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Saliva Protein Composition Relates with Interindividual Variations in Bread Sensory Ratings

Elsa Lamy, Vera Santos, Sara Barrambana, Carla Simões, Laura Carreira, Paulo Infante, and Fernando Capela e Silva*

Sensory perception of starch-based products associates with salivary α-amylase enzymatic activity. Besides this, other proteins relate to taste sensitivity and oral food processing. As such, the participation of different salivary constituents in starch-rich food's sensory evaluation cannot be excluded. This study aims to identify salivary proteins altered by bread mastication and correlated with sensory ratings. In Experiment 1 the effect of bread mastication in α-amylase enzymatic activity and SDS PAGE profiles between is assessed ($N = 64$). In Experiment 2, a sub-sample of these individuals ($N = 22$) is subjected to sensory tests and the sensory ratings obtained are correlated with saliva protein composition. Salivary α-amylase activity, in the supernatant of saliva collected after bread mastication, is negatively correlated with sweetness and saltiness ratings. Moreover, saltiness is positively correlated with the expression levels of carbonic anhydrase VI. Bread roughness presented a positive association with α-amylase enzymatic activity and a negative association with S-type cystatin expression levels. Despite further studies are needed to clarify the negative association between salivary amylase enzymatic activity and sweetness ratings, observed in this study, these results reinforce the role of α-amylase and highlights that other salivary proteins can also influence starch-based sensory perception.

1. Introduction

Food intake is a complex process influenced by a variety of homeostatic, behavioral, sensory and hedonic factors. Among the aspects that influence food preferences and choices, the perception of food in the oral cavity is one of the most important.[1]

Cereals are dominant crops in world agriculture, with wheat being processed into a range of foods, including bread. In turn, bread is consumed by billions of people worldwide,[2] making up roughly 10% of the adult caloric intake.[3] Despite the importance of cereals as a source of many essential and beneficial components of the human diet, overconsumption of starch-rich foods, together with a sedentary lifestyle, can lead to overweight and diabetes.[4] Moreover, starch is an important ingredient in the food industry, influencing food taste and texture and, consequently, acceptance and preference.

Saliva has been increasingly recognized as having an important role in oral food sensing. The salivary protein α-amylase is involved in the digestion of starch in the oral cavity, hydrolyzing it. This hydrolysis results in the production of maltose, maltotriose and bounding dextrins.[5] These molecules have particular sensory properties, such as sweetness. The salivary enzyme α-amylase is encoded by the AMY1 gene, which is known to show extensive copy-number variation, which can associate with the dietary levels of starch in regular diets.[6,7,8] Moreover, the levels of this salivary enzyme change throughout the day and according to satiety state.[9] Salivary α-amylase has been linked to sensory perception: interindividual differences in the amounts of this protein were associated with sweet taste sensitivity.[10] Also, an influence of α-amylase in the sensory perception of starchy foods, as well as in the general flavor of these foods, has been reported.[11] Different studies support the assumption that salivary α-amylase influences the way starchy products are sensed in the mouth: i) differences in sensory evaluation were referred to depend on the amount of time food is in the mouth and on the levels of α-amylase;[12] ii) a positive correlation between the activity of this salivary enzyme and the levels of sweet-tasting molecules present in the mouth was observed.[13]

Prof. E. Lamy, Dr. C. Simões, Dr. L. Carreira, Prof. F. Capela e Silva
MED (Mediterranean Institute for Agriculture, Environment and Development), Institute for Advanced Studies and Research (IIFA)
University of Évora
Évora 7002-554, Portugal
E-mail: ecsl@uevora.pt

Dr. V. Santos, Dr. S. Barrambana, Prof. F. Capela e Silva
University of Evora
Department of Biology, School of Sciences and Technology (ECT)
University of Évora
Évora 7002-554, Portugal

Prof. P. Infante
CIMA (Research Centre for Mathematics and Applications), IIFA
Department of Mathematics, School of Sciences and Technology (ECT)
University of Évora
Évora, Portugal

