Proteomic profiles of the acquired enamel pellicle formed *in vitro, in situ* or *in vivo*

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Running title: Proteome of the acquired enamel pellicle

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Abstract

This study compared the protein profile of the acquired enamel pellicle (AEP) formed under three conditions: *in vitro*, *in situ* and *in vivo*. Nine volunteers participated in all procedures. In the *in vitro* condition, the volunteers donated saliva, in which specimens were incubated to form the AEP. In the *in situ* condition, the volunteers used an oral device containing specimens where the AEP was formed. In the *in vivo* condition, the AEP was collected from the volunteers own teeth. All AEPs were formed for 120 minutes, collected and processed by Mass spectrometry. Considering all conditions, in total 321 proteins were identified, being 37 proteins commonly considered typical in the AEP. For each condition were identified 66, 174 and 170 proteins for *in vitro*, *in situ* and *in vivo*, respectively. For the *in vitro* condition, 17 pellicle-typical proteins were not identified. Furthermore, several proteins with important functions within the AEP presented differences in expression in the three conditions. The qualitative profile of the proteins, especially the typical ones, is different in the *in vitro* condition. In addition, there are important quantitative differences, which may interfere in the extrapolation of *in vitro* results to an *in situ* and *in vivo* condition.

Keywords: Proteomics; Saliva; Mass spectrometry

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Introduction

The acquired enamel pellicle (AEP) is a bacteria-free layer composed chiefly of saliva-derived proteins and glycoproteins that adsorb to the dental surface, with carbohydrates, neutral lipids, phospholipids and glycolipids also contained in minor amounts (1-4). The AEP executes important functions to the teeth, such as acting as a diffusion barrier, decreasing the direct contact of the acids with the tooth surface and protecting against dissolution (5-7). The effectiveness of this protection depends on its composition and physical properties (5,8,9). It is generally accepted that the AEP is composed of a tightly bound basal layer, with proteins strongly adhered to the tooth surface, and a more loosely packet globular layer, formed mostly by protein-protein interactions.

In an attempt to further understand which proteins are present in the AEP and their role in protecting against acid dissolution, proteomic tools have been widely employed in the last decade to evaluate protein profiles. To date, more than 300 proteins have been identified in the AEP (10), considering both the basal and globular layer. However, one of the main limiting factors of proteomic analysis is undoubtfully the tiny amount of proteins that can be recovered from a single dental surface. Due to this, in most *in vivo* studies proteins from different tooth surfaces across a whole dental arch are collected. This, on the one hand, allows for the recovery of larger amounts of proteins to be analyzed (11-13), but on the other hand, the composition of the AEP changes according to its location in the dental arches (10). In this regard, some *in situ* studies have also been performed (14-17), which collect the AEP only from some tooth specimens that can be precisely placed at defined locations within the volunteer's mouth, thus lowering the variations arising from location in the dental arches. Though *in situ* condition can decrease AEP variations, these studies are more difficult to conduct because they require specialized preparation of dental specimens as well as a team of volunteers who are willing to wear oral appliances largely for longer periods. As the latter can be problematic to arrange, in *in vitro* experiments the AEP can be formed in the laboratory from saliva donated by the volunteers. Though these *in vitro* conditions are easier to perform, they can still be challenging, because the AEP formed in the laboratory does not mimic the normal salivary flow observed in *in situ* and in *in vivo* conditions. Moreover, the number of proteins obtained from *in vitro* formed AEP is very limited, highlighted by two studies published so far (to the best of our knowledge) analyzing the proteomic composition formed under these conditions, where the authors identified 45 and 55 proteins (18,19).

The usefulness of *in vitro* and *in situ* conditions to analyze proteomic profiles of AEP can, therefore, be questioned, especially because *in vivo* conditions provide more substantial results and are more relevant from the clinical point of view. However, there are some

situations, in which these experimental designs are more difficult or virtually impossible to perform, for example, when we are interested in pellicles formed on dentin specimens or in cases where mechanistic effects are investigated; more specifically, when the interest lies not only on the effect that a certain product has on the pellicle, but also its effect on the tooth structure. In such cases, both the pellicle as well as the underlying tooth surface should be analyzed. Another example is a screening assay, when several treatment groups are tested (with different concentrations and times of application of the active agents). In this case, *in vitro* experiments should be performed, since they do not require the participation of volunteers, and can help in the initial screening, in order to select the best groups to be further tested in *in situ* or *in vivo* conditions.

