)

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* StN15:water
Q8TAX7	**Mucin-7**	**257**	**0,44**
Q9UGM3	**Deleted in malignant brain tumors 1 protein**	**75**	**0,44**
Q9UGM3	**Deleted in malignant brain tumors 1 protein**	**75**	**0,44**
P13647	**Keratin_ type II cytoskeletal 5**	**101**	**0,37**
P02538	**Keratin_ type II cytoskeletal 6A**	**147**	**0,36**
P04259	**Keratin_ type II cytoskeletal 6B**	**147**	**0,36**
P48668	**Keratin_ type II cytoskeletal 6C**	**147**	**0,35**
P60660	**Myosin light polypeptide 6**	**498**	**0,35**
P27797	**Calreticulin**	**163**	**0,29**
P12273	**Prolactin-inducible protein**	**155**	**0,24**
P19961	**Alpha-amylase 2B**	**1394**	**0,22**
P04746	**Pancreatic alpha-amylase**	**836**	**0,18**
P04745	**Alpha-amylase 1**	**1445**	**0,17**
Q13131	5'-AMP-activated protein kinase catalytic subunit alpha-1	192	StN15*
P54646	5'-AMP-activated protein kinase catalytic subunit alpha-2	97	StN15*
O14561	Acyl carrier protein_ mitochondrial	575	StN15*
Q96CM8	Acyl-CoA synthetase family member 2_ mitochondrial	427	StN15*
P10696	Alkaline phosphatase_ germ cell type	95	StN15*
Q13367	AP-3 complex subunit beta-2	80	StN15*
A6NEK1	Arrestin domain-containing protein 5	94	StN15*
O43252	Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase 1	78	StN15*
Q8IV04	Carabin	110	StN15*
Q6ZRK6	Coiled-coil domain-containing protein 73	127	StN15*
Q9H6Q4	Cytosolic iron-sulfur assembly component 3	80	StN15*
Q13561	Dynactin subunit 2	84	StN15*

Table 3. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with statherin-derived peptide (StN15) (1.88 x 10^{-5}M) for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(continued)

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* StN15:water
O95208	Epsin-2	84	StN15*
Q969U6	F-box/WD repeat-containing protein 5	368	StN15*
Q9NSN8	Gamma-1-syntrophin	485	StN15*
Q12879	Glutamate receptor ionotropic_ NMDA 2A	77	StN15*
P69905	Hemoglobin subunit alpha	256	StN15*
P01834	Immunoglobulin kappa constant	209	StN15*
P11215	Integrin alpha-M	109	StN15*
P78413	Iroquois-class homeodomain protein IRX-4	284	StN15*
Q53EV4	Leucine-rich repeat-containing protein 23	84	StN15*
Q9C0I9	Leucine-rich repeat-containing protein 27	89	StN15*
Q14676	Mediator of DNA damage checkpoint protein 1	119	StN15*
A0JLT2	Mediator of RNA polymerase II transcription subunit 19	671	StN15*
P01106	Myc proto-oncogene protein	152	StN15*
P29597	Non-receptor tyrosine-protein kinase TYK2	196	StN15*
Q9GZN6	Orphan sodium- and chloride-dependent neurotransmitter transporter NTT5	100	StN15*
O60664	Perilipin-3	273	StN15*
P20618	Proteasome subunit beta type-1	79	StN15*
Q8IXR5	Protein FAM178B	67	StN15*
Q8TDP1	Ribonuclease H2 subunit C	135	StN15*
Q8WVD5	RING finger protein 141	164	StN15*
Q8TE82	SH3 domain and tetratricopeptide repeat-containing protein 1	68	StN15*
Q1XH10	SKI/DACH domain-containing protein 1	65	StN15*
P0DMW4	Small integral membrane protein 10-like protein 2A	255	StN15*

Table 3. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with statherin-derived peptide (StN15) (1.88 x 10^{-5}M) for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(continued)

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* StN15:water
P0DMW5	Small integral membrane protein 10-like protein 2B	255	StN15*
Q8WXA9	Splicing regulatory glutamine/lysine-rich protein 1	95	StN15*
P59095	StAR-related lipid transfer protein 6	101	StN15*
Q9BZW7	Testis-specific gene 10 protein	193	StN15*
Q969Y2	tRNA modification GTPase GTPBP3_ mitochondrial	66	StN15*
			StN15*
P08631	Tyrosine-protein kinase HCK	324	StN15*
Q96KH6	Uncharacterized protein C18orf12	192	StN15*
Q86WZ6	Zinc finger protein 227	309	StN15*
P51508	Zinc finger protein 81	208	StN15*
Q96DA0	Zymogen granule protein 16 homolog B	373	StN15*
P31947	14-3-3 protein sigma	269	water*
P02708	Acetylcholine receptor subunit alpha	77	water*
Q9UJX3	Anaphase-promoting complex subunit 7	149	water*
Q01484	Ankyrin-2	189	water*
Q5T2E6	Armadillo-like helical domain-containing protein 3	96	water*
Q6PL18	ATPase family AAA domain-containing protein 2	64	water*
O75185	Calcium-transporting ATPase type 2C member 2	66	water*
P0DO97	Coiled-coil domain-containing protein 192	98	water*
Q96A83	Collagen alpha-1(XXVI) chain	111	water*
Q02246	Contactin-2	172	water*
Q9UBG3	Cornulin	105	water*
Q86XP0	Cytosolic phospholipase A2 delta	68	water*
Q8TEA8	D-aminoacyl-tRNA deacylase 1	152	water*
Q9Y485	DmX-like protein 1	83	water*
Q8N136	Dynein assembly factor with WDR repeat domains 1	86	water*

