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Mastication of Carrots with Different Shapes Affects the Composition of the Salivary Proteome—A Pilot Study

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Abstract: Chewing is a sensorimotor activity that aims to prepare food for swallowing, in which saliva intervenes, moistening food particles, assisting in bolus formation, enzymatic digestion, and swallowing. This study investigated the effect of chewing carrots of different shapes on salivary secretion and protein profiles using 2-DE gel electrophoresis. Fifteen participants chewed sliced or grated carrots, or parafilm (non-food control), in a crossover design, with saliva samples collected before and after mastication. The results showed significant differences in salivation and saliva composition when chewing carrots vs. the control, with sliced carrots inducing greater changes: 37 protein spots, including amylase and immunoglobulin spots, 9 protein spots, and 1 protein spot were significantly altered after chewing sliced carrots, grated carrots, and parafilm, respectively. Mastication combined with the sensory properties of food had a greater effect on saliva secretion and salivary protein levels than mastication alone. Among carrot shapes, the results suggest that harder food textures, which require more chewing effort, lead to more pronounced changes in salivary protein profiles. These findings contribute to the understanding of how food shape and texture influence salivation and salivary proteome dynamics, with potential implications for oral digestion, food-related sensory experiences, and the personalisation of diets for individuals with a compromised chewing capacity.

Keywords: food oral processing; mastication; chewing; salivary secretion; salivary proteome; food texture; amylase; food matrix; food-related sensory experiences; food form

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1. Introduction

The first step in the digestive process is chewing, which helps to prepare the food for swallowing and further processing in the digestive system. During chewing, the food bolus or food particles are reduced in size, saliva is produced to moisten the food [1], and flavours are released [2]. Salivary proteins, the major organic constituents of saliva, are essential for initiating the digestive process and for modulating the sensory experience of food. In addition, saliva is a highly dynamic system that can be influenced by several factors simultaneously, such as diet patterns [3].

Regarding oral digestion, some salivary proteins and enzymes (e.g., amylase, lipase) play well-known roles in breaking down food nutrients so that they can be easily absorbed in later phases of digestion. During oral processing, nutrients are released from the food matrix as it disintegrates during mastication and bolus formation. In this context, the proteins present in saliva might also modulate the release of nutrients from the food matrix, leading to variations in their subsequent bioavailability during digestion [4–6].

Additionally, mastication also stimulates the secretion of saliva. Several masticatory muscles (the masseter, temporalis, medial pterygoid, and lateral pterygoid muscles) work together to enable the complex process of chewing by controlling the movement of the temporomandibular joint (TMJ) [7]. Disorders such as bruxism (a rhythmic involuntary movement of the jaw muscles) can impact the dynamics of chewing [8]. In terms of consumption, foods with more fibrous textures or dry foods, for example, often require more effort to break down and may lead to increased saliva production [9,10]. In addition to the characteristics of food, the shape of food can also influence the oral processing behaviour, energy intake, and metabolism [1,11–13]. For instance, Chen and colleagues (2024) showed that carrot strips required a longer chewing time than carrot cubes, with carrot julienne producing more and smaller carrot bolus particles than carrot cubes. Although these differences in oral processing did not affect the amount of carrot nutrients released (β-carotene) [14], less is known about how the shape of foods affects their oral sensory perception and consequent intake behaviour (e.g., food preferences, amount of food eaten). Other authors have also investigated how the consumption of different types and sizes of foods (Melba toast with and without margarine, and three sizes of cake and cheese) affects the salivary flow rate without finding significant differences in this salivary parameter among the different foods and forms [9]. However, despite these interesting studies, the effect of food shape on the salivary proteome is still largely unknown.

Differences in salivary secretion due to mastication of foods with different textures or shapes might modify the concentration and composition of proteins in saliva. In a recent systematic review, the impact of mastication on the total protein concentration and levels of different salivary proteins (alpha-amylase, peroxidase, lysozyme, sIgA, and mucin concentration) was addressed [15]. The authors found that there is no consensus about chewing impact, as some studies reported no or minimum effect, while others showed an increase and others a decrease in salivary protein amounts. These differences could be due to the different experimental approaches and methods used (analytical procedures, chewing material, etc.). Most of these studies used parafilm, plastic pieces, or gums with different compositions as chewing materials, while only two studies used foods (celery and bread). These studies evaluated the impact of chewing on the activity and/or concentration of specific proteins individually, but they did not consider the complete salivary proteome. In fact, the relationship between different food textures and the salivary proteome has been little explored. As far as the authors know, only one previous study has presented results about the impact of food mastication (bread and rice) on the salivary proteome, observing differences in the level of salivary proteins, depending on the type of food [16].

Among the effects that variations in saliva protein composition may have, the oral sensory perception of food is one of them. Salivary proteins can intervene in the release and perception of food flavour components (taste and aroma), as well as in the perception of tactile sensations (e.g., astringency, spiciness) and texture [17–19]. Texture being a major determinant for food acceptance, highly related to food palatability and satiation, and consequently food selection and intake patterns [20], it may be particularly important to know how it influences the saliva characteristics related to sensory perception.

The aim of this pilot study is to evaluate how the mastication of carrots in two distinct forms—sliced and grated—affects the salivary proteome. Carrots were selected as an example of a food that is usually found in different shapes and textures depending on culinary preparation (e.g., salads, stick snacks). The null hypothesis is that chewing carrots in different forms will not induce different changes in salivary parameters.

