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# Mechanisms of astringency: Structural alteration of the oral mucosal pellicle by dietary tannins and protective effect of *b*PRPs

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ABSTRACT

The interaction of tannins with salivary proteins is involved in astringency. This paper focussed on saliva lining oral mucosae, the mucosal pellicle. Using a cell-based model, the impact of two dietary tannins (EgC and EgCG) on the mucosal pellicle structure and properties was investigated by microscopic techniques. The role of basic Proline-Rich-Proteins (bPRPs) in protecting the mucosal pellicle was also evaluated.

At low (0.05 mM) tannin concentration, below the sensory detection threshold, the distribution of salivary mucins MUC5B on cells remained unaffected. At 0.5 and 1 mM, MUC5B-tannin aggregates were observed and their size increased with tannin concentration and with galloylation. In addition, 3 mM EgCG resulted in higher friction forces measured by AFM. In presence of bPRPs, the size distribution of aggregates was greatly modified and tended to resemble that of the "no tannin" condition, highlighting that bPRPs have a protective effect against the structural alteration induced by dietary tannins.

## 1. Introduction

Astringency, mostly considered as an unpleasant sensory attribute, is an organoleptic tactile sensation (Green, 1993) mediated by the trigeminal nerve (Schobel et al., 2014). It is defined as the sensation of drying and puckering of oral mucosa. Astringency can be experienced during the consumption of plant food products, such as green tea, red wine or berries. In those products, proanthocyanidins, a group of tannins, have been identified as responsible for this sensation. Tannins are phenolic compounds (*syn.* polyphenols) and have the ability to precipitate proteins. They can be divided into three groups, proanthocyanidins (*syn.* condensed tannins), which are oligomers and polymers of flavan-3-ols, hydrolysable tannins, which are polyesters of sugars and gallic or ellagic acids, and complex tannins in which both types are covalently bound. Tannins are plant secondary metabolites, which play a role in plant defence mechanisms through their deleterious effects for plant predators (i.e. herbivores and omnivores). For instance in mammalian herbivores, tannins can reduce digestibility, damage the gastrointestinal mucosa and epithelium, and lead to kidney or liver failure or endogenous nitrogen loss (Shimada, 2006). In rodents, a tannin-rich diet induces weight loss, which is continuous in hamsters but reversible after three days in rats and mice. In parallel, feeding on tannins induces in rats and mice a dramatic increase in salivary proline-rich proteins (PRPs) within three days, while this is not observed in hamsters (Shimada, 2006).

Indeed in mammals, the presence of PRPs in saliva appears to be linked to the consumption of tannins. PRPs are particularly abundant in human saliva and may constitute up to 70% of parotid saliva proteins (Bennick, 1982). PRPs are classified in three groups depending on their isoelectric point and their degree of glycosylation: acidic, basic and glycosylated PRP (aPRP, bPRP and gPRP, respectively). aPRPs play a role in calcium binding and gPRPs in oral lubrication, while the main function of bPRP is the scavenging of tannins. Thus, investigations on tannin-protein interactions have shown that PRPs have a particular affinity for tannins (Shimada, 2006). Therefore, according to the Red Queen hypothesis, it can be postulated that bPRPs are part of a defence mechanism selected to protect organisms against the detrimental effects of tannins. Astringency is also probably a chemosensory signal for the detection of tannin-rich foods, leading to a shunning behaviour.

Astringency is thought to be due to a loss in the lubrication capacity

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of salivary proteins and more particularly of the proteins composing the mucosal pellicle (Nayak & Carpenter, 2008). The mucosal pellicle is a thin biological structure, whose thickness can reach 100 nm (Morzel, Siying, Brignot, & Lherminier, 2014), made of salivary proteins anchored onto oral epithelial thanks to covalent and non-covalent bonds (Bradway et al., 1992; Gibbins, Yakubov, Proctor, Wilson, & Carpenter, 2014). The mucosal pellicle contains MUC5B, MUC7, cystatins and IgA (Gibbins et al., 2014) and also amylases and PRPs (Bradway et al., 1992). Among these salivary proteins, the mucins MUC5B have been identified as major components. Davies et al. have reported that tannins aggregate the salivary mucins MUC5B and MUC7 (Davies et al., 2014). Therefore, interaction of astringent compounds with adsorbed mucins could play an important role in astringency sensation (Biegler, Delius, Käsdorf, Hofmann, & Lieleg, 2016).

In this context, the two hypotheses tested in this study are that 1 – tannins aggregate the mucosal pellicle proteins, with an impact on lubricating properties of this structure and 2 – PRPs play a protective role by scavenging tannins, precluding their access to the mucosa and its consequent structural alteration. As a result, astringency would be perceived only when tannin concentration is high enough to overcome the protective capacity of PRPs. To test these hypotheses, an *in vitro* cell-based model of oral mucosa with a mucosal pellicle previously developed (Ployon et al., 2016) was used. First, to investigate the effect of tannins on the mucosal pellicle structure, the model was exposed to two dietary monomers of proanthocyanidins, Epigallocatechin (EgC) and Epigallocatechin gallate (EgCG), differing in their structure by the presence of a galloylated moiety on EgCG (Fig. 1) and which is detected sensorially at lower concentrations. The mucosal pellicle structure and properties were investigated using three complementary microscopic techniques, fluorescent immunostaining of salivary MUC5B, Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). Second, in order to evaluate the role of bPRP in the protection of the oral mucosa, the model was covered by a liquid film containing IB5 (a human bPRP) prior to exposure to EgCG. The size of the aggregates with and without PRPs was evaluated by image analysis and compared.

## 2. Material and methods

### 2.1. Saliva collection

The study was performed in agreement with the guidelines laid out in the declaration of Helsinki. Written informed consent was obtained from the participants. Unstimulated saliva was obtained by the spitting method from fifteen volunteers who declared to be in good oral health. Subjects were instructed to refrain from smoking, eating or drinking for at least two hours before saliva collection. All samples were pooled and centrifuged at 14,000*g* for 20 min at 4 °C. The resulting pool of clarified saliva was divided into aliquots of 4 ml. Samples were immediately frozen at −80 °C.

