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Start looking at saliva: Effect of visualization of food images on salivary proteome

Erica Marques [a,1], Carla Simões [a,1], María Pérez-Jiménez [a,*], Fernando Capela e Silva [a,b], Elsa Lamy [a,*]

[a] MED – Mediterranean Institute for Agriculture, Environment and Development & CHANGE – Global Change and Sustainability Institute, Institute for Advanced Studies and Research, Universidade de Évora, Pólo da Mitra, Ap. 94, 7006-554 Évora, Portugal
[b] Department of Medical and Health Sciences, School of Health and Human Development, Universidade de Évora, Rua Romão Ramalho 59, 7000-671 Évora, Portugal

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ABSTRACT

This study aims to assess the influence of exposure to different visual food stimuli, on the salivary proteome, and relate them to the perception that participants had about those stimuli. For this purpose, participants were exposed to three food images: pizza, chocolate cake and salad. Unstimulated saliva was collected, before and during the image presentation, and the affective reactions evoked were assessed in a 9-point scale. Salivary secretion rate, total protein concentration and changes in the salivary proteome, by uni-dimensional (SDS-PAGE) and two-dimensional electrophoresis (2-DE), were studied. Results showed that salad image elicited a lower mouthwatering sensation than pizza and chocolate cake. Regarding salivary proteins, albumin increased, while amylase decreased during pizza visualization, carbonic anhydrase VI (CA-VI) increased in the visualization of the chocolate cake, while type S cystatins increased with salad image. Amylase showed a positive correlation with positive affective reactions produced by food images, while light chain of immunoglobulin, prolactin-inducible protein and type S cystatins correlated with negative reactions. Finally, CA-VI and short-palate lung and nasal epithelium carcinoma associated protein 2 (SPLUNC2) levels increased in the group that positively reacting to chocolate cake (cake +), compared to the group that react negatively to the chocolate cake (cake –) and control, contrarily to Ig alpha1 chain C region. This study showed the variations in saliva in response to pre-ingestive stimuli, and its relationship with affective reactions suggesting that the affective reactions that food triggers, might affect more the changes in salivary proteome than the type of food.

1. Introduction

The cephalic phase is considered the first phase of digestion (Lasschuijt et al., 2020), and is characterized by a series of physiological, endocrine, and autonomic responses that occur in anticipation of food intake, known as cephalic phase responses (CPRs) (Zafra et al., 2006). CPRs minimise the internal disturbances caused by food intake, thereby preparing the body for the digestion and absorption of nutrients (Smeets et al., 2010). According to some authors, CPRs allow for greater food intake, reduce the time between consumption and nutrient absorption, and consequently increase in the amount of nutrients that can be absorbed (Skvortsova et al., 2021), and play a significant role in regulating appetite and satiety (Power & Schulkin, 2008; Skvortsova et al., 2021).

CPRs are triggered by exposure to the sensory properties of food, such as sight, smell, taste, or even the mere thought or anticipation of eating (Carreira et al., 2020). It has been suggested that the greater the complexity of the stimulus, the greater the response generated. Therefore, there seem to be stimuli that produce more significant responses than others do, which, according to Mattes (1997), can be ranked as follows: chewing and swallowing > taste > smell > vision or sound > cognitive signals.

Among the various CPRs, salivary responses are particularly noteworthy, as the sensory stimuli of food or the anticipation of eating can induce a rapid release of saliva in the oral cavity (Pavlov, 2010). However, unlike other CPRs that are mediated by the vagus nerve, salivary CPRs result from interactions between the sympathetic and parasympathetic nervous systems (Mattes, 2000). The parasympathetic

* Corresponding authors.
E-mail addresses: maria.jimenez@uevora.pt (M. Pérez-Jiménez), ecsl@uevora.pt (E. Lamy).
[1] E. Marques and C. Simões, both authors contributed equally to this paper.

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system primarily increases blood flow to the salivary glands and stimulates saliva production by acinar cells, while the sympathetic system modulates the composition of saliva, particularly its protein content (Mattes, 2000).

The salivary responses vary with different types of food; for instance, dry foods tend to stimulate more salivation compared to moist foods (Pavlov, 2020). The saliva secretion could also be affected by the hardness and microstructure of foods (Pu et al., 2021). The physical and chemical properties of food also influence the magnitude of salivary responses, being well-known that acidic substances stimulate greater salivation to protect the oral mucosa.

Regarding pre-ingestive stimuli, which lead to cephalic phase responses, various studies have also investigated their impact on salivary secretion. For instance, Keesman et al. (2016) found that exposure to different foods (appealing, neutral, and sour) by simulating their consumption (seeing, feeling, smelling and thinking about the taste and mouthfeel), resulted in higher salivary secretion responses compared to the exposure to non-food stimuli (wood), with the highest secretion rate observed for appealing (small bag of chips) and sour foods (lemon slice) (Keesman et al., 2016). Similarly, (van der Waal et al., 2021) showed that the sensory properties of chocolate (vision and odour) increased salivation of participants, which also reported a higher desire, especially in a hungry state (compared to in a satiated state).

Despite of the studies in salivary secretion, the information about the changes in salivary composition induced by pre-ingestive stimuli as odour or vision is still limited. Some studies investigated the changes induced by food odour in salivary composition. For instance, Morquecho-Campos et al. (2019) studied changes induced by the odour of foods with varied taste properties (sweet, salty, and sour) and macronutrients (foods rich in protein, carbohydrates, fat, and low in calories). They observed an increase in salivary secretion due to exposure to food odour. However, for the salivary parameters analysed (amylase, lipase, and mucin 5B), they did not observe changes due to odour stimulation (Morquecho-Campos et al., 2019). On the other hand, Carreira et al. (2020) studied changes in saliva induced by the bread odour and observed, in addition to increases in the salivary secretion rate, changes in the salivary proteome (increases in the levels of type S cystatins, immunoglobulins, and two forms of amylase).

While the role of vision in food choices is well-documented, the specific effects of visual pre-ingestive stimuli on salivary composition remain highly underexplored. Previous studies, such as those by Lai et al. (2021), have shown that visual stimuli like food photos and videos can cause changes in salivary secretion and pupil diameter, an indicator of attention, stress, or emotion. These findings suggest that visual food stimuli can trigger significant emotional and physiological responses, including changes in salivation. However, the impact of these visual stimuli on the composition of saliva is not yet fully understood.