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Besides α-amylase, other salivary proteins have been associated with taste perception.[14–16] Carbonic anhydrase VI (CA VI) was the salivary protein first associated with bitter taste sensitivity,[17] with different authors reporting a relationship between the level of this protein and bitterness.[18,16] Also, cystatins[15,16] and proline-rich proteins[14,19] have been shown to be linked to this basic taste. Additionally, not only taste, but also food aroma[20] and tactile perception[21] are influenced by saliva protein profile. The potential role of saliva in oral food perception appears to be even more complex, as the relationship between saliva and oral sensing is also influenced by other factors such as sex and body mass index.[22]

Despite what was stated above and from our knowledge, studies relating the salivary protein profile with the oral sensory evaluation of starchy foods such as bread have not yet been performed. As such, the aims of the present study were to assess: 1) the changes that bread chewing induces in salivary α-amylase activity and salivary protein composition; 2) the relationship between these salivary parameters and bread sensory ratings.

2. Experimental Section

2.1. Participants, Saliva Collection and Sensory Collection

The study consisted of two parts permitting to assess salivary content changes due to bread mastication (Experiment 1) and the possible relationship between salivary protein content and sensory ratings (Experiment 2). Before the beginning of each experiment, 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.

2.1.1. Experiment 1

Sixty-four young adults (males, $N = 16$; females, $N = 48$, with 18–30 years old), healthy, without signs of oral or nasal health problems and not taking medications participated in the study. All were asked to restrain from eating or drinking anything other than water for, at least, 2 h before tests. Each of the participants received 30 g of refined wheat bread (type 65 wheat flour, water, 1.5% salt). Immediately before bread distribution, individuals were asked to clean their mouths from residual saliva with water and unstimulated saliva was collected by passive droll into tubes, maintained on ice, during 4 min (collection 1: R1). After this, individuals chewed the bread (ingesting it), in 2–3 bites, cleaned any residues from the mouth, with water, and collected saliva as described before (collection 2: R2). All saliva collected was immediately stored at −20 °C until laboratory analysis.

2.1.2. Experiment 2

Twenty-two of the individuals participating in experiment 1 (males $N = 11$; females $N = 11$, with 18–30 years old) performed sensory tests for the bread. These individuals, who were chosen randomly, with the only aim of keeping the same number of men and women, were first instructed about each sensory descriptor, to minimize misinterpretation. Individuals were asked to chew a piece of bread of 30 g, in 2–3 bites, for at least 15 s and to record the level of the intensity perceived for each sensation/descriptor (stickiness, hardness, roughness, sweetness, saltiness, bitterness, overall preference), using a categorical 8-point scale (1 = least, 8 = most intense).

2.2. Laboratory Saliva Analysis

In order to remove mucins and cell and/or food residues, one day after collection, saliva samples were thawed on ice and centrifuged at 13 000 *g* for 30 min at 4 °C. The supernatant was transferred to polyethylene tubes and stored at −20 °C for subsequent analysis. Although this protocol has the limitation of resulting in a freeze-thaw cycle, it allows the recovery of a homogeneous supernatant for analytical work.

2.2.1. Saliva Flow Rate and Total Protein Concentration

Saliva flow rate was assessed by assuming that saliva density is 1.0. Tubes containing saliva were weighed and the empty tube weight was subtracted. The final value was divided by 4 (minutes of collection). Total protein concentration was determined by the Bradford method, using bovine serum albumin (BSA) as standard, and plates were read at 600 nm in a microplate reader (Glomax, Promega).

