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ORIGINAL ARTICLE

Anticaries activity of egg ovalbumin in an experimental caries biofilm model on enamel and dentin

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Abstract

Objectives Limited evidence suggests a putative inhibitory effect of dietary proteins on demineralization during the carious process. The aim was to explore a potential anticaries activity of the egg protein ovalbumin on a relevant in vitro approach.

Materials and methods Biofilms of *Streptococcus mutans* UA159 were formed on saliva-coated enamel and dentin bovine slabs. Biofilms were challenged with 10% sucrose followed by either a 200 μg/mL solution of ovalbumin or 1:10, 1:100, and 1:1000 (*v*/*v*) serial dilutions of that ovalbumin solution, for the entire length of the experiment. Biofilms exposed to 10% sucrose followed only by 0.9% NaCl served as caries-positive control. Once completed the experimental phase, biofilms were analyzed for biomass, viable bacteria, and polysaccharide formation. Final surface hardness (SH) was obtained to calculate %SH loss (demineralization). Two independent experiments were conducted, in triplicate. Data were analyzed by ANOVA and a post hoc test at the 95% confidence level.

Results A reduction ($p < 0.05$) in biomass and extracellular polysaccharide formation, but not in the number of viable cells, was observed for both dental substrates. All ovalbumin concentrations tested showed lower demineralization than the positive control ($p < 0.05$), in a dose-dependent manner. The highest concentration showed a reduction in the %SH loss of about 30% for both enamel and dentin.

Conclusion Egg ovalbumin presented to sucrose-challenged biofilms of *Streptococcus mutans* seems to reduce cariogenicity of a biofilm-caries model.

Clinical relevance Ovalbumin may counteract the cariogenic effect of sugars. If these findings are clinically confirmed, novel preventive approaches for caries are warranted.

Keywords Dental caries · Caries model · Oral biofilm · *Streptococcus mutans* · Ovalbumin · Dietary proteins

Introduction

Compromising quality of life [1] and affecting more than one third of the world's population, dental caries remains a public health concern worldwide [2]. The canonical restorative approach in caries treatment has resulted inefficient, expensive, and with low coverage. Preventive efforts, therefore, appear more rational to cope with the disease. Although fluoridated agents have been widely acknowledged as efficient for caries prevention [3], supplementary novel preventive and therapeutic approaches are needed to help alleviate the burden of disease imposed by caries. Moreover, health concerns about some potential side effects of fluorides have made some people less prone to use fluoridated products for caries prevention, yet, without supporting evidence.

Dietary factors are key in the onset of caries [4]. When sugar-rich food components are frequently ingested, a cariogenic dental biofilm will result, which in turn creates environmental conditions for the growth and proliferation of a cariogenic biofilm [5]. For a long time, the main focus in diet-associated research has been restricted to cariogenic nutrients, mainly sucrose and other fermentable carbohydrates [6]. The role played by other nutrients, ubiquitous to any diet, remains largely unknown (for a review, the reader is referred to [7]). Most of the research centered in other nutrients or foods different from sucrose has been carried out on dairy products. It has been argued that bovine milk inhibits bacterial adherence on the teeth [8]. We have reported that unsaturated free fatty acids may have an anticaries potential [9]. Furthermore, we

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have suggested a role of those fatty acids in the reduced demineralization induced by whole milk [10]. With regard to dietary proteins, higher consumption of the nutrient has been associated with lower caries incidence and with a favorable microbiological shift [11]. Protein milk components, including casein, have been claimed to reduce demineralization by the formation of a thin layer that would inhibit bacterial adhesion [12]. Most of the studies, however, have been performed in experimental animals with protein supplementation in a mixed diet [13, 14].

The mechanism by which proteins would inhibit the carious process may derive from alkali production by oral bacteria from dietary peptide substrates. Indeed, these peptides have been implicated in biofilm pH homeostasis and its consequent caries inhibition [15]. Several oral bacterial species, such as *S. sanguinis*, *S. gordonii*, and *S. salivarius* may contribute to saliva alkalization by metabolizing peptides to ammonium through the urease and arginine deiminase enzymes [16, 17]. Little research has been performed using dietary peptides, such as albumins from egg, nevertheless.