Regarding saliva and its composition, various studies have suggested that saliva is one of the main contributors taste, aroma and texture perception (Criado et al., 2021; Guichard & Le Quéré, 2022; Lamy et al., 2020; Pérez-Jiménez et al., 2020). Among its components, salivary proteins might have a crucial role for oral perception, since specific proteins in saliva are associated with the sensitivity to different flavour attributes. For example, bitter taste perception has been related with the levels of carbonic anhydrase VI (CA VI), cystatins, and proline-rich proteins (PRPs) (Cabras et al., 2012; Dsamou et al., 2012). While α-amylase has been negatively associated with sweet taste sensitivity (Rodrigues, Costa, et al., 2017), and more recently, by using electronic tongue, the amylase activity has been related with an enhanced perception of bitter, sweet, and sour taste but with a significantly reduced umami perception (Pu et al., 2024). Salivary proteins can also influence the perception of food texture, as it has been observed that different levels of α-amylase among different individuals seem to be responsible for variations in the texture perception of starchy foods (de Wijk et al., 2004). Furthermore, salivary proteins (e.g. PRPs, histatins, cystatins) can interact with food components like polyphenols, influencing the perception of astringency (Lamy et al., 2020). Additionally, salivary proteins can also affect the release of aroma compounds from food, thus affecting to their perception. Some mechanisms described have been the binding capacity of salivary mucins and alpha-amylase to retain aroma compounds from food (Pérez-Jiménez et al., 2020).

On the other hand, salivary proteome can also have effects on initial and further food digestion and nutrients/bioactive bioavailability (Berkel Kasikci et al., 2024; Lavoisier et al., 2024). As such, potential variations in saliva biochemical profile, due to different factors, may affect further ingestive and digestive aspects.

This study aims to investigate how the exposure to different visual food stimuli affects the salivary proteome and the associated reaction/emotion responses triggered by food images. This research seeks to provide a deeper understanding of the complex interplay between pre-ingestive stimuli and physiological changes in the context of food consumption.

2. Material and methods

2.1. Participants

A sample of 20 healthy participants (10 females and 10 males), from 21 to 49 years old, were recruited. Exclusion criteria included being smoker, being pregnant or breastfeeding women, having reported smell or taste related diseases, or any other chronic disease related with food behavior or medication that affect metabolism. Before the beginning of the study, all subjects read and signed an informed consent form. All procedures were performed according to the Declaration of Helsinki for Medical Research Involving Human Subjects and had ethical approval from the Ethical Committee of the University of Évora (Reference number: 22012).

At the beginning of the study session, a socio-demographic questionnaire was passed to participants asking about some personal information (e.g. age, gender, weight, height, and taste preferences). The aim of this questionnaire was to characterise the sample population to better understand the context of participants. However, this information was not consider for statistical analysis.

2.2. Visual stimuli and affective reactions assesment

Each participant was exposed to 4 visual stimuli (images) through virtual reality (VR) glasses (Oculus Quest) (Images in Supplementary Material). Three of these were food: pizza, chocolate cake and salad, and one was non-food (car), used as a control. The image of pizza was a slice of pizza with melted cheese and pepperoni commonly associated with a palatable food. The image of chocolate cake showed a slice of dark chocolate desert with various layers to evoke a sweet desert. Salad image was a bowl of fresh mixed lettuce, tomatoes, and cucumbers, representing a natural vegetable and less palatable option. The selection of these three foods (pizza, chocolate cake, and salad) was based on their distinct sensory characteristics and their expected ability to elicit varied emotional/reaction responses. Pizza and chocolate cake represent high-palatability, caloric foods with savory and sweet tastes, respectively, while salad was chosen as a generally consider low-palatability healthier option, although this generalisation might vary depending on the salad presentation and on the individual food preferences. Food stimuli were presented in random order, while the non-food image was always the last one. Between stimuli, a break of 10 min was allowed.

Following the presentation of each visual stimulus, participants assessed, on 9-point scales, the intensity of eight defined affective reactions that visual stimuli evoked: triggered appetite; desire; mouth-watering; well-being/calm; fascination/excitement; joy; repulsion; apathy/indifference. The questions were made in the way “seeing this image, how many ‘*affective reaction*’ you feel”, with 1 being “nothing” and 9 being “extremely”. Additionally, participants were asked (also on

a 9-point scale) to indicate their level of appetite at the moment for the consumption of the specific food from the picture (pizza, chocolate cake and salad) they observed. These scales were not applied to the control image (car) as it was a non-food image.

The food images presented were based on images in the FoodPic image database (https: //osf.io/av6he/), which have already been validated for the Portuguese population in terms of the effect they produce (Prada et al., 2017). Since these images did not meet the required quality standards for viewing in VR glasses, new photos were taken, as close as possible to the images tested in the above-mentioned study. The images were taken by professionals in the field (from the School of Arts of the University of Évora) with the specific intention to be used in this study.

2.3. Saliva collection and total protein concentration

Prior to experiments, each participant was instructed not to eat nor drink anything, except water, for at least one hour and a half before the beginning of each session, which occurred always between 10:30–12:30 a.m. Unstimulated saliva from participants was collected before and during each stimulus, in both cases for a period of 4 min. Participants were requested to accumulate all saliva produced in the mouth and spitting it into a tube only at the end of the collection period, or in case of discomfort, at each time they need it during the collection period (Beltzer et al., 2010).

Saliva samples were maintained on ice during the collections, and then they were stored at −28 °C, by no longer than 1 month. Before analysis, saliva samples were centrifuged at 13,000*g* for 15 min at 4 °C to remove mucins, cells and/or food residues.

Saliva flow rate was assessed by assuming a saliva density of 1.0 g/mL. Total protein concentration was determined by the Bradford method, using bovine serum albumin (BSA) as standard, and plates were read at 600 nm in microplate reader (Glomax, Promega, Madison, WI, USA).

2.4. One-dimensional electrophoresis (SDS-PAGE)

All saliva samples were subjected to SDS-PAGE for protein separation, following Laemmli protocol (Laemmli, 1970). Briefly, for each saliva sample, the volume corresponding to 7 μg of total protein concentration was used. Then, saliva samples were mixed with a Laemmli sample buffer (Laemmli, 1970) and run in 14 % polyacrylamide gels (Protean xi, Bio-Rad, CA, USA). Saliva samples were analysed in duplicate. A molecular mass marker (Millipore MPSTD4) was applied to one of the wells of each gel.

The electrophoresis run at a constant voltage of 150 V until the front of the run reached the end of the gel. Following this, the gels were placed in fixing solution (40 % methanol, 10 % acetic acid), then staining solution (1 % CBB R-250, 50 % methanol, 10 % acetic acid) and finally washing solutions (10 % acetic acid) for various times, until the removal of background. Then, gels were scanned using the ImageScanner III scanner (Epson) and Labscan software (GE Healthcare). The gel images were analysed using ImageLab software (BioRad), that automatically converted the bands into relative volume (% volume relative to the total volume of each lane). These values were then used for statistical analysis.

