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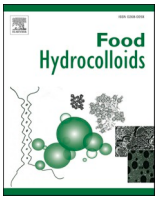

Oral impairments decrease the nutrient bioaccessibility of bread in the elderly

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ABSTRACT

In the elderly, oral functions are modified by changes in muscular force or saliva production among others, resulting in inadequate food fragmentation which potentially impacts on oral and gastrointestinal digestion. The purpose of this work was to evaluate the consequences of oral deficiencies on the starch and protein digestibility of bread. *In vitro* boluses were prepared with the AM^2 masticator using normal and deficient mastication programming. Normal mastication (NM) and deficient mastication in terms of force (DfM), saliva (DsM), and their combination (DfsM) were performed. Static *in vitro* digestion, simulating physiological conditions in the elderly, were carried out. Bolus particle size, starch and protein digestibility, Fourier-transform infrared (FTIR) spectroscopy, and microstructure after *in vitro* oral and gastrointestinal digestion were analysed. More compacted boluses were observed after deficient mastication combined with greater particle sizes. The poorly fragmented boluses obtained with deficient mastication affected the oral digestion of starch, probably due to lower saliva impregnation. Digesta from deficient mastication boluses exhibited lower D-glucose release and degree of protein hydrolysis. FTIR results in the carbohydrates region also revealed weaker initiation of oral digestion of starch in DsM and DfsM boluses. These results on bread demonstrate for the first time how oral deficiencies modify nutrient bioaccessibility and, therefore, stress the importance of designing foods for specific populations such as the elderly.

1. Introduction

Growing life expectancy and decreasing birth rate have provoked a continuous demographic transformation represented by higher proportions of elderly people (Huang, Liu, Muo, & Chang, 2021). The percentage of people over 65 years-old was 9.3% in 2020 and it is estimated to reach 16% in 2050, corresponding to 1.5 billion seniors worldwide (United Nations, 2020).

Ageing is a natural process characterised by various changes in the human body and oral elements age in the same way as all other organs (Amarya, Singh, & Sabharwal, 2018, chap. 1; Rashid, Tiwari, & Lehl, 2020). Ageing itself does not have a high impact on masticatory performance, although masticatory muscle mass and bite force decrease with age, but becomes more deleterious when the oral cavity is affected by severe oral impairments (Gaszynska, Kopacz, Fronczek-Wojciechowska, Padula, & Szatko, 2017; Mishellany-Dutour, Renaud, Peyron, Rimek, & Woda, 2008). Indeed, it is often combined with dental loss, substantial decrease in saliva, and decline in motor skills and tongue motility. These impairments worsen with age and impact on masticatory performance and the ability to disrupt hard foods into small particles sufficiently lubricated by saliva (Müller, Naharro, & Carlsson, 2007; Peyron, Santé-Lhoutellier, François, & Hennequin, 2018; Steele, Treasure, Pitts, Morris, & Bradnock, 2000). In this sense, the swallowable boluses formed when oral functions are impaired are characterised by low levels of food breakdown, resulting in a great proportion of large particles that increase food bolus hardness (Peyron et al., 2018, 2021). Furthermore, several studies have suggested that food boluses insufficiently fragmented during mastication reduce oral release of nutrients, as well as their bioaccessibility (Peyron et al., 2018, 2021), which could impact on the nutritional status of the elderly (Mabiama et al., 2021; N'Gom & Woda, 2002).

Nutrition is a set of complex physiological phenomena in which both

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the type and quantity of nutrients ingested are crucial, as well as their digestion and absorption levels, especially for carbohydrates and proteins (Freitas, Le Feunteun, Panouillé, & Souchon, 2018). The connection between carbohydrates and proteins configures the primary structure of numerous cereal-based products including wheat bread, which is one of the principal sources of carbohydrates for humans such as starch (Aleixandre, Benavent-Gil, & Rosell, 2019; Freitas et al., 2018). Starch is considered the main source of digestible carbohydrates in the human diet (Gropper & Smith, 2013). In bread, starch digestion begins in the oral cavity due to the action of salivary α-amylase once the saliva is blended with the food particles, and continues until early gastric digestion (Freitas et al., 2018; Hoebler et al., 1998; Pentikäinen et al., 2014). In contrast, protein digestion starts in the stomach owing to the acidic conditions and action of enzymes, and it is completed in the small intestine due to the activity of peptidases and proteases (Gropper & Smith, 2013). Nevertheless, it is important to highlight that oral impairments produce food boluses with larger particle sizes that could impede the initiation of oral digestion of starch. Besides, ageing alters the physiology of the upper part of the digestive tract, impacting protein digestibility as a consequence of a reduced motility and acidification (Russell, 1992). Some studies have investigated the impact of deficient mastication on the starch or protein digestibility of meat and pasta products (Blanquet-Diot, François, Denis, Hennequin, & Peyron, 2021; Peyron et al., 2021), but no studies have reported the impact of diverse oral deficiencies on the glucose release and protein digestibility of bread and their effect on the structure of this product and/or its nutrients.

Hence, this work aimed to evaluate the effects of several oral impairments on the nutrient bioaccessibility of bread in the elderly. To this end, the impact of mastication on starch and protein digestibility of bread after *in vitro* oral and gastrointestinal digestion, as well as its effect on the structural changes of nutrients were investigated. Concerning the *in vitro* digestive processing, it is important to highlight that digestive conditions in the elderly were simulated. Fourier-transform infrared (FTIR) spectroscopy, which is recognised as a non-invasive and valuable tool for the examination of bread composition and for monitoring structural changes (Kong & Yu, 2007; Ozkoc, Samnu, Sahin, & Turabi, 2009), was used to study the effect of oral impairments on the structural changes of bread nutrients after *in vitro* oral and/or gastrointestinal digestion.

2. Materials and methods

2.1. Materials

The bread employed in this study (*baguette tradition*) was bought in a local French bakery. For static *in vitro* oral and gastrointestinal digestion, salivary α-amylase from human saliva, pepsin from gastric porcine mucosa, lipase A from *Aspergillus niger*, pancreatin from porcine pancreas, and bile extract porcine were employed. Analytical grade salts (potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, magnesium chloride hexahydrate, ammonium carbonate, and calcium chloride dihydrate) were also used. For analytical determinations, boric acid, hydrochloric acid (37%), sulphuric acid (95–97%), sodium hydroxide, L-methionine, DL-dithiothreitol solution 1 M, o-phthaldialdehyde, sodium tetraborate, sodium dodecyl sulphate, trichloroacetic acid, and phosphotungstic acid were employed. All the reagents used for conducting the static *in vitro* oral and gastrointestinal digestion and the analytical determinations were provided by Sigma-Aldrich Co. (St. Louis, MO, USA).

Enzymatic kits (Digestible Starch and Resistant Starch K-DSTRS, Maltose/Sucrose/D-glucose K-MASUG) were from Megazyme® (Bray, Ireland).