Given the limited evidence and to the fact that this mechanism arises as a promising approach to prevent dental caries, more research appears necessary to elucidate whether dietary proteins are indeed caries-protective and if so, to explore the potential mechanisms associated with the effect. The aim of this study, therefore, was to investigate the caries inhibitory effect of egg ovalbumin on a relevant experimental single-species caries model of *Streptococcus mutans* (*S. mutans*).

Materials and methods

Experimental overview

To test the hypothesis that ovalbumin inhibits the cariogenicity of biofilms of *S. mutans* UA159 under a highly cariogenic environment, a validated experimental caries model was used [18]. Biofilms were formed on bovine enamel and dentin slabs for 5 and 4 days, respectively. To induce a cariogenic system, biofilms formed on the slabs were exposed three times per day to 10% sucrose (*w*/*v*) during 5 min. Immediately after sucrose, biofilms were exposed to serial dilutions of an ovalbumin solution for five additional minutes. *S. mutans* biofilms exposed to 10% sucrose followed by 0.9% NaCl and to 0.9% NaCl followed also by 0.9% NaCl were included in the experimental design as positive and negative caries controls, respectively, each condition in triplicate. With the purpose of creating a pH-cycling environment that resembles clinical conditions, culture medium was changed twice per day and the pH was registered as a surrogate for biofilm acidogenicity. After the experimental phase, biofilms were separated from the dental hard tissues to measure the biofilm biomass, the number of live cells, and the polysaccharide production. In addition to the biofilm analysis, enamel and dentin slabs were examined before and after the experimental phase by surface Knoop microhardness (SH) to evaluate the demineralization that occurred during the cariogenic challenges with and without the presence of ovalbumin. The entire experiment was independently repeated to end up with six replicates per experimental condition.

Enamel and dentin slabs and biofilm formation

Enamel and dentin slabs of 7 × 4 × 1 mm were formed using diamond disks from crowns and roots of bovine incisors, stored for no longer than 30 days in 0.9% NaCl (*w*/*v*). After polishing with disks (Soflex, 3M, St. Paul, MN, USA), slabs were tested to obtain an initial SH value (SHi) with a microhardness tester (402 MVD, Wolpert Wilson Instruments, USA) at 50 g on enamel and 10 g on dentin, both for 5 s [9]. To avoid variability from using slabs too different in terms of SH, only those with a SH within the range of 369.52 ± 22.04 for enamel and 60.81 ± 8.07 for dentin ($n = 36$) were included. After sterilization under ethylene oxide [19], slabs were held by orthodontic wire and located into a 24-well cell culture plate that contained ultrafiltered (0.22 μm) pooled human saliva for 30 min with a protease inhibitor cocktail [20], which resembles the clinical acquired pellicle. Once the adherent surface was created by salivary proteins, biofilms were formed on the enamel and dentin slabs by adding an inoculum comprising *S. mutans* cultures (OD 0.8 at 600 nm) and 1% sucrose-containing medium [20], incubated for 8 h. Biofilms were transferred to 0.1 mM glucose-supplemented BHI medium for additional 16 h, thus completing 24 h, where biofilms were mature to start treatment exposure.

Experimental exposures

Mature biofilms grown on the slabs were exposed to 10% sucrose for 5 min three times per day at 8:30 AM, 12:30 PM, and 4:30 PM, except for the negative control exposed to 0.9% NaCl instead. Immediately after the cariogenic challenge with 10% sucrose, slabs and the biofilms were treated for five additional minutes with the protein ovalbumin (Sigma-Aldrich, Saint Louis, MO, USA) at either 200 μg/mL, 20 μg/mL, 2 μg/mL, or 0.2 μg/mL dilutions in 0.9% NaCl. Both controls were treated with 0.9% NaCl. After each exposure, biofilms were washed with 0.9% NaCl and relocated in the plate. Culture medium was changed before the first and after the last daily experimental exposure.