2.5. Two-dimensional electrophoresis (2-DE)

2-DE analysis was used only to assess the effect of the chocolate cake image on the salivary protein profile. Chocolate cake was chosen as a palatable and comforting food and two sub-groups were constituted, according to the type of reaction to chocolate cake image: positive reaction (cake +) vs. neutral reaction (cake –). To classify individuals into cake + or cake – affective reactions, the criteria of saliva flow rate and subjective reported desire for ingesting were used. Thus, individuals with increases in salivary flow rate and desire ≥ 5 (on a 9-point scale) were classified as positively reacting to chocolate cake (cake +; $N = 9$) and individuals with no increases in saliva flow rate and desire levels <5 were considered as neutral (cake –; $N = 9$). The saliva collected for non-food image (car) presentation was used as control ($N = 9$; being these 9 of the 18 individuals described earlier, which presented neutral reaction to car image). Saliva samples were mixed in 18 pools, with each pool composed by the saliva from 3 different individuals: 3 pools for before and 3 pools for during the presentation of chocolate cake with positive reaction (cake +); 3 pools for before and 3 pools for during the presentation of chocolate cake with neutral reaction (cake -); 3 pools for before and 3 pools for during the presentation of car image (control). Each pool was tested in duplicate.

For 2-DE analysis a previously published protocol optimized for saliva samples was used (Carreira et al., 2020). For that, a volume of saliva pools corresponding to 125 μg of total protein was aliquot and lyophilised.

The first dimension was performed by using gel strips (IPG strips 3-10NL, GE Healthcare). After rehydration, the strips were placed in the Multiphor II system for isoelectric protein focusing. Focusing was performed at 12 °C, and according to the following programme: step 1 - rise to 100 V (0:01 h), step 2 - 300 V (1:00 h), step 3 - rise to 3500 V (4:00 h), step 4 - 3500 V (3:30 h).

The second dimension (separation by molecular masses) was run by using 14 % acrylamide gels, following the same procedure described in the previous section (section 2.5). Then, gels placed first in fixing solution, then in colouring solution and finally in several changes of washing solution, as previously described. Then, gels were digitalised using the ImageScanner III scanner (Epson) and Labscan software (GE Healthcare).

The protein profiles were analysed using SameSpots software (TotalLab). For that, each gel was first manually aligned to the reference gel, which served as the basis for the subsequent automatic alignment. The detection of spots according to the aligned gels was corrected and those spots in which the labelling was wrong were edited. Statistical analysis was performed directly in the software.

2.6. Statistical analysis

Different parameters were tested for normal distribution (Shapiro-Wilk) and homoscedasticity (Levene test). In order to test for differences among food images in their effects at the level of the affective reactions in response to stimuli, one-way ANOVA was used.

GLM (General Linear Model) within-subject comparison was used to evaluate the effect of each image in each of the salivary parameters tested (flow rate, protein concentration and percentage of volume of each protein band). Period was set as factor, with 2 levels: before and during visualization. Since GLM does not allow to assess the effect of each individual food image, a paired-samples *t*-test was used, whenever this information was of interest.

In order to evaluate the existence of a relationship between changes in salivary parameters and affective reactions triggered by seeing the images, the analysis was done in 2 steps. First, through Principal Component Analysis (PCA) the variables concerning the affective reactions triggered by seeing the foods were reduced to components. For this, the correlation matrix of the standardized variables was examined, and the number of components to retain was based on eigenvalues, total explained variance and Scree plot examination. The Quartimax rotation with Kaiser normalization was performed, and the overall Kaiser-Meyer-Olkin (KMO) measure and Bartlett's test of sphericity were examined as assumptions of the test. In the second step, Pearson correlation was used to access the relationship between each of the extracted components with the variations in the salivary parameters. This "variation" in the salivary parameters was calculated as the value of the salivary parameter during image visualization minus the value of the salivary parameter in the period before visualization.

For analysis of the 2-DE salivary profiles, the protein spots obtained for each pool were compared through one-way ANOVA test, for the 3 situations: control (car), cake + (when seeing the cake produced effect at saliva volume level) and cake – (when seeing the cake had no effect at saliva volume level).

Statistics was run using SPSS software (IBM, v.25), except for protein spots, obtained by 2-DE, for which SameSpots (TotalLab) software was used. In all cases, a confidence interval of 95 % was considered.

3. Results

3.1. Affective reactions triggered by food images

In average, the intensity with which the affective reactions triggered by the different food pictures were perceived did not present significant differences among them. Except for mouthwatering sensation, for which the salad image elicited significantly ($p = 0.006$) lower levels (4.25) than pizza (6.60) and chocolate cake (6.30) images (Table 1). In spite of the lack of statistical differences in other affective reaction, some interesting trends can be obtain from these results. For instance, for anxiety higher standard deviation (SD) was observed for pizza and chocolate cake images, comparatively to the salad image, indicating higher variability among individuals in the way they react to the first two food images (Table 1).

3.2. Effect of food images on saliva secretion and composition

Using GLM model it was observed that salivary flow was significantly reduced after the control (car) image visualization from 1.42 mL/min before to 0.97 mL/min during visualization (Fig. 1A). Nevertheless, there were not significant differences in salivary flow during the visualization of any of the food images, comparatively to the situation before (Fig. 1A).

Regarding the total protein concentration (TPC), it decreased significantly during the visualization of the salty food images: pizza (from 620 to 503 μg/mL) and salad (from 716 to 601 μg/mL) (Fig. 1B).

When salivary proteins were separated by SDS-PAGE, it was possible to observe 25 protein bands, between 14 and 200 kDa, consistently present in the different profiles from all individual saliva samples (Fig. 2).

Percentage volume of each protein band, obtained from image analysis of the different SDS-PAGE gels were tested using GLM test to evaluate the effect of the image visualization on the level of each protein band. Image analysis of the different gels resulted in the observation of statistically significant changes in 6 bands (b1, b, d, e3, h, j) from saliva SDS-PAGE protein profile when comparing the period before and during visualization of images (Table 2). Individual data (heat maps) is presented as supplementary Fig. 1).

In the case of band b (albumin), it increased significantly from 9.1 to 10.5 (before vs during) during the pizza image visualization, but it hardly change for the other visual stimuli (cake, salad and control). By opposite, a tendency for decreases in the levels of band d (alpha-amylase) during the visualization of all images were observed. Although this decrease was statistically significant only in the case of pizza observation from 14.66 to 12.97 (before and during, respectively) and in the visualization of the control image (car), that decreased from 15.57 to 12.56 (Table 2).

The band e3 (carbonic anhydrase VI + zinc-alpha2-glycoprotein), increased significantly during the observation of the chocolate cake image (the sweet image), from 1.30 (before) to 1.65 (during). While remains with little variations for the other images representing the other stimuli (pizza, salad and car). Regarding band j (type S cystatins) it increased significantly ($p < 0.05$) during the visualization of salad image, from 9.51 to 11.47, and during the visualization of the control image (car) from 10.53 to 12.44, but there were hardly any changes for the pizza and cake images (Table 2).

3.3. Relationship between SDS-PAGE salivary profile changes and affective reactions to images

In order to further investigate whether the changes in the salivary protein profile were related with the affective reactions triggered by the visualization of the image, regardless of the type of food, the different affective reactions rated by participants, for each image, were submitted to a PCA, since it allows to consider the overall variance among participants and possible underlying patterns in the whole dataset. PCA (KMO = 0.794; $P < 0.001$ for Bartlett's test) resulted in two components, which together explain 74.5 % of variance: Component 1, respecting to positive affective reactions and Component 2, respecting to negative affective reactions (Table 3, Supplementary Fig. 2). In this analysis, anxiety was not included in the model, since it remained constant among participants.