Given the importance of all of these experimental designs at different stages of research, it is not known to which extent the protein compositions of the acquired pellicles formed *in vitro*, *in situ* and *in vivo* are similar. In addition, we need to be able to extrapolate the results obtained from *in vitro* and *in situ* conditions to the more clinically relevant *in vivo* condition. Thus, the aim of the present study was to identify the differences in the protein profile of the AEPs formed under *in vitro*, *in situ*, and *in vivo* conditions by nano Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI-MS/MS). The null hypothesis was that there are no differences in the protein profile of the acquired enamel pellicles formed under *in vitro*, *in situ* and *in vivo* conditions.

Material and methods

This study is made up of 3 parts, where AEPs from the same volunteers are formed on enamel surfaces under 3 experimental conditions: *in vitro*, *in situ*, and *in vivo*. All the experiments were conducted at Bauru School of Dentistry, University of São Paulo.

Selection of volunteers and ethical aspects

Based on previous studies involving proteomic analysis of AEP (11-13,20), we selected 9 adult volunteers (average age: 25 years) of both genders to participate in all three parts of the present study after signing an informed consent. The inclusion criteria were healthy adults, willing to participate in all parts of the study, with none of the following characteristics described as exclusion criteria: smokers, low salivary flow (unstimulated > 0.3 mL/min and stimulated > 1.0 mL/min) or using medications that could change the salivary flow, presence of caries lesions, gingivitis and periodontitis. The participating volunteers received a kit containing a toothbrush, a toothpaste (Sorriso, 1100 ppm F as NaF, Colgate Palmolive, São Paulo, SP, Brazil) and floss (Colgate Palmolive), to standardize the oral hygiene throughout the study. This work was approved by the Research Ethics Committee of Bauru School of Dentistry, University

of São Paulo, SP, Brazil (CAAE 86774018.6.0000.5417) and it followed the guidelines of good clinical practice and conformed to the Declaration of Helsinki.

Preparation of human enamel specimens

For the *in vitro* and *in situ* parts, a total of 216 enamel specimens were prepared from human third molars obtained from the surgery clinics of Bauru School of Dentistry, University of São Paulo, after the donors provided written consent. The teeth had been disinfected and kept at 0.1% buffered thymol solution (pH 7.0) (Sigma-Aldrich, St. Louis, MO, USA) until the time of specimen preparation. Visual inspection was performed to evaluate the presence of caries, stains and cracks, which were exclusion criteria for the teeth. After separating the crown from the root, the enamel specimens were prepared by cutting a 4 mm × 4 mm enamel block from the buccal surface of the crowns (ISOMET Low Speed Saw Buehler, Lake Bluff, IL, USA) and grinding the dentin surface until the blocks had a 2 mm height. The buccal enamel surface was left untreated, and the experiments were performed on this native enamel surface. The specimens were then cleaned for 7 min at 25 °C by ultrasonication (T7 Thornton, a Unique Ind. E Com. Ltda., São Paulo, SP, Brazil), and stored with wet gauze in a refrigerator at 4 °C until the time of the experiment.

Collection of unstimulated saliva

For the *in vitro* part, the AEP was formed in the laboratory using the saliva from the volunteers. For that, unstimulated saliva was collected always during the morning (to avoid circadian effects). The nine volunteers remained seated and at rest, with their heads slightly leaning forward, allowing saliva to drip into vials kept in ice (21). Then, the saliva was centrifuged (14,000 g, 4 °C, 15 min), and the supernatants were collected and pooled. Finally, protease inhibitors (Phenylmethane sulfonyl fluoride - PMSF, N-Ethyhlmaleimide - NEM and Phenantroline - Sigma-Aldrich) were added to the saliva in a volume ratio of 1:100 (inhibitor : saliva) (14) and immediately the saliva supernatants were stored at -80 ºC, in three separate tubes, until the time of the experiment. In total, the volunteers provided 81 mL saliva.