Table 3. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with statherin-derived peptide (StN15) (1.88 x 10^{-5}M) for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(continued)

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* StN15:water
Q8NHG8	E3 ubiquitin-protein ligase ZNRF2	113	water*
Q5MNV8	F-box only protein 47	156	water*
P14136	Glial fibrillary acidic protein	87	water*
Q4G0P3	Hydrocephalus-inducing protein homolog	192	water*
Q9NVH2	Integrator complex subunit 7	229	water*
P04264	Keratin_ type II cytoskeletal 1	140	water*
P08729	Keratin_ type II cytoskeletal 7	87	water*
P05787	Keratin_ type II cytoskeletal 8	87	water*
Q6KB66	Keratin_ type II cytoskeletal 80	87	water*
Q86W92	Liprin-beta-1	202	water*
Q9BY66	Lysine-specific demethylase 5D	77	water*
Q99972	Myocilin	96	water*
Q06710	Paired box protein Pax-8	168	water*
Q9UKI3	Pre-B lymphocyte protein 3	355	water*
Q9HCU5	Prolactin regulatory element-binding protein	148	water*
Q9H714	Protein associated with UVRAG as autophagy enhancer	58	water*
Q9HAT1	Protein ERGIC-53-like	400	water*
Q9Y5H0	Protocadherin gamma-A3	140	water*
Q9BRP9	Putative uncharacterized protein MGC13053	148	water*
Q86TS7	Putative UPF0730 protein encoded by LINC00643	411	water*
A5PLK6	Regulator of G-protein signaling protein-like	139	water*
Q8N122	Regulatory-associated protein of mTOR	102	water*
Q9Y2P8	RNA 3'-terminal phosphate cyclase-like protein	184	water*
Q96PQ1	Sialic acid-binding Ig-like lectin 12	242	water*
Q9BSK2	Solute carrier family 25 member 33	122	water*
Q15772	Striated muscle preferentially expressed protein kinase	327	water*

Table 3. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with statherin-derived peptide (StN15) (1.88 x 10^{-5}M) for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

(conclusion)

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* StN15:water
Q9NXG6	Transmembrane prolyl 4-hydroxylase	166	water*
Q7L1V2	Vacuolar fusion protein MON1 homolog B	511	water*
O43895	Xaa-Pro aminopeptidase 2	188	water*
Q8WYQ9	Zinc finger CCHC domain-containing protein 14	55	water*

[a]Identification is based on proteins ID from UniProt protein database, reviewed only (http://www.uniprot.org/).
[b]Proteins with expression significantly modified are organized according to the ratio.
*Indicates unique proteins in alphabetical order.
Proteins highlighted in bold are enhanced or decreased more than 2-fold.

Table 4. Proteins with significantly modified expression that dwelled in the acquired enamel pellicle (AEP) after wash with mixture (MIX) of the 3 proteins (Cane, StN15 and HB) at the same concentrations already described for 1 minute in comparison with control (water), followed by formation of the AEP (2 hours) and later acid challenge (0.01 M hydrochloric acid pH 2) for 10 seconds.

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* MIX:water
P59666	Neutrophil defensin 3	1578	1,42
P06702	Protein S100-A9	3052	1,28
P63261	Actin_ cytoplasmic 2	1073	0,68
P60709	Actin_ cytoplasmic 1	1073	0,65
P04083	Annexin A1	183	0,59
Q5QNW6	Histone H2B type 2-F	292	0,55
O60814	Histone H2B type 1-K	292	0,54
P62807	Histone H2B type 1-C/E/F/G/I	292	0,54
Q93079	Histone H2B type 1-H	292	0,54
P02538	Keratin_ type II cytoskeletal 6A	147	0,53
P06899	Histone H2B type 1-J	292	0,53
Q8N257	Histone H2B type 3-B	292	0,53
Q16778	Histone H2B type 2-E	292	0,53
P04259	Keratin_ type II cytoskeletal 6B	147	0,52
P23527	Histone H2B type 1-O	292	0,52
P48668	Keratin_ type II cytoskeletal 6C	147	0,51
Q99877	Histone H2B type 1-N	292	0,51
Q99879	Histone H2B type 1-M	292	0,50
P57053	Histone H2B type F-S	292	0,50

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(continued)