### 2.2. Cell culture and formation of in vitro mucosal pellicle

TR146/MUC1 cells (Ployon et al., 2016) were grown in DMEM/F12–GlutaMAX medium (1:1) supplemented with 10% Foetal Bovine Serum (FBS), 1% penicillin/streptomycin (P/S) and 2.5 mg/ml of neomycin G418 (Gibco® by Life Technologies). Culture conditions were maintained at 37 °C and 7.5% $CO_2$. Cells were cultured in T75 flasks and the medium was changed every two days. Sub-cultures were prepared at 80% confluence using Trypsin-EDTA. Cells were seeded into eight-chamber glass slides for MUC5B immunostaining, and on 10 mm diameter glass slips for SEM and AFM imaging. All supports were coated with Cell-Tak™ (Corning) prior to seeding at a density of $4 \times 10^6$ cells/cm$^2$. In these conditions, confluence was reached in 48 h. Three days after confluence, a mucosal pellicle was deposited on the cells' surface by incubating cells for 2 h with clarified saliva diluted into growth medium (1:1) (Ployon et al., 2016). After incubation, samples were washed twice with PBS in order to eliminate the non-adsorbed saliva.

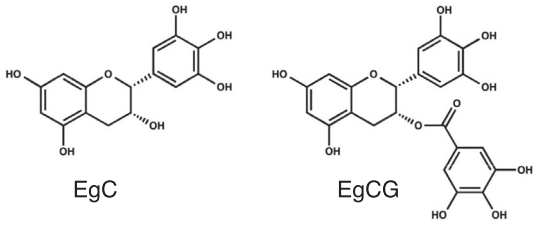


**Fig. 1.** Structure of Epigallocathechin (EgC) and Epigallocatechin gallate (EgCG).

### 2.3. Tannins solution

Epigallocatechin (EgC) and Epigallocatechin gallate (EgCG) were purchased from Santa Cruz Biotechnology (Texas, US). EgC and EgCG were diluted in PBS (pH 7.5) immediately before use to avoid compounds oxidation.

### 2.4. IB5 production

IB5 was produced and purified according to the method described by Boze et al. (2010) and was a kind gift from Dr. Cheynier (INRA Montpellier). The concentration of IB5 was adjusted to 0.66 mM in PBS.

### 2.5. Viability assay

Cells were cultured into 96-wells plates. When confluence was reached, cells were incubated for 2 h with clarified saliva diluted in growth medium as described above. After two washes with PBS, samples were exposed to tannins solutions (0.05–10 mM) or IB5 solution at 0.33 mM for 1 h. Cytotoxic effects were assessed using the Neutral Red assay, using a fluorimetric method. Briefly, cells were incubated for 3 h at 37 °C with 200 µl of medium containing neutral red at 50 µg/ml, washed twice with PBS and incubated at room temperature for 1 h in neutral red eluent (ethanol:$H_2O$:acetic acid, 50:49:1) with gentle agitation. Reading of the fluorescence was performed with Victor3V microplate reader (PerkinElmer) with excitation and emission wavelengths fixed at 544 nm and 595 nm, respectively. 80% of viability was considered as an indicator of non-cytotoxicity. Assays were performed in triplicate.

### 2.6. Exposure of the model to EgC and EgCG

Three concentrations of EgC and EgCG were tested for MUC5B immunostaining and SEM: 0.05, 0.5 and 1 mM. These concentrations were chosen taking into account the sensory detection thresholds of EgC and EgCG which were measured at 0.54 mM and 0.19 mM, respectively (Scharbert, Holzmann, & Hofmann, 2004). For AFM imaging, two concentrations of EgCG (1 and 3 mM) were tested. The cell-based model was covered with a tannin solution in PBS, or PBS alone as a control. After 5 min, the liquid was removed and cells were fixed prior to microscopical observations. Each condition was tested in triplicate.

### 2.7. Exposure of the model mucosa to EgCG in presence of IB5

The effect of EgCG at 1 mM was evaluated in presence of IB5 at 0.33 mM. The cell-based model was preliminarily covered by 62.5 µl of IB5 solution at 0.66 mM. Then, 62.5 µl of EgCG at 2 mM or 1 mM was added, resulting in final concentrations of 1 mM or 0.5 mM in EgCG and 0.33 mM in IB5. In the control condition, 62.5 µl of PBS were added to the 62.5 µl of IB5 solution.

### 2.8. Fluorescence immunostaining of MUC5B

Cells were fixed in 4% paraformaldehyde (PFA) for 30 min. Autofluorescence of PFA was blocked with a solution of $NH_4Cl$ at 50 mM. Non-specific binding was prevented using 0.3% free fat milk/5% goat serum in PBS. A primary antibody anti-MUC5B F2 (1:50 in PBS) produced in mouse was used (kind gift from Prof. Veerman, Free University of Amsterdam). Alexa 488 Goat anti-mouse IgG from Invitrogen was used as secondary antibody (1:400 in PBS). The samples were mounted into Prolong Gold antifade reagent with DAPI (Invitrogen). MUC5B immunostaining was observed with a NIKON Eclipse E600 microscope (Nikon Instruments, Melville, New York, USA) after excitation with high-pressure mercury-vapor lamp. Images were acquired using a Nikon Dxm1200C camera. The Nikon NISBR software was used for data acquisition. Three images, taken on different areas of the sample, were acquired for each of the triplicate.

### 2.9. Scanning Electron Microscopy

Fixation of TR146/MUC1 cells was carried out with 2.5% glutaraldehyde in PBS for 30 min, followed by 0.4% osmium tetroxide in PBS for 30 min. Dehydration was done through graded baths of ethanol (from 30 to 100%). Drying was performed by the critical point drying (CPD) method using Leica CPD 030. Samples were then coated with a thin carbon layer using a CRESSINGTON 308R and observed with a scanning electron microscope JEOL JSM 7600F (JEOL Ltd). SEM was operated at 2 kV and samples were observed at a working distance of 5 mm.