***In vitro* part**

The experiment was conducted as previously described (19). A total of 108 enamel specimens were used for this part. Before each AEP formation, the specimens underwent prophylaxis with pumice slurry (Pedra Pomes, S.S. While Artigos Dentários, Rio de Janeiro, RJ, Brazil) and they were individually placed in microtubes with 250 µL of saliva and kept at 37 °C under agitation. The AEP was allowed to form during 120 min, and during this period, the saliva aliquot was replaced by a new one every 30 min (this was made to mimic the salivary

flow and clearance rate present in *in situ* and *in vivo* conditions). Afterwards, the specimens were immediately washed with deionized water then dried. For AEP harvesting, a 5 mm × 10 mm electrode filter paper (Electrode Wick, Bio-Rad, Hercules, California, CA, USA) was soaked in 3% citric acid (Sigma-Aldrich). The excess was removed with absorbent paper and rubbed on the surface of the enamel specimens (without pressure) (13). One electrode filter paper was used for every six specimens. The filter papers were stored in tubes and frozen at -80°C until the preparation for the proteomic analyses. The experiment was repeated two more times, being conducted during three consecutive days.

***In situ* part**

A total of 108 enamel specimens were used for this part. Individual oral appliances for the mandibular arches of each volunteer where prepared based on models prepared from alginate impressions, as described previously (Bauru *in situ* pellicle model) (16). Each appliance contained 12 enamel specimens. The appliance was placed on the lower jaw of the volunteers, with the enamel specimens on the buccal surface of the premolars/molars (six specimens on each side). Before each AEP formation, the specimens underwent prophylaxis with pumice slurry. The AEP was allowed to form during 120 min in the morning to avoid circadian effects (21). During this period, the volunteers were not allowed to eat or drink. The device was then removed from the mouth; the specimens were washed with deionized water and dried (17). For AEP harvesting, the same procedure was performed as described for the *in vitro* condition. The experiment was repeated two more times, being conducted during three consecutive days.

***In vivo* part**

The volunteers received a professional prophylaxis with pumice slurry prior to AEP formation and were asked not to eat or drink for the 120 min of AEP formation, which took place in the morning to avoid circadian effects (21). For AEP harvesting, the vestibular surfaces of the mandibular teeth were rinsed with deionized water, dried with compressed air and isolated with cotton rolls. Twelve 4 mm × 4 mm areas (6 from each side of the mandibular arch) were selected, and they corresponded to the exact same location where the enamel specimens in the *in situ* experiment were located (buccal surface of the premolars/molars). The AEP was collected from these 12 areas, using the same procedures as for the *in vitro* and *in situ* parts. The experiment was repeated two more times, being conducted during three consecutive days.

Preparation of the AEP for proteomic analysis and protein concentration measurement

All the electrode filter papers, containing the AEP of the three days (triplicate collection) of the experiment were pooled according to each experimental condition (*in vitro*, *in*

situ and *in vivo*). The AEPs were prepared as previously described (10). Briefly, proteins were extracted using a solution containing 6 M urea, 2 M thiourea in 50 mM NH_4HCO_3 (Sigma-Aldrich), pH 7.8. Samples were placed in Falcon Amicon tubes (Amicon Ultra - 15 Centrifugal Filter Units - Merck Millipore, Tullagreen, CO, IRL), centrifuged and concentrated to approximately 150 µL. Protein concentrations were quantified using the Bradford method (Bio-Rad, Hercules, California, CA, USA). Then the samples were reduced (5 mM dithiothreitol - Sigma-Aldrich), alkylated (10 mM iodoacetamide - Sigma-Aldrich) and digested for 14 h at 37 ºC by adding 2% (w/w) trypsin (Promega, Madison, WI, USA). C18 Spin columns (Thermo Scientific, Rockford, IL, USA) were used to desalt and purify the samples. Protein concentrations were quantified again using the Bradford method (Bio-Rad, Hercules, California, CA, USA) and total amount of proteins obtained for each condition calculated. After that, all samples were adjusted to the same concentration and resuspended in a solution containing 3% acetonitrile (Sigma-Aldrich) and 0.1% formic acid (Sigma-Aldrich) to be submitted to nano LC-ESI-MS/MS.