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* MIX:water
Q99880	**Histone H2B type 1-L**	**292**	**0,49**
P01876	**Immunoglobulin heavy constant alpha 1**	**213**	**0,46**
P01877	**Immunoglobulin heavy constant alpha 2**	**132**	**0,41**
P25311	**Zinc-alpha-2-glycoprotein**	**914**	**0,41**
P04264	**Keratin_ type II cytoskeletal 1**	**140**	**0,37**
P05164	**Myeloperoxidase**	**133**	**0,34**
P04746	**Pancreatic alpha-amylase**	**836**	**0,31**
P04745	**Alpha-amylase 1**	**1445**	**0,29**
P19961	**Alpha-amylase 2B**	**1394**	**0,26**
Q01813	ATP-dependent 6-phosphofructokinase_ platelet type	170	MIX*
P27482	Calmodulin-like protein 3	125	MIX*
Q8TCT0	Ceramide kinase	78	MIX*
P36222	Chitinase-3-like protein 1	238	MIX*
P42773	Cyclin-dependent kinase 4 inhibitor C	114	MIX*
P09228	Cystatin-SA	307	MIX*
Q969U6	F-box/WD repeat-containing protein 5	296	MIX*
Q7L622	G2/M phase-specific E3 ubiquitin-protein ligase	53	MIX*
Q9NSN8	Gamma-1-syntrophin	248	MIX*
Q13439	Golgin subfamily A member 4	103	MIX*
P01834	Immunoglobulin kappa constant	336	MIX*
Q9UGB7	Inositol oxygenase	226	MIX*
O95232	Luc7-like protein 3	303	MIX*
O95222	Olfactory receptor 6A2	105	MIX*
Q96AQ6	Pre-B-cell leukemia transcription factor-interacting protein 1	156	MIX*
Q8IWL2	Pulmonary surfactant-associated protein A1	197	MIX*
Q86UX6	Serine/threonine-protein kinase 32C	66	MIX*
Q66K14	TBC1 domain family member 9B	108	MIX*

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(continued)

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* MIX:water
Q9C035	Tripartite motif-containing protein 5	208	MIX*
Q92834	X-linked retinitis pigmentosa GTPase regulator	107	MIX*
Q8N8L2	Zinc finger protein 491	122	MIX*
P31947	14-3-3 protein sigma	269	water*
P02708	Acetylcholine receptor subunit alpha	77	water*
Q9UJX3	Anaphase-promoting complex subunit 7	149	water*
Q01484	Ankyrin-2	189	water*
Q5T2E6	Armadillo-like helical domain-containing protein 3	96	water*
Q6PL18	ATPase family AAA domain-containing protein 2	64	water*
O75185	Calcium-transporting ATPase type 2C member 2	66	water*
P27797	Calreticulin	163	water*
P0DO97	Coiled-coil domain-containing protein 192	98	water*
Q96A83	Collagen alpha-1(XXVI) chain	111	water*
Q02246	Contactin-2	172	water*
Q9UBG3	Cornulin	105	water*
Q86XP0	Cytosolic phospholipase A2 delta	68	water*
Q8TEA8	D-aminoacyl-tRNA deacylase 1	152	water*
Q8N1I0	Dedicator of cytokinesis protein 4	397	water*
Q9UGM3	Deleted in malignant brain tumors 1 protein	75	water*
Q9Y485	DmX-like protein 1	83	water*
Q9UNA4	DNA polymerase iota	537	water*
Q8N136	Dynein assembly factor with WDR repeat domains 1	86	water*
Q8NHG8	E3 ubiquitin-protein ligase ZNRF2	113	water*
Q5MNV8	F-box only protein 47	156	water*
P14136	Glial fibrillary acidic protein	87	water*
A0A2R8Y619	Histone H2B	106	water*
Q96A08	Histone H2B type 1-A	106	water*

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(continued)

[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* MIX:water
Q4G0P3	Hydrocephalus-inducing protein homolog	192	water*
P01857	Immunoglobulin heavy constant gamma 1	90	water*
P01859	Immunoglobulin heavy constant gamma 2	33	water*
P01860	Immunoglobulin heavy constant gamma 3	38	water*
P01861	Immunoglobulin heavy constant gamma 4	38	water*
P0CG04	Immunoglobulin lambda constant 1	191	water*
P0DOY2	Immunoglobulin lambda constant 2	191	water*
P0DOY3	Immunoglobulin lambda constant 3	191	water*
P0CF74	Immunoglobulin lambda constant 6	98	water*
A0M8Q6	Immunoglobulin lambda constant 7	98	water*
B9A064	Immunoglobulin lambda-like polypeptide 5	191	water*
Q9NVH2	Integrator complex subunit 7	229	water*
P13645	Keratin_ type I cytoskeletal 10	140	water*
Q99456	Keratin_ type I cytoskeletal 12	75	water*
P13646	Keratin_ type I cytoskeletal 13	146	water*
P19013	Keratin_ type II cytoskeletal 4	336	water*
P13647	Keratin_ type II cytoskeletal 5	101	water*
P08729	Keratin_ type II cytoskeletal 7	87	water*
Q86Y46	Keratin_ type II cytoskeletal 73	229	water*
P05787	Keratin_ type II cytoskeletal 8	87	water*
Q6KB66	Keratin_ type II cytoskeletal 80	87	water*
P02788	Lactotransferrin	122	water*
Q86W92	Liprin-beta-1	202	water*
P61626	Lysozyme C	519	water*
Q8TAX7	Mucin-7	257	water*
Q99972	Myocilin	96	water*
P60660	Myosin light polypeptide 6	498	water*
Q06710	Paired box protein Pax-8	168	water*
Q9UKI3	Pre-B lymphocyte protein 3	355	water*