Acid production by the biofilm

Acidogenicity induced by *S. mutans* biofilms was tested by measuring the culture medium pH throughout the entire length of the experiment. A microelectrode (HI 1083B, Hanna

Instruments, Rumania) coupled to a portable pH meter (HI 9126-02, Hanna Instruments, Rumania) was used to register pH, directly from the wells of the spent culture medium. Readings were taken before each medium change [18].

Microhardness test on enamel and dentin

To determine the demineralization occurred during the cariogenic challenges and the potential inhibitory effect induced by the presence of egg ovalbumin after the sucrose challenge, SH was obtained from each sample of enamel and dentin before and after the experimental phase. SH has been vastly used as a surrogate measurement of demineralization [21] and previously validated [22]. Slabs were washed three times with 0.9% NaCl and biofilms were detached from the slabs by shaking the slabs for 30 s with a vortex mixer in a tube containing 1 mL 0.9% NaCl, making a visual confirmation that no biofilm was retained on the slabs. Before the experimental phase, each slab was indented three times with a separation of 100 μm between each indentation, to originate the initial SH (SHi). After the experimental phase, slabs were indented again to obtain a final SH (SHf) reading (Kg/mm^2) next to the initial three indentations. Mean values from the initial and final measurements were used to obtain the percentage of SH loss (%SHL) calculated by the formula: (SHi − SHf) × 100/SHi.

Analysis of the biofilms

Using the biofilms recovered from the previous steps (as indicated above), the suspension created in 1 mL 0.9% NaCl was aliquoted into smaller volumes to determine the following: biomass, viable microorganisms [18], and intra- and extracellular polysaccharides [23]. To obtain the biomass from the biofilms, a previously described protocol was used [20]. Briefly, 200 μL from the biofilm suspension was transferred to a pre-weighted tube and incubated with 100% ethanol at − 20 °C for 15 min, centrifuged (10 min at 5000*g* and 4 °C). The procedure was repeated and the pellet was washed with 500 μL of 75% ethanol and dried for 24 h in a desiccator. Biomass value was calculated subtracting the final dry weight to the initial weight of the empty tube expressed as milligrams per milliliter of biofilm suspension.

To estimate the number of viable cells of *S. mutans* in each of the biofilms, suspensions were serially diluted in 0.9% NaCl (*v*/*v*) and plated onto BHI agar plates in duplicate. Agar plates were anaerobically incubated (24 h at 37 °C), and the resulting colonies were counted at the dilution that allowed visualization of isolated colony forming units (CFU). Viable bacterial cells from the biofilms were expressed as CFU/mg of biofilm dry weight after correcting by the dilution factor, as previously described [23].

To determine the insoluble fraction from the biofilm polysaccharides, a previously reported protocol was used [23] that allows to quantify soluble and insoluble polysaccharides from the biofilm. Briefly, to calculate soluble extracellular polysaccharides (SEPS), suspensions were centrifuged (10,000*g* for 5 min at 4 °C) and the supernatant was maintained apart for further use. To estimate insoluble polysaccharides (IEPS), the pellet from the previous step was resuspended into 200 μL of 1 M NaOH, homogenized, and centrifuged. The supernatant was separated for additional analysis. Three volumes of cold 100% ethanol were added to each different supernatant fraction, incubated for 30 min at − 20 °C, and centrifuged, and the supernatant was discarded. The resulting pellet was washed with cold 70% ethanol and centrifuged again. Polysaccharide concentration was determined by the total carbohydrate concentration using the sulfuric phenol method [24]. Results were normalized by biofilm dry weight and expressed as percentage of either SEPS and IEPS by milligrams of biomass.

Statistical analysis of the data

Parametric distribution of the data was verified by the Kolmogorov-Smirnov test. ANOVA test was carried out to detect differences among the experimental conditions. Bonferroni post hoc test was used to detect statistical differences between each pair of experimental groups. The SPSS 15.0 statistical software was used to manipulate the data. Differences were considered significant at $p < 0.05$.