Shotgun label-free quantitative proteomic analysis

Peptide identification was performed by Xevo Mass spectrometer (MS) coupled to the nanoACQUITY UPLC (Ultra-Performance Liquid Chromatography) (Waters, Manchester, NH, UK), as previously described (10). The nanoACQUITY UPLC, was equipped with a nanoACQUITY HSS T3, analytical reverse phase column (75 µm × 150 mm, 1.8 µm particle size, Waters). *PROTEINLYNX GLOBAL SERVER™ (PLGS)* version 3.0 was used to process and search the continuous by liquid chromatography mass spectrometry in data-independent analysis mode (LC-MS^E). Samples from each condition were analyzed in triplicate (technical triplicates). Proteins were identified with the embedded ion accounting algorithm in the software and a search of the *Homo sapiens* database downloaded on April 2017 from UniProtKB (http://www.uniprot.org/). The proteins were classified according with biological function (within AEP) (21,22), origin and molecular interaction (http://www.uniprot.org/).

For the label-free quantitative proteome, three MS raw files from each pooled group (according to the experimental condition: *in vitro*, *in situ*, or *in vivo*) were analysed using the *PLGS* software. This software, specially designed for proteomics purposes by Waters Corporation, is coupled to the mass spectrometer and uses Monte Carlo algorithms. These are algorithms that attempt to explore the possible solutions through use of (pseudo-)random numbers. Furthermore, the algorithm attempts to navigate through the space of possible solutions by accepting or rejecting steps based partly on its current state. Such algorithms are referred to as Markov Chain Monte Carlo (MCMC) methods. All the proteins identified with a score with confidence greater than 95% were included in the quantitative analysis. Identical peptides from each triplicate by sample were grouped based on mass accuracy (<10 ppm) and

on time of retention tolerance <0.25 min, using the clustering software embedded in the PLGS. Difference in expression among the groups was expressed as $p<0.05$ for down-regulated proteins and $1\text{-}p>0.95$ for up-regulated proteins (10-12,19,20). The following relevant comparisons were made: *in vitro vs. in situ*, *in vitro vs. in vivo* and *in situ vs. in vivo*.

Results

A total of 321 proteins were identified in the present study (Table S1). The number of proteins identified in each experimental condition was 66, 174 and 170 for *in vitro*, *in situ* and *in vivo*, respectively (Table S1). The amount (µg) of proteins obtained by Bradford method at each condition was 8.88 µg, 52.04 µg and 49.08 µg for *in vitro*, *in situ* and *in vivo*, respectively. Only 23 proteins were common to AEPs from all three conditions (Fig. 1A), from which 15 are commonly considered typical proteins of the AEP, namely two isoforms of alpha-amylase, two isoforms of basic salivary proline-rich protein (PRP), three isoforms of Cystatin, *Histatin-3*, *Lysozyme C*, *Mucin-7*, *Pancreatic alpha-amylase*, *PRP 4*, *Salivary acidic proline-rich phosphoprotein ½*, *Statherin* and *Submaxillary gland androgen regulated protein 3b* (Fig. 1B).

In the *in vitro* condition, 31 proteins were not identified that where common in the other two conditions. Among those, 17 are usually regarded as typical proteins of the AEP, namely *Cystatin-D*, *Histatin-3*, eleven isoforms of Immunoglobulin, *Lyzozyme*, *Proline-rich protein 27*, two isoforms of Protein S100. As for the *in situ* condition, 10 proteins were not found, however none of them is regarded as typical protein of the AEP (Table S1).

A total of 37 proteins considered typical in the AEP were identified in the study (highlighted in bold in the table S1). The numbers in each experimental condition were 15, 37 and 32 for *in vitro*, *in situ* and *in vivo*, respectively (Table S1). The number of proteins identified exclusively in only one of the conditions were 31, 118 and 106, for *in vitro*, *in situ* and *in vivo*, respectively (Fig. 1). Two isoforms of Cystatins (B and C), two isoforms of Immunoglobulin and *Lysozyme A* were only found in the *in situ* condition (Table S1 and Fig. 1B).