The existence of relationship between each of the components and the changes in salivary protein profile (considered, for each protein band as the difference between the percentage of volume of the band before and during images observation) was assessed. The changes in the protein bands b1 (n.i.) and d (alpha-amylase) correlated positively (0.397 and 0.401, respectively) with Component 1 (referred as positive affective reactions). Whereas the changes in the protein bands h1 (light chain of immunoglobulin) and i1 (prolactin inducible protein) correlated positively (0.284 and 0.492, respectively) with Component 2 (referred as negative affective reactions) and band j (type S cystatins) correlated negatively (−0.341) with this component 2 (Table 4, Table 2).

3.4. Effect of visual stimuli in 2-DE saliva profile

To evaluate the effect of food images' visualization, on salivary 2-DE profile, and due to limited amount of individual saliva, pools of saliva samples were constituted. For 2-DE profiles, only one food image was considered, in this case, the chocolate cake. Since results from salivary flow rate and SDS-PAGE suggested that the effect of cake image might differ among individuals, existing also variation in the reported desire for ingesting this food, two groups of participants ($n = 9$ per group) were created, namely 9 participants for whom the visualization of the chocolate cake image increased salivary flow rate (cake +) and 9 participants for whom the chocolate cake image did not produce changes in salivary flow rate (cake -). For the same participants ($N = 18$), saliva obtained

Table 1
– Intensity reported by participants on 9-point scales to affective reactions (mean ± SD) triggered by the different food pictures.

Reaction/emotion	Food Image			P
	Pizza	Chocolate cake	Salad	
Appetite for the specific food	6.45 ± 2.14	6.35 ± 2.23	5.50 ± 2.46	0.357
Desire for ingesting that food	6.45 ± 2.40	5.95 ± 2.40	4.90 ± 2.63	0.139
Mouthwatering	6.60 ± 2.30[a]	6.30 ± 2.06[a]	4.25 ± 2.81[b]	0.006*
Wellbeing	4.85 ± 2.62	4.50 ± 2.24	4.90 ± 2.65	0.860
Excitement	4.25 ± 2.92	4.45 ± 2.31	4.90 ± 2.65	0.245
Anxiety	2.40 ± 2.11	2.30 ± 2.23	1.35 ± 0.81	0.145
Happiness	5.15 ± 2.54	5.05 ± 2.28	4.15 ± 2.74	0.393
Repulsion	1.05 ± 0.22	1.00 ± 0.00	1.10 ± 0.45	0.552
Indifference	2.30 ± 1.98	2.20 ± 2.04	2.80 ± 2.41	0.643

Note: Different letters mean differences between food images; * statistically significant for $P < 0.05$.

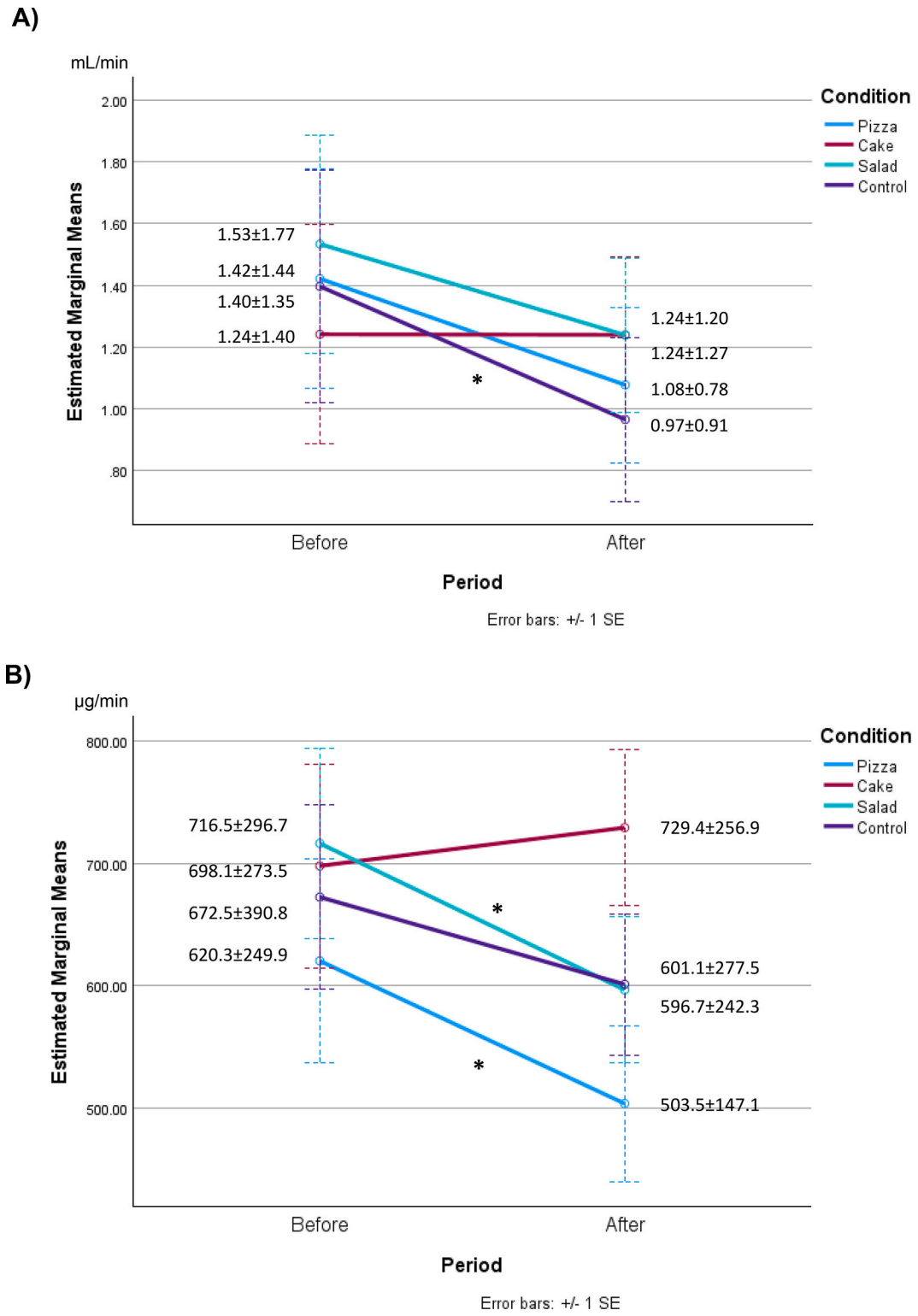

Fig. 1. – Salivary flow rate (A) and total protein concentration (B) before and during observation of the studied images (values are mean +/− SD). Asterisk (*) above the lines indicated statistically significant differences. Error bars indicated +/− standard error (SE).

during car visualization was used as control. As such, 2-DE were compared among three conditions: i) non-food image (control); ii) chocolate cake image inducing increased salivary flow rate (cake +); iii) chocolate cake image inducing no changes in saliva flow (cake -).