2.2.2. SDS-PAGE Separation and Mass Spectrometry Protein Identification

Each saliva sample was run in duplicate. For each sample, a volume corresponding to 7.5 µg total protein was mixed with sample buffer and run on each lane of a 14% polyacrylamide mini-gel (Protean xi, Bio-Rad, CA, USA) using a Laemmli buffer system, as described elsewhere.[23] An electrophoretic run was performed at a constant voltage of 140 V until front dye reached the end of the gel. Gels were fixed for 1 h in 40% methanol/10% acetic acid, followed by staining for 2 h with Coomassie Brilliant Blue (CBB) G-250. Gel images were acquired using a scanning Molecular Dynamics densitometer with internal calibration and LabScan software (GE Healthcare), and images were analyzed using GelAnalyzer software (GelAnalyzer 2010a by Istvan Lazar, www.gelanalyzer.com) for the volume percentage of each protein band. Molecular masses were determined in accordance with molecular mass standards (Bio-Rad Precision Plus Protein Dual Color 161-0394) run with protein samples.

The protein bands whose levels were associated with sensory parameters were excised from well-resolved gels and tryptic digested with porcine trypsin (Sequencing Grade Modified Trypsin, Promega) following the protocol previously described elsewhere.[23] Protein identification was performed in a MALDI TOF-TOF mass spectrometer (AB Sciex 4800 Plus) using 4000 Series Explorer v. 3.5.3.3 analysis software (Applied Biosystems), as previously described.[24] Briefly, samples were desalted and

concentrated using reversed-phase Poros R2 (Applied Biosystems, CA, USA) and eluted directly to the MALDI target with matrix solution (α-cyano-4-hydroxycinnamic acid, CHCA; Fluka).

The monoisotopic masses of the peptides were used to search for protein identification through the use of Protein Pilot v. 4.5 software (AB Sciex) with the Mascot search engine (MOWSE algorithm). The Swiss-Prot database, restricted to *Homo sapiens*, was used for all searches, considering the parameters: i) minimum mass accuracy of 50 ppm; ii) mass tolerance of 0.3 Da; iii) two missed cleavages in peptide mass; iv) carbamidomethylation of Cys and oxidation of Met, as fixed and variable amino acid modifications, respectively.

2.2.3. Determining the Enzymatic Activity of Salivary α-Amylase

A Salimetrics kit was used to determine the enzymatic activity of salivary α-amylase according to the manufacturer's recommendations. Briefly, saliva samples were diluted 200× and applied on the microplate in duplicate, followed by application of a substrate (2-chloro-*p*-nitrophenol) preheated to 37 °C. The mixture was incubated at 37 °C for 1 min, absorbance values were read at 405 nm in a plate reader spectrophotometer, followed by incubation for an additional 2 min at 37 °C and a new reading at 405 nm. The enzymatic activity of α-amylase (U mL^{-1}) was calculated by the following formula: (ΔAbs./min × TV × DF)/(MMA × SV × LP), where ΔAbs./min is absorbance variation per minute, TV is total test volume (0.287 mL), DF is dilution factor, MMA is millimolar absorbance of substrate 2-chloro-*p*-nitrophenol (12.9), SV is sample volume (0.007 mL), and LP is light path (0.97, specific for plate received with kit).

2.3. Statistical Analysis

The values of total protein concentration, salivary secretion rate, protein band amount (volume percentage) and salivary α-amylase enzymatic activity were analyzed statistically. Descriptive statistics were performed and data normal distribution and homoscedasticity were tested through Shapiro–Wilk and Leven tests, respectively.

Paired *t*-test was used to compare salivary protein bands between the periods before (R1) and after bread mastication (R2). In the case of bands for which normality and homoscedasticity assumptions were observed, the non-parametric equivalent Wilcoxon test was used.

In order to assess the relationship between salivary α-amylase response to bread mastication and sweetness ratings, groups of high versus low ratings were constituted, considering as "high sweet tasters" the individuals with ratings higher than the median and "low sweet tasters" the ones with ratings equal or lower than the median. The differences in the salivary α-amylase enzymatic activity response to bread chewing, between these two groups, were accessed using repeated measures ANOVA (within subjects factor: period (R1 and R2); between subjects factor: group (high and low sweet tasters)).