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[a]Number of acession	Proteins	Score of PLGS	[b]*Ratio* MIX:water
P07737	Profilin-1	134	water*
Q9HCU5	Prolactin regulatory element-binding protein	148	water*
Q9H714	Protein associated with UVRAG as autophagy enhancer	58	water*
Q9HAT1	Protein ERGIC-53-like	400	water*
Q9Y5H0	Protocadherin gamma-A3	140	water*
Q9BRP9	Putative uncharacterized protein MGC13053	148	water*
Q86TS7	Putative UPF0730 protein encoded by LINC00643	411	water*
P14618	Pyruvate kinase PKM	383	water*
A5PLK6	Regulator of G-protein signaling protein-like	139	water*
Q8N122	Regulatory-associated protein of mTOR	102	water*
Q9Y2P8	RNA 3'-terminal phosphate cyclase-like protein	184	water*
Q96PQ1	Sialic acid-binding Ig-like lectin 12	242	water*
Q9BSK2	Solute carrier family 25 member 33	122	water*
Q15772	Striated muscle preferentially expressed protein kinase	327	water*
Q9NXG6	Transmembrane prolyl 4-hydroxylase	166	water*
Q7L1V2	Vacuolar fusion protein MON1 homolog B	511	water*
O43895	Xaa-Pro aminopeptidase 2	188	water*
Q8WYQ9	Zinc finger CCHC domain-containing protein 14	55	water*

[a]Identification is based on proteins ID from UniProt protein database, reviewed only (http://www.uniprot.org/).
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3-Discussion

3 DISCUSSION

ETW is progressive, i.e., the prevalence and severity progress with age (Jaeggi et al., 2014) so that preventive measures are extremely important. Moreover, the prevalence of this clinical condition has been increasing, which has led to an increase in the research in this area (Kreulen et al., 2010; Donovan et al., 2021; Rocha et al., 2022; Chatzidimitriou et al., 2023).

The demineralization involved in ETW happens partially on the tooth surface, due to frequent exposure to intrinsic, extrinsic acids or their combination (Donovan et al., 2021). Within the ETW there is no fixed characteristic “critical” pH, a value at which the erosive solution is exactly saturated in relation to a specific solid, in this study in question, that of dental enamel. This depends both on the solubility of the enamel (the solid of our interest) and on the activities of the relevant mineral constituents of the solution (calcium, phosphate and fluoride, to a lesser extent) (Lussi et al., 2014). Thus, the continuous loss of dental tissue occurs due to undersaturation both in relation to hydroxyapatite and in relation to fluorapatite, since the pH of the causative agents is lower than 4.5 (Lussi et al., 2011).

An important protective factor against ETW, mainly due to its extensive biological properties, is the saliva present in the oral cavity. It is formed by the secretion of the salivary glands, presenting a neutral pH. Among its various functions, saliva maintains oral health and also participates directly in the remineralization of dental tissue, as it is a source of calcium and phosphate ions (Buzalaf et al., 2012). In addition, saliva is responsible for contributing to the formation of a protein-rich pellicle, the AEP. This pellicle is responsible for protecting the tooth surface against chemical and mechanical agents. The thickness of the film varies according to the surface and is proportional to the contact with saliva. Research has detected hundreds of proteins in the composition of the AEP in vivo, such as mucin, amylase, lysozyme, PRPs, statherin, varying according to its location in the dental arches (Ventura et al., 2017), as well as several peptides (Siqueira et al., 2007). Among these proteins, the presence of intact histatin in the acquired pellicle was confirmed, which is resistant to degradation when adsorbed to hydroxyapatite, which may give it the potential to protect the surface of dental enamel against acid attacks (Siqueira

et al., 2010; McDonald et al., 2011). Thus, AEP works as a mechanical barrier against acid dissolution (Buzalaf et al., 2012; Vukosavljevic et al., 2014).

Our research group in recent years, through AEP engineering, has focused on the search for proteins that resistant to removal by erosive acids (Delecrode et al., 2015; Cassiano et al., 2018; Taira et al., 2018), so that enrichment of the AEP with these proteins might help protect against ETW. Several candidates have been found out, which have remained in the AEP even after severe erosive attacks. Among them, we highlight HB (Martini et al., 2019), statherin (Taira et al., 2018) and CaneCPI-5 (Santiago et al., 2017), which are target proteins evaluated in this study.

The study involved the participation of 10 volunteers, of both genders, aged between 18 and 35 years. They were selected after using the inclusion and exclusion criteria and also the analysis of stimulated and non-stimulated salivary flow. An average of 4 samples per treatment group were collected from 3-4 volunteers. Considering the low proteins count normally retrieved from AEP in vivo, the number of 10 volunteers is enough to compose pools (biological triplicates) for the proteomic analysis of AEP. This sample size (n=10) was calculated based on previous studies that presented a similar methodology (Ventura et al., 2017; Cassiano et al., 2018; Taira et al., 2018, 2020).

Thus, it was an in vivo, triple-blind (volunteer, researcher and analyst) and crossover study, i.e., all volunteers went through all phases of the study (5 types of mouthwashes X 2 types of erosive challenges, totaling 10 phases. The great advantage of this experimental design was to reproduce a clinical picture, so that in the future, we can have a dental product focused on the prevention of ETW.