Comparing the normalized volume (% volume) of each spot, through ANOVA, it was possible to observe statistically significant differences in the expression levels of eleven spots (Figs 4 and 5). Among these, 7 spots (spots *94, 106, 161, 201, 205, 315* and *319*) increased with chocolate cake image observation, in the group to which this image induced increased salivation (cake +), having the lowest levels when the non-food image (control) was presented. On the opposite, 3 spots (spots *280, 299* and *300*) were decreased during chocolate cake visualization in the same group (cake +). Spot *279* was the one increased during chocolate cake image observation, in the group showing no changes in saliva

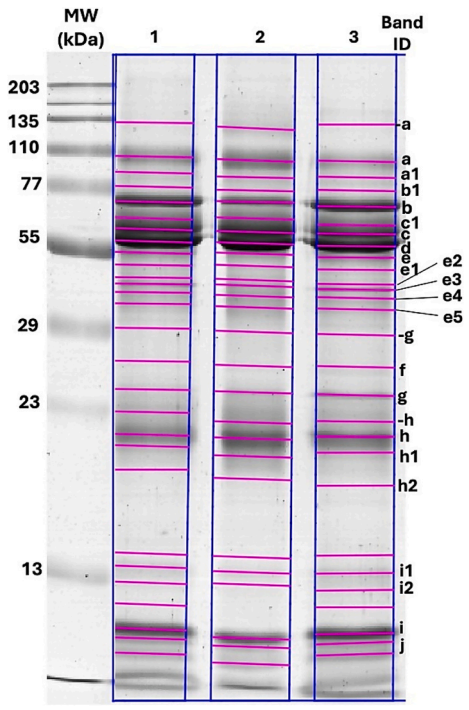

Fig. 2. – Salivary protein profile (SDS-PAGE) representative of the saliva under analysis (letters represent the 25 protein bands subjected to analysis; 1–3 – different samples run in the gel, as example).

flow rate in response to this image (cake -).

4. Discussion

The objective of this study was to evaluate the influence of the exposure to different visual food stimuli, on the salivary proteome, and relate them to the affective reactions that participants perceived about those stimuli. For this purpose, participants were exposed to three food images: a fast-food savory palatable food (pizza), a sweet palatable food (chocolate cake) and generally considered low palatable food (salad). As control the partcipants were exposed to a non-food image, a car.

First, the affective reactions evoked by the visualization of food images were evaluated. As observed salad image elicited lower levels of mouthwatering sensation than pizza and chocolate cake images. As mouthwatering sensation is related with the sensory pleasure that palatable foods produce (Jiang et al., 2014), this could be an explanation of the higher intensity of mouthwatering sensation reported in pizza and cake, which are expected to be more palatable foods, compared to salad. Mouthwatering sensation has been explained by the activation of facial muscles which are under voluntary control, compressing the ducts that transport saliva from the glands to the mouth, resulting in a short-lasting saliva flow (Carpenter, 2013). The increased mouthwatering sensation reported for pizza and chocolate cake might reflect this muscle activation in response to more palatable and caloric stimuli, triggering the secretion of saliva and mouthwatering sensation. Additionally, and as previously reported, high calorie foods have shown to be more associated to positive affective reactions (as mouthwatering) than low calorie food, as they activate cerebral regions involved in emotions, motivation and responses related to cognitive behavior (Killgore et al., 2003). Results did not show significant differences in other "positive" affective reactions (e.g. happiness, appetite, desire), although they tend to be perceived with higher intensity when more palatable foods (pizza and cake) are presented than in the lower palatable food (salad). Overall, these results suggests that specific affective responses might vary in their intensity depending on the sensory properties of the food stimuli, providing more information about of how visual cues influence emotions.

The impact of food images visualization on salivary secretion and composition was assessed. Results showed that salivary flow rate decreased when individuals hold the VR glasses, as observed by the significant differences in the salivary flow rate before and during the non-food image presentation used as control (car). However, this decrease in salivary flow rate was not observed when the images were food images, suggesting that the increase in salivation produced by them

Table 3
Component loadings of affective reactions triggered by the different food pictures, with Quartimax rotation with Kaiser normalization.

Explained variance (%)	Component	
	1	2
	57.6	*16.9*
Appetite for the specific food	0.793	−0.454
Desire for ingesting that food	0.891	
Mouthwatering	0.880	
Wellbeing	0.610	
Anxiety	0.880	
Happiness	0.874	0.349
Repulsion		0.841
Indifference	−0.510	0.646

Coefficients <0.300 were omitted.

Table 2
–Bands observed (mean ± SD) in SDS-PAGE salivary profiles ($N = 20$) that significantly differ between periods (before and during) and the type of image visualized.

Band	Protein ID*	Food Image								P (int)[2]
		Pizza		Chocolate cake		Salad		Control (non-food image)		
		Before	During	Before	During	Before	During	Before	During	
B1	n.i.	0.84 ± 0.31[a]	1.26 ± 0.21[b]	1.26 ± 0.31	0.81 ± 0.21	1.37 ± 0.32	0.81 ± 0.22	0.93 ± 0.31	0.77 ± 0.21	0.042*
B	Albumin	9.1 ± 0.95[a]	10.5 ± 0.90[b]	10.32 ± 0.95	10.40 ± 0.90	8.65 ± 1.00	8.34 ± 0.96	8.85 ± 0.95	9.31 ± 0.90	0.092
D	Alpha-amylase	14.66 ± 1.67[a]	12.97 ± 1.34[b]	14.29 ± 1.67	13.66 ± 1.34	15.63 ± 1.77	13.25 ± 1.42	15.57 ± 1.67[a]	12.56 ± 1.34[b]	0.319
E3	Carbonic anhydrase VI + zinc-alpha2-glycoprotein	1.35 ± 0.12	1.45 ± 0.14	1.30 ± 0.12[a]	1.65 ± 0.14[b]	1.16 ± 0.13	1.27 ± 0.15	1.29 ± 0.12	1.34 ± 0.14	0.393
G	n.i.	1.70 ± 0.19	1.79 ± 0.23	1.48 ± 0.17	1.50 ± 0.13	1.71 ± 0.16[a]	1.38 ± 0.15[b]	1.52 ± 0.18	1.79 ± 0.17	0.213
J	Type S cystatins	11.52 ± 1.51	12.38 ± 1.74	12.46 ± 1.25	11.68 ± 1.45	9.51 ± 1.25[a]	11.47 ± 1.45[b]	10.53 ± 1.21[a]	12.44 ± 1.40[b]	0.110

*Protein identification based on previous mass spectrometry analyses (Carreira et al., 2020); n.i. - not-identified – n.i.; [1]p-value for the interaction period*image; different upper letters represent statistically significant differences ($P < 0.05$) between before and during image presentation.

Table 4
– Statistically significant correlations between saliva SDS-PAGE bands and reactions/emotion components.