Correlations between sensory ratings and the parameters of saliva collected after bread tasting were tested by Spearman's Rho coefficient. Taking into account the interindividual variability in salivary protein concentration and flow rate, the relationship between salivary α-amylase and bread sensory evaluation was accessed, considering enzymatic activity not only per volume of saliva (U mL^{-1}), but also corrected for total protein (U μg^{-1}) and flow rate (U min^{-1}). Similar results were obtained in all cases. For salivary parameters, such as flow rate, total protein concentration and α-amylase enzymatic activity, tests were also performed separately for each sex.

Statistical analysis was performed using SPSS v. 24, with significance level set at 5%.

3. Results

3.1. Influence of Bread Mastication on Saliva Composition

Before testing the relationship between bread sensory evaluation and salivary protein composition, the effects of bread mastication, on salivation, were assessed. When saliva collected immediately before bread presentation (R1) was compared with saliva collected immediately after bread mastication (R2), no significant differences were observed in total protein concentration (425.39 ± 24.5 for R1 and 447.6 ± 27.1 μg mL^{-1} for R2, $p = 0.403$). In terms of the amount of saliva secreted, differences were statistically significant, with the observation of a significant increase in salivary flow rate (0.49 ± 0.03 versus 0.63 ± 0.04 mL min^{-1}, R1 and R2, respectively, $p = 0.005$). For α-amylase enzymatic activity, no statistically significant variations occurred after bread chewing (55.7 ± 6.7 versus 59.2 ± 5.3 U mL^{-1}, R1 and R2, respectively, $p = 0.579$).

Looking at salivary SDS-PAGE profiles, 18 protein bands, in the range of 10–250 kDa, were consistently observed in the gels (**Figure 1**). Band C, identified as containing Ig polymeric receptor was significantly decreased after bread mastication (9.20 ± 0.61 versus 8.00 ± 0.71, before and after, respectively; $p = 0.008$). Details identification of protein bands are presented in **Table 1**.

3.2. Saliva Relationship with Bread Sensory Ratings

1) *Bread sensory ratings*. Sensory analysis of bread samples, by the participants, resulted in the mean evaluation present in **Table 2**. Roughness was the sensory quality rated with higher values, with sweetness and biterness being the qualities rated with lower intensity.
2) *Total protein concentration, flow rate, α-amylase enzymtic activity and SDS-PAGE profile*.
   In this study, the association between salivary parameters and bread sensory ratings was studied for the saliva collected after bread mastication (i.e., saliva stimulated by bread: R2). These samples were chosen, instead of basal saliva (R1), since R2 saliva is the one directly contacting with food. Salivary flow rate was positively correlated with the reported intensity of sweet taste.

The statistically significant associations between salivary protein profile, in R2, and sensory parameters are detailed in **Table 3**.

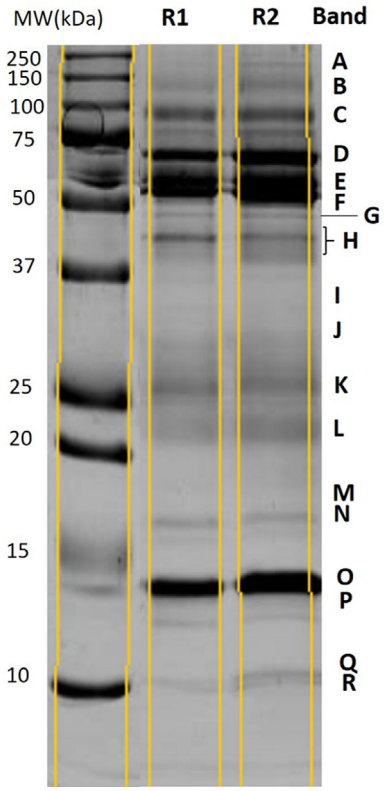

Figure 1. Representative SDS-PAGE salivary profile of individuals studied in two saliva collection periods (R1 before and R2 after bread mastication; letters on the right side represent the protein bands compared; MW, molecular mass (kDa)).