Results

Acidogenicity from the biofilms exposed to sucrose was affected by the presence of ovalbumin when evaluated by medium pH measurements. After 104 h for biofilms formed on enamel and 80 h for those formed on dentin, pH values showed great variation across treatment groups (Fig. 1). When compared to sucrose alone (caries control), biofilms exposed to sucrose followed by 200 μg/mL, 20 μg/mL, and 2 μg/mL of egg ovalbumin led to a medium with higher pH ($p < 0.05$). Conversely, biofilms treated with 0.2 μg/mL of ovalbumin failed to counteract pH decrease after sucrose exposure ($p > 0.05$), in enamel and dentin. A dose-dependent decrease in biofilm formation measured through biomass (Fig. 2) was observed in the biofilms exposed to sucrose followed by ovalbumin ($p < 0.05$) in enamel and dentin. Unlike the other three dilutions, the highest egg ovalbumin dilution at 1:1000 did not show a biomass reduction ($p > 0.05$) in enamel and neither 1:100 or 1:000 reduced biomass in dentin. Exposure of the *S. mutans* biofilms to sucrose followed by egg ovalbumin did not affect the count of viable bacterial cells at any of the dilutions tested (Fig. 3). When the formation of IEPS was assessed, only biofilms exposed to sucrose and ovalbumin at 200 μg/mL showed a significant decrease, both in those formed on enamel and in those formed

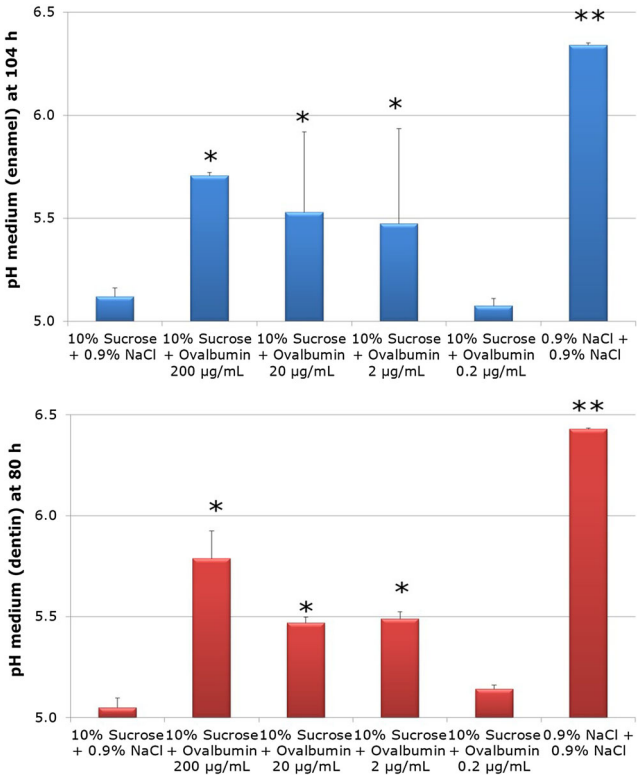

Fig. 1 Acidogenicity elicited from the *S. mutans* biofilms formed on enamel (upper panel) and on dentin (lower panel), in response to sucrose and the dilutions of egg ovalbumin, as indicated. Plot shows mean pH registered in the spent culture medium at 104 h for enamel- and 80 h for dentin-formed biofilms. Error bars represent the standard deviation (SD) of the mean for each experimental condition. **p* value < 0.05 and ***p* value < 0.001, with respect to the first bar of the caries-positive control with 10% sucrose