By investigating the texture-induced changes in salivary protein composition, this study aims to provide a better understanding of how the physical properties of food might influence oral processing and digestion. The findings could inform personalised dietary strategies for individuals with compromised chewing capacity, enhance food sensory experiences, and guide the development of innovative food products that align with both health and enjoyment. This research addresses a significant gap at the intersection of food science, nutrition, and oral health.

2. Materials and Methods

2.1. Individuals

A total of 15 healthy individuals (9 women and 6 men) between 20 and 46 years old and of normal weight (BMI between 18.5 and 24.9 kg/m^2) participated in this study. All participants followed an omnivore diet. As this was a pilot study, no sample size calculation was used [21]. Young and middle-aged adults were selected as the population who commonly consume foods with different textures and to avoid age-related physiological decline or chewing difficulties affecting mastication. Individuals from both genders were included to ensure the identification of representative trends in the results. However, the higher proportion of women (60%) compared to men (40%) was due to the availability of volunteers. Participants were recruited from the University of Évora facilities. Participants were not screened specifically for temporomandibular disorders or bruxism.

Inclusion criteria included adults aged at least 18 years, not taking any medications, and living in Évora for the duration of the study.

Exclusion criteria included a history of allergies or intolerance to carrots, a manifest chronic disease (e.g., cancer) affecting their physiology or metabolism, and a history of oral diseases that involve difficulties in chewing (e.g., lack of teeth, xerostomia). Prior to their voluntary participation, they were informed about the aim and methods of this study, and they signed an informed consent. The study was approved by the Ethical Committee of the University of Évora (GD/2746/2021).

2.2. Study Procedure

A flowchart of the study procedure is shown in Figure 1. Participants were randomly allocated to 3 different mastication conditions: 4 g of raw carrot slices (CS), 4 g of raw carrot grated (CG), or a small piece (2 × 2 cm) of parafilm (P) (Amcor, Oshkosh, WI, USA) as a control. The same carrots were used to slice or grate to avoid differences in the sensory characteristics of the two conditions due to differences in the carrots used. As such, on each experimental day, carrot slicing and grating were undertaken using the same batch of carrots. Participants were instructed to have their regular lunch and come to our laboratory 2 h 30 min later (between 16 and 17 h) without eating or drinking anything other than water after lunch.

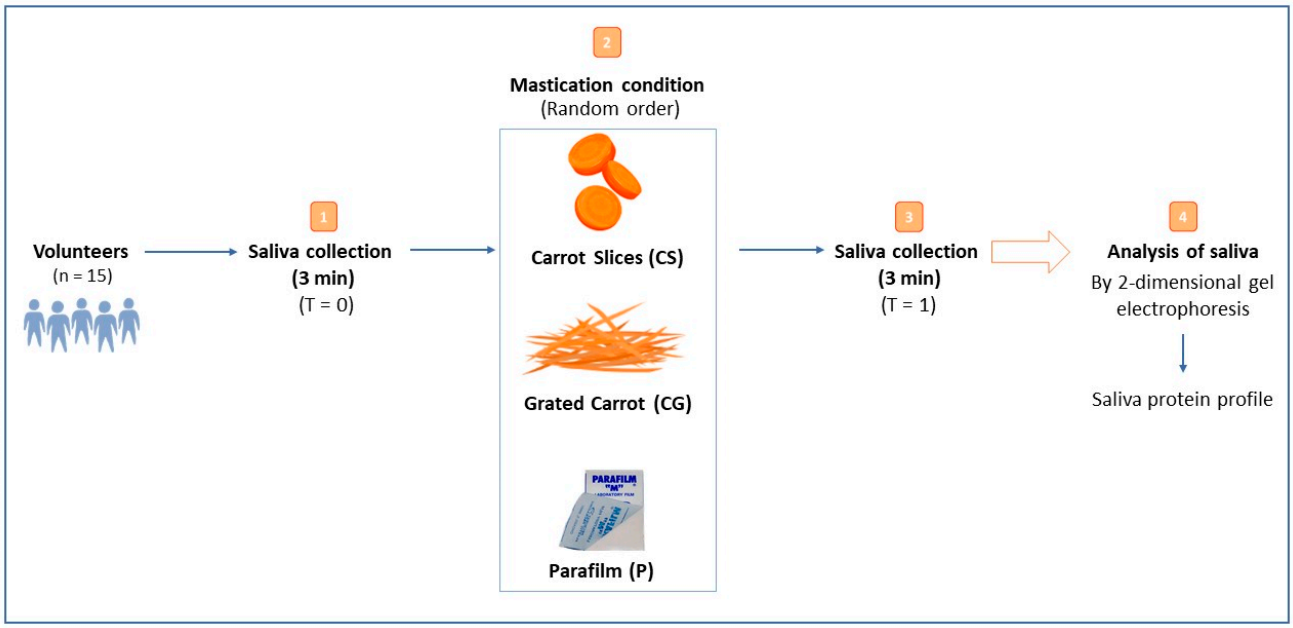

Figure 1. Flowchart with a timeline of the study procedure.

Over three different non-consecutive days, the 15 participants were divided into groups of 5, with each of these 3 groups being submitted to each mastication condition in a random order. Each individual tested only one condition per day, ensuring that ultimately, all participants tested all 3 conditions. This study followed a full cross-over design where each participant experienced all conditions, acting as their own control.