When the conditions *in vitro* and *in situ* were compared, 25 proteins were common to both, among which 15 proteins are commonly considered typical of the AEP (two isoforms of Alpha-amylase, two isoforms of Basic salivary proline-rich protein, three isoforms of Cystatin, *Histatin-1*, *Lysozyme C*, *Mucin-7*, *Pancreatic alpha-amylase*, *Proline-rich protein 4*, *Salivary acidic proline-rich phosphoprotein ½*, *Statherin* and *Submaxillary gland androgen-regulated protein 3B*). Comparing the *in vitro* and *in vivo* condition, 32 proteins were common to both conditions, where 15 of them are commonly considered typical of the AEP (two isoforms of Alpha-amylase, two isoforms of Basic salivary proline-rich protein, three isoforms of Cystatin, *Histatin-1*, *Lysozyme C*, *Mucin-7*, *Pancreatic alpha-amylase*, *Proline-rich protein 4*, *Salivary acidic prolinerich phosphoprotein ½*, *Statherin* and *Submaxillary gland androgen-regulated*

protein 3B). When the *in situ* and *in vivo* conditions were compared, they presented 54 proteins in common, among which 32 are commonly typical AEP proteins, namely two isoforms of Alpha-amylase, two isoforms of Basic salivary proline-rich protein, four isoforms of Cystatin, two isoforms of Histatin, eleven isoforms of Immunoglobulin, two isoforms of Lysozyme, *Mucin-7*, *Pancreatic alpha-amylase*, two isoforms of Proline-rich protein, two isoforms of Protein S100, *Salivary acidic proline-rich phosphoprotein ½*, *Statherin* and *Submaxillary gland androgenregulated protein 3B*) (Table S1 and Fig. 1A).

Three comparative analyzes were performed to evaluate the difference in expression: *in vitro vs. in situ*, *in vitro vs. in vivo* and *in situ vs. in vivo* (using $p<0.05$ for down-regulated proteins and $1\text{-}p>0.95$ for up-regulated proteins). In the *in vitro vs. in situ* comparison, only 2 proteins were increased in the *in vitro* condition, *Statherin* being the most important for the composition of the AEP, while 23 proteins were reduced *in vitro*, including two isoforms of Alpha-amylase, two isoforms of basic salivary PRP, three isoforms of Cystatin, *Histatin-1*, *Lysozyme C*, *Mucin-7*, *Pancreatic alpha-amylase*, *Proline-rich protein 4*, *Salivary acidic proline-rich phosphoprotein ½* and *Submaxillary gland androgen-regulated protein 3B* (Table 1).

When the *in vitro* condition was compared with the *in vivo*, 9 proteins were increased in the first, including 5 isoforms of Hemoglobin and *Statherin*. In addition, 23 proteins were decreased in the *in vitro* condition, such as two isoforms of Alpha-amylase, two isoforms of basic salivary PRP, three isoforms of Cystatin, *Histatin-1*, *Lysozyme C*, *Mucin-7*, *Pancreatic alpha-amylase*, *Proline-rich protein 4*, *Salivary acidic proline-rich phosphoprotein ½* and *Submaxillary gland androgen-regulated protein 3B* (Table 2).

Comparing the *in situ vs. in vivo* conditions, 38 proteins were increased in the *in situ* experiment, among which are two isoforms of alpha-amylase, two isoforms of basic salivary PRP, four isoforms of Cystatin, two isoforms of Histatin-1, nine isoforms of Immunoglobulin, two isoforms of Lysozyme, *Mucin-7*, *Pancreatic alpha-amylase*, *Protein S100-A9*, *Submaxillary gland androgen-regulated protein 3B*, two isoforms of Proline-rich protein and *Salivary acidic proline-rich phosphoprotein ½*. Also, 12 proteins were reduced, one of which was *Statherin* (Table 3).