### 2.10. Atomic Force Microscopy imaging

Fixation of TR146/MUC1 cells was carried out with 2.5% glutaraldehyde in PBS for a minimum of 30 min. Atomic force microscopy experiments were performed using a Multimode 8 AFM microscope (Bruker, Santa Barbara, CA, USA). The topography and friction images were collected in $10 \times 10\ \mu m^2$ of scan size and 1 Hz of scan rate in high resolution ($512 \times 512$ pixel$^2$) using contact mode. The surfaces of samples were scanned in trace and retrace directions with V-shaped silicon nitride cantilevers with spring constant of 0.12 N/m (DNP-S, Bruker, Santa Barbara, CA, USA) in PBS medium (pH 7.8 ± 0.2, Bio-Rad). After obtaining a satisfactory topographical image in contact mode, the scan angle switched to 90° from 0° in order to record friction force images. The amount of tracking force was fixed for all samples to be able to compare the friction properties of different samples. In lateral force microscopy, the cantilever scans over the sample surface laterally (perpendicular to their lengths) and is torqued depending on the friction properties of substrate. The lateral forces are recorded by monitoring the horizontal deflection on the 4-segmented photodetector. The measured relative friction forces were in Volts and directly proportional to absolute friction force (in N). The friction force images visualize the high- and low-friction sites on the sample surface and they can provide a comparative analysis of the friction properties of different samples. Topography and friction force images of each sample were acquired in three conditions: 0, 1 and 3 mM EgCG (n = 7 per condition). For each sample, the median friction force was extracted for trace and retrace images and the mean value was calculated. The difference between the 1 and 3 mM conditions and the control (no tannin) was tested by a Student *t*-test.

### 2.11. Image analysis and statistical analysis

Images of MUC5B immunostained samples were analyzed using IgorPro software (WaveMetrics, Tigard, Oregon). 32-bits coloured images were converted into 8-bits grey level images. Then a thresholding operation, converting grey images into binary images, was performed. Finally, the binary images were submitted to an operation of image particle analysis and particles area in pixels were extracted and converted in mm$^2$.

The distribution of the total particle area was calculated and the particle sizes, representing 25 and 75% of the total particle area distribution (PS25 and PS75), were determined.

The differences between the different conditions were analyzed using a non-parametric Kruskal-Wallis test, followed by a post hoc Dunn-Holland-Wolfe test for pairwise comparison.

## 3. Results

### 3.1. Viability assay

For the exposure time of 1 h, EgC did not affect cell viability at any of the concentrations tested, while EgCG exhibited a cytotoxic effect for a concentration between 6 and 10 mM. The 0.33 mM IB5 solution was not toxic to cells.

### 3.2. Effect of tannins on the mucosal pellicle structure

The salivary mucin MUC5B, a major constituent of the mucosal pellicle, was immunostained on the *in vitro* model of oral mucosa in the presence, or not, of EgC or EgCG (Fig. 2). In the absence of tannins, salivary MUC5B was detectable as a layer exhibiting some between-cell variability, in accordance with previous observations (Ployon et al., 2016). The appearance of the MUC5B layer was hardly, if at all, affected by EgC and EgCG at 0.05 mM. By contrast, treatment with 1 mM EgC and EgCG (and to a lesser with 0.5 mM EgC and EgCG) was associated with the presence of some larger MUC5B deposits with higher fluorescence intensity (see arrows on Fig. 2), which correspond probably to MUC5B-tannin aggregates. The structure of these objects was investigated by Scanning Electronic Microscopy (SEM) in control conditions (no tannin) and at 0.05 and 1 mM of EgC and EgCG (Fig. 3). In the control condition (Fig. 3A), saliva deposit was made of a thin, loose, filamentous network as previously described by Ployon et al. (2016). When the model was exposed to EgC at 0.05 mM (Fig. 3B), the structural difference was limited, while addition of EgCG at the same concentration resulted in a deposit that appeared slightly thicker (Fig. 3C).

By contrast, when both tannins were added at the higher concentration (1 mM), larger and denser deposits were present on the cells' surface (3D and 3E). The size of these aggregates could reach several μm.

### 3.3. Effect of tannins on aggregates' population

Fig. 4 presents data obtained by image analysis of MUC5B immunostaining images, translating the size of MUC5B-tannins aggregates: cumulative particle area as a function of particle size (left) and PS75 determined from the cumulative curves (right) for both tannins and each concentration. For both tannins, differences in the cumulative curves can be observed depending on tannin concentration. PS25 was almost identical for the three concentrations while PS75 increased strongly with tannin concentration. In addition, large particles ($> 100$–$120\ \mu m^2$) were present only at the 1 mM concentration. Therefore, the higher the tannin concentration, the larger were the aggregates. For both tannins, PS75 was not significantly different from the control at 0.05 mM, while the difference became significant from 0.5 mM with p-values below 0.05, 0.001, 0.05 and 0.001 for EgC 0.5 mM, EgC 1 mM, EgCG 0.5 mM and EgCG 1 mM, respectively. Comparing now the two tannins, and although the difference was not significant, PS75 values tended to be higher for EgCG than for EgC at the two higher concentrations. In addition, the highest particle sizes ($> 200\ \mu m^2$) were observed for EgCG at 1 mM. In other words, EgCG generated overall larger particles than EgC, at least at 0.5 and 1 mM. These trends were also observable on mean particle areas, with values of 1.08 (control) 1.33, 1.34 and 2.04 $\mu m^2$ for EgC at 0.05, 0.5 and