2.2. Masticatory trials

In vitro masticatory trials were run by means of using the AM^2 masticator, which was designed and validated for producing food boluses presenting similar granulometric properties to those observed in boluses collected after normal *in vivo* mastication (Peyron et al., 2018, 2021). Programming of the AM^2 masticator was based on previous *in vivo* experiments on bread products to obtain the dynamic parameters of the masticatory sequence and to determine the particle size distribution of the *in vivo* bread boluses recovered. Programming was then adjusted progressively and finally validated when the *in vivo* and *in vitro* particle size distribution curves of both bread boluses overlapped. The duration of the *in vivo* masticatory sequence was 27 s. The *in vitro* normal mastication (NM) boluses were prepared using the *in vivo* mastication data obtained from 10 young subjects, whereas the *in vitro* simulation of several oral impairments was performed by modifying certain parameters of the AM^2 masticator so as to mimic mastication with deficiency in force (DfM) and saliva (DsM), as well as their combination (DfsM). Table 1 summarises the conditions used when programming the AM^2 masticator to mimic the *in vitro* NM and the different oral impairments. Tap water was added to the chamber of the AM^2 masticator, in place of saliva, to simulate mouth coating and to avoid uncontrolled oral digestion by salivary α-amylase; however, saliva was subsequently used (section 2.4). At the end of each masticatory sequence, the *in vitro* food boluses produced were recovered by means of using a spatula and the granulometric analysis was carried out. The *in vitro* boluses produced to determine nutrient bioaccessibility after *in vitro* oral and gastrointestinal digestion, as well as their structural changes, were stored at - 80 °C until further analysis. Ten boluses per condition were prepared for the granulometric analysis, six boluses per condition were produced for oral digestion exclusively, and three other boluses per condition were prepared for the oral and gastrointestinal digestion.

2.3. Granulometric analysis of food boluses

The granulometric assays of the *in vivo* and *in vitro* bread boluses were performed using a mechanical dry sieve shaker for 3 min at a vibratory amplitude of 1.7 mm (Retsch Gmbh, AS 200 digit CA, Düsseldorf, Germany). To this end, the bread boluses were poured onto a nylon cloth of 0.3 mm (Sefar, Switzerland) and rinsed with tap water to achieve a great particle spreading and to remove saliva in the case of *in vivo* bread boluses. After that, the boluses were dried in a ventilated oven for 30 min at 37 °C and the dried particles were mechanically sieved by employing 10 sieves with apertures of 7.1, 6.3, 4.0, 2.5, 2.0, 1.4, 1.0, 0.8, 0.4, and <0.4 mm (Saulas, France). The particles retained on each sieve were weighted and the results were expressed as the cumulative particle weight curve falling through each specific sieve. Finally, each curve gave the median particle size (d_{50}) defined as the aperture of a theoretical sieve throughout which the 50% of the bread bolus particle weight could pass (Peyron et al., 2018). The assays were conducted on all the bread boluses recovered after performing the *in vivo* and *in vitro* masticatory experiments (10 *in vivo* boluses and 10 *in vitro* boluses per condition tested).

Table 1
Conditions used when programming the AM^2 masticator to mimic the *in vitro* normal mastication and the different oral deficiencies.

Type of *in vitro* mastication	Number of masticatory cycles	Spring stiffness (N/mm)	Tap water addition (mL)
NM	30	13.80	3
DfM	30	10.38	3
DsM	30	13.80	2
DfsM	30	10.38	2

NM: normal mastication; DfM: deficient mastication in terms of force; DsM: deficient mastication in terms of saliva; DfsM: deficient mastication in terms of force and saliva.

2.4. In vitro oral and gastrointestinal digestion

In vitro oral and gastrointestinal digestion was studied by following the standardised static INFOGEST protocol described by Minekus et al. (2014) and Brodkorb et al. (2019), but with minor changes to simulate digestive conditions in elderly people (Denis et al., 2016; Duval et al., 2020). Briefly, a shaking incubator chamber (NB-205 L, Kisker Biotech GmbH & Co. KG, Steinfurt, Germany) at 180 rpm and at 37 °C was employed to run the analysis in triplicate. To imitate the oral phase (pH 7), simulated salivary fluid and α-amylase enzyme (75 U/mL) were blended with the NM and DfM samples in a ratio 1:1 (v/w) and with the DsM and DfsM in a ratio 0.5:1 (v/w) for 30 s. The duration of the oral phase was established considering the duration of the *in vivo* mastication test (section 2.2); however, it was slightly increased by some seconds (10% higher compared to the duration of the *in vivo* mastication assay). Afterward, the pH of the samples was adjusted to 3.54 with HCl (6 M) and simulated gastric fluid, pepsin (1200 U/mL), and gastric lipase (12 U/mL) were mixed with the oral boluses (1:1, v/v) for 120 min. An aliquot of 1 mL was withdrawn from each reaction tube at the end of the gastric phase and was immediately mixed with 1 μL of NaOH (40 M) to stop the enzymatic reaction. In the intestinal phase, the pH was adjusted to 7 by adding NaOH (1 M) and the simulated intestinal fluid, the pancreatin suspension (66.3 U/mL trypsin activity), and the bile solution (6.7 mM) were blended with the gastric chyme (1:1, v/v) for 120 min. At the end of the intestinal phase, 10 μL of Pefabloc® (500 mM) per mL of digesta was added to inhibit the proteolysis. Blank samples (without bread but with all enzymes and bile) and undigested samples at zero time (with bread boluses, all the simulated fluids and enzymes, the bile solution, and the Pefabloc® (500 mM) to inhibit the proteolysis) were also prepared. All samples were stored at −80 °C until further analysis.

2.5. Determination of starch digestion products

2.5.1. In vitro starch digestibility

The *in vitro* starch digestibility of the different bread boluses obtained after simulating *in vitro* oral digestion was determined following the manufacturer's instructions (Megazyme® K-DSTRS kit). After *in vitro* oral digestion of the boluses, as described in section 2.4, α-amylase activity was immediately inhibited by adjusting the pH to 3 with HCl (6 M). The samples were then centrifuged (4000 g, 10 min, 4 °C) and the pellet was recovered. The supernatants were also kept at −80 °C for further analysis. Subsequently, 0.5 g of each sample (pellet) was incubated at 37 °C with pancreatic α-amylase (40 KU/g) and amyloglucosidase (17 KU/g) and different aliquots were collected at 20 min, 120 min, and 240 min to estimate rapidly digestible starch, slowly digestible starch, and total digestible starch, respectively. Moreover, the starch remaining after 240 min of digestion, which is defined as resistant starch was also determined. To this end, samples were dissolved in NaOH (1.7 M) and digested using amyloglucosidase (3300 U/mL). Lastly, the release of glucose was analysed using GOPOD reagent, the absorbance of which was measured at 510 nm with a Multiskan™ Spectrum Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). All determinations were performed in triplicate.

2.5.2. Sugar analysis

Sugar (maltose and D-glucose) analyses were conducted in triplicate as described by the provider (Megazyme® K-MASUG kit), with minor changes. The supernatants collected after mimicking the *in vitro* oral digestion of the boluses (section 2.5.1) and the centrifuged aliquots recovered at the end of the gastric and intestinal phases (4000 g, 10 min, 4 °C) were employed to determine the amounts of maltose and D-glucose released during *in vitro* digestion. For that, the amount of nicotinamide-adenine dinucleotide phosphate formed in the presence of the enzyme glucose-6-phosphate dehydrogenase was measured by the increase in absorbance at 340 nm with a Multiskan™ Spectrum Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA).

2.6. Determination of protein digestion products

2.6.1. Analysis of nitrogen fractions

The total nitrogen content of *in vitro* bread boluses and digesta was determined by means of using a micro-Kjeldahl (VELP Scientifica Srl, Usmate, Italy). In the case of digesta, trichloroacetic acid-soluble nitrogen (accounting for all peptides and free amino acids) and phosphotungstic acid-soluble nitrogen (accounting for small peptides and free amino acids) were also determined. To this end, the trichloroacetic acid-soluble nitrogen fraction was quantified by adding 3.3 mL of trichloroacetic acid (48%, w/v) to 10 mL of digesta. The mixture was kept for 30 min at 20 °C, centrifuged (4000 g, 20 min, 20 °C), and the supernatant was recovered. The phosphotungstic acid-soluble nitrogen fraction was also determined by adding 4.9 mL of sulphuric acid (3.95 M) and 2.1 mL of phosphotungstic acid (33.3%, w/v) to 7 mL of digesta. The mixture was maintained overnight at 4 °C and subsequently centrifuged (4000 g, 20 min, 20 °C). Aliquots of 5 mL from both centrifuged extracts were analysed with the micro-Kjeldahl. Non-digested nitrogen, expressed as the difference between total nitrogen content and all peptide and free amino acid content, was also reported. All measurements were done in triplicate and a nitrogen-to-protein conversion factor of 5.70 was employed (AOAC, 2000, p. 950:36).