		Band b1	Band d	Band h1	Band i1	Band j
Component 1 (positive affective reactions)	Rho	0.397	0.401			
	p-value	0.005**	0.003**			
Component 2 (negative affective reactions)	Rho			0.284	0.492	−0.341
	p-value			0.041*	0.0005**	0.017*

* Band protein identification: Band b1 - n.i.; Band d - alpha-amylase (native form); Band h1- light chain of immunoglobulin; Band i1 PIP (prolactin-induced protein); Band j - type S cystatins. (Carreira et al., 2020).

masked this "glasses" effect. These results agree with previous studies in which the exposure to food visualization increased salivary flow rate (Kershaw & Running, 2018; Wooley & Wooley, 1973). Even so, it is possible to hypothesize that the sight of images, instead of real foods, have lower power of stimulation (Morquecho-Campos et al., 2020). Moreover, another explanation for not observing significant increases in the average salivary flow rate, during food image visualization could be the higher variability in the salivary flow rates among individuals, with variation coefficients higher than 90 % among them.

The potential effect of food images on the amount of saliva secreted is reinforced by the changes in total protein concentration (TPC) that were observed (Fig. 1B). The decreased TPC suggests increases in salivary flow rate. A decrease in TPC during visual stimulation with bread smelling has been previously reported (Carreira et al., 2020), which is agree with our findings. Other authors have also reported a lower TPC in saliva with anticipatory stimulation (visual and visual+odour) without changes in salivary flow rate in comparison to non-stimulated saliva (Morquecho-Campos et al., 2020), which agree with our results. Additionally, in the referred study, the authors found that saliva protein secretion rate during visual and visual+odour stimulation was reduced compared to protein secretion rate in non-stimulated saliva (Morquecho-Campos et al., 2020). Thus, according to the authors' findings, the reduction in salivary protein secretion rate could be also possible explanation to the decrease in saliva TPC.

Results obtained from SDS-PAGE showed specific changes in salivary protein profiles in response to visual stimuli, such as albumin, amylase, carbonic anhydrase VI (CA-VI), type S cystatins, prolactin-induced protein (PIP) and immunoglobulins. For instance, albumin (band b1) increased during the pizza visualization. In previous studies, higher levels of salivary albumin have been associated to the perception of bitter taste in hypersensitive individuals towards this taste (Fábián et al., 2015). Salivary albumin seems to increase the availability of low soluble polyphenols for taste cells, improving the perception of bitter taste (Fábián et al., 2015). Thus, the increase in the levels of this protein during observation of a food image, like pizza, leads to hypothesize that it can affect the sensitivity to bitter taste.

This potential effect of food image visualization in taste perception is reinforced by the variations observed in the levels of other salivary proteins also previously showed to be associated with taste perception. Is the case of alpha amylase, which was observed to be related with sweet taste sensitivity (Rodrigues, da Costa, et al., 2017) and CA- VI (also called gustin), linked to bitter (Patrikainen et al., 2014) and sweet taste (Rodrigues, Costa, et al., 2017). In previous studies it has been reported a decrease in alpha-amylase concentration and secretion rate as the stimulation levels increased, from odour, odour+vision, odour+visiton+taste, and odour+vision+taste+mastication (Morquecho-Campos et al., 2020), which agree with present results, for pizza image.

Concerning CA-VI, one of its functions is to neutralize the acids in the mouth during ingestion and protect against plaque and caries (Kivelä et al., 1999). In fact, it have been found higher levels of CA-VI in saliva from individuals with poor oral hygiene (Arabacı et al., 2015). Therefore, the increased levels of this protein by the visualization of a sweet food, such as cake (Table 2), may represent the increase as a protective agent in the mouth against the effect that sugars from cake can have on teeth. However, more studies are needed to confirm if the increase in CA-VI in saliva occurs for the visualization of other types of sweet foods, to support this potential protective pre-ingestive response to sweet foods.

Another group of salivary protein, originating in the salivary glands, that was previously associated with food oral perception and that were observed to change under a visual food stimuli was type S cystatins. These proteins have been associated with the oral perception of bitter taste (Rodrigues, da Costa, et al., 2017) and astringency (Dsamou et al., 2012), as well as with the consumption of foods of plant origin, especially those that contain phenolic compounds (eg. tannins, flavanols) (Louro et al., 2021). In regard to present study, as salad contains foods of plant origin (rich in polyphenols) and taking into account the findings from the above-mentioned studies, the increase in type S cystatins with salad image (but not with pizza nor cake; Table 2) might be hypothesized as an anticipatory response due to the salad visualization. However, this hypothesis needs to be further studied in future assays. Moreover, being a salivary protein associated with aversive sensations, such as bitterness and astringency, its changes in response to salad visual stimulation may mean that the perception of these sensations may be modulated by visualizing these foods before consuming them.

Despite the different effect of the different images in the salivary protein bands, when affective reactions to visualization were taken into account, it was observed that salivary protein changes correlated with more positive or more negative feelings (Table 4). The native form of alpha-amylase (band d), although being decreased, in average, during pizza visualization, showed positive correlation in its change levels with the positive affective reactions produced by food images. I.e., for the individuals to which the food images were more positive, higher increases (or lower decreases) in the relative amounts of this protein.

It has been observed increases in salivary amylase in situations where the sympathetic nervous system is activated (due to a stressor) (Rohleder et al., 2004). However, in a general way, food stimuli (in anticipation to ingestion) are received in hypothalamus, which send signals that later will result in vagus nerve activation of parasympathetic system in some organs, such as stomach and salivary glands (Smeets et al., 2010). This would lead to the expectation of a decrease, and not an increase, in alpha-amylase. However, the increased level of SDS-PAGE amylase band (band d) means a higher proportion of this protein, in relation to the total salivary proteins and not its absolute amount, meaning that the greater the positivity triggered by the stimuli, higher proportion of native alpha-amylase in the total of salivary proteins. A previous study, from our team, also observed increases in the proportion of alpha-amylase in response to anticipatory food stimuli – bread smell (Carreira et al., 2020). Other authors also have reported increase in alpha-amylase caused by high arousal emotions regardless of whether they were positive or negative emotions (Adam et al., 2011; Sánchez-Navarro et al., 2012). However, in the present study, the arousal was not measured and, since there were no food images inducing strong rejection, further studies will be necessary to confirm whether these variations of alpha-amylase are related to just positive affective reactions, or rather, with arousal, independently of it being associated with positive and negative affective reactions.

On the other hand, other proteins correlated with negative reactions. Particularly, higher increases (or lower decreases) of light chain of immunoglobulin (band h1) and prolactin inducible protein (band i1), and higher decreases (or lower increases) of type S cystatins (band j) tend to occur when the stimuli induce higher negative reactions (Table 4). Thus, these results suggest that more aversive reactions/emotion such as low desire of ingestion or disgust increased the levels of

light chain of immunoglobulin and prolactin inducible protein (PIP), while decreased the levels of type S cystatins in saliva.