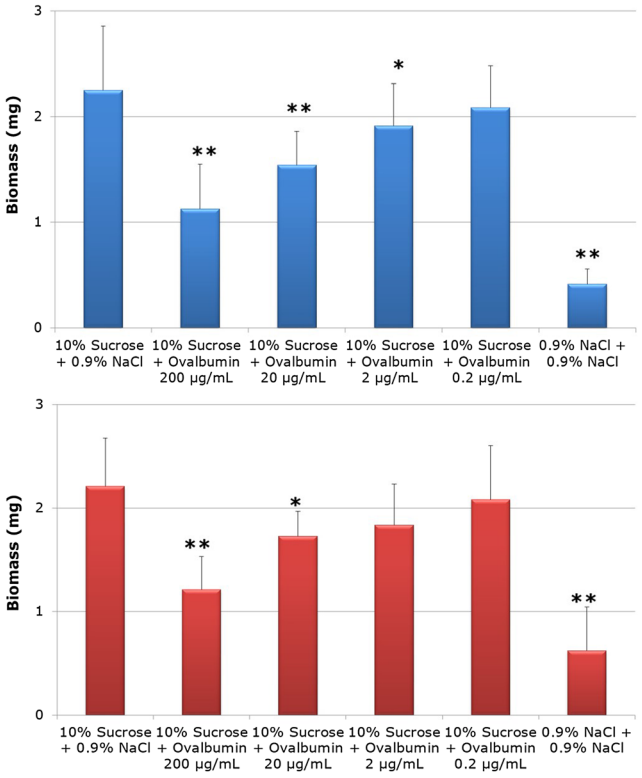

Fig. 2 Biomass formed by *S. mutans* biofilms on enamel (upper panel) and on dentin (lower panel), in response to sucrose and the dilutions of egg ovalbumin, as indicated. Plot shows mean values for biomass in milligrams. Error bars represent the standard deviation (SD) of the mean for each experimental condition. **p* value < 0.05 and ***p* value < 0.001, with respect to the first bar of the caries-positive control with 10% sucrose

on dentin (Fig. 4). The SEPS fraction showed a similar behavior than the IEPS (data not shown). Demineralization measured by %SHL was reduced when the cariogenic challenge with sucrose was followed by the exposure to a solution of egg ovalbumin to the biofilms formed on enamel as well as those formed on dentin. Reduction was dose dependent, but in enamel, a significant reduction was observed until a 1:100 (*v*/*v*) dilution, whereas the effect was verifiable only up to a 1:10 (*v*/*v*) dilution in dentin (Fig. 5).

Discussion

Despite the permanent efforts of the dental profession and public policy, caries has not been controlled in most countries [2]. The most traditional approach to prevent caries has been the implementation of fluoride programs with an individual or community focus. Water fluoridation, fluoridated dentifrices and mouthwashes, and fluoridated varnish applications are the sources of fluoride with the highest coverage in the population. Caries is a sugar-caused disease, nevertheless [25]. Hence, preventive measures should emphasize dietary control over fluoride applications. Changing dietary patterns, however, has been identified as a rather challenging task. In fact, evidence shows that ono-to-one interventions aimed at changing behavior are moderately effective for fruit, vegetable, or alcohol consumption, but not for modifying sugar consumption [26]. As an attractive prevention approach, natural dietary components may represent a different and novel approach to cope with caries. Herein, we demonstrated a potential strong caries inhibitory effect of ovalbumin, the most abundant protein in egg white.

Consumption of higher amounts of dietary proteins has been associated with lower caries incidence and with balance microbial composition of the dental biofilm [11]. It has been suggested that the milk protein casein reduces enamel demineralization by the formation of a thin layer that would inhibit bacterial adhesion [12]. In our approach, we formed the biofilms on the hard tissues, before the exposure to the treatments. So, arguing about a protein-induced protective layer is not convincing in this case. Although there are only few studies dealing with the potential anticariogenic effect of dietary proteins, most of them were carried out on experimental animals, fed with a protein-rich diet [13, 14]. Those early results showed that rats fed with egg white or casein developed less