Table 1. MS identification of salivary proteins present in SDS-PAGE bands associated with bread mastication and sensory ratings.

Band	Protein	Accession number (Uniprot)	MW [kDa] (estimated/ theoretical)
C	Ig polymeric receptor	P01833	125.0/84.4
	Lactotransferrin	P02788	125.0/80.0
D	Serum albumin	P02768	71.0/71.3
E	α-Amylase 1	P04745	66.0/58.4
F	α-Amylase 1	P04745	60.0/58.4
G	α-Amylase 1	P04745	50.0/58.4
H	Zinc-α2-glycoprotein	P25311	41.0/34.5
	Carbonic anhydrase VI	P23280	41.0/35.5
K	Zymogen granule protein 16 homolog B	Q96DA0	28.0/22.7
	Immunoglobulin kappa constant	P01834	28.0/11.9
L	Immunoglobulin kappa constant	P01834	23.5/11.9
M	Prolactin inducible protein	P12273	16.5/16.8
O	Cystatin-SN	P01037	14.0/16.6
	Cystatin-S	P01036	14.0/16.5

Table 2. Sensory ratings (mean ± standard deviation) of bread samples.

Bread descriptors	Total ($N = 22$)
Sweetness	2.59 ± 1.18
Saltiness	4.05 ± 1.46
Bitterness	2.73 ± 1.61
Stickiness	4.36 ± 1.33
Roughness	5.00 ± 1.02
Hardness	4.32 ± 1.04

Sweet ratings of bread were associated with both α-amylase enzymatic activity and protein expression levels. The enzymatic activity of this protein was negatively correlated with sweetness ratings, in line with what was observed for one of the α-amylase containing bands (band G). In contrast, band M (identified as prolactin-inducible protein) presented a strong positive correlation with sweetness ratings.

Individuals with different sweet taste rating levels (low- versus high-sweet ratings) were compared to access the effect of bread mastication in α-amylase enzymatic activity. ANOVA repeated measures were used to test if changes in the enzymatic activity of this protein were similar. Significant interaction between period and group was observed ($p = 0.034$), where low sweet-rating individuals did not change (48.6 ± 9.5 and 59.8 ± 13.6 U mL^{-1}, before and after, respectively; $p = 0.181$) and high sweet-rating individuals tended to decrease (from 37.8 ± 5.7 to 25.8 ± 6.5 U mL^{-1}, before and after, respectively; $p = 0.090$) in the enzymatic activity of this salivary protein. Although these two groups did not differ in their enzymatic activity at R1($p = 0.356$), they differed, at R2, where high sweet rating individuals had significant lower enzymatic activity levels ($p = 0.044$) (**Figure 2**).

Besides sweetness, saltiness ratings also presented relationship with protein composition. These ratings were positively correlated with protein bands K (identified as containing a mixture of zymogen granule protein 16 homolog B and immunoglobulin kappa constant) and H (identified as containing CA-VI and zinc-α2-glycoprotein) and negatively correlated with protein bands F (identified as containing α-amylase) and L (identified as containing Immunoglobulin kappa constant) (Table 3).

Bitter taste ratings were correlated with one protein band, from the SDS-PAGE salivary profile. In this case, band D (identified as containing albumin) presented negative correlation with the intensity level of bitter taste perceived in bread (Table 3).

Besides basic tastes, textural parameters, such as thickness, roughness and stickiness were also assessed. Only roughness presented a statistically significant association with saliva, being positively correlated with salivary α-amylase enzymatic activity and negatively correlated with the expression level of band O (identified as containing cystatins type S) (Table 3).

4. Discussion

During chewing, saliva participates both in bolus formation and in the initial digestion of food constituents. Moreover, during this phase, saliva can interact with food constituents and influence food sensory evaluation.[25]

Table 3. Statistically significant correlations ($p < 0.05$) between salivary parameters (R2) and bread sensory ratings.