Discussion

The main aim of the present study was to provide the basis for the appropriate comparison between studies conducted employing different designs, e.g. *in vitro*, *in situ* and *in vivo*. However, some considerations are important that might explain the distinct results found when different designs were employed. The *in vitro* salivary pellicle was formed using unstimulated saliva, while *in situ* and *in vivo* pellicles were formed using saliva in

the oral cavity. The latter could have some more influence from the crevicular fluid, but this is probably a smaller portion of the pellicle, especially localized in the buccal surface of the teeth, because the AEP was collected from the middle third of the tooth surface. So the influence of the crevicular fluid in the present experiment was probably minor. However, some proteins of the pellicle are likely originated from crevicular fluid, such as serotransferrin (23).

One fundamental aspect of the present study was that the same volunteers took part in all the 3 conditions. This allowed a decrease in variations in function of the composition of the saliva (24). Moreover, all experiments were conducted in the morning (including collection of saliva for the *in vitro* experiment), also reducing circadian differences and decreasing variations between the conditions (21). Another important point was that the location of AEP collection in the *in vivo* condition corresponded to the exact same location where the enamel specimens in the *in situ* experiment were located, which allowed for less variations in the proteomic profile due to differences in locations within an individual's oral cavity (10). One downside was that different teeth were used. In the *in vivo* condition, AEP was collected from the volunteer's own teeth, but the *in vitro* and *in situ* conditions, the AEP were collected from other enamel specimens, and it could be argued that the different teeth could have different pellicles. However, a previous study showed that there are no differences in the proteomic profile of AEP even between human and bovine enamel (16), so no differences can be expected in the present study, when human teeth were used in all conditions of AEP formation. In fact, using human enamel specimens actually prompted an experimental model as close as possible to the clinical condition.

It is important, however, to address a limitation of the *in vitro* condition, where the natural salivary flow is virtually impossible to reproduce and could impact the amount of proteins recovered during pellicle harvesting. In order to address this limitation, we changed the saliva every 30 min in the *in vitro* condition (19), which led to the recovery of around 9 µg of proteins, enough for the proteomic analysis that allowed identification of 66 proteins. This figure is higher than that reported in the study by SIQUEIRA ET AL. (18) (45 proteins) and in the study by PELA ET AL. (19) (55 proteins). Moreover, from these 66 proteins, we identified 15 that are typically found in the acquired enamel pellicle. It is important to highlight that the quantification results obtained by method Bradford demonstrated a difference between the groups. However, all samples were calculated and adjusted to the same concentration (8.88 µg) before being submitted for proteomic analysis, allowing a real comparison between groups.

As for the *in situ* study, we identified 174 proteins, what is much higher than the one obtained in our previous experiment (37 proteins) (16). This can be attributed to the higher number of enamel specimens employed in the present study in comparison to the previous one (108 *versus* 54 proteins). In addition, from the 174 proteins identified in the *in situ* study, 37 are typical of the acquired enamel pellicle. In a recent *in situ* study, 498 proteins were identified in

individual pellicles samples formed on bovine enamel. The higher number of identified proteins is due to the much bigger surface area (8 cm^2) and to the fact that the pellicle is collected using solutions that cannot be employed in the clinical condition (25). The total number of proteins identified *in situ* was virtually identical to the one *in vivo* (170 proteins), which is lower than what was previously reported by VENTURA and coworkers (10). However, in the latter, the AEP was collected from all the volunteers' teeth, while in the present study the pellicle was only collected from the selected sites.

We identified a considerably lower number of proteins in the *in vitro* AEP compared to the *in situ* and *in vivo* conditions (Fig. 1), furthermore several of the proteins identified *in situ* and *in vivo* AEPs are intracellular proteins, whose function within the AEP are not known so far. Moreover, 23 proteins were common to the 3 conditions, from which 15 are typical of the AEP, with important functions in this integument. However, in the *in vitro* condition, some important typical proteins of the AEP were not identified, namely eleven isoforms of Immunoglobulin (participates in the defense response to bacterium, *Histatin-3* (exhibit antibacterial and antifungal activities), two isoforms of *Protein S100* (calcium and zinc binding and antibacterial function) and *Lysozyme*. The latter protein has the ability to degrade bacterial peptidoglycans, resulting in a strong anticariogenic potential (24). The absence of these proteins can influence in studies related to dental caries. This is an emerging field of research, since recently, 23 proteins were identified with different expression in the initial pellicle of caries-free and caries-active individuals (25).