At each testing session, non-stimulated saliva samples were collected for 3 min before mastication (T0). Immediately after, a piece of carrots or parafilm was provided, and participants were asked to chew for 30 s. Carrot pieces were swallowed, and parafilm was spit out. Then, another 3 min period was dedicated for saliva collection (T1).

2.3. Saliva Collection

Unstimulated saliva samples from each individual were collected before (T0) and after (T1) each mastication condition (CS, CG, or P) during periods of 3 min, as referred to in the previous point. For this, participants remained in a comfortable position, relaxed, and were instructed to accumulate all the saliva produced in the mouth for 3 min, spitting it out into plastic tubes (VWR, Radnor, PA, USA) at the end of this time. Only in cases where the accumulation of saliva in the mouth for 3 min became uncomfortable, participants were allowed to spit before 3 min (althought they continuedto accumulate for the remaining time). This procedure was chosen to avoid variability in the number of spitting events throughout the collection interval, which would represent different levels of mechanical stimulation among individuals. Tubes containing saliva were kept on ice during collection, and then were stored at −28 °C until analysis.

The salivary secretion rate (mL/min) was calculated considering a saliva density of 1 g/mL and dividing the result by the collection period (3 min).

Before chemical analysis, saliva samples were centrifuged at 13,000× *g* and 4 °C for 15 min to remove more dense material. Supernatants were kept for analysis, and precipitates were discarded. The total protein content of saliva supernatants was determined by the Bradford method in m-96 microplates and using BSA (Biowest, Nuaillé, France) as a standard. Microplates were read at 600 nm in a microplate reader (Glomax, Promega, Madison, WI, USA).

2.4. 2-DE Analysis of Saliva

For 2-DE analysis, a sub-sample of 6 participants (5 women and 1 man) was selected, based on the amount of salivary protein available to allow 2-DE gel analysis. A previously published protocol optimised for saliva samples was used [16]. Briefly, a volume of each saliva sample, corresponding to 250 µg of total protein was aliquotted, desalted, and concentrated using 3 kDa cut-off ultra-filtration microfuge tubes (Nanosep 3K omega, PALL Corporation, New York, NY, USA). The recovered volume (always inferior to 50 µL) from the fraction higher than 3 kDa was mixed with rehydration buffer to a final volume of 250 µL. Thirteen-centimetre pH 3–10 NL IPG strips (Cytiva Europe GmbH—Freiburg im Breisgau, Germany) were passively in-gel rehydrated overnight in a Multiphor Reswelling Tray (GE Healthcare, Chicago, IL, USA) and subsequently focused using the previously described focusing program [22], using a Multiphor II isoelectric focusing system (GE Healthcare). Following focusing, gel strips were equilibrated and then applied on the top of a 12% SDS-PAGE gel (1 × 160 × 160 mm) for vertical separation, using a Protean II xi cell (Bio-Rad, Hercules, CA, USA) at a constant voltage of 150 V until the end of the run. Gels were stained with CBB-R250 (0.1% in 40% methanol (VWR), 10% acetic acid (VWR)) and de-stained with several washes of 10% acetic acid. The de-stained gels were scanned using the ImageScanner III scanner (Epson, Suwa, Japan) and Labscan software version 6.0. (GE Healthcare, Tokyo, Japan), and the protein profiles were analysed using SameSpots software 4.5 (TotalLab, Newcastle upon Tyne, UK). A first manual alignment of each gel with respect to the reference gel was made, 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.

2.5. Western Blot

In order to validate previous mass spectrometry (MS) identifications (presented in the Section 3, Section 3.2) and mainly to have a full vision of the different spots of amylase present in whole-saliva 2-DE profiles, saliva samples from 12 healthy volunteers (both sexes and the same age range as those tested for chewing) were collected and treated under similar conditions as described previously in Sections 2.2 and 2.3. The total protein concentration of the supernatants of the centrifuged samples was determined following the Bradford method, and 2-DE gels were run under the conditions described above (Section 2.4).

After the second dimension in-gel separation, proteins were transferred to a PVDF membrane (Amersham, UK), and proteins were blocked using the procedure described before [23], by using BSA instead of non-fat milk for blocking. After that, membranes were incubated with a primary monoclonal antibody anti-amylase (WH0000276M4, Sigma-Aldrich, Algés, Portugal; dilution 1:500) overnight at 4 °C, and spots were detected with a secondary antibody linked to alkaline phosphatase (anti-mouse; S372B, Promega, Madison, WI, USA; dilution 1:10,000) using a chemifluorescent substrate (ECF Plus Western Blotting Detection Reagents, GE Healthcare). The contact between the membrane and the substrate was monitored at 1.5 min for all membranes. Membranes were revealed in a transilluminator (Bio-Rad Gel-doc system), and a semi-quantitative analysis of band expression was carried out using the software Bio-Rad Image Lab 6.1.

2.6. Statistical Analysis

Normality and homoscedasticity were evaluated through Shapiro–Wilk and Levene tests, respectively.

To compare the effect of the different masticatory conditions, GLM repeated measures within subjects were used, considering the period (before and after) and the condition (CS, CG, P) as factors. Given the significant interaction between period and condition, a paired T-test was used to compare the periods before and after chewing in each of the 3 different conditions.