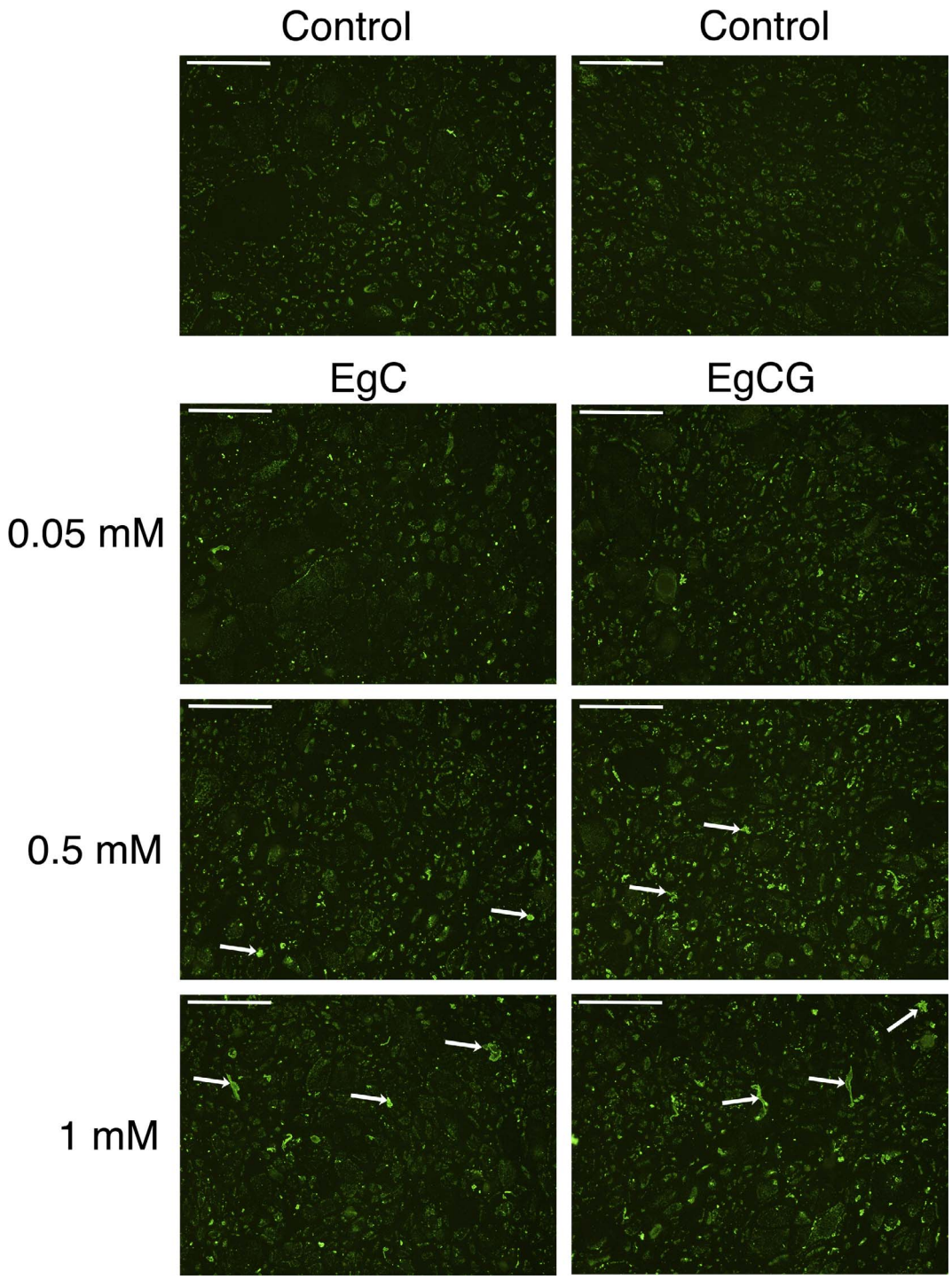


**Fig. 2.** Immunostaining of MUC5B on the *in vitro* model (TR146/MUC1 cells with a mucosal pellicle) after exposure to tannins (EgC and EgCG) at four concentrations: 0 (Control), 0.05, 0.5 and 1 mM. Scale bar = 100 μm.

1 mM, and 1.17, 1.57 and 2.61 $\mu m^2$ for EgCG at 0.05, 0.5 and 1 mM. This corresponds to radii from 600 to 900 nm approximately.

### 3.4. Effect of EgCG on friction force

Fig. 5 presents atomic force microscopy (AFM) images of topography (left) and friction forces (right) of the oral mucosa model's surface in control condition (no tannin) and in presence of EgCG at 1 and 3 mM. The topography of the *in vitro* model exposed to 1 or 3 mM EgCG was not drastically modified compared to the control condition (Fig. 5, left). In all conditions, the cells' surface was characterized by the presence of microplicae, as also observed on Fig. 3. These structural features are typical of stratified epithelial cells' surface. The microplicae height was in the range of a few hundreds of nm. In contrast to topography, friction force (Fig. 5, right) appeared influenced by EgCG concentration. At 3 mM especially, some areas were characterized by much higher friction force (see arrows) than in the other conditions. Considering the 7 replicates per condition, the average friction force recorded were 0.603 ± 0.186 V (no tannin), 0.560 ± 0.086 V (1 mM EgCG) and 1.313 ± 0.376 V (3 mM EgCG). The 3 mM condition was statistically different from the control ($p = 0.002$).

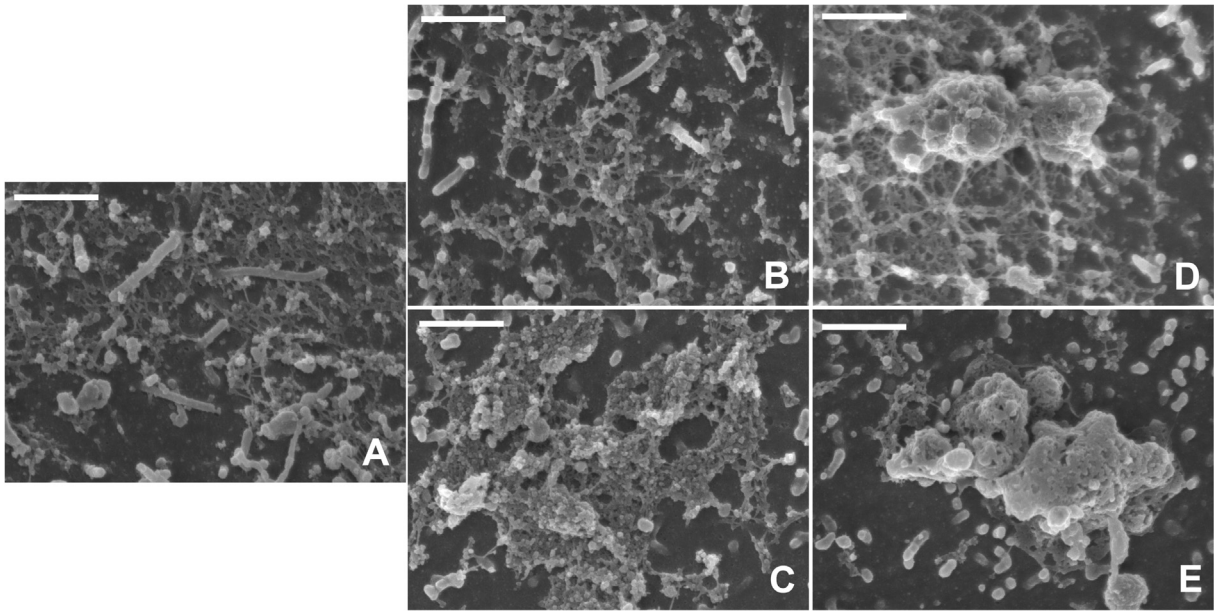


**Fig. 3.** SEM observation (×25,000) of the *in vitro* mucosal pellicle after exposure to tannins. A. Control (no tannins), B. EgC 0.05 mM, C. EgCG 0.05 mM, D. EgC 1 mM, E. EgCG 1 mM. Scale bar = 1 µm.