The proteins evaluated both in the extrinsic and intrinsic study were: 1- commercially available lyophilized human hemoglobin (#H7379) (Sigma-Aldrich); 2- CaneCPI-5 that was recombinantly produced under the coordination of Prof. Dr. Flávio Henrique Silva, at UFSCAR. A bacterial strain of E. coli Rosetta (DE3) was used, altered utilizing plasmid pET28aCaneCPI-5 (Miguel, 2014). Purification of expressed protein from the soluble fraction of bacterial cultures induced was achieved by IPTG (Isopropyl-beta-D-Thiogalactosidio), subjected to centrifugation and sonication. Purification was performed by affinity chromatography, using Ni-NTA Superflow nickel resin columns (Qiagen); 3-

Peptide containing 15 N-terminal residues of statherin (DSSEEKFLRRIGRFG), with phosphorylated serines two and three (StN15pSpS), synthesized chemically by solid phase method (Merrifield, 1963; Amblard et al., 2005), based on the standard protocol that employs the FMOC group as a protector of the α-amino groups and t-butyl derivatives for protection of side chains of trifunctional amino acid residues (Chan et al., 2000) in the laboratory of Prof. Reinaldo Marchetto, from Biochemistry and Chemical Technology Department, Institute of Chemistry – UNESP, Araraquara. A Wang type resin containing the first amino acid of the prior incorporated sequence was utilized as starting polymer. This peptide was synthesized because research reported that at least 15 N-terminal residues or more are required in statherin-derived peptides to lower demineralization of the enamel (Shah et al., 2011). Still thinking about a future commercial application of our findings, the use of peptides derived from statherin with 15 N-terminal residues is much more attractive than the use of the whole protein, due to the low cost and easier of storage.

The factors involved in the research design were the type of protein solution utilized as mouthwash, in five levels and the type of erosive challenge in 2 levels. In each phase, dental prophylaxis was performed, the volunteers rinsed (10 mL for one min) utilizing control (deionized water) (1), 0.1 mg/mL CaneCPI-5 (2), 1.0 mg/mL HB (3), 1.88×10^{-5}M StN15 (4) solutions or mixture of the three proteins/peptide (5). Concentrations of CaneCPI-5 (Santiago et al., 2017), HB (Martini et al., 2020) and StN15 (Taira et al., 2020) were based on prior in vitro researches by our team. The protein solutions pH was native and the proteins/peptides were diluted in deionized water. Participants were deprived for two hours of eating and drinking, so AEP could form. This in vivo study protocol was recently used by Araujo et al., 2022, for the evaluation of the protein profile of the AEP formed for 3 min, with the same protein mouthwashes with the CaneCPI-5, HB and StN15.

After 2 hours of formation of the AEP, an enamel biopsy was performed on the buccal surface of tooth 21, for 10 s (10 µl of extrinsic or intrinsic acid, depending on the phase), for subsequent calcium analysis, using the Arsenazo III colorimetric method (Vogel et al., 2006). Then, we also for 10 s, we performed the erosive challenge (extrinsic or intrinsic, depending on the phase) on upper and lower teeth buccal surfaces, with a pipette (200 µl of acid). It is important to

highlight that all these procedures were performed during the morning, to avoid the influence of the circadian rhythm. Subsequently, the remaining AEP proteins were harvested with an electrode filter paper and the samples were analyzed quantitatively by label-free proteomics.

Extrinsic factors are very often related to eating habits, such as consumption of soft drinks, fruit juices, with low pH value, lifestyle, drug use (Zero, 1996). In contrast, intrinsic factors involve the contact of acids from the stomach with the oral cavity, exceeding the existing buffer function in saliva (Salas et al., 2015). On the other hand, intrinsic ETW involves the clinical conditions of GERD, bulimia nervosa and chronic alcohol consumption (Zero, 1996; Carvalho et al., 2015; Salas et al., 2015). We evaluated the potential of mouthwashes with distinct proteins/peptide that were previously identified with acid-resistant properties to change the proteome of the AEP, persist in this integument after intrinsic or extrinsic erosive challenges and protect enamel against demineralization, which was directly evaluated by enamel biopsies. It is also worth highlighting that this study was awarded among all the USP units, and several videos submitted referring to researches carried out involving master's and/or PhD studies, with honorable mention at Prêmio Vídeo Pós-Graduação USP 2021, da Grande Área: Ciências da Saúde II (**Annex E**)

Since two difference types of erosive challenges were performed, and their results were distinct, and also considering that clinically the extrinsic and intrinsic erosive challenges are very different conditions the discussion of the thesis will be carried out separately for each type of acid.

Extrinsic erosive enamel demineralization

Due to its novelty, this part of the study received the Science Award from the Cariology Group of the International Association for Dental Research (IADR) in 2020 (**Annex F**). Each year, the Cariology Group selects up to 3 abstracts submitted for presentation in the general session of the IADR to be awarded, but our study was awarded by itself that year, being selected among 450 abstracts submitted for this award. It is important to highlight that the IADR is the biggest and most important research congress in Dentistry.

The new eating habits have turned more towards a diet rich in acidic foods and these, in contact with the dental surface, can cause a continuous demineralization of the teeth, and a loss in volume of this structure may occur, through ETW (Lussi et al., 2014). This has been subject of great concern in dentistry (Schlueter et al., 2020; Pedrosa et al., 2020), so it is important that studies are focused on this area.

In the present study, to simulate the extrinsic erosive challenge, 1% citric acid pH 2.5 was used. This protocol has been successfully employed in other in vivo studies (Pela et al., 2023; Delecrode et al., 2015).