Concerning 2-DE data, since the normalised volumes of each protein spot did not follow a normal distribution, the Wilcoxon test was used to compare between the two collection times (T0, before; and T1, after) in each mastication condition (CS, CG, P) separately. The effect size was calculated as the ratio of the spot volume percentages after and before chewing (T1/T0) to allow inferences about the practical significance of the differences.

SPSS software (IBM SPSS Statistics version 29.0.1.0(171), IBM Corp©, Armonk, NY, USA) and a significance level of 0.05 were used for statistics.

3. Results

3.1. Differences in Salivation Depending on Mastication Condition (CS, CG, P)

The variations in salivation depending on the mastication condition are illustrated in Figure 2. GLM repeated measures showed a significant effect of period ($p = 0.021$) and a significant interaction between period and condition ($p = 0.006$). As can be observed, there were no significant increases in the saliva flow rate, after chewing parafilm or CG ($p = 0.664$ and $p = 0.178$, respectively), but a significant increase in salivation was observed for the CS condition ($p = 0.004$).

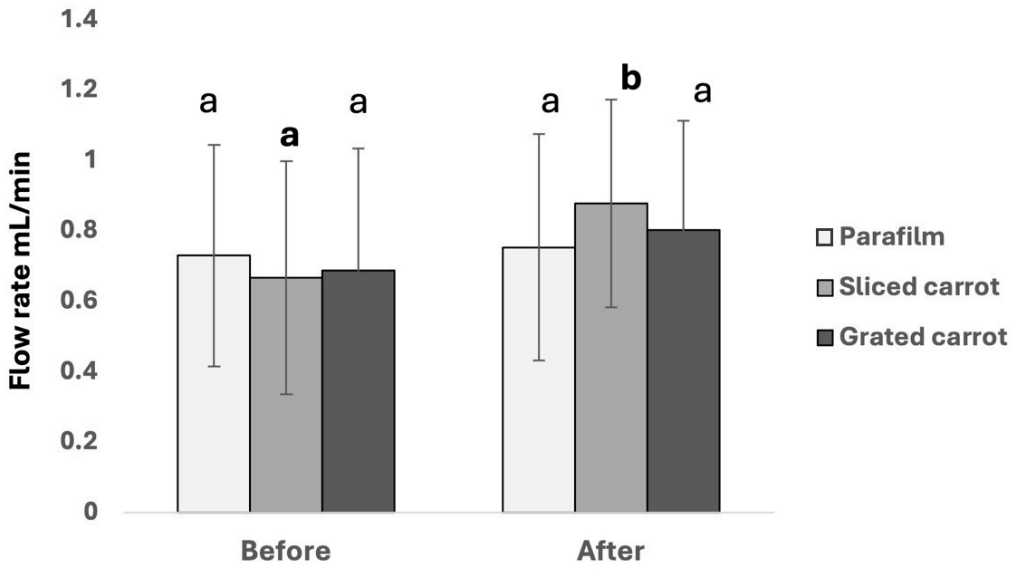

Figure 2. Saliva flow rate variation between the periods before (T0) and after (T1) each chewing condition (mean ± standard deviation; different upper letters (a ≠ b) represent differences between periods for $p < 0.05$).

3.2. Differences in 2-DE Spots Depending on Mastication Conditions (CS, CG, P)

An example of a gel with the protein spots quantified for analysis is shown in Figure 3.