### 3.5. Effect of EgCG on the mucosal pellicle structure in presence of IB5

In order to assess the protective role of PRP toward tannin, the model of mucosa was preliminarily covered by a solution of the bPRP, IB5, prior to MUC5B immunostaining and image analysis. Addition of IB5 alone did not impact on the structure of the pellicle (data not shown).

Following the same format as Fig. 4, Fig. 6 presents data translating the size of MUC5B-tannins aggregates. There were differences in the shape of the cumulative curves. In particular, the presence of PRP modified the curves obtained with EgCG alone in such a way that they tended to resemble the control curve. The difference was especially

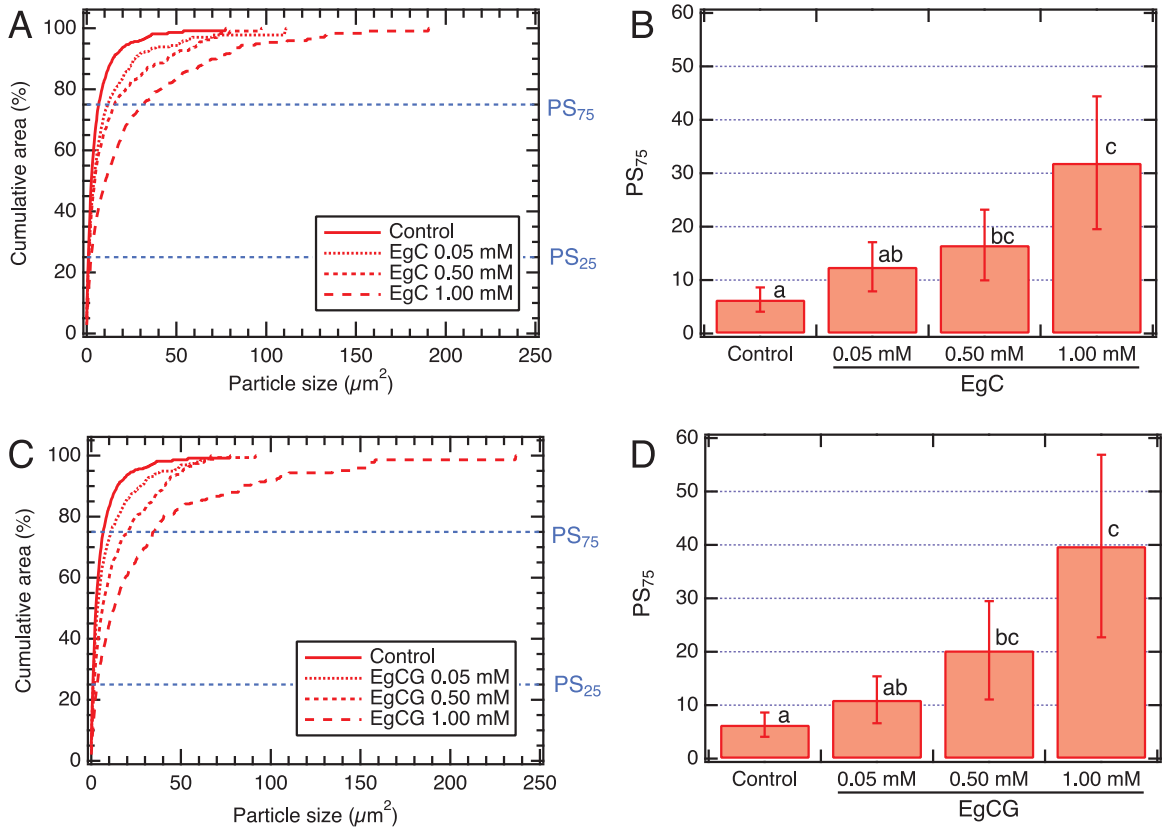


**Fig. 4.** Impact of the tannin type and concentration on MUC5B-tannins aggregates size. Distribution of particle size and particle size at 75% of the cumulative particle area after exposure of the *in vitro* model to EgC (top) and EgCG (bottom) at 0.05, 0.5 and 1 mM.