Sensory parameter	Flow rate [mL min^{-1}]	α-Amylase [U mL^{-1}]	Band D	Band F	Band G	Band H	Band L	Band M	Band O
Roughness		0.512							−0.533
Sweetness	0.513	−0.479			−0.553			0.792	
Saltiness				−0.638		0.594	−0.623		
Bitterness			−0.626						

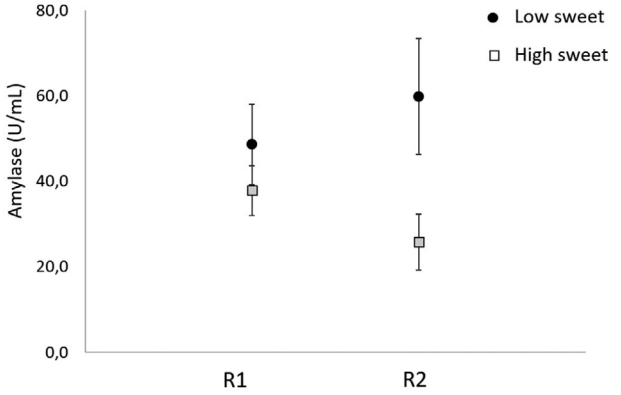

Figure 2. Variation in salivary amylase levels (mean ± standard error) of individuals rating low versus high levels of sweet taste. *Significant for $p < 0.05$.

In the present study, the influence of bread mastication on saliva composition was assessed, and an increase in salivary flow rate was observed. This increase in salivary flow rate, induced by food flavor and chewing is well known.[26]

Regarding protein profile, only one protein band was decreased immediately after bread mastication, being this identified as containing Ig polymeric receptor. Since salivary IgA (S-IgA) is secreted linked to a part of the receptor, it is expected that their levels represent the levels of S-IgA. The reason why this protein decreased with bread chewing, comparatively to the other salivary proteins, is not known. It was shown that the autonomic regulation of S-IgA secretion is different from the major stored secretory proteins of salivary glands and that stimuli that increase the levels of secretory granule salivary proteins may not increase the levels of S-IgA at the same level.[27] Although increases in S-IgA induced by mastication have been reported,[28] it is possible that the increase levels are lower than the ones from the other salivary proteins. Since, in the present study, saliva SDS PAGE profiles were compared considering the same amount of total protein, in each sample, this reduced levels of the protein band may only represent a lower increase comparatively to the other salivary proteins. However, further studies are aimed to clarify this point.

In recent years, different authors provided evidence that salivary proteome is associated with oral food perception.[10,15,29] However, the studies consisted mainly in comparisons between individuals with different levels of responsiveness to single-stimulus solutions. In the present study, we were able to show that interindividual variations in salivary proteome can also be associated with variations in food matrix sensory evaluation.

Salivary α-amylase was found to be negatively correlated to saltiness and sweetness ratings. In the case of saltiness, the relationship was particularly concerning protein expression levels, whereas for sweetness the main association was at enzymatic activity level. The influence of salivation on salt release during bread consumption was already reported:[30,31] higher enzymatic degradation of starch is associated with decreased efficiency in mixing the food with the saliva aqueous phase, leading to reduced transport of sodium to the palate.