Proteomic analysis revealed that all solutions of the proteins/peptides caused marked changes in the protein profile of the AEP that remained on the tooth surface after the extrinsic erosive challenge and also significantly reduced the extrinsic enamel erosive demineralization. Recently, our findings with the CaneCPI-5 solution were corroborated in another study by our group that showed that rinsing with CaneCPI-5 solution was as effective as rinsing with Elmex Erosion™ to reduce erosive demineralization (Pela et al., 2023). It is important to mention that in the latter study, another response variable (reflectometry) was employed, besides Ca analysis, which gives additional confidence to the data.

Results also demonstrated that dental surface in vivo treatment with hydroxyapatite bonding proteins expressively increased proteins count immobilized within AEP following erosive challenge (8.2 µg for the control group versus 16.2-21.7 µg for the experimental groups). In addition, a notable increase of numerous proteins within AEP that are potentially displacement resistant by acids (Supplementary Tables 1-4, article 1) was observed, protecting against erosive demineralization (Figure 1, article 1). Intriguing, proteins families with expression changes as well as the ratios discovered were distinct for the different proteins analyzed (Supplementary Tables 1-4, article 1). Therefore, considering the experiment main outcome (erosive demineralization prevention), these differences do not seem to be of relevant, since all proteins/peptide isolated appointed analogous protection. The 3 proteins combined, however, was not capable of providing protection in comparison to water. This might be a result of the 3 proteins combined concentrations' being identical to the ones utilized when the proteins were isolated. Taking in

consideration that all of them have attraction for hydroxyapatite, they might have contested for binding sites. Further examination of these proteins combination effect at lower concentrations could be of interest, since distinctive proteins could alter AEP composition in a unique manner (Supplementary Tables 1-3, article 1).

A notable finding of the current research is that although some increased expression proteins or found solely within proteins/peptide treatment are those released by salivary glands, which are usually noticed within AEP (for example, PRPs, cystatins, amylase, lactotransferrin, histatins), nearly all of which are intracellular proteins (e.g., keratins, histones, actins and profilins, tubulins, 14-3-3 proteins). It is known that predecessor proteins observed within AEP basal layer presents the highest erosive demineralization prevention (Hannig et al., 2009). Therefore, boosting the number of proteins with high hydroxyapatite affinity in the basal layer, as tested in the current work, may also enhance other proteins number that do not link to hydroxyapatite, but bind to predecessor proteins, what could have been the case of intracellular proteins. An example of this is the actin enhance, noted in HB and StN15 treatments and in the combined proteins. Within the same research groups, profilin (an actin-binding protein) also increased. Thus, actin and profilin may formulated a portion of the protein aggregates captured in the second phase of AEP formulation (Hannig, 1999). It is important to mention that the proteins/peptides treatment enhanced numerous antimicrobial proteins within AEP, such as countless isoforms of immunoglobulins, cystatins, histatins and lysozyme. Thus, it is possible that they also prevent dental caries. In fact, CaneCPI-5 was shown in vitro to significantly decreases microcosm biofilms activity, the counts of lactobacilli and mutans streptococci, as well as the enamel demineralization (integrated mineral loss; transverse microrradiography) (Araújo et al., 2021).

These findings are very promising as it is the first time that an in vivo study has tested the pellicle engineering concept for enamel surface enrichment with CaneCPI-5, HB or StN15 after extrinsic erosive challenges. The tested treatments notably increase acid-resistant proteins in AEP and are potentially protective against extrinsic erosive enamel demineralization.

Intrinsic erosive enamel demineralization

This part of this study was awarded at the International Association for Dental Research (IADR) in 2021 for the Colgate Research in Prevention Travel Award (**Annex G**). Each year, 6 people from around the world are selected for this award.

In this work, to simulate intrinsic ETW, we used HCl 0.01 M as it corresponds to pH 2 (Taira et al., 2018), and the pH of the gastric juice typically ranges between 1 and 3 (Milosevic et al., 1997). Our intention when using this acid was two-fold, as follows: 1) identify in the AEP proteins that resist to intrinsic acid (HCl) and 2) to evaluate which of the protein/peptide would protect against the intrinsic erosive challenge. It is very important to identify proteins in the AEP that are resistant to removal by HCl. This acid, in question, comes from the stomach and is found in the oral cavity after episodes of gastroesophageal reflux, vomiting and bulimia nervosa (Moazzez et al., 2014).

Results demonstrated that tooth surface in vivo treatment utilizing proteins with hydroxyapatite affinity hydroxyapatite enhanced the number of proteins immobilized within AEP following erosive attack (6.4 µg for the control group versus 8.6-13.6 µg for the experimental groups). In addition, a boost in numerous AEP proteins that are potentially displacement resistant by acids was observed (Complementary Tables 1-4, article 2). Intriguing, proteins families' presenting changes in expression, as well as the reasons noticed, were distinct for various proteins studied (Complementary Tables 1-4, article 2). An expressive finding of the current research is that several proteins that are not typically described in AEP, but that bind calcium or other proteins, were identified exclusively in the groups treated with the tested proteins / peptides, isolated or mixed (MIX). In addition, some increased expression proteins or found solely in protein/peptide treatment are released by salivary glands, which are usually noticed within AEP (e.g., PRPs, cystatin, lysozyme-C, submaxillary gland androgen-regulated protein 3B), evidenced by the Cane-CPI-5 and StN15 peptide treatment group. The 3 proteins mixed, however, was not capable to provide protection in comparison to water. This might be a result of the 3 proteins combined concentrations' being identical to the ones utilized when the proteins were isolated. Taking in consideration that all of them have attraction

for hydroxyapatite, they might have contested for binding sites. Further examination of these proteins combination effect at lower concentrations could be of interest, since distinctive proteins could alter AEP composition in a unique manner (Complementary Tables 1-4, article 2).