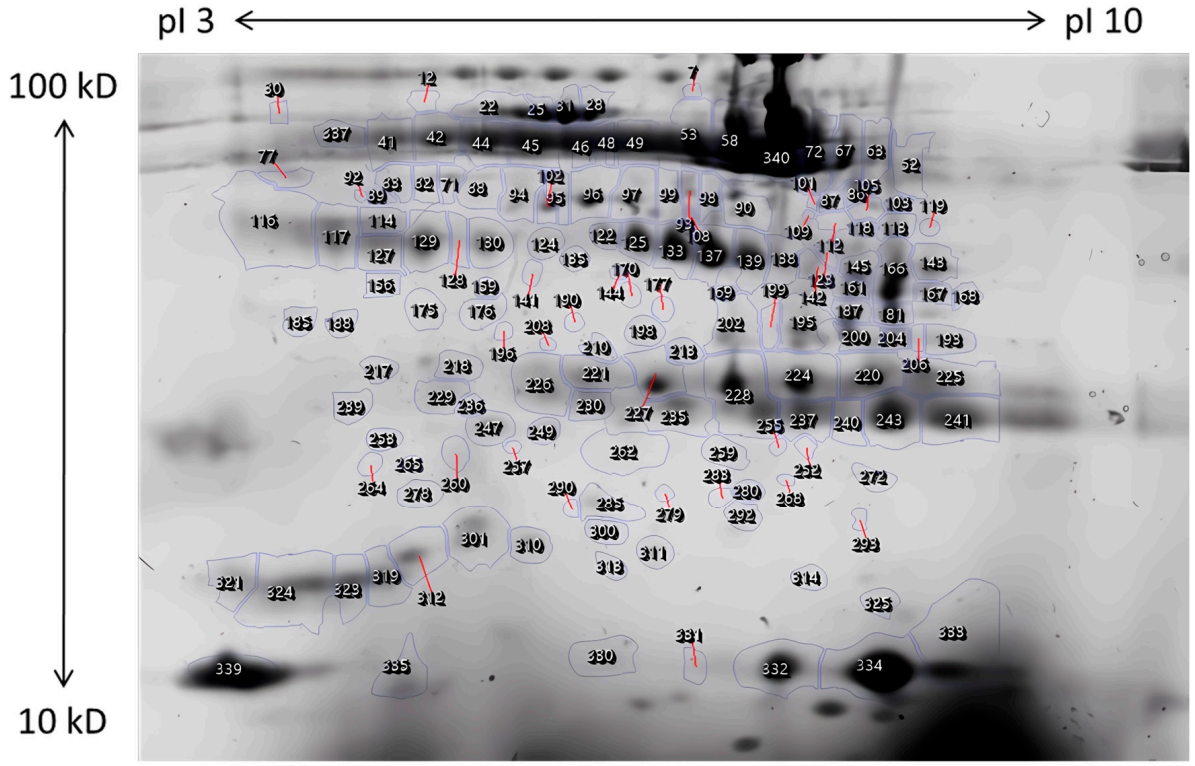

Figure 3. Example of a representative 2-DE gel and the selected spots for their quantification and comparison among conditions.

The changes in the salivary protein spots among the three mastication conditions, as well as the identification of spots by MS, are shown in Table 1.

Table 1. List of protein spots, previously identified in Rodrigues et al. (2017), (2017), and (2019) [23–25], and variation for the different mastication conditions: carrot slices (CS), grated carrot (CG), or parafilm/control (P). *p*-values obtained from the Wilcoxon test when comparing the protein spots before (T0) and after (T1) mastication in each condition (CG, CS, P).

Spot No.	CG			CS			P			Identification
	p-Value	Change	Ratio (T1/T0)	*p*-Value	Change	Ratio (T1/T0)	*p*-Value	Change	Ratio (T1/T0)	
25	0.5		0.96	0.028	↓	0.55	0.753		0.98	Serum albumin
28	0.043	↓	0.54	0.028	↓	0.42	0.463		0.97	Serum albumin
31	0.138		0.83	0.028	↓	0.42	0.345		0.88	Serum albumin
41	0.345		0.81	0.046	↓	0.70	0.345		0.94	Ig α-1 chain C region
42	0.138		0.77	0.046	↓	0.71	0.345		0.95	Ig α-1 chain C region
44	0.08		0.83	0.046	↓	0.61	0.075		0.86	Ig α-1 chain C region
45	0.345		1.05	0.046	↓	0.60	0.028	↓	0.79	Ig α-1 chain C region
90	0.043	↑	1.58	0.028	↑	**2.41**	0.6		1.27	n.i.
93	0.043	↑	1.53	0.028	↑	1.68	0.173		0.93	n.i.
108	0.08		1.54	0.046	↑	**2.16**	0.463		1.06	n.i
118	0.686		1.09	0.046	↑	**2.10**	0.249		0.81	Alpha-amylase (potential) #
123	0.225		1.40	0.046	↑	**2.23**	0.345		1.06	n.i.
141	0.5		0.92	0.028	↓	0.59	0.6		1.03	n.i.
143	0.043	↑	1.79	0.028	↑	**2.93**	0.463		0.92	Alpha-amylase (potential) #
166	0.225		1.47	0.028	↑	1.90	0.249		0.97	Alpha-amylase (potential) #
167	0.08		1.52	0.046	↑	**3.04**	0.173		0.79	Alpha-amylase (potential) #
181	0.225		1.61	0.028	↑	**2.47**	0.249		0.93	Alpha-amylase (potential) #
187	0.5		1.33	0.028	↑	**2.40**	0.6		1.01	Alpha-amylase (potential) #
195	0.138		1.97	0.028	↑	**3.49**	0.753		1.31	Alpha-amylase (potential) #
198	0.043	↑	1.88	0.028	↑	**3.21**	0.173		1.45	n.i.
200	0.686		1.36	0.028	↑	1.92	0.249		0.88	Alpha-amylase (potential) #
202	0.043	↑	2.55	0.028	↑	**4.27**	0.249		1.23	Alpha-amylase (potential) #
213	0.345		1.22	0.028	↑	**3.55**	0.249		0.93	n.i.
220	0.893		1.20	0.028	↑	1.87	0.075		0.75	Ig kappa chain C region
221	0.893		1.09	0.046	↑	1.48	0.249		0.91	Ig kappa chain C region
224	0.138		1.58	0.028	↑	**2.61**	0.249		0.90	Ig kappa chain C region
227	0.043	↑	1.23	0.028	↑	**2.02**	0.6		1.07	Ig kappa chain C region
228	0.043	↑	1.40	0.028	↑	**2.04**	0.345		0.91	Ig kappa chain C region
239	0.345		0.93	0.046	↓	0.76	0.6		1.14	n.i.
259	0.225		1.28	0.028	↑	**2.05**	0.173		1.45	n.i.
262	0.345		1.82	0.028	↑	**3.96**	0.345		1.22	n.i.
285	0.225		1.31	0.028	↑	**2.20**	0.917		1.13	n.i.
300	0.686		1.17	0.028	↑	**3.01**	0.345		1.70	n.i.