2.6.2. OPA assay and degree of hydrolysis

The free primary amino groups of the digested and undigested samples were determined using the OPA (o-phthaldialdehyde) method (Nielsen, Petersen, & Dambmann, 2001). The OPA reagent was composed of SDS (20%, w/v), DL-dithiothreitol solution (1 M), OPA solution in ethanol (10 mg/mL), and sodium tetraborate (20 mM). The samples were centrifuged (4000 g, 10 min, 4 °C) prior to being analysed and then 50 μL of each sample was added to 100 μL of the OPA reagent. The mixture was incubated at 18 °C for 40 min and the absorbance was measured at 340 nm with a Multiskan™ Spectrum Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA). The free primary amino groups were quantified by employing a L-methionine standard curve with concentrations ranging from 0 mM to 2 mM. The assays were performed in triplicate.

The degree of hydrolysis (DH), which corresponds to the proportion of cleaved peptide bonds within samples (Halabi, Croguennec, Bouhallab, Dupont, & Deglaire, 2020), was calculated according to Equation (1).

$$\mathrm{DH}\ (\%) = \frac{[\mathrm{NH_2}\ \text{digested samples}] - [\mathrm{NH_2}\ \text{undigested samples}]}{[\mathrm{NH_2}\ \text{digested samples}]}\ \mathrm{x}\ 100 \qquad (1)$$

where [NH_2 digested samples] represents the free primary amino group content in the samples at the end of the gastric or intestinal phases (mg/L) and [NH_2 undigested samples] represents the free primary amino group content in undigested samples.

2.7. Fourier-transform infrared (FTIR) analysis

2.7.1. Liquid sample measurements

FTIR analysis of the liquid phase of bread boluses (section 2.5.1) and digesta was performed using a Bruker Tensor II spectrometer equipped with OPUS 7.5 software (Bruker, Bremen, Germany). The spectral resolution was established at 4 cm^{-1} and 32 scans over a wavelength range of 4000–600 cm^{-1} were employed for each measurement. The three best spectra from six were selected, treated by applying the second derivative, and cut to estimate the areas of interest (Amide I region: 1710–1590 cm^{-1}) after atmospheric compensation and baseline adjustment.

2.7.2. Solid sample measurements

The *in vitro* boluses collected after mimicking *in vitro* oral digestion (section 2.5.1) were cryofixed in isopentane, cooled with liquid nitrogen (−196 °C), and kept at −80 °C until further analysis. A cryomicrotome (Leica CM 1950; Leica Biosystems, Nussloch, Germany) was utilised to cut serial sections of 6 μm and 10 μm thick of each bolus, which were then collected on glass slides and kept at – 20 °C until analysis.

Bolus sections of 6 μm collected on BaF2 window were used for IR analysis. The IR spectra were acquired using a FTIR microscope (Thermo Scientific, Nicolet iN10) scanning over a wavelength range of 4000–675 cm^{-1}, with a spectral resolution of 4 cm^{-1} and an aperture size fixed at 30 μm × 30 μm. Each spectrum come from 64 accumulated scans and the cumulative spectra were averaged and subtracted from a background spectrum determined at the beginning of the scan by compiling 128 scans (Renaud et al., 2022).

2.8. Microstructure analysis

The microstructural characteristics of samples were evaluated by light microscopy. Bolus sections of 10 μm thick collected on glass slides were stained for 3 min with a Lugol solution (0.3%, w/v) and for 3 min more with a Light Green solution (0.1%, w/v) to stain starch in purple and proteins in green, respectively. Successive 30-s rinses with deionised water were used to remove excess dye. Micrographs were acquired at 20x magnification using an Olympus BX 61 microscope equipped with a high-resolution digital camera (Olympus DP 71) and an Olympus Cell Sens software (Olympus France SAS, Rungis, France).

2.9. Statistical analysis

The results obtained were analysed by using one-way ANOVA or repeated measures ANOVA, and post-hoc comparisons between *in vitro* NM boluses and *in vitro* deficient mastication boluses after simulating *in vitro* oral and gastrointestinal digestion were made employing Dunnett's multiple comparison test. The results were statistically processed by XLSTAT 2020.3.1 software (XLSTAT statistical and data analysis solution, Addinsoft, New York, USA, displayed as the mean of replicates ± standard deviations (SD), and the statistically significant limit was established at $p < 0.05$.

3. Results

3.1. Programming of the AM^2 masticator

Fig. 1 presents the granulometric properties of the *in vivo* bread boluses and the *in vitro* boluses produced by the AM^2 masticator. As can be seen, the programming of the masticatory apparatus for the preparation of the different *in vitro* bread boluses was validated given that the particle size distribution curve of the *in vitro* NM boluses was not significantly different ($p > 0.05$) from that obtained in the case of *in vivo* boluses (Fig. 1A). Furthermore, no significant differences ($p > 0.05$) were observed for the median particle size (d_{50}) values of the *in vivo* bread boluses and *in vitro* NM bread boluses, these values being 4.2 ± 0.6 mm and 4.4 ± 0.5 mm, respectively.

When deficiencies were mimicked, the particle size distribution curves of all the *in vitro* bread boluses prepared with the AM^2 masticator were significantly different from those of NM ($p < 0.05$) (Fig. 1B). A