Thus, from a qualitative point of view, the *in vitro* condition did not identify some important proteins of the AEP, suggesting that this condition is not completely reproducible in relation to the *in vivo* condition. In contrast, the *in situ* condition showed a similarity of the total and proteins commonly typical of the AEP with the *in vivo* condition.

From the quantitative point of view, relevant differences were also detected between the different conditions, using the expression for down-regulated and up-regulated proteins. When the *in vitro* condition was compared with the *in situ* and *in vivo* ones, there was a great reduction in important proteins such as Cystatins, *Mucin-7* and *Alpha-amylase*, which can have an impact in studies involving caries and dental erosion, where the protective role of these proteins is evaluated (14) (Table 1 and 2). For illustration, the increase in *Statherin* in the *in vitro* condition was only 37% in relation to the *in situ* one, while all proteins reduced in the *in vitro* condition compared to the *in situ* one showed a reduction greater than 50%. In addition, when comparing the *in vitro* and *in vivo* conditions, the increase in *Statherin* was modest (29%) and regarding the different isoforms of hemoglobin, it was moderate (61 to 68%) in the *in vitro* condition. However, the reductions in important proteins such as *Cystatin, Mucin-7, Alpha-amylase* and *Lysozyme C* were large. These results possibly indicate that the performance of treatments with these proteins in the *in vitro* condition might be underestimated with respect to the *in situ* and *in*

vivo conditions, when the treatments aim at increasing the concentrations of these proteins in the acquired pellicle.

On the other hand, when the *in situ* condition was compared with the *in vivo* one, the opposite was observed, i.e., there was a great increase in important proteins of the AEP in the *in situ* condition, such as *Alpha-amylase*, various isoforms of PRPs, Cystatins, Histatins, *Protein S-100* and *Lysozyme* (Table 3). This implicates that in *in situ* studies, the performance of treatments evaluating these proteins might be highly comparable with *in vivo* conditions.

Based on our results, the null hypothesis was rejected. Both proteomics profiles (qualitative and quantitative) were different, especially in respect to the proteins commonly typical of the AEP in the *in vitro* condition when compared with *in situ* and *in vivo* condition. The differences between the conditions might interfere in the extrapolation of the results obtained in *in vitro* studies to *in situ* and *in vivo* conditions. Our results raise a new perspective for the interpretation of studies where the protective role of the acquired enamel pellicle should be taken into account. Especially in the case of erosive tooth wear, the recommendations regarding preventive and therapeutic measures are based on *in vitro* and *in situ* studies, due to the lack of suitable response variables to evaluate the progression of the condition clinically. In addition, the absence of some proteins related to bacterial activity can have strong impacts in *in vitro* studies related to dental caries. Thus, the results obtained here should be taken into account for the appropriate interpretation of these studies.

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Role of the authors: VTP, TB, TSC, AL and MARB. conceived the experiments; VTP, JGQL, TMOV and GDC conducted the experiments; VTP, MARB, TB, TSC and AL analysed the results; VTP, MARB, TB, TSC and AL drafted the manuscript. All authors reviewed the manuscript.

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Figure 1. Venn Diagram with the numbers of the exclusive proteins from each group and proteins common to 2 or more group. (**A)** represents total proteins. (**B)** represents the proteins commonly typical of the acquired enamel pellicle.

Table 1. Relative quantification of proteins identified in the acquired enamel pellicle collected in *in vitro* and *in situ.*

Table 2. Relative quantification of proteins identified in the acquired enamel pellicle collected in *in vitro* and *in vivo*.

Table 3. Relative quantification of proteins identified in the acquired enamel pellicle collected in *in situ* and *in vivo*.

Table S1. Classification of proteins identified in the acquired enamel pellicle collected *in vitro, in situ* and *in vivo.*