Table 1. *Cont.*

Spot No.	CG			CS			P			Identification
	p-Value	Change	Ratio (T1/T0)	*p*-Value	Change	Ratio (T1/T0)	*p*-Value	Change	Ratio (T1/T0)	
301	0.893		1.13	0.028	↑	**2.38**	0.463		1.29	Prolactin-inducible protein
311	0.225		1.39	0.028	↑	**2.49**	0.116		1.54	n.i.
312	0.345		1.87	0.028	↑	**2.84**	0.917		1.11	Prolactin-inducible protein
313	0.345		0.92	0.028	↑	**2.02**	0.6		1.09	n.i.
334	0.043	↑	2.73	0.917		0.99	0.917		1.05	Cystatin SN

Spots identified as alpha-amylase by Western blot analysis with human anti-alpha-amylase (AMY1) antibody. Details presented in Figure 4. Bold—effect sizes lower than 0.5 or higher than 2.0 are signalled, as these represent decreases to less than half or increases to more than double, respectively.

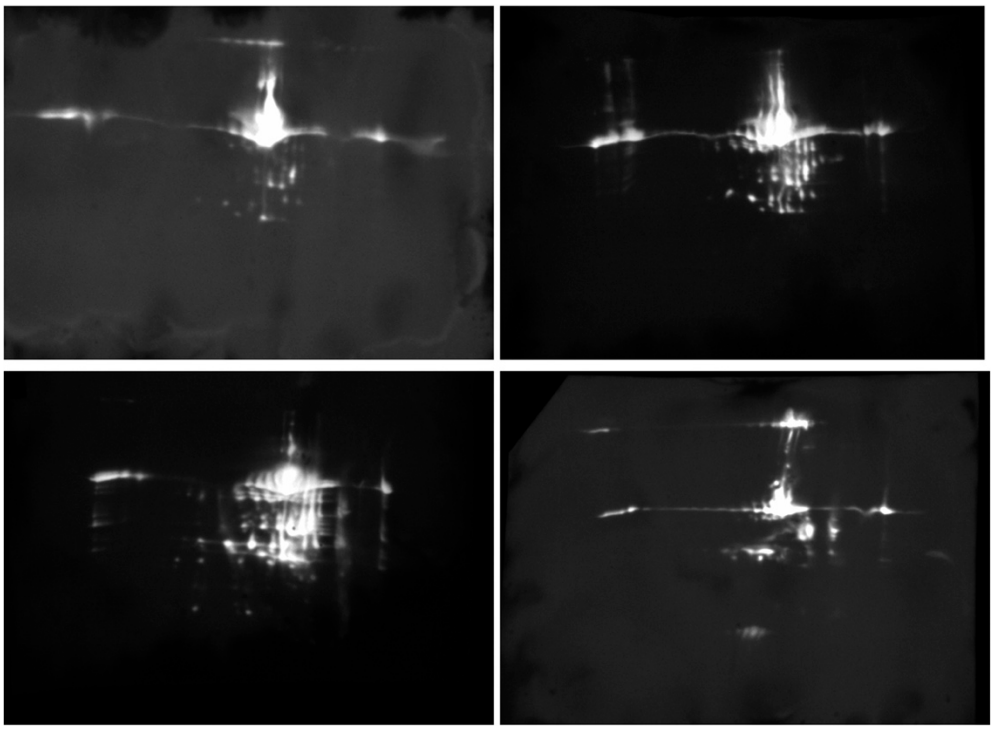

Figure 4. Examples of images of membranes incubated with anti-alpha-amylase primary antibody, highlighting the presence of several different spots of this protein.

As can be observed, the effects of chewing on the salivary protein profile were much more pronounced for carrots (CS and CG) than for the non-flavoured control parafilm (P). In fact, only one protein spot (spot 45, previously identified as the Ig α-1 chain C region) showed significant decreases due to the mastication of parafilm, while the mastication of carrot slices (CS) and grated carrot (CG) resulted in significant differences in the levels of 37 and 9 protein spots, respectively. From these nine protein spots significantly affected by grated carrot mastication, eight of them also changed after the mastication of sliced carrots, while they were not affected by parafilm mastication. Of those, the levels of seven spots increased in saliva after carrot mastication, whereas the levels of one spot (spot 28, previously identified as serum albumin) decreased after carrot mastication (CG, CS). Of the seven spots that increased due to mastication of both shapes of carrots, two of them (spots 143 and 202) were identified as amylase (from Western blot analysis, as further detailed) and two of them (spots 227 and 228) as the Ig kappa chain C region, while three of them (spots 90, 93, and 198) were not identified.

Among the 37 spot proteins that showed significant differences after the consumption of CS, 9 of them presented significant decreases after the consumption of slices of carrot, whereas 28 spots presented significant increases. Considering the proteins identified thus far (Table 1), chewing CS resulted in decreased levels of four spots of the Ig α-1 chain C region (spots 41, 42, 44, and 45) and three spots of albumin (spots 25, 28, and 31). Increased levels were observed for nine protein spots of amylase (spots 118, 143, 166, 167, 181, 187, 195, 200, and 202), five spots of Ig kappa C chains (spots 220, 221, 224, 227, and 228), and two spots of prolactin-inducible proteins (spot 301 and 312). Twelve other protein spots were observed to increase after CS chewing, although they were not previously identified by MS (Table 1). It was possible to observe that the most pronounced variations (with reductions by half or less, or increases by double or more) were observed for sliced carrots (Table 1—highlighting relevant effect-size in bold).