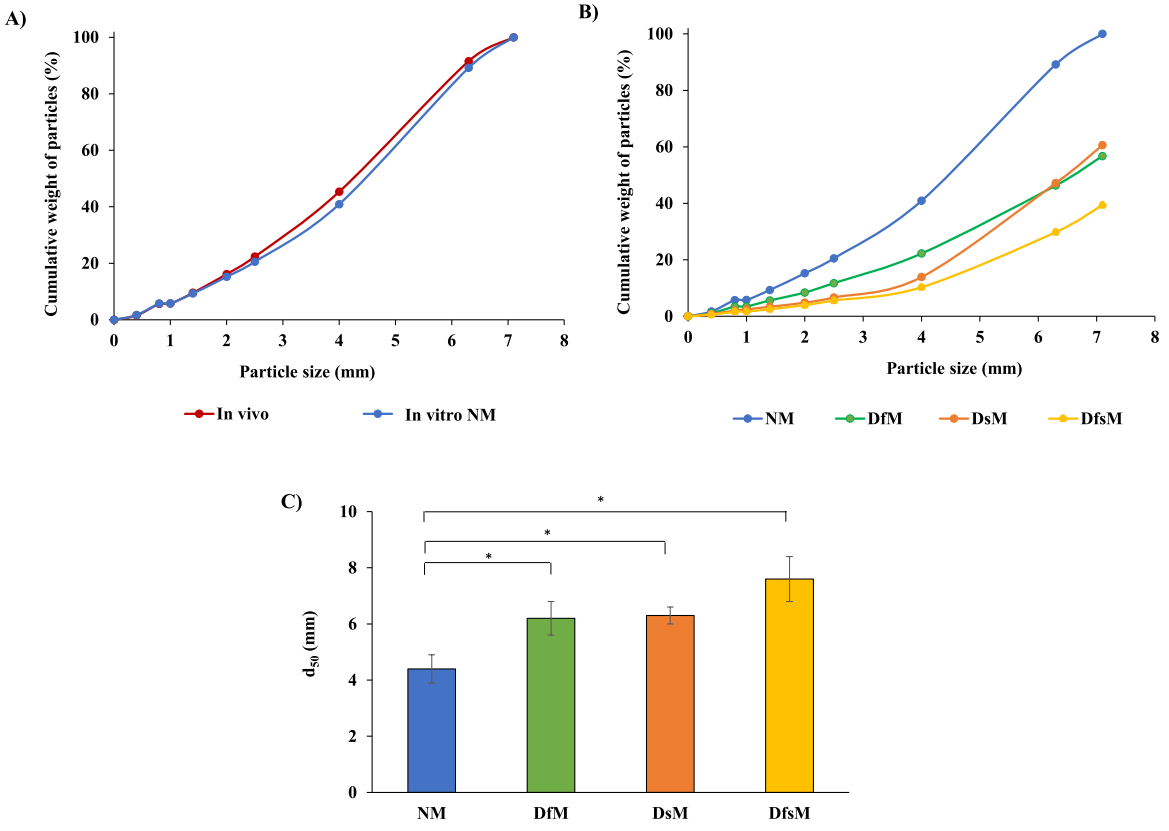

Fig. 1. Granulometric properties of the bread boluses: (A) overlapped particle size distribution curves of *in vivo* and *in vitro* normal mastication boluses used to validate the programming of the masticator; (B) particle size distribution curves of the boluses recovered after performing *in vitro* normal or deficient masticatory experiments with the AM^2 masticator. Curves are presented as mean cumulative percentages of particle weight passing through each sieve; (C) median particle size values expressed as d_{50} in mm. Mean values (n = 10) ± SD. NM: normal mastication; DfM: deficient mastication in terms of force; DsM: deficient mastication in terms of saliva; DfsM: deficient mastication in terms of force and saliva. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$.

greater proportion of large particles was observed when simulating the different oral impairments (DfM, DsM, and DfsM). In these cases, the cumulative weight did not reach 100% because of the presence of particles larger than the greater sieve aperture. Besides, the d_{50} values of the *in vitro* bread boluses presenting several oral deficiencies were significantly ($p < 0.05$) higher than those observed in the case of *in vitro* NM boluses (Fig. 1C), indicating the formation of insufficiently fragmented food boluses. For instance, DfM and DsM boluses exhibited d_{50} values of 6.2 ± 0.6 mm and 6.3 ± 0.3 mm, respectively. However, it is important to highlight that these differences were more pronounced in the case of DfsM boluses, showing that these samples had the greatest proportion of large particles (7.6 ± 0.8 mm).

3.2. Determination of starch digestion products

The release of starch and its hydrolysis into small glucose polymers begin with the disruption of the food matrix and saliva impregnation produced during mastication. Fig. 2 presents the starch digestibility results of the different bread boluses tested after simulating the *in vitro* oral digestion. Regarding the rapidly digestible starch content, significantly ($p < 0.01$) lower values were observed in the case of DfsM boluses (5.6 ± 0.2%) compared to NM boluses (7.6 ± 0.6%) due to their higher particle sizes and the lower saliva content used to simulate the saliva deficiency. The slowly digestible starch values of all the boluses evaluated ranged from 4.0 ± 0.2% to 6.6 ± 0.2%, whereas the resistant starch content was lower than 0.5%. Lastly, the total digestible starch and the total starch contents of DsM and DfsM boluses significantly increased ($p < 0.001$; $p < 0.05$) in comparison with the NM boluses, but it is important to mention that in both cases the same saliva deficiency was mimicked.

Fig. 3 shows the maltose and D-glucose contents present in the liquid phase of NM, DfM, DsM, and DfsM boluses after simulating *in vitro* oral and gastrointestinal digestion. Part of the starch released when simulating *in vitro* oral digestion was hydrolysed into maltose due to the action of salivary α-amylase, while a small quantity of D-glucose was detected (Fig. 3A–B). Concerning the maltose content, no significant differences ($p > 0.05$) were observed among samples, whose values ranged from 4.9 ± 0.5 g/L to 5.7 ± 0.9 g/L. In contrast, significant differences ($p < 0.001$) in D-glucose content were noted between boluses presenting different oral impairments (DfM, DsM, or DfsM boluses) and NM boluses. In this sense, DfM, DsM, and DfsM boluses exhibited lower D-glucose contents (0.2 ± 0.1 g/L) than NM boluses after the *in vitro* oral digestion, probably due to their higher particles size, which likely decreases the accessibility of salivary α-amylase.

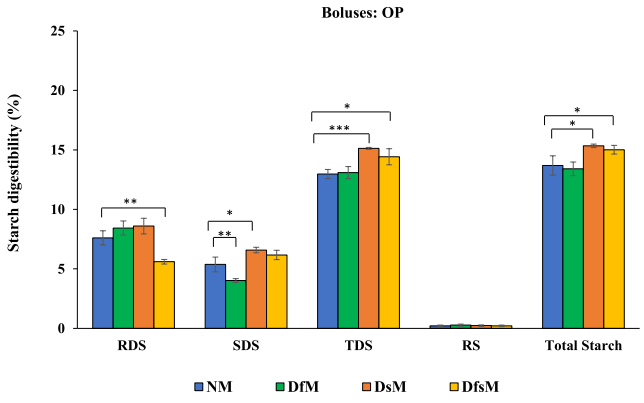

Fig. 2. Percentage (%) of rapidly digestible starch (RDS), slowly digestible starch (SDS), total digestible starch (TDS), resistant starch (RS), and total starch values of the different *in vitro* bread boluses obtained after simulating *in vitro* oral digestion. Mean values (n = 3) ± SD. NM: normal mastication; DfM: deficient mastication in terms of force; DsM: deficient mastication in terms of saliva; DfsM: deficient mastication in terms of force and saliva; OP: Oral phase. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$.

At the end of the gastric phase, a significantly ($p < 0.05$) higher maltose content was observed in DsM and DfsM samples compared to NM samples, suggesting a delay in starch digestibility probably owing to saliva deficiency. For instance, the maltose content in DsM and DfsM samples was 1.9 ± 0.3 g/L and 1.8 ± 0.4 g/L, respectively (Fig. 3C). However, a significantly ($p < 0.05$) lower D-glucose release was noted in DfsM samples (0.2 ± 0.0 g/L) in comparison with NM samples (0.3 ± 0.1 g/L) due to cumulative deficiency in mastication (force and saliva impairments) (Fig. 3D). At the end of the intestinal phase, most of the initial starch and maltose contents were hydrolysed into D-glucose (Fig. 3F), this being significantly ($p < 0.05$) less marked in the case of DfsM samples (1.3 ± 0.2 g/L) because of the cumulative deficiency in mastication. No significant differences ($p > 0.05$) were observed among samples regarding the amounts of residual maltose in the intestinal phase (Fig. 3E).

3.3. Determination of protein digestion products

The total protein content and the nitrogen fractions of the different samples at the end of the *in vitro* oral and gastrointestinal digestion (intestinal phase) are shown in Fig. 4. The total nitrogen content and the total protein content of the different *in vitro* bread boluses recovered at the end of the *in vitro* oral digestion were close to 1.412 g/100 g and 8.047 g/100 g, respectively (Fig. 4A–B).

At the end of the intestinal phase, the total nitrogen content of samples was between 0.262 ± 0.003 g/100 g and 0.274 ± 0.002 g/100 g, while the total protein content values of the different samples ranged from 1.496 ± 0.015 g/100 g to 1.561 ± 0.011 g/100 g (Fig. 4C–D). Additionally, the content of all peptides and free amino acid content of the different samples was close to 0.188 g/100 g. Concerning the small peptides and free amino acid fraction, significant differences ($p < 0.01$; $p < 0.001$) were observed between the NM samples and the samples presenting different oral impairments (DfM, DsM, or DfsM), suggesting the impact of particle size on their protein digestibility (Fig. 4F). It is well-known that larger particles minimise protein surface exposure, making access of enzymes to cleavage sites difficult. A similar trend was observed when evaluating the non-digested nitrogen content of the different samples (Fig. 4G). Generally, those samples containing larger particles exhibited significantly ($p < 0.001$; $p < 0.05$) greater values of non-digested nitrogen, denoting once again the impact of particle size on the protein digestibility of samples.