Among the treatments tested with the proteins/peptides, several intracellular proteins (for example, keratins, histones and actins) were evidenced. It is known that predecessor proteins observed within AEP basal layer are the highest erosive demineralization preventive (Hannig et al., 2009). Thus, boosting proteins with high hydroxyapatite affinity in the basal layer, as examined in the current research, may also enhance other proteins that do not link to hydroxyapatite, but link to predecessor proteins, which could have been the case of intracellular proteins, which is in agreement with the result found in a recent work by our research group, in which these proteins were highly expressed in the group treated with HB in the AEP formed for 3 min (Araujo et al., 2022). In the CaneCPI-5 protein treatment group, pyruvate kinase (a cadherin-binding protein, which belongs to the calcium-dependent transmembrane glycoprotein family) showed a greater increase among the identified proteins. Thus, pyruvate kinase may formulate portion of the protein aggregates that are captured in the second phase of AEP formation (Hannig, 1999). It is important to mention that the proteins/peptides treatment enhanced numerous antimicrobial proteins within AEP, such as countless isoforms of immunoglobulins, cystatins, histatins and lysozyme. Thus, it is possible that they also prevent dental caries. As mentioned above, AEP engineering, at least with CaneCPI-5 was shown to reduce microcosm biofilm formation and reduce dental caries. The anticaries effect of these proteins/peptide should be evaluated in further studies, employing protocols that more closely mimic clinical condition.

In this work, in which a stronger erosive challenge was performed with HCl, only HB was able to protect enamel against erosive demineralization. These findings are in-line with an in vivo study by our group that found that GERD patients without ETW have HB levels 3-fold higher in the AEP (Martini et al., 2019) and 22-fold higher in saliva (Martini et al., 2020) when compared to GERD patients with ETW. It was also recently found that after rinsing with HB, there was an increase of up to 18x in the subunits of this protein in the AEP

formed for 3 minutes (Araujo et al., 2022). As mentioned above, HB has strong affinity for hydroxyapatite, and hydroxyapatite columns show an excellent performance for purification of HB (Kawasaki et al., 1985). It is important to highlight that the adsorption of HB to hydroxyapatite increases as the pH decreases (Yu et al., 2017). Patients with erosive tooth wear have a pH in the oral cavity typically lower than that found in healthy patients (Bartlett et al., 1996). Thus, the lower pH in the oral cavity in patients with GERD may increase the chance of HB adsorption to tooth surfaces, since it confers a positive charge on this protein.

In summary, our results showed that protein/peptide treatment, alone or combined, increased acid-resistant proteins in AEP, but only HB was able to protect against intrinsic erosive demineralization. These findings open the avenue for a new preventive approach to intrinsic erosive demineralization, employing acquired pellicle engineering procedures involving the incorporation of HB in dental products for the prevention of intrinsic erosion. Due to the fact that for intrinsic erosion only HB was protective, we decided to use bioinformatic tools in attempt to better understand the differences among the proteins/peptide evaluated in the intrinsic erosion condition.

The biological and/or molecular processes found in this comparison of the 1.0 mg/mL group of Hemoglobin (HB) vs control (water) were: humoral immune response (37%), keratinocyte differentiation (22%), humoral immune response antimicrobial peptide-mediated activity (13%), focal adhesion cluster (11%), alpha hemoglobin binding (8%), regulation of epidermal development (6%), and alpha-amylase activity (3%) (Figure 2, article 2).

Among the evidenced processes, 37% of them refer to the humoral immune response. This process is directly related to the body's defense, in which we have the participation of immunoglobulins. Through the proteomic approach, the differential expression of several immunoglobulins was identified, which were reduced or absent in the HB group, compared to the water group (Complementary Table 2, article 2). It has been reported that immunoglobulins contribute to protection against intrinsic erosive challenges, in addition to having antimicrobial action (Araujo et al., 2022). In addition, 13% of the processes presented through this bioinformatics analysis are linked to the antimicrobial humoral immune response mediated by antimicrobial peptide, which is an

important factor of antimicrobial action when we associate it with carious lesions and the intrinsic erosive challenge (Araujo et al., 2022).