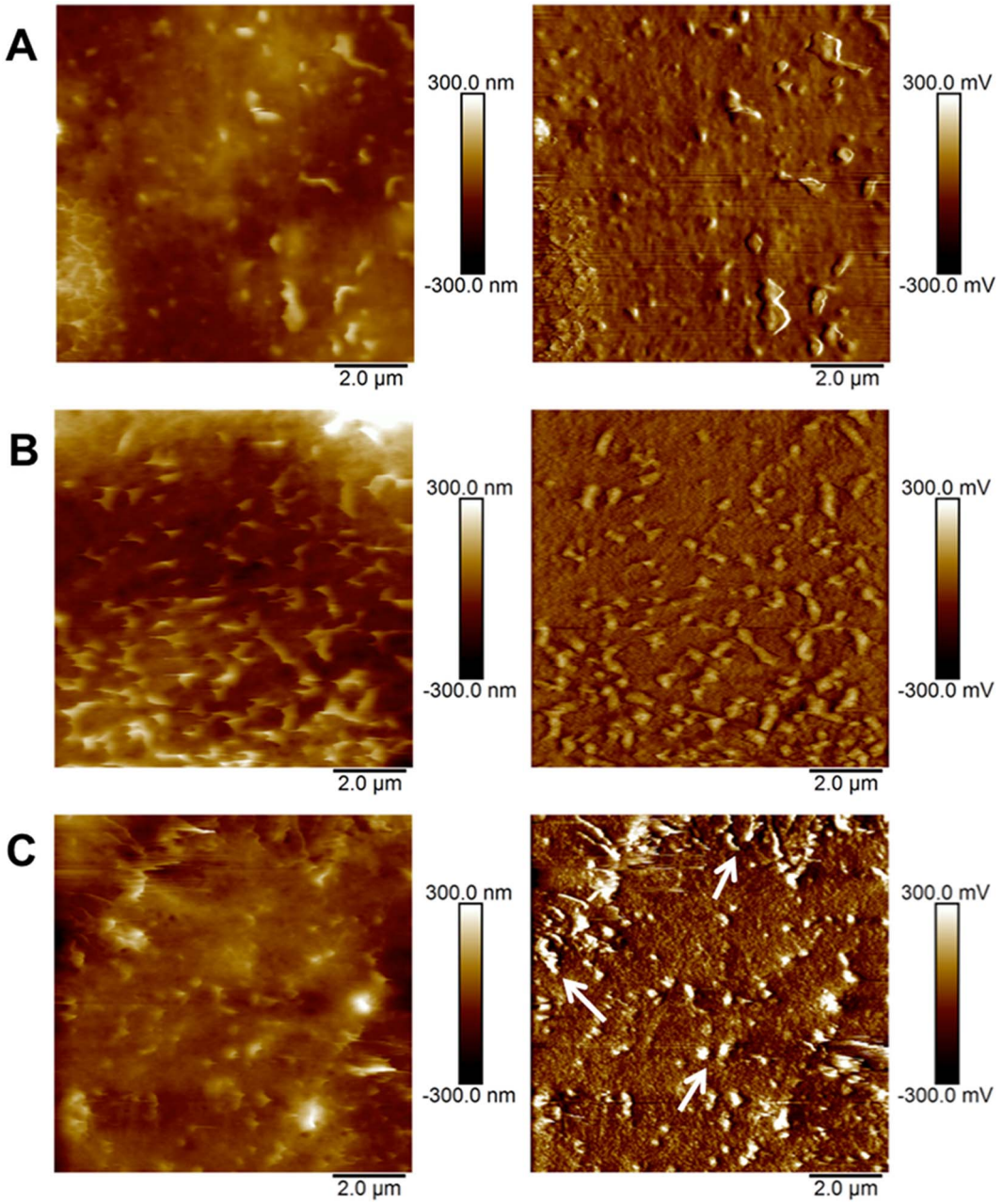


**Fig. 5.** AFM topography (left) and friction (right) images of the *in vitro* model (TR146/MUC1 cells with a mucosal pellicle). a. Control (no EgCG), b. EgCG 1 M, c EgCG 3 mM.

striking for the 1 mM EgCG: PS75, which was significantly increased by exposure to EgCG alone ($p \leq 0.001$), became non-significantly different from the control when PRP were added.

## 4. Discussion

The aim of this work was to contribute to the understanding of oral mechanisms involved in the sensation of astringency. Overall, it was found that tannins altered the mucosal pellicle by generating aggregates whose size depended on tannins' structure and concentration. At elevated concentrations, increased friction was measured on oral cells lined with a mucosal pellicle. Finally, we demonstrated that the bPRP IB5 could counteract the effect of tannins in terms of structural alteration.

The choice of focussing on the mucosal pellicle to decipher the mechanisms of astringency was guided by the fact that this sensation is perceived predominantly on mucosal surfaces, which are described as dry and puckered upon exposure to astringents. The mucosal salivary pellicle is a thin protein layer bound onto epithelium cells' surface. It is composed of epithelial and salivary proteins selectively adsorbed onto the cells' surface (Hannig, Hannig, Kensche, & Carpenter, 2017). MUC5B (Gibbins et al., 2014; Morzel et al., 2014; Ployon et al., 2016), amylase, cystatin and acidic proline-rich proteins (aPRPs) (Bradway et al., 1992) but also secretory component and IgA (Gibbins & Carpenter, 2013) have been identified in mucosal pellicles. Structurally, it has been described as organized in two layers (Macakova, Yakubov, Plunkett, & Stokes, 2010): an anchoring layer, constituted of the membrane-bound mucin MUC1 (Ukkonen et al., 2017) and small salivary proteins, and an external layer mainly composed of MUC5B (Macakova et al., 2010). The MUC5B-rich moiety is consistently thought to be chiefly responsible for the lubrication properties of this structure. Therefore, in order to focus on astringency, which is characterized as a loss of lubrication, the impact of tannins of the MUC5B layer was specifically studied.

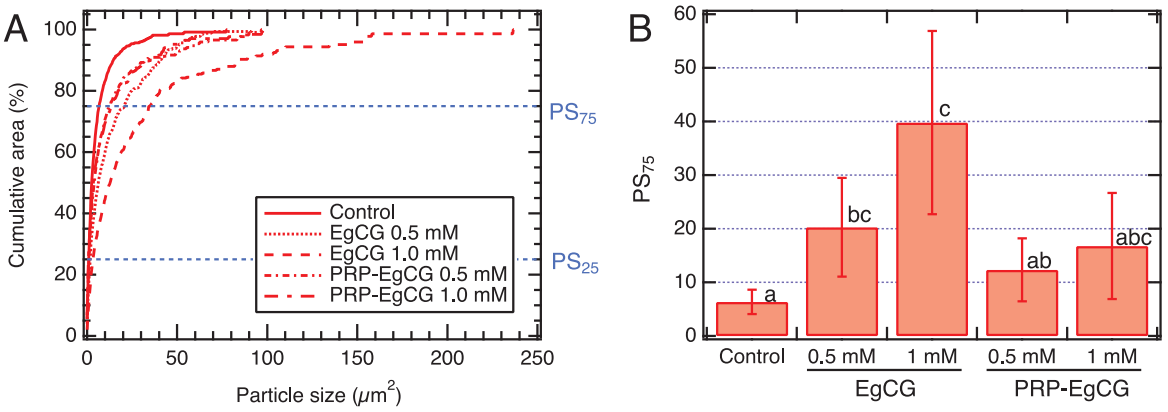


**Fig. 6.** Impact of PRP on MUC5B-tannins aggregates size. Distribution of particle size and particle size at 75% of the cumulative particle area after exposure of the *in vitro* model to the tannin EgCG at 0.5 mM and 1 mM, in presence or not of the bPRP IB5 (0.33 mM).