Fig. 5 presents the degree of hydrolysis (DH, %) of the different samples at the end of each stage of *in vitro* gastrointestinal digestion. Regarding the gastric phase, significant differences ($p < 0.001$) were noted between the NM samples and the samples presenting different oral impairments (DfM, DsM, or DfsM), hydrolysis being more limited in the case of DfsM samples. This could be attributed to the large peptides formed during the gastric digestion as a consequence of cumulative deficiency in mastication. Furthermore, at the end of the intestinal phase, the NM samples presented a DH of 43 ± 2%, being the DH of DfM, DsM, and DfsM samples (*ca.* 30%) significantly ($p < 0.001$) lower (Fig. 5B). These results are in line with those observed when determining the small peptides and free amino acid fraction of each sample at the end of the intestinal phase.

3.4. Fourier-transform infrared (FTIR) analysis

The secondary structure of gluten plays an important role in the characteristics of bread products (Georget & Belton, 2006). In order to link the results obtained during the protein digestibility of samples to their protein secondary structure composition, FTIR analysis was performed. Proteins present characteristic absorption bands in the mid infra-red spectrum. The most sensitive spectral region of proteins is the Amide I band (1750-1590 cm^{-1}), which favours the analysis of the protein secondary structure composition due to its high sensitivity to

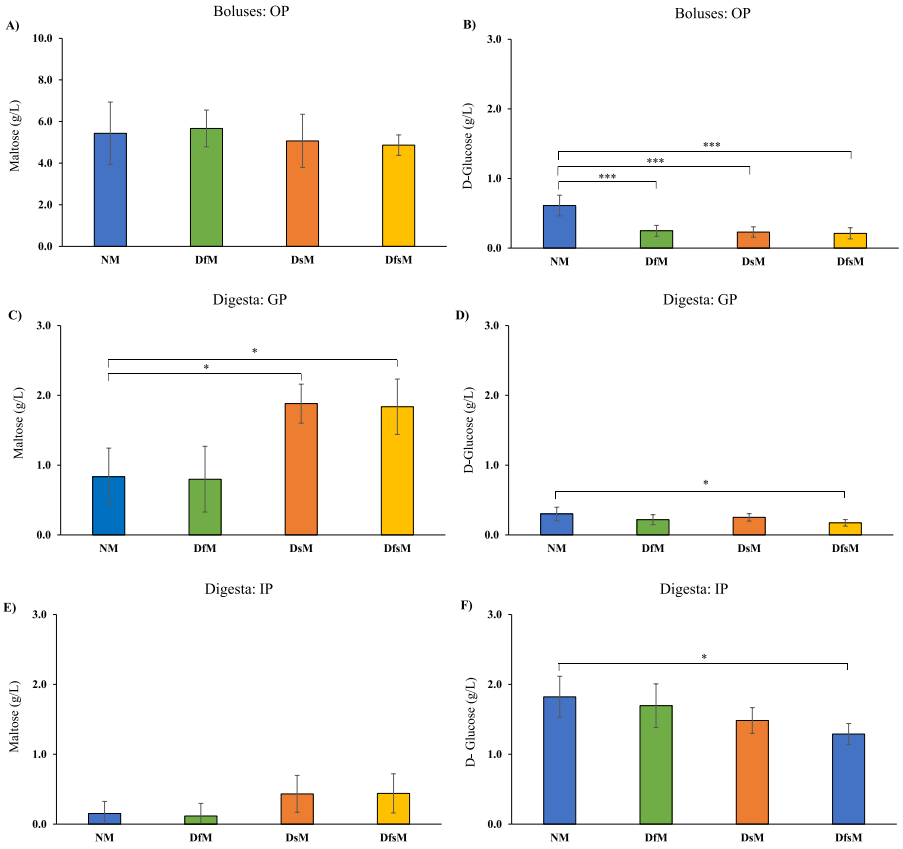

Fig. 3. Maltose (g/L) and D-glucose (g/L) contents present in the liquid phase of the different samples after simulating *in vitro* oral (A and B) and gastrointestinal digestion (C and F). Mean values (n = 3) ± SD. OP: oral phase; GP: gastric phase; IP: intestinal phase; NM: normal mastication; DfM: deficient mastication in terms of force; DsM: deficient mastication in terms of saliva; DfsM: deficient mastication in terms of force and saliva. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$.

slight modifications in molecular geometry and hydrogen bonding patterns. The components of Amide I band like β-sheet (<1643 cm^{-1} and 1689-1699 cm^{-1}), β-turn (1667-1687 cm^{-1}), 3.10-helix (1660-1666 cm^{-1}), and random (1646-1650 cm^{-1}) structures can be highlighted by evaluating the frequency with which they exist and contribute to protein secondary structure.

The protein secondary structure composition (%) of NM, DfM, DsM, and DfsM samples after mimicking *in vitro* oral and gastrointestinal digestion is shown in Fig. 6 (A-C). β-sheet is the main protein secondary structure present in all the samples with values close to 60%, meanwhile the β-turn, 3.10-helix, and random structures contributed to their composition with values lower than 25%. The protein secondary structure composition of the samples cannot be linked with their protein digestibility data.

FITR analysis was used to study the starch-related variations produced in the boluses at the end of *in vitro* oral digestion (Fig. 6D). To this end, the carbohydrates region (1200-900 cm^{-1}) was selected for a detailed investigation of the starch digestibility of bread boluses. The FTIR spectrum of the *in vitro* bread boluses exhibited a band related to the C–O vibration of the glycosidic units (1027 cm^{-1}), this vibrational band being greater in those boluses where saliva deficiency was simulated (DsM and DfsM boluses), which suggests a weaker initiation of oral digestion due to lower saliva content. These results accord with those observed when investigating the starch digestibility of the *in vitro* bread boluses (section 3.2).

3.5. Microstructure of in vitro boluses

Fig. 7 presents the micrographs of the different *in vitro* bread boluses recovered after simulating *in vitro* oral digestion. After mastication, all the samples were disintegrated and the protein-starch network appeared to be compacted, leading to the formation of bread boluses consisting of aggregates of starch (in purple) and proteins (in green). It is important to highlight that these structures appeared more compacted in the case of DfM, DsM, and DfsM boluses, probably due to their greater particle sizes.

4. Discussion

Ageing, which is not a pathology *sensu stricto*, leads to several physiological changes in cells, tissues, and organs. The oral condition of the elderly is often defective, although oral health and comfort are prerequisites for correct masticatory function and, therefore, good nutrition. Ageing is frequently associated with dental loss and oral deficiencies, including decreased salivation and xerostomia. In the elderly, these oral impairments play an important role in the formation of swallowable boluses, which are characterised by greater proportions of large particles that could modify the bioaccessibility of nutrients (Peyron et al., 2018, 2021). In the present study, the effects of several oral impairments on the nutrient bioaccessibility of bread in the elderly were investigated. To this end, the effect of mastication on the starch and protein digestibility of bread after *in vitro* oral and gastrointestinal digestion, as well as its impact on the structural variations of nutrients were considered. The AM^2 masticator was employed to perform *in vitro* boluses. It is well-known that *in vitro* assays can lead to approximative conclusions. Nevertheless, the use of the AM^2 masticator to focus oral step in digestion positions this work in a realistic context. Regarding *in vitro* digestive processing, it is important to highlight that digestive conditions in the elderly were simulated. Static *in vitro* digestion models have been shown to be very useful in predicting outcomes of *in vivo* digestion (Bohn et al., 2017; Sanchón et al., 2018). Combination of both