AEP is typically composed of proteins, being considered an important protective factor against ETW (Carvalho et al., 2020). Among the biological processes affected when comparing the HB vs control (water) group, we found alpha-amylase activity (3%) and binding to alpha hemoglobin (8%), which reinforces the protective effect of treatment with the solution containing HB. Alpha-amylase protein is typically found in the basal layer of AEP (Martins et al., 2013) and two alpha-amylase subunits were decreased upon HB treatment (Complementary Table 2, article 2), which indicates that HB, due to its high affinity for hydroxyapatite, must have preferentially bound to this mineral, leaving fewer binding sites for other basal layer proteins. HB is a tetramer composed of two pairs of globins, each linked to a heme group. In adult humans, HBs consist of 96.5-98.5% HbA1 (α2β2 dimer) and 1.5-3.5% HbA2 (α2δ2 dimer), while Hb ε replaces the β chain in embryos. The different HB subunits have unique amino acid sequences (Gell, 2018). Amidst them, the α chain is the one with the most elevated serine, threonine and tyrosine residues content that could be phosphorylated, giving the protein a negative charge, thus enhancing its hydroxyapatite binding capability (Kawasaki et al., 1986, 1987).

Another finding that caught our attention was that 11% of the processes were related to focal adhesion assembly. These structures are multiprotein complexes containing integrins, which form mechanical links among intracellular actin bundles and the extracellular substrate in various cell types. Intracellular proteins were also highly expressed in the proteomic analysis of part of this previously published study (Carvalho et al., 2020). We believe they are part of some important structural arrangement within the AEP. The fact that 22% of biological processes are related to the differentiation of keratinocytes and 6% are related to the regulation of epidermal development, may be related to proteins from desquamated epithelial cells, which are also reported as constituents of AEP (Martins et al., 2013).

In conclusion, the bioinformatics analyses presented helped to better understand the results obtained with the proteomic analysis, corroborating previous studies and bringing new insights. All the proteins/peptide evaluated increased acid-resistant proteins in the AEP, regardless the type of erosive

challenge, but only HB protected enamel against intrinsic erosion. Our findings open new avenues for the development of dental products to prevent ETW based on AEP engineering procedures.

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Annexes

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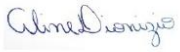
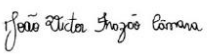
Aline Dionízio NOME 4	 Assinatura
João Victor Frazão Câmara NOME 5	 Assinatura

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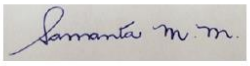

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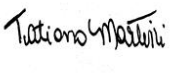

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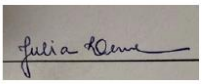

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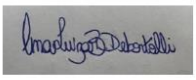

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Bauru, 28 de agosto de 2023.

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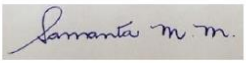
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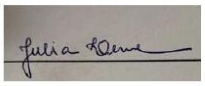
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Associate Editor comments:
Thanks for submitting a revised version of this paper. It addresses the comments made in the initial review, however there is still a need for English style/grammar review. Below are only some examples/suggestions (from a non-native English speaker), but I advise the authors to seek proper assistance for a thorough review of the paper:
1. The first sentence of the Abstract is extremely long and grammatically incorrect.

2. The second sentence of the Abstract could start with the subject (Ten volunteers) for clarity.
3. Line 111-113: these sentences are repetitive and could be merged.
4. Line 117-120: these sentences are difficult to read. Perhaps better construction would be: 'The vestibular surfaces of the remaining lower and upper teeth were coated with....' ; 'Each quadrant of the mouth was flushed with deionized water for acid removal, gently dried with compressed air and isolated with cotton rolls to avoid lip contamination'.
5. Line: 123-126: There is a lot of redundancy between these two sentences and they can be merged.
6. Line 193: the sentence 'This can be observed…' is unnecessary in the context and can be deleted.
7. Line 195: the sentence 'On the other hand…' is difficult to follow and needs to be revised for clarity.

Others:
1. Line 10: what does '10s' refer to? It can be deleted.
2. Line 76: numbers should have the commas replaced by periods.
3. Line 109: would the number indicate diameter? If so, please add.
4. Line 117: please clarify how this procedure was done. Was a specific surface area for each tooth isolated? If so, how? If not, how even distribution of the acid was achieved?

Reviewer 1 report:
Comments to authors
The manuscript was improved, all my comments were addressed. I have no further comments on it.

Reviewer 2 report:

Reviewer 2 report:
Comments to authors
The authors have addressed all issues raised in the review. Thank you.

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ANNEX E- Honorable mention award

PRÊMIO VÍDEO PÓS-GRADUAÇÃO USP 2021

A Universidade de São Paulo concede menção honrosa no Prêmio Vídeo Pós-graduação USP 2021
Grande Área: Ciências da Saúde II a

Thamyris de Souza Carvalho

pelo vídeo "Engenharia de película adquirida do esmalte para proteção contra a erosão dentária: avaliação in vivo do efeito protetor da cistatina derivada da cana-de-açúcar (CaneCPI-5), hemoglobina e/ou peptídeo derivado da estaterina"
Programa: Ciências Odontológicas Aplicadas - FOB
Orientadora: Marília Afonso Rabelo Buzalaf

VAHAN AGOPYAN
Reitor

CARLOS G. CARLOTTI JR.
Pró-reitor de Pós-graduação

ANNEX F- Cariology Research Group Award

IADR
International Association for Dental Research

and the
Cariology Research Group

AWARD CERTIFICATE
Presented to

Thamyris Souza Carvalho

"Acquired pellicle engineering by treatment with proteins/peptide protects against erosion"

Recipient of
Science Award

IADR 98th General Session & Exhibition
IADR Centennial
March 18-21, 2020

ANNEX G- IADR Colgate Research in Prevention Travel Award