The impact of tannins on coating of oral surfaces by mucins has been previously studied. Thus, by quantifying mucins in solutions of tannins swirled in mouth and expectorated, Lee and Vickers (2012) reported that the mucus coating was not substantially altered, and suggested that tannins-mucins complexes may remain on mucosal surfaces. Another study reported that mouth rinsing with tannic acid modified the dental pellicle ultrastructure by making it distinctly more electron dense (Hertel et al., 2017), and the authors proposed that the dental pellicle might be strengthened by the inclusion of PRP-tannic acid complexes. Together, these two studies suggest that mucins are not extensively removed from oral surfaces by tannins. What we observed, namely the formation of aggregates, rather suggests that the spatial distribution of mucins is impacted by tannins. Aggregation of MUC5B by EgCG, and thus modification of the MUC5B network, has been previously reported (Davies et al., 2014). For a concentration of purified MUC5B of 15 $\mu g.ml^{-1}$ (i.e. ~0.027 μM), an increase of aggregate size was observed by AFM for EgCG at 0.075 and 0.375 mM in a dose-dependent manner with an average area reaching ~36,000 $nm^2$ at the high EgCG level, i.e. an average radius in the range of 100–120 nm (assuming that aggregates are circular-shaped). Using another mucin (porcine gastric mucin) at much higher concentration (~1.8 μM) with also much more concentrated EgCG (up to 22 mM), there was also an increase of aggregates' size with a tannin dose-effect and the average aggregate radius measured either by Dynamic Light Scattering or AFM was not substantially different from the previous study, in the range of 200 nm for the highest EgCG concentration (McColl, Horvath, Yakubov, & Ramsden, 2017). Another study using a much lower EgCG:mucin molar ratio (90 μM EgCG:1.8 μM pig gastric mucin) reported that no large aggregates were present, and estimated the radius of mucin-EgCG blobs (measured by TEM) at ~12–20 nm (Zhao et al., 2012). In our present study, and although the method of measurement (particle detection by image analysis on fluorescent images) may slightly over-estimate the size of aggregates due to the diffusion of fluorescence, the average aggregate size is quite clearly higher (radii range: 600–900 nm) than in the three studies quoted above. The EgCG:mucin molar ratio is not known here, especially since the MUC5B are not in solution but adsorbed to the cells' surface. However, another major difference between our study and the three others is that we worked on whole saliva and not purified mucin. Therefore, the presence of other salivary proteins likely has an effect on the formation of aggregates.

Protein aggregates can be formed in presence of tannins due to the multidentate properties of the latter, allowing to bridge two proteins together (Canon et al., 2013). The aggregation process induced by tannins has been extensively described for bPRPs, where aggregation occurs when at least three tannins are bound per protein and where aggregate size increases with tannin concentration, with radii reaching up to 1000 nm (Canon et al., 2013). In addition, glycosylation of PRPs prevents their aggregation by tannins (Sarni-Manchado, Canals-Bosch, Mazerolles, & Cheynier, 2008), precluding bridging of the peptidic chains due to steric hindrance. This last element is of interest for the process of aggregation of mucins, since these large proteins present a central region that is heavily glycosylated, flanked by the C and N terminal protein domains with relatively little glycosylations. Indeed, Davies et al. (2014) reported the aggregation by EgCG of MUC5B N- and C-terminal protein domains, but not of oligosaccharide-rich regions. Therefore, it is likely that aggregation expands from the unglycosylated regions. We also propose that other salivary proteins may be involved in this process and possibly facilitate the aggregation, especially if their degree of glycosylation is null or limited. The co-precipitation of mucins and other proteins by various astringent compounds has recently been reported and characterized, and it was found that amylase, cystatins and PRPs were particularly abundant in the precipitates formed upon incubation of saliva with EgCG (Delius, Medard, Kuster, & Hofmann, 2017). Cystatins have been described by several authors as constituents of the mucosal pellicle (Bradway et al., 1992; Gibbins & Carpenter, 2013), and MUC5B is known to form heterotypic complexes with salivary proteins, such as amylase and PRPs (Iontcheva, Oppenheim, & Troxler, 1997). This suggests that the MUC5B aggregates which we observed are more precisely aggregates of several types of proteins with tannins, and that the inclusion of other proteins with a propensity to aggregation could explain the larger average size that we observed compared to studies based on purified mucins.

The size of the aggregates was also influenced by the tannin structure, with higher values for EgCG than for EgC. Generally, interaction between the phenolic hydroxyl groups of EgC and EgCG and the amide groups of proteins is responsible for binding of these tannins to saliva proteins (Haslam, Lilley, Magnolato, & Warminski, 1992). However, EgCG differs structurally from EgC by an extra galloyl group and it has been previously shown that galloylation, representing one additional potential site for interaction, enhances aggregation (Poncet-Legrand, Cartalade, Putaux, Cheynier, & Vernhet, 2003). Davies et al. (2014) also observed different behaviours of salivary mucins in the presence of epicatechin (EC) or EgCG, in particular the absence of MUC5B aggregation when exposed to EC as opposed to EgCG. Our results are also in general agreement with those of Schwarz and Hofmann (2008) who measured the protein binding activity of 13 different astringents and found that binding of whole saliva proteins was highest for the compounds containing at least one galloyl group (Schwarz & Hofmann, 2008). Binding being a prerequisite to aggregation, it is consistent with the effect of galloylation on aggregate size that we observed.

Having clearly demonstrated that EgC and EgCG can alter the mucosal pellicle by inducing mucin aggregates, one may wonder what the sensory consequences of such structural modifications are. Overall, saliva is considered responsible for the lubrication between oral