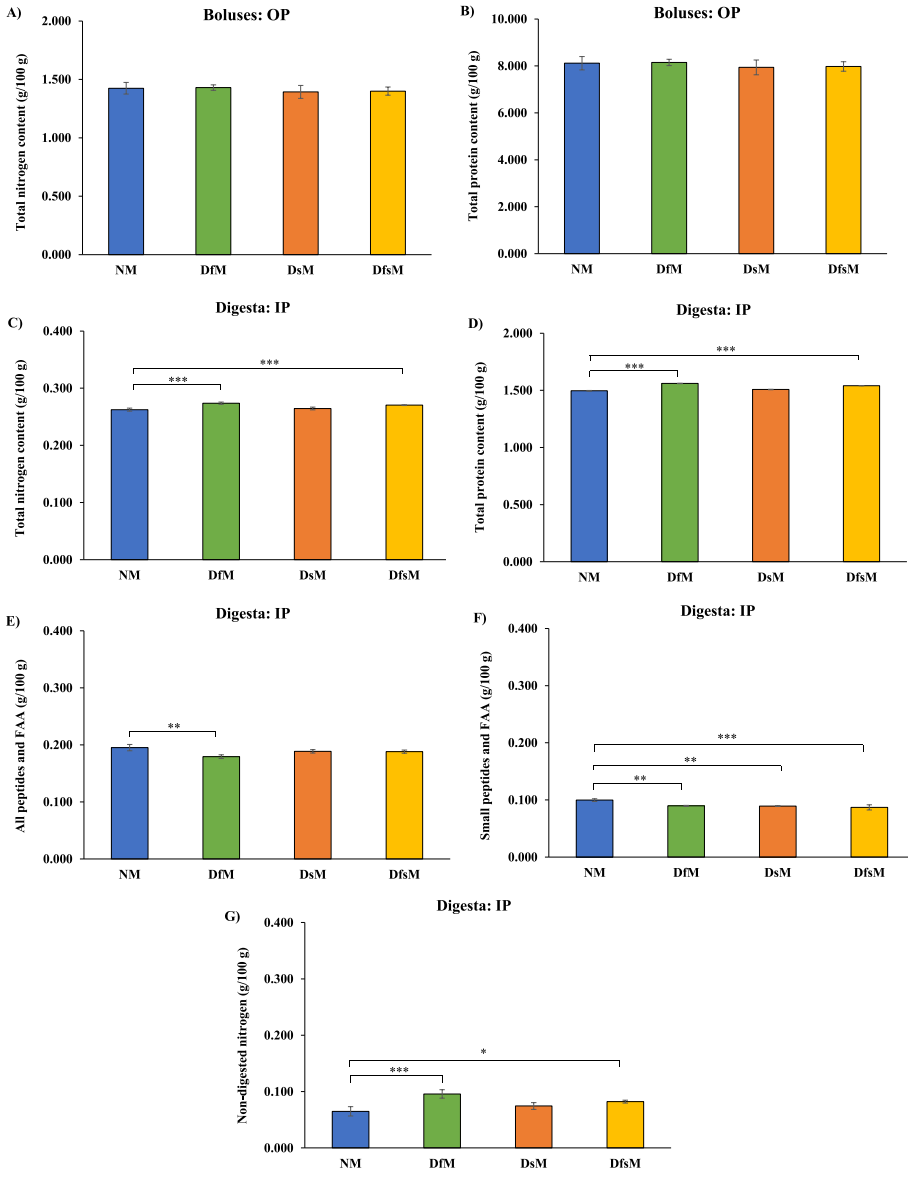

Fig. 4. Total nitrogen content (g/100 g) and total protein content (g/100 g) of *in vitro* bread boluses and intestinal digesta (A–D). All peptides and free amino acids (g/100 g), small peptides and free amino acids (g/100 g), and non-digested nitrogen (g/100 g) of the different samples at the end of the intestinal phase of *in vitro* digestion (E–G). OP: oral phase; IP: intestinal phase; FAA: free amino acids; NM: normal mastication; DfM: deficient mastication in terms of force; DsM: deficient mastication in terms of saliva; DfsM: deficient mastication in terms of force and saliva. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$.

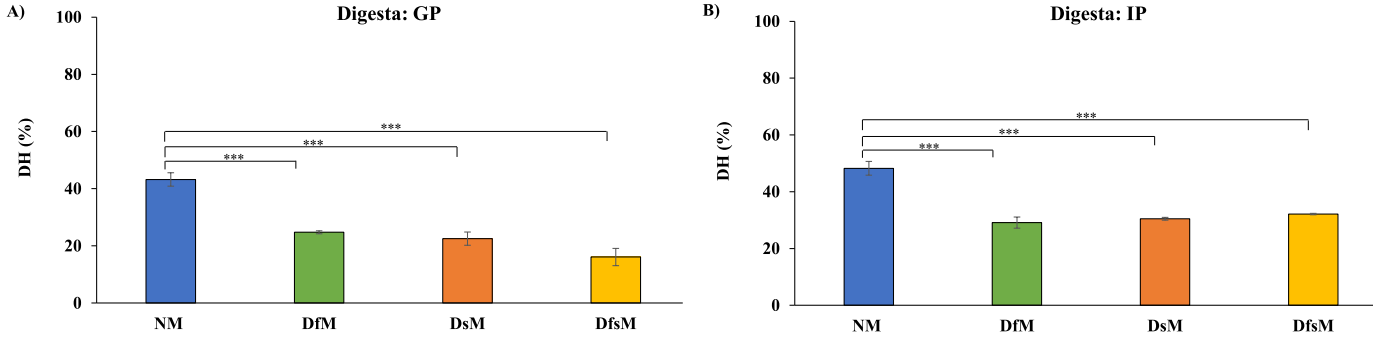

Fig. 5. Degree of hydrolysis (DH, %) of the different samples at the end of the gastric and intestinal phases of *in vitro* digestion. GP: gastric phase; IP: intestinal phase; NM: normal mastication; DfM: deficient mastication in terms of force; DsM: deficient mastication in terms of saliva; DfsM: deficient mastication in terms of force and saliva. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$.

models in the present work proposes proper interpretations without excessive approximations since the oral phase represents the main targeted focus.