Regarding PIP, increased levels of this protein have been observed after the oral simulation with 6-gingerol, described as pungent (Lorenz et al., 2011), which suggest that this protein in saliva may respond to disagreeable/aversive stimuli. Additionally, it has been suggested that acute stressors can increase the release of these proteins in saliva (Trueba et al., 2012), although this lacks consensus, as some authors showed a decreased in PIP in response to a stress test (a simulated oral exam) (Zallocco et al., 2021).

Immunoglobulins in saliva have also been affected by stressing events. For instance, a decreased in immunoglobulin free light chains in saliva have been observed in university students due to psychological stress (a period of exams at the end of semester) (Irshad et al., 2020), while other studies showed an increase in salivary light chain IgA due to acute psychosocial stress (a Trier Social Stress Test) (Trueba et al., 2012). Additionally, higher levels of immunoglobulins in saliva have been associated to an increased bitter taste perception (Dsamou et al., 2012), which could be considered an aversive sensation. However, and to the authors' knowledge, the impact of pre-ingestive visual stimulation on salivary immunoglobulins has not yet been investigated.

Regarding salivary cystatins, they have been associated with aversive stimuli as bitterness or astringency (Dsamou et al., 2012; Rodrigues, Costa, et al., 2017). Specifically, higher levels of type S cystatins have been shown in saliva from high sensitive individuals to bitter taste (Rodrigues et al., 2019). Additionally, an immediate increase in the levels of type S cystatin has been observed as response of acute stress (produced by the Trier Social Stress Test) (Trueba et al., 2012). However, our results showed lower increases of type S cystatins when negative affective reactions were more intense (Table 4). Being S-type cystatins from submandibular gland origin (Henskens et al., 1994; Kaufman & Lamster, 2000), one possible explanation is a lower activation of the parotid glands and/or greater activation of the submandibular glands when the images trigger more intense negative affective reactions. Further research is needed to confirm this hypothesis.

Overall, these results showed correlations between salivary protein changes and emotional responses, underscoring the interplay between physiological and psychological factors. Various salivary proteins showed significant changes in their SDS-PAGE profiles during the visualization of the different food stimuli: pizza (albumin, amylase), cake (CA-VI) and salad (type S cystatins). Moreover, when considering the affective reactions triggered by food images, results showed that positive reactions (such as desire to eat, mouthwatering or happiness) were linked to proteins like amylase or CA-VI, which at the same time are associated with the sensory modulation of bitter (Patrikainen et al., 2014) and sweet taste (Rodrigues, da Costa, et al., 2017). Conversely, negative reactions, such as disgust or indifference, were associated with proteins like immunoglobulins, PIP or type S cystatins, that have been associated to the perception of aversive stimuli like pungent (PIP) (Lorenz et al., 2011), bitter taste (type S cystatins and immunoglobulins) (Dsamou et al., 2012; Rodrigues, Costa, et al., 2017), astringency (type S cystatins) (Dsamou et al., 2012), and with stressing events (type S cystatins and immunoglobulins) (Irshad et al., 2020; Trueba et al., 2012), reflecting more aversive or stress-related responses. Therefore, these findings suggest that salivary responses to visual stimuli are not only determined by the food type, but are strongly influenced by the individual's emotional and sensory experience.

Results from 2-DE analysis are in line with those observed through the one-dimensional profiles (SDS-PAGE), which showed increases in CA-VI levels induced by the chocolate cake image. CA-VI spots increased in the group cake +, compared to the group cake – and control (Fig. 3). Additionally, the increase in CA-VI spots occurred for various proteoforms with different isoelectric points, and not only for a specific proteoform. The different CA-VI proteoforms in saliva and their different functions have been little investigated yet (Yrjänäinen et al., 2022). Despite this, it is interesting to note that the increases in CA-VI levels were observed in the individuals who reported more desire to eat the cake and who salivated more (cake + group), and not in the individuals for whom the chocolate cake image did not produce this effect (cake -). This reinforces the idea, already discussed, that salivary responses, in response to pre-ingestive stimuli, might be associated more with a reaction/emotional response than with a cognitive association with the type of food in question.

Regarding the short-palate lung and nasal epithelium carcinoma associated protein 2 (SPLUNC2), its levels were higher in cake + group compared to control and cake – groups (Fig. 3), suggesting that this protein increase in response to more desire of ingestion and more salivation produced by the food. Although some studies have observed an increase of SPLUNC2 with oral stimulation with tastants (Bader et al., 2018; Lorenz et al., 2011) and food mastication (Carreira et al., 2020), as

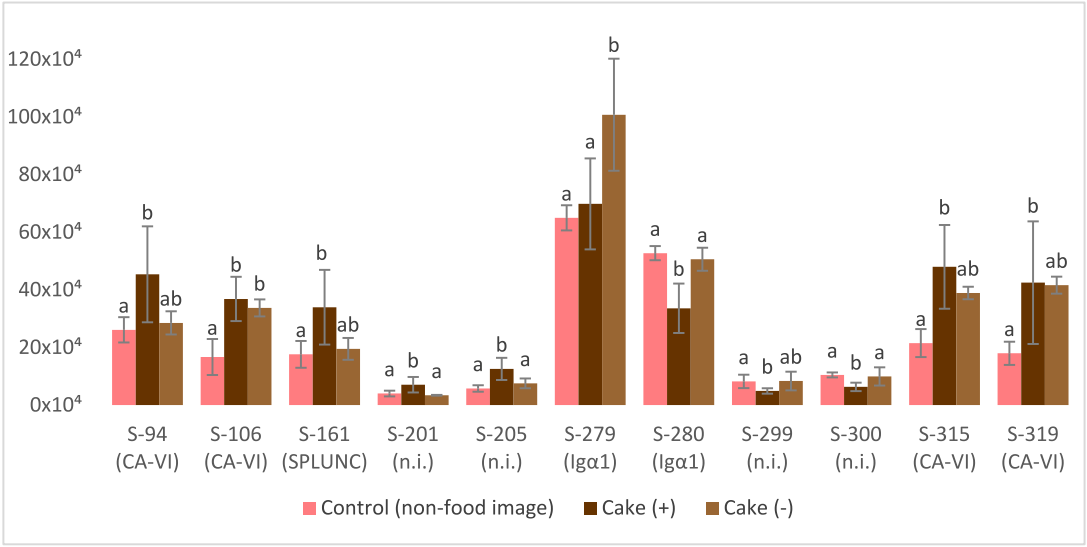

Fig. 3. – Volume of spots (in percentage) expressed in the saliva secreted in response to the chocolate cake images in the group ($n = 9$) who the visualization of the image increased salivary flow rate (cake +), in the group (n = 9) for who image did not produce changes in salivary flow rate (cake -), and in the control group (non-food image). Error bars represent standard deviation. Letters above bars represent levels of LSD test from ANOVA.
Protein identification of spots (in parenthesis) based on (Jessie, Pang, Haji, Rahim, & Hashim, 2010).