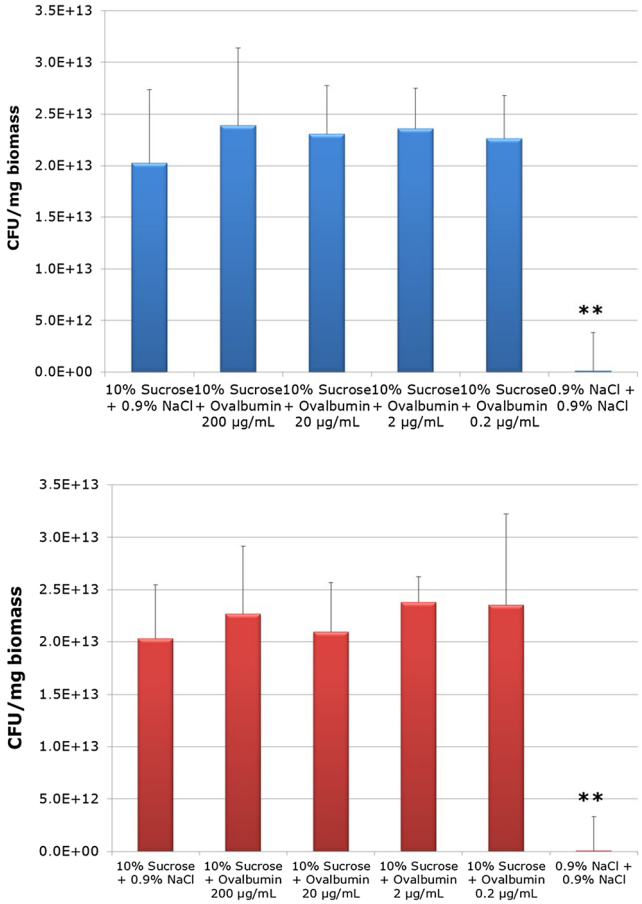

Fig. 3 Viable bacterial cells recovered from the *S. mutans* biofilms on enamel (upper panel) and on dentin (lower panel), in response to sucrose and the dilutions of egg ovalbumin, as indicated. Plot shows mean values of colony forming units (CFU) by milligrams of biomass. Error bars represent the standard deviation (SD) of the mean for each experimental condition. $^{**}p$ value < 0.001, with respect to the first bar of the caries-positive control with 10% sucrose

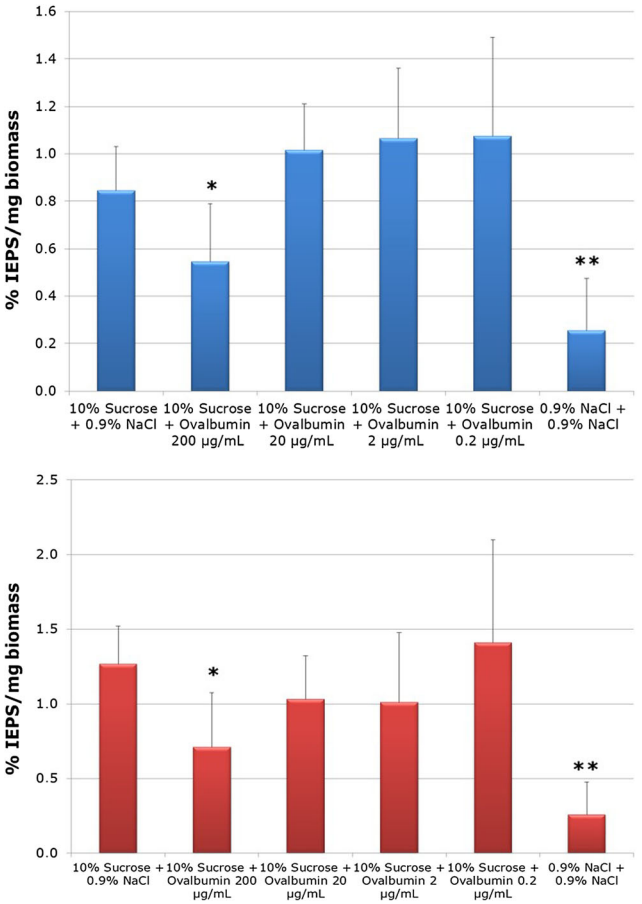

Fig. 4 Insoluble extracellular polysaccharide (IEPS) formation by the *S. mutans* biofilms on enamel (upper panel) and on dentin (lower panel), in response to sucrose and the dilutions of egg ovalbumin, as indicated. Plot shows the percentage of IEPS by milligrams of biomass. Error bars represent the standard deviation (SD) of the mean for each experimental condition. $^{*}p$ value < 0.05 and $^{**}p$ value < 0.001, with respect to the first bar of the caries-positive control with 10% sucrose