4.1. Impact of mastication on nutrient bioaccessibility

Food digestion is a complex process comprising oral, gastric, and

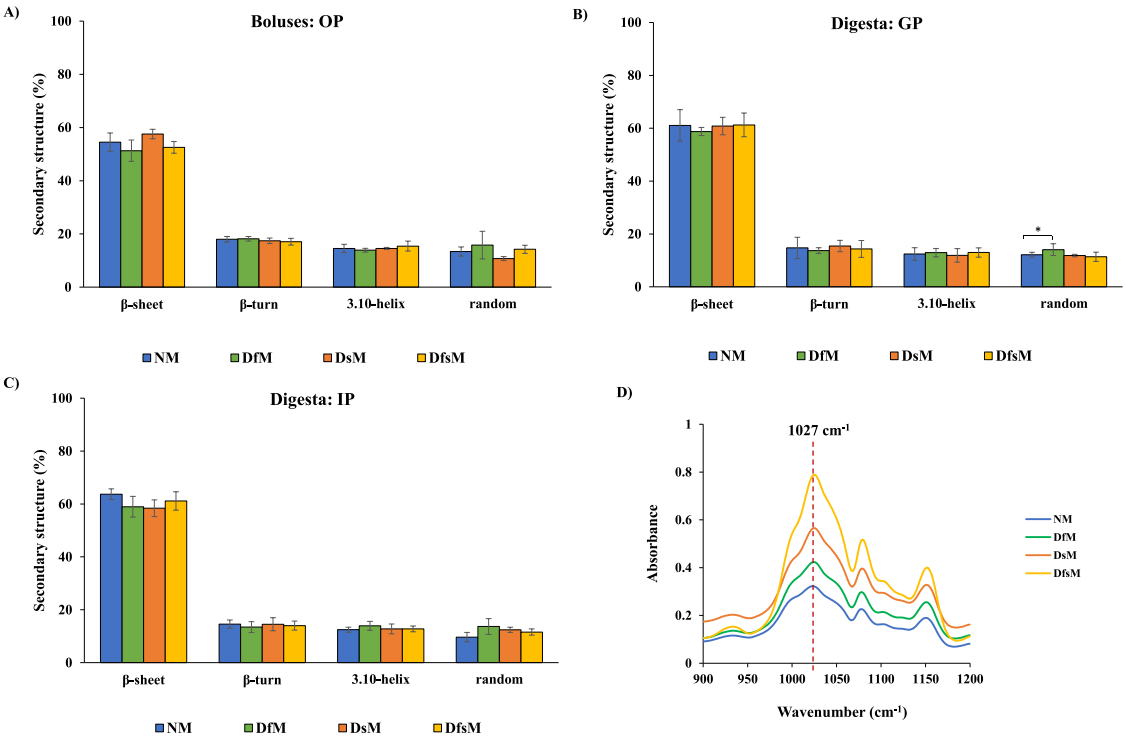

Fig. 6. FTIR spectroscopy analysis of protein and starch: protein secondary structure composition (%) of NM, DfM, DsM, and DfsM samples after mimicking *in vitro* oral and gastrointestinal digestion (A–C); normalised spectra of the different *in vitro* boluses in the carbohydrates region after simulating *in vitro* oral digestion (D). OP: oral phase; GP: gastric phase; IP: intestinal phase; NM: normal mastication; DfM: deficient mastication in terms of force; DsM: deficient mastication in terms of saliva; DfsM: deficient mastication in terms of force and saliva. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$.

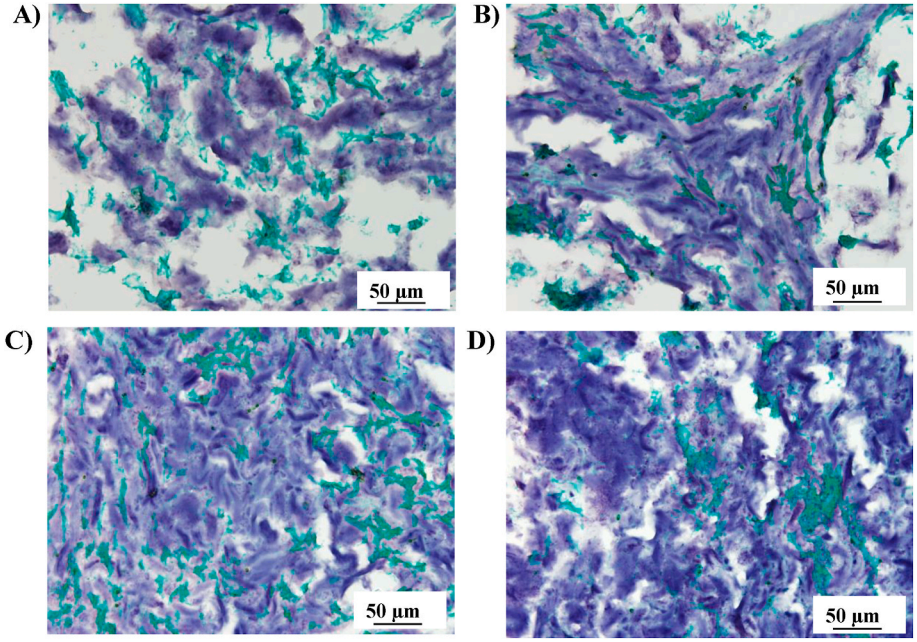

Fig. 7. Micrographs recorded at 20x magnification of the different *in vitro* bread boluses after simulating *in vitro* oral digestion. Protein appears green (stained with Light Green) and starch appears purple (stained with Lugol). A) NM: normal mastication; B) DfM: deficient mastication in terms of force; C) DsM: deficient mastication in terms of saliva; D) DfsM: deficient mastication in terms of force and saliva. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

intestinal phases, in which several mechanical, chemical, and enzymatic actions occur. The digestion process starts in the mouth where the breakdown of foods into smaller particles by mastication and the formation of swallowable boluses by saliva incorporation take place (Peyron et al., 2018). Food fragmentation favours transportation of the boluses to the stomach and also extends their surface area, promoting the efficiency of digestion and the release of aroma and taste compounds (Chen, 2015). Furthermore, saliva allows particles to be compacted, as well as enzymatic breakdown, destabilisation of colloidal complexes, generation of aggregates and precipitates, and merging of aroma compounds (Mosca & Chen, 2017). The most important salivary enzyme is α-amylase, which initiates the starch hydrolysis (Freitas et al., 2018; Mosca & Chen, 2017).

Oral deficiencies are involved in the decline of masticatory function,

particularly the decrease of muscular force, reduced motility and agility of oral components, and reduced salivary content (Peyron et al., 2018). In the present study, different oral impairments were simulated using the AM^2 masticator, which was designed to produce fractures in food matrices similar to those observed during *in vivo* food bolus formation (Peyron & Woda, 2016). The *in vitro* bread boluses recovered after mimicking the oral deficiencies showed greater proportions of large particles compared to the *in vitro* bread boluses obtained by normal mastication. These boluses are obtained as a consequence of deficient mastication and are frequently noted in elderly people with impaired dental status and oral functions (Peyron et al., 2021). Similar results with bread were observed by Vanhatalo et al. (2022), who performed a mastication trial involving 26 normal-weight adults. Moreover, the proportion of large particles significantly increased when simulating saliva reduction together with loss of force, which lowers the exchanges between bread boluses and saliva and impedes or delays the initiation of oral digestion of starch (Alam et al., 2019; Blanquet-Diot et al., 2021; Bornhorst, Kostlan, & Singh, 2013). In this sense, Freitas et al. (2018) reported that salivary α-amylase released about 80% of the starch in bread before its inactivation by low gastric pH, the starch being hydrolysed into oligosaccharides. It is important to mention that this percentage was reached within the first 20 min of gastric digestion. In the present study, the kinetics of starch and oligosaccharides release in the gastric compartment was not determined, as well as the buffering capacity of bread. It refers to the resistance of a solution to pH changes by adding an acid or a base and is influenced by bolus particle sizes. Mennah-Govela, Singh, and Bornhorst (2019) elucidated that large cube gels of a protein-based model system had lower buffering capacity compared to dispersions. Additionally, Sicard, Mirade, Portanguen, Clerjon, and Kondjoyan (2018) pointed out that the bolus particle size and the buffering capacity of meat played a key role in protein digestibility. Despite some works suggested the importance of implementing the known buffering capacity of meals during *in vitro* gastric digestion tests, further studies are needed to obtain a standardised method to experimentally determine this capacity in a wide variety of foods.

Following with the starch hydrolysis of samples, the results showed that after 120 min (end of the gastric phase), there was more maltose and less D-glucose when deficient masticatory conditions combining saliva and force alterations were mimicked. This outcome confirmed a delay in starch digestion. However, greater D-glucose contents were obtained at the end of the intestinal phase due to the action of pancreatic α-amylase, which favours the amylolytic process (Freitas et al., 2018), being the levels of D-glucose lower when simulating saliva deficiency along with loss of force. In this regard, it is important to remark that an increase in blood glucose concentrations is associated with slower gastric emptying, contributing to fullness and short-term satiety (Azlan et al., 2022). Thus, cumulative oral deficiencies could also favour the prevalence or development of metabolic syndromes, obesity, and cardiovascular diseases, which are linked to gastric emptying, fullness, and satiety.