Several low-abundance protein spots, with a basic *pI*, showed increases, particularly, after CS chewing. Being low-abundance, these spots usually fail identification by MS, although they are in a region where several spots have been identified as salivary amylase by MALD-TOF [26]. By performing Western blot analysis, to identify the different proteoforms of amylase separated by 2-DE, it was possible to observe that several protein spots present in that part of the profile bind to the anti-amylase antibody (Figure 4). This reinforces the hypothesis of increased levels of amylase (with a lower molecular mass than the native protein) after chewing carrot slices (CS), which is not as evident after chewing grated carrot (CG) and not observed after parafilm (P) chewing.

4. Discussion

This pilot study explored how the mastication of carrots in different shapes, sliced (CS) and grated (CG), affects the salivary secretion and proteome (proportions of salivary proteins) compared to a non-food control (parafilm).

The results showed almost no changes in salivation after parafilm mastication and an increase in salivation after carrot mastication, although this was statistically significant only in the case of CG. These results indicated that chewing food (carrots) tends to induce higher increases in salivation compared to non-food (parafilm), with the grated carrot tending to induce higher salivation after mastication. Previous studies have observed similar results with a lower secretion of saliva after the mastication of non-food products (parafilm) compared to foods (e.g., celery, bread, toast, cake), with no statistical differences in salivation among food types [9,27]; see the review in Forde and Bolhuis, 2022 [1].

When looking at the changes in salivary protein profiles, it was interesting to observe that chewing the non-flavoured material (parafilm) produced almost no effects at the salivary protein profile level, with only one spot (Ig α-1 chain C region protein) presenting statistically significant differences between the period before and after chewing parafilm. These results suggested that the chewing itself has minor effects on the salivary proteome. Although different studies showing the relevance of chewing force to healthy salivary gland function [28] and despite it being known that chewing results in increased salivary secretion due to activation of the masticatory–salivary reflex [29], as parafilm is an inert material, which has neither nutrients nor flavours, it appears to be less effective in stimulating the active secretion of salivary proteins that usually occurs in food consumption.

The chewing of carrots, both in slices (CS) and grated (CG), induced variations in the levels of serum albumin (decrease) and amylase and Ig kappa chain C regions (increase) in saliva. Thus, chewing real foods such as carrots produced more changes in the salivary proteome than chewing parafilm. This could be explained by a higher activation of the salivary glands by the mechanical stimulation of mastication combined with sensory and nutritional stimulation from real foods, which has previously been observed to result in a higher secretion of saliva and proteins [30].

In addition to that, when comparing the differences in salivary protein levels depending on the shape of carrots (CS vs. CG), higher differences were observed for CS than for CG, as 9 protein spots changed in CG compared to 37 spots in CS due to mastication.

These results indicated that chewing CS, which presented a harder texture and involved more chewing movements, affected the salivary proteome more than the consumption of CG, which could explain why more protein changes were observed in CS than in CG. A higher mastication need is well accepted to induce a higher saliva volume, with researchers such as Lashley (1916) and Kerr (1961) (both cited by Hector, 1999 [30]) demonstrating that receptors within the periodontal ligament were responsible for afferent information inducing salivary secretion (for more detail, please see Hector, 1999, and Garrett et al. (1999) [30,31]). Since CS and CG are associated with similar sensory stimulation, higher mastication results in a higher amount of saliva produced, possibly also resulting in more changes at the salivary protein profile level.

Among salivary proteins, increased levels of some proteoforms (molecular forms of the same protein) of amylase, Ig chains, and prolactin-inducible protein (PIP) were observed due to CS mastication. Regarding amylase, although some proteoforms of this protein increased due to the mastication of both types of carrot, a higher number of spots increased only after chewing CS. These results were not surprising, as amylase is a key enzyme in saliva involved in oral digestion, as it begins to break down carbohydrates in the mouth. The relatively increased levels of amylase in CS than in CG suggest that the food-shape characteristics have a significant influence on its secretion, and not only the composition of the chewing product (food vs. non-food). Foods that are in harder and larger portions (sliced vs. grated) and that required a higher chewing effort exhibited greater increases in the levels of amylase in the saliva. In a previous study evaluating the impact of non-food (parafilm) and food chewing (celery or bread) on salivary amylase, the authors also found a higher amylase activity in the saliva after the consumption of foods than of non-foods [27]. Among foods, they found more amylase secretion after bread than celery consumption, which was higher after mastication of larger pieces of bread than smaller ones [27], which agrees with the findings from this study. Thus, the increase in amylase levels in saliva could be explained not only by the food composition itself (e.g., the starch content), but also due to the physical characteristics of the food (shape or hardness) and the mastication force.

In spite of this, and according to a previous systematic review, other authors have found a different impact of chewing on salivary amylase [15]. For instance, some authors have observed a decrease, other authors an increase, and most authors have found no effect of mastication on amylase activity [15]. Thus, no consensus has been reached about the impact of mastication on amylase activity, although these studies used different chewing materials (such as chewing gum, plastic pieces, and dental prints) that were not always food. In addition, most of these studies evaluated the total amylase activity present in saliva using diverse kits, but they did not evaluate the variations in the amylase proteoforms present in saliva, not all of which necessarily present the same enzymatic activity [32]. Indeed, these proteoforms may present slightly different structures due to modifications (e.g., glycosylation, phosphorylation) which might lead to variations in substrate affinity or catalytic activity, thus affecting their enzymatic activity efficiency [32]. Thus, it is not to be discarded that the diverse results about the impact of mastication on salivary amylase could be due to different effects that chewing may have on amylase proteoforms, influencing the total salivary amylase activity in a different way, depending on the chewing stimuli (e.g., food properties). In addition, and interestingly, the spots that increased with CS are spots with a lower molecular mass and higher isoelectric point than the native form of amylase. A previous study from Hirtz et al. (2005) [26] used mass spectrometry to study the different protein spots of amylase separated by 2-DE. The authors observed that the spots in the gel region corresponding to the spots of amylase which increased with CS mastication are truncated forms of amylase, resulting from protease activity. The unresolved question is whether proteolysis occurs before or after secretion in the mouth. In addition to the effect that amylase modifications may have on its enzymatic activity [26], it was previously observed that different stimuli result in increases in different amylase proteoforms [32], reinforcing the different stimulation that the different types of chewing produce. The complexity of salivary-alpha amylase is increased by the high variation among individuals