Concerning the participation of salivary α-amylase in sweet taste evaluation, it was not a complete surprise. A potential effect of this salivary protein in the sweet taste intensity of bread has also been referred.[32] The arising question is why the relationship between this protein and bread sweet taste ratings was negative, in the present study. This is contrary to data from a recent work, where higher activity of salivary α-amylase was associated with higher levels of salivary maltose, which, in turn, was associated with higher sweetness reports.[13] In the present work, the individuals reporting higher ratings of sweetness presented a tendency for decreases in salivary α-amylase enzymatic activity in supernatant, as opposed to the ones with low sweetness ratings. We can hypothesize that individuals who rated bread as more sweet had higher levels of the salivary α-amylase enzyme hydrolyzing bread starch and, consequently, lower levels of the enzyme available to hydrolyze the substrate used in the laboratory tests for enzymatic activity determination. In fact, the differences in enzymatic activity of α-amylase between low and high sweet rating individuals were not observed in saliva collected in R1. Although we requested participants to chew the bread for a fixed amount of time, factors not controlled in this study, as different mastication force or rate, can be responsible for different bolus insalivation and consequent access of α-amylase to starch molecules. Nevertheless, further studies need to be done to test this hypothesis. In addition to this hypothesis, the evidence of a negative relationship between salivary α-amylase enzymatic activity and sweet taste sensitivity, previously reported,[10] also lead to the supposition that individuals with higher α-amylase enzymatic activity levels may be less sensitive to sweetness and, for that reason, can report lower rates of this taste in bread. Curiously, among the protein bands identified as α-amylase, only band G showed a correlation with sweetness similar to the one observed for the enzymatic activity of the protein. On the contrary, the most abundant bands of α-amylase (bands E and F) were not correlated with sweetness ratings, suggesting that different α-amylase isoforms may be differently correlated with enzymatic activity levels and, consequently, may have different roles in oral sensory sensitivity. In line with our results, a study with pig saliva showed that the enzymatic activity of this salivary protein is not equally correlated with all the forms of the protein present in a SDS-PAGE gel.[33]

Besides basic tastes, textural attributes of bread were also correlated with salivary protein profile. In the case of α-amylase, individuals with higher enzymatic activity rated higher roughness, which is in line with reports of reduced viscosity when starch degradation by α-amylase is increased.[11,30,34] On the contrary, S-type cystatins band was observed to be positively correlated with this sensory parameter. S-type cystatins are mainly a product of the submandibular glands, which are mixed glands that secrete a more viscous fluid containing mucins, which will reduce the friction between food particles and oral surfaces.[35] As such, it is possible that the positive relationship between roughness and α-amylase and the negative correlation between this textural parameter and cystatins does not mean a direct cause–effect action of these proteins, but rather indicates a higher proportion of saliva from submandibular glands in individuals who perceive bread as less rough.

5. Conclusions

The present study allowed the confirmation that bread sensory ratings are influenced by interindividual differences in saliva composition. Although salivary α-amylase was already suggested to be involved in starch-rich food products sensory perception, our study presents novel results, not only by demonstrating that the levels of this salivary enzyme, in saliva collected after chewing, are negatively correlated with sweetness and saltiness, but particularly by suggesting the involvement of other salivary proteins in bread sensory perception. This participation of salivary proteome in the sensory evaluation of a food product should be taken into account with a deeper investigation in further works. These results, showing that individual variations in saliva proteome contribute to variations in the intensity with which food sensory attributes are sensed, can be particularly important in the understanding of consumer foods acceptance and dietary choices.

The present study has some limitations that must be considered: the study was performed in individuals not trained for food sensory evaluation, so it cannot be excluded that some of the characteristics evaluated may have slightly different meanings for each individual. Also, the reduced number of individuals involved in Experiment 2 (bread sensory ratings), does not allow to access the effect of sex in sensory evaluation and/or in saliva composition. Finally, and despite instructions were given to all participants to chew the bread in 15 s, individuals were not controlled for mastication rate nor force, which can be factors affecting the level of insalivation of food. Despite these limitations, our results point to inter-individual differences in saliva protein composition that relate to differences in food sensory rating, highlighting the importance of knowing saliva composition to understand food acceptance.

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Author Contributions

E. L. and F. C. S. conceptualized the work. V. S., S. B., C. S, and L. C. provided the methodology. Formal analysis was carried out by V. S., S. B., and E. L. The investigation of the work was carried out by V. S., S. B., and E. L. Data curation was performed by P. I. and E. L. The original draft preparation was carried out by L. V. S. and E. L. and E. L., P. I., and F. C. S. completed the review and editing of the paper. E. L. was involved in supervision, project administration and funding acquisition.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

bread, salivary proteome, sensory evaluation, α-amylase

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