carious lesions. When individual amino acids were incorporated in the diet, no effect was detectable. We speculate, therefore, that the biochemical structure of the protein may be involved in the caries-protective effect of the protein. Alternatively, it has been reported that *Streptococcus*, *Actinomyces*, and *Lactobacillus* can metabolize proteins and amino acids into ammonia, maintaining pH balance within the biofilm [27]. Furthermore, host and bacterial proteases can degrade proteins present in the oral environment, either from saliva or from the diet, and transport them into the bacterial cell, serving as fermentative substrate. Another potential mechanism of action of ovalbumin is to be degraded and converted into short-chain fatty acids by deamination [28]. Fatty acids have been shown to inhibit the cariogenic activity of sucrose. Thus, ovalbumin could act through an indirect pathway in the caries process. Finally, a non-specific mechanism can also be argued. The isoelectric point of ovalbumin is pH 4.7, at which the protein has a net of charge zero. Lower pH values turn the protein cationic and higher anionic [29]. This pH-dependent characteristic of changing electric charges according to the medium properties confers any protein with a buffer capacity. Hence, there is biological plausibility in that a potential anticariogenic effect may be reached with several proteins, not exclusively ovalbumin. Indeed, we conducted previous experiments and set up the model using serum bovine albumin, obtaining similar results (data not shown). Furthermore, ovalbumin denatures more easily against pH or temperature variations, leading to serine saponification. This change releases phosphate to the medium, inhibiting salivary pH drop and consequently biofilm acidification [30].

The non-infectious current conception of dental caries has led to a new definition. Thus, caries is currently better understood as a disease characterized by a process of demineralization of the hard dental tissues, caused by frequent fermentable carbohydrate exposure to the dental biofilm, which shifts the ecological balance towards a non-infectious polymicrobial dysbiosis [31]. In this new conceptual scenario, the powerful anticariogenic potential effect of egg ovalbumin shown here

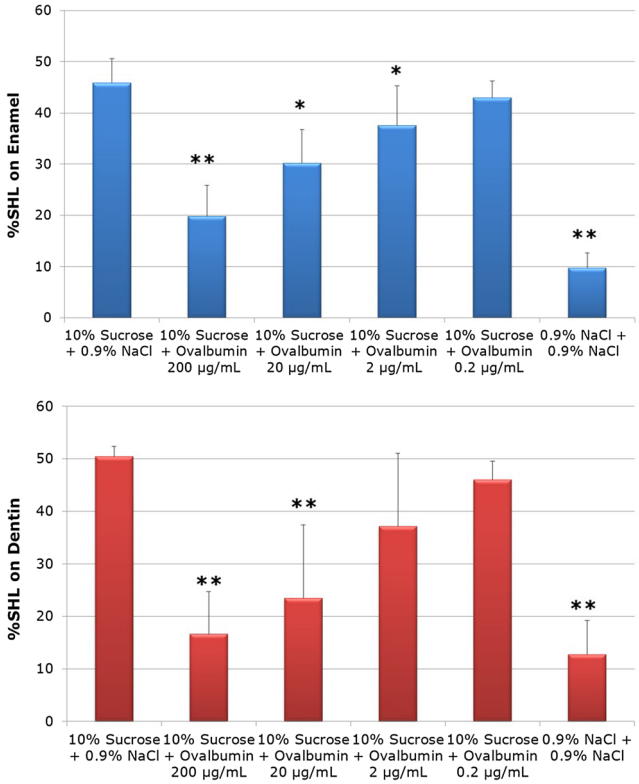

Fig. 5 Enamel demineralization induced by the cariogenic challenge with 10% sucrose followed by dilutions of egg ovalbumin, as indicated, on enamel (upper panel) and on dentin (lower panel). Plot shows the percentage of surface hardness loss (%SHL). Error bars represent the standard deviation (SD) of the mean for each experimental condition. *p value < 0.05 and **p value < 0.001, with respect to the first bar of the caries-positive control with 10% sucrose