in the number of copies of the gene that codifies this protein (AMY1), which has been linked to human dietary evolution for starch intake [33].

On the other hand, and concerning regions of Ig kappa, they are associated with immune functions in the oral cavity. Their increase due to CS mastication could be solely due to the active chewing movements, which could reflect the activation of the immune response associated with the protection of the oral mucosa against food mastication, food particles, or potential pathogens. In a similar way, PIPs are involved in the immune response and oral homeostasis; thus, their increase after chewing CS could also be related to their involvement in the protection of mucosal surfaces from increased chewing activity. Regarding PIPs, in a previous study using 2-DE analysis to evaluate the impact of rice and bread mastication on the salivary proteome, the authors also found increases in two and one spot of PIP after rice and bread mastication, respectively [16], which is consistent with the present results.

Finally, decreased levels of serum albumin and regions of sIgA due to CS mastication were observed. sIgA is involved in oral immune response functions, while serum albumin is associated with protective and maintenance functions in the oral cavity [34]. The impact of chewing on those proteins has been poorly investigated. In the case of sIgA, different effects (increase, decrease, or no effect) of mastication have been reported when evaluating their concentration in saliva by ELISA [15]. In humans, sIgA secretion has been reported to increase with chewing compared to resting levels [35], but in our previous study, a decrease in chains of sIgA with bread and rice mastication was observed, which was explained by the gustatory stimulation that chewing involves, together with the mechanical stimulation [16]. Nevertheless, there are also reports of increased sIgA secretion induced by gustatory stimuli [36], highlighting that the effect of food chewing and oral processing on sIgA and salivary proteins needs to be better understood. In 2002, Proctor and Carpenter reviewed the neural control of sIgA secretion, from which the authors concluded that both parasympathetic and sympathetic nervous system activation results in sIgA increases in saliva, although the effects of the two branches of the autonomic nervous system have different strengths [37]. The authors also state that the effect of autonomic innervation in the secretion of this protein is different from the one at the level of salivary proteins stored in acinar cell granules. As such, it is possible that the decrease observed in sIgA chains after chewing CS does not represent a true decrease in the sIgA concentration in saliva, but rather a lower proportion of this protein in relation to other salivary proteins. In fact, for electrophoretic separation, the same amount of total protein was used for all samples, independently of whether some samples had a higher total protein concentration than others, allowing only a comparison of the proportions among salivary proteins between treatments and not the absolute amounts of those proteins in saliva.

The increased proportion of some immunoglobulins (the Ig kappa chain C region) might, at first sight, appear contradictory to the decreased proportion of sIgA chains with CS chewing. However, the Ig chain that is observed to increase after CS chewing is a light chain (kappa light chain). Whereas heavy chains are specific and determine the class of an immunoglobulin, the same light chains can be present in all Ig classes. Hence, the increase in the kappa light chain proportion after chewing the carrot that required a higher mastication effort may indicate a higher passage of Ig from blood sources to the saliva (e.g., Riis et al. (2020) [38], and for more details about Ig in saliva, please see Brandtzaeg, 2013 [39]).

5. Conclusions

This pilot study evaluated the impact of food shape during mastication on the salivary proteome by comparing carrot in slices (CS), grated carrot (CG), and parafilm (non-food control). The results showed that chewing carrots resulted in higher variations in the protein profile than parafilm mastication, with variations in the levels of specific proteins including amylase and immunoglobulin chains. This effect was even more pronounced in CS, observed by the changes in a higher number of salivary proteins in response to CS

mastication (n = 37 protein spots) compared to the changes in response to CG mastication (n = 9 protein spots).

These findings suggest that both the composition (nutrients and taste compounds) and the physical properties (e.g., portion size and hardness) of food modulate salivary protein secretion. These results also reinforce the thought that shape and texture influence salivary gland stimulation and consequently the type of secretion. This underlines the importance of considering food shape and texture when evaluating the salivary response during oral food digestion.

Overall, this pilot study provides evidence that the mastication of carrots in different shapes significantly influences the composition of the salivary proteome. In spite of this, further research with larger sample sizes and varied food textures will be necessary to confirm these preliminary findings and expand our understanding of the relationship between mastication, salivary proteins, and sensory perception.

From our knowledge, this is the first study where a proteomics approach was used to access the effects of food shape and size on saliva secretion. Despite the interesting information observed, including the different effect for different proteoforms of the same protein, there are limitations that need to be considered, namely the reduced number of participants selected for proteomics analysis. Moreover, mastication force can only be inferred from the forms of carrot used, since no direct assessment of masticatory movements and masticatory force was made for each participant.

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