surfaces, by maintaining wetness and reducing abrasion. For example, adsorption for 1 h of human saliva to synthetic surfaces reduces the friction coefficient by a factor of 20 (Berg, Rutland, & Arnebrant, 2003). Among the different proteins thought to participate to the rheological and tribological properties of saliva, the gel-forming MUC5B has received special attention. Mucins are highly hydrated, amphiphilic glycoproteins that facilitate lubrication in each of the three lubrication regimes that can occur in the presence of a fluid (boundary, mixed or hydrodynamic lubrication) (Coles, Chang, & Zauscher, 2010). Boundary lubrication of oral surfaces, which is characterized by a low film thickness (Coles et al., 2010), occurs when the surfaces (e.g. tongue and palate, cheek mucosa and teeth) are in full contact, which we believe occurs inevitably in the context of food oral processing. In addition, salivary pellicles formed on hydrophobic synthetic surfaces result in friction coefficients that remain low, even in the boundary regime (Macakova, Yakubov, Plunkett, & Stokes, 2011). Finally, direct interaction of tannins with proteins of the mucosal pellicle increased the perceived astringency of tea (Nayak & Carpenter, 2008). These two elements (role of mucins in boundary lubrication, importance of salivary pellicles in instrumentally-measured or sensorially-perceived friction) suggest that the MUC5B mucins included in salivary pellicles most likely contribute to the mouthfeel perceived on mucosal surfaces during eating or drinking. Their aggregation induced by tannins, as we measured in this study, would contribute to the loss of lubrication and to the typical dry and rough feeling of astringency. This is consistent with the increase of the friction coefficient of pre-adsorbed salivary pellicle formed onto synthetic surfaces when exposed to EgCG (Rossetti, Bongaerts, Wantling, Stokes, & Williamson, 2009). In the present study, we also measured at the 3 mM EgCC concentration an increased friction force at the surface of the epithelial cells. Besides this loss of lubrication, it is possible that aggregation can also result in a discontinuous and patchy mucosal pellicle, exposing the underneath epithelial cells. Tannins can interact directly with and bind to epithelial cells (Soares et al., 2016), which has been proposed to be another mechanism at the origin of astringency (Green, 1993). The possible involvement of chemoreceptors (Schobel et al., 2014; Sirk, Friedman, & Brown, 2011) or the binding to lipid of cell membranes (Sirk et al., 2011) have, for example, been suggested. Therefore, alteration of the mucosal pellicle by tannins, as documented here, could lead to astringency through these different mechanisms.

It is interesting at this stage to link our observations with the sensory perception of tannins. The intensity of the astringency sensation elicited by tannins depends on both their concentration and structure, for example their degree of polymerization (Vidal et al., 2003) or the stereochemistry of monomers (Thorngate & Noble, 1995). When it comes to sensory thresholds, data are very scarce. This is probably due to the methodological challenge constituted by the relatively slow development of astringency after ingestion and its long persistence and the fact that the perceived intensity of the same astringent solution increases upon repeated exposures (Green, 1993). For these reasons, the classical sensory methods for determination of detection or recognition thresholds are not well adapted. Nevertheless, recognition thresholds of 0.52 and 0.19 mM were reported for EgC and EgCG, respectively (Scharbert et al., 2004). These values are consistent with the fact that the mucin aggregate sizes were overall larger for the more astringent compound EgCG. Therefore, although this would need to be further confirmed, this suggests that the aggregate size distribution (particularly the presence and proportion of larger aggregates) is correlated with the perceived astringency of this class of tannins. One should note that the friction measured by AFM was significantly increased only for a higher concentration (3 mM). This is not entirely surprising since, as we suggested above, the loss of lubrication is probably only one consequence of the mucosal pellicle alteration. Other mechanisms (interactions of tannins with the membrane lipids, activation of specialized receptors) may participate to the astringency sensation, as also suggested in another study focussing on friction coefficients of salivary films (Rossetti et al., 2009). This can explain that human oro-sensory perception is more sensitive than the instrumental measure of only one aspect of the astringency sensation.

Another difference between the various *in vitro* studies and the human physiological situation is the presence of free flowing saliva in the oral cavity when a subject consumes a tannin-containing beverage. Based on this, we tested a second hypothesis, namely that PRPs in solution play a protective role towards tannins, precluding their access to the mucosa and its consequent structural alteration. We indeed observed that in the presence of the human PRP IB5 at physiological concentration, the formation of mucin aggregates induced by EgCG was limited. PRPs belong to the intrinsically disordered proteins, with an unusual extended conformation (Boze et al., 2010). This structure enables bPRPs to bind several tannins but also different types of tannins (Canon, Giuliani, Paté, & Sarni-Manchado, 2010), which could lead to an unfolded to folded structural transition (Canon et al., 2011). bPRP-tannin interaction occurs in three steps: formation of soluble non-covalent complexes, formation of aggregates from these supramolecular edifices and ultimately precipitation (Canon et al., 2013). At 0.33 mM IB5, the use of Small Angle X-ray Scattering enabled to determine that aggregation starts when EgCG concentration exceeds 0.5 mM approximately (Canon et al., 2013). In this study, we also observed visually that the threshold of precipitation of IB5 by EgCG was situated between 0.5 and 1 mM (data not shown). Therefore, in our conditions, it is expected that at 0.5 mM EgCG, aggregates are formed but they do not necessarily precipitate onto the mucosal pellicle. At 1 mM in contrast, aggregates are formed and they also precipitate. In both cases, the interactions between PRP and tannins limit the concentration of tannins which can interact with and alter the mucosal pellicle, as we observed based on mucin aggregates' size. However, the sensory implications are not straightforward, since astringency of tannins results probably from several mechanisms: loss of lubrication properties of free flowing saliva induced for example by salivary proteins aggregation (Lu & Bennick, 1998), direct tactile perception of large aggregates/precipitates of proteins from free saliva (Canon et al., 2013) and/or alteration of the mucosal pellicle (as we demonstrated here) with the several possible consequences discussed above. To sum up, we propose that the different following events occur in the oral cavity, depending on tannin concentration: around the threshold of aggregation of PRP by tannin, soluble aggregates are formed and it is possible that the consequent modification of the lubricating power of saliva is already perceived. Around the threshold of precipitation, in addition to the reduced lubrication properties of free flowing saliva, PRP-EgCG complexes precipitate onto the mucosal surfaces with certainly an impact on the lubricating function of the mucosal pellicle (although it is not at this stage structurally altered to a very large extent). At even higher concentrations, when the tannin concentration exceeds the capacity of interactions between PRPs and tannins, tannins can also alter the mucosal pellicle structure. From a sensory point of view, these different events combine to form the complex astringency sensation.

One should finally keep in mind that these different mechanisms are intimately dependent on the initial concentration of PRPs in saliva, which shows high inter-individual variability (Cabras et al., 2012), but also on the initial mucosal pellicle structure. This topic of inter-individual variability deserves further attention in order to gain a deeper understanding of variability in flavour perception.

## Conflict of interest statement

The authors declare no conflict of interest.

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