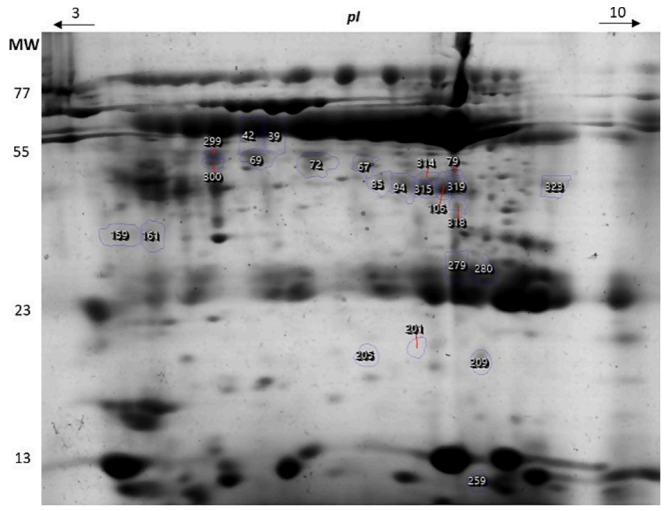

Fig. 4. – 2-DE protein profile representative of saliva samples. The spots observed to change significantly among image conditions are numbered. ($N = 9$ individuals per group). (MW – molecular mass marker (kDa); pI –isoelectric point).

far as the authors know, there are no studies where the levels of this protein have been studied in response to affective reactions and salivation evoked by visual food stimuli.

On the other hand, the spot 279, which correspond to Ig alpha1 chain C region, was the one that showed higher increases in cake – group (Fig. 3). As previously mentioned, salivary IgA was suggested as a marker that increased in acute stress (Castro-Quintas et al., 2023; Trueba et al., 2012), although chronic stress may have an opposite effect (Engeland et al., 2016). In the present study, it was not assessed if the individuals who responded to cake image without saliva increase liked cakes, or if this is a type of food usually rejected or less preferred, to understand if higher levels of this protein mean higher stress during visualization. This protein has been observed at higher levels in the saliva of super taster individuals to bitter taste, compared to non-tester individuals (Rodrigues, da Costa, et al., 2017), reinforcing that alterations induced by pre-ingestive stimuli may affect taste perception.

Finally, other protein spots (201, 205, 299 and 300) also showed variations in their levels depending on the group (cake +, cake – or control) (Fig. 3). Thus, it will be of interest to identify these salivary proteins in future studies in order to go deeper on the understanding of the effect of pre-ingestive stimuli.

5. Strengths and limitations

The main strength of this study is the evaluation of different food images, presenting different sensory characteristics, in terms of the effect they have in the protein composition of saliva, and using VR glasses, to isolate images. From our knowledge, this is the first study going deep in saliva protein composition responses to visual pre-ingestive stimuli. However, some limitations need to be considered, as the use of images instead of real food to evaluate the impact of the stimulation. Additionally, the use of VR glasses, which was good to obtain a better quality image, isolating it from outside visual elements, is not an element frequently used, being not discarded the possibility of producing some stress/discomfort in participants, thus affecting their salivary composition. As well, and to better understand the changes in saliva composition, it would be interesting to have objective measures of arousal response. Finally, the impossibility to identify some SDS-PAGE bands and 2-DE spots, and the strategy of pooling of samples for 2-DE analysis, also limit the impact of present findings. Despite these limitations, this study provides novel findings in this field. Future studies overcoming these limitations would be of interest to better comprehend the changes in salivary proteome in response to pre-ingestive visual stimuli and the consequences that they may have in food behavior and/or digestive physiology.

6. Conclusions

This study aims to bridge the gap in knowledge regarding the effects of visual food stimuli on the salivary proteome and the associated reaction/emotional responses, by examining how exposure to different food images (pizza, chocolate cake and salad) affects saliva protein composition. From our knowledge, this is the first time that food images are presented through VR glasses and salivary proteome analysed in response to that. Results from this study showed a lower mouthwatering sensation elicited by salad than pizza and chocolate cake. Regarding the changes in salivary proteins due to visual stimuli, albumin increased, while amylase decreased during pizza visualization, CA-VI increased in the visualization of the chocolate cake, while type S cystatins increased with salad image. Thus highlighting that visual stimuli alone can modulate salivary protein composition and flow, simulating the anticipatory physiological changes occurring in saliva that prepare the body for digestion.

Additionally, salivary amylase showed a positive correlation with positive affective reactions produced by food images, while light chain of immunoglobulin, prolactin-inducible protein and type S cystatins correlated with negative reactions. The observed associations between salivary proteins and emotional responses suggest a potential link between pre-ingestive stimuli and the modulation of taste perception, which might influence food preference and sensory experience.

Finally, results showed that CA-VI and SPLUNC levels increased in the group that positively reacting to chocolate cake (cake +), compared to the group that react negatively to the chocolate cake (cake –) and control, contrarily to Ig alpha1 chain C region. These results showed the correlation between positive or negative affective reactions in response to food stimuli and the changes in salivary proteins.

The findings from this study showed that it is not only the total volume of saliva, nor the total protein concentration that responds to pre-ingestive stimulation, but also the proportion among the different types of proteins are changed. By confirming that emotional responses to food images correlate with salivary protein changes, our study highlights the relationship between visual food cues, physiological oral reactions, and potential behavioural outcomes, such as food choices and appetite regulation.

One relevant aspect, that deserves to be detailed, in the future, is the relationship that salivary protein changes have with affective reactions, as it seems that the affective effect that a food triggers, on the individual, can be more effective in changing salivary proteome than the exact type of food.

Future research investigating the effects of different visual food stimuli on saliva protein composition, examining the role of specific salivary proteins in taste perception and emotional responses, exploring how salivary components influence individual differences in responses to food stimuli, and assessing the impact of the food composition and properties (e.g. dryness) on salivary protein composition during food observation would be of interest to better understand the role of saliva in response to food stimuli.

On the other hand, the findings of this study suggest potential implications that could be applied to enhance the gastronomic experience and/or health of consumers. By observing how visual food stimuli influence salivary proteins, emotional responses and possibly oral perception, these results might contribute to develop marketing strategies or product design to maximise consumer appeal, or to make healthier foods more attractive for consumers, thus tailoring the food experiences to meet sensory and health goals.

CRediT authorship contribution statement

Erica Marques: Writing – original draft, Methodology, Investigation. **Carla Simões:** Writing – original draft, Methodology, Investigation, Conceptualization. **María Pérez-Jiménez:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Fernando Capela e Silva:** Writing – review & editing, Supervision, Formal analysis, Data curation, Conceptualization. **Elsa Lamy:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ethical statement

This study involved human subjects.

All participants signed an informed consent before their participation in this study. The privacy rights and confidentiality of participants and their data was always assured. All procedures were performed according to the Declaration of Helsinki for Medical Research Involving Human Subjects and had ethical approval from the Ethical Committee of the University of Évora (Reference number: 22012).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.116301.

Data availability

Data will be made available on request.

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