and the lack of a killing effect appear quite appealing as a preventive tool and are consistent with the way caries is understood. Egg ovalbumin reduced pH drop, demineralization, biomass formation, and polysaccharide production, without affecting the number of bacterial cells (Fig. 4). Egg ovalbumin, therefore, would not be antibacterial, but anticaries, nonetheless. Using a metabolomic approach, new ideas have been developed on how the dental biofilm may maintain symbiosis or switch to dysbiosis in the presence or absence of proteins [27]. For example, proteolytic and amino acid–degrading bacteria of the dental biofilm can cleave protein structure and degrade them to produce components, such as ammonia, with buffer capacities against acidogenic species, leading to a more alkaline environment. Caries inhibition mediated by proteins may be the result of the use of the peptides by oral bacteria. Several streptococci can turn saliva alkaline by metabolizing peptides to ammonium through the urease and the arginine deiminase system (ADS) enzymes [32, 33]. Ammonia can neutralize pH, even in the presence of fermentable carbohydrates. The hydrolysis of urea and the catabolism of arginine are the primary sources of bacteria-generated alkali in dental biofilms. Recently, arginine has been proposed as an anticaries substance. It was reported that high-caries individuals have lower levels of free arginine in saliva, as compared with caries-free people [34]. Furthermore, the use of arginine in the clinic has shown promising results [35]. Since our model is a single-species biofilm with *S. mutans*, the latter mechanism is not possible, as *S. mutans* is not an alkali-producing bacterium. Another potential explanation comes from the activity of certain inhibitory components contained in the egg white. For example, 37% of the hen's egg vitelline membrane proteins is lysozyme [36]. Lysozyme has shown strong antibacterial and antifungal activity and is abundantly expressed in saliva [37]. This protein, along with other molecules, is part of the salivary innate immunity, which is reported to control the growth and proliferation of the dental biofilm [38]. More precise mechanisms to explain the activity observed against biofilm bacteria deserve further research and are of great interest.

Ovalbumin was capable of efficiently counteracting the cariogenic activity of sucrose, in a dose-dependent manner. Although the results in almost all the outcomes were similar between biofilms grown on enamel and dentin, the highest dilutions retained an effect on enamel, but not in dentin-formed samples. Dentin is structurally different than the enamel, and the carious process has particularities that make it more prone to demineralization [39]. Indeed, our results are consistent with the reported higher lability of the dentin tissue than the enamel, to the activity of bacterial acids [40].

Given the fact that no similar study has been reported, we had to provide a rationale for an experimental approach like this. We intended to model a situation where, despite counseling on restricting sugars in the diet, cariogenic food is being consumed. Thus, ovalbumin would need to be effective under a highly cariogenic environment, with low compliance. The election of the 200 μg/mL, as the highest egg ovalbumin dilution was based on preliminary experiments. One voluntary subject ate cooked egg white, providing saliva samples every minute for up to 30 min. Salivary total protein concentration (DC Protein Assay Kit, Bio-Rad, Hercules, CA, USA) was quantified. The highest protein concentration detected was 200 μg/mL at the first minute (data not shown). This concentration, therefore, represents a realistic amount of protein present in the mouth after consuming protein-rich food, like egg ovalbumin. Yet, it was very interesting to observe that a 100-fold in enamel and a 10-fold dilution of the original ovalbumin solution strongly inhibited dentin demineralization, suggesting anticaries effect at very low ovalbumin doses.

We acknowledge the limitations of this first study. The use of a single-species biofilms excluded the influence of several intervening factors in caries, such as saliva, fluoride, remineralizing substances, and the presence of a metabolically active and complex dental biofilm. Yet, we believe that a proof-of-principle like the present research opens the field for the discussion of diet, beyond sugars [7], as potential

alternative to caries control. Further research should be conducted to test the anticaries effect of egg ovalbumin in a clinical situation, whereby all the other modulating factors are present. If the protection is sustained, dietary interventions and programs incorporating dietary components rather than restricting consumption may emerge as an attractive alternative for caries prevention and control.

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Author's contributions RAG and CM conceived the experiments and the research questions. RAG and CM designed the experiments. PJ and CM performed the experiments, collected the data, and analyzed them. PJ drafted the first manuscript. RAG wrote the final version of the article. RAG, PJ, and CM approved the submitted version.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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