In addition to the data on the protein digestibility of samples, the results obtained also demonstrate that lack of food fragmentation during deficient mastication plays an important role in the release of peptides and free amino acids during the gastrointestinal digestion in the elderly. It is well-known that higher particle sizes minimise the protein surface exposure, thus hindering the access of enzymes to cleavage sites (Paz-Yépez, Peinado, Heredia, & Andrés, 2019). Therefore, the gastric disintegration of the bread boluses depends on the mechanical status in which they arrive into the stomach (Peyron et al., 2021), affecting the gastric digestive events by retarding the release of peptides and free amino acids during digestion. Peyron et al. (2021) showed that the cumulative effect of oral decline and the digestive conditions in the elderly people significantly reduces the levels of meat peptides available in the gastric compartment. Additionally, Ranawana, Clegg, Shafat, and Henry (2011) demonstrated that smaller particles sizes significantly decrease the duration of gastric emptying, increase the glucose release, and improve insulin responses.

Lastly, the hydrolysis of macronutrients is concluded in the intestinal phase, while non-digestible food constituents can be fermented in the large intestine by bacterial microflora. In the present work, lower peptides and free amino acids contents were noted at the end of the intestinal phase in the case of poorly fragmented bread boluses compared to normal mastication boluses, indicating once again the effect of mastication on the protein digestibility of samples, this effect being linked to starch digestibility and glucose release. Freitas and Le Feunteun (2019) observed that, in the intestinal phase, pancreatic α-amylase moves the amylolytic procedure forward and profits from the previous (pepsin) and simultaneous (pancreatic proteases) hydrolysis of the protein network, which enhances starch accessibility. Furthermore, higher contents of non-digested nitrogen were detected at the end of gastrointestinal digestion in those samples simulating deficient mastication, which could possibly be fermented by the bacterial microflora of the large intestine as previously indicated. Diverse studies have suggested that products from microbial protein fermentation are harmful for health and are associated to multiple disorders as cancer, diabetes, and obesity (Rodríguez-Romero et al., 2022; Zhao et al., 2018).

4.2. Impact of mastication on the structural changes of nutrients of bread

Mastication turns the food products into a lubricated and cohesive swallowable bolus by several complex mechanical and chemical transformations. During mastication, food products are mechanically disintegrated to smaller fragments and enzymatically disintegrated by digestive enzymes through digestion (Panouillé, Saint-Eve, Déléris, Le Bleis, & Souchon, 2014; Stokes, Boehm, & Baier, 2013).

The food matrix macrostructure and microstructure and the mechanics of its oral disruption are considered the principal factors favouring or impeding the exchanges with the different salivary enzymes, the release of nutrients during mastication, and the successive digestive processes (Al-Rabadi, Gilbert, & Gidley, 2009; Blanquet-Diot et al., 2021; Bornhorst & Singh, 2014). Furthermore, dense structures, tortuous protein food matrices, starch entrapment, and starch- or enzyme-protein interactions have also been elucidated such as possible limiting factors during oral and gastrointestinal digestion (Zou, Sissons, Gidley, Gilbert, & Warren, 2015).

Freshly made bread has a sponge crumb, which consists of a continuous phase containing a gluten network and leached starch molecules, and a discontinuous phase of partially gelatinised starch granules (Gray & Bemiller, 2003). The secondary structure of the gluten fraction defines the bread's properties and can be determined by FTIR analysis. No changes in the secondary structure of gluten were observed, because of oral impairments and the β-sheet conformation was the most abundant in all the samples after *in vitro* oral and gastrointestinal digestion. Therefore, the decreased protein digestibility of poorly fragmented boluses could not be explained by the secondary structure. Conversely, Carbonaro, Maselli, and Nucara (2012) revealed that the protein digestibility of cereals was negatively correlated with the fraction of protein aggregates plus β-sheets in the secondary structure. Furthermore, Alvarez-Ramirez et al. (2018) pointed out that the β-turn fraction, a configuration offering less conformational obstruction to the action of enzymes, changed depending on the part of the bread digested (crumb or crust), which could be connected with food structure and particle size.

The starch-related variations that occurred after mastication were confirmed by IR analysis conducted with a FTIR microscope. In this sense, a greater vibrational band corresponding to the C–O vibration of the glycosidic units was noted in those boluses where saliva deficiency was simulated, this being more pronounced in the case of the cumulative effect (loss of force and saliva deficiency). As previously indicated, lower exchanges between the bread boluses and saliva could impede or delay the initiation of oral digestion of starch (Alam et al., 2019; Blanquet-Diot et al., 2021; Bornhorst et al., 2013). It is also important to remark that

salivary α-amylase is crucial in the starch hydrolysis of samples as it attacks the α-1,4 glycosidic bonds. When interacting with starch, α-amylase employs a multiple attack mechanism wherein during one enzyme-substrate complex formation, several α-1,4 glycosidic bonds are cleaved successively after the first attack (Sharma, Pico, Martinez, & Duizer, 2020). This cleavage results in the production of a series of branched oligosaccharides and obtention of sugar molecules, including dextrin, maltose, and glucose (Zhang, Chen, & Chen, 2022). However, a spatial rearrangement can occur that limits the access of salivary α-amylase to starch. In a study based on steamed rice bread, Tang et al. (2021) showed that the addition of okara changed the multiscale structure of rice bread. As a result, the sites of action of salivary α-amylase were effectively shielded, thus restricting the α-amylase-s-tarch interactions.

Finally, to further elucidate the impact of mastication on bolus structure, microscopy images of the *in vitro* bread boluses recovered after simulating *in vitro* oral digestion were performed. After mastication, all the samples were disintegrated and the protein-starch network appeared to be compacted, leading to the formation of bread boluses consisting of starch-protein aggregates. Similarly, Johansson, Vázquez Gutiérrez, Landberg, Alminger, and Langton (2018) noted that during mastication, the protein-starch matrix of wheat bread appeared to be compacted, forming a bolus containing aggregates of starch granules and proteins. Nevertheless, the *in vitro* bread boluses obtained after mimicking deficient mastication showed larger and more compact particles than normal masticated bread boluses. The quantity of salivary α-amylase in a food bolus is crucial for the overall breakdown of starch in starch-based products as breads (Bornhorst & Singh, 2012; Vanhatalo et al., 2022). The larger particles noted in poorly fragmented boluses might impair the access of salivary α-amylase to starch granules, impeding or delaying oral digestion of starch and D-glucose release. Besides, the large differences in particle size could influence the gastric emptying rates of foods and also influence the rate of starch and protein digestion in the duodenum (Bornhorst et al., 2013; Bornhorst & Singh, 2014). That is, the whole digestive procedure would be slowed down, delaying D-glucose release and protein digestion owing to the limited contact of enzymes with bolus particles (Peyron et al., 2018). Therefore, oral deficiencies are unfavourable in the digestion of food and nutrient metabolism, and better oral health care is needed for good nutritional management in those populations with masticatory impairments and oral disabilities.

5. Conclusions

This work demonstrates that oral impairments limit, to some extent, the nutrient bioaccessibility of bread in the elderly, thereby possible altering their nutritional status. The degree of food fragmentation and salivation during mastication plays an important role in protein digestion and starch hydrolysis. In addition, reduced digestibility exposes the elderly to more protein escaping the upper part of the digestive tract and being fermented in the colon. Furthermore, increasing knowledge of food oral processing and digestion in the elderly will provide help in designing novel food products, as well as usual foods like bread to cover the needs of this specific population, especially in terms of texture.

Author statement

Conceptualisation: S.R.,V.S.-L., and M.A.P.; Methodology: S.R., M.G., L.A., and A.V.; Formal Analysis: S.R., V.S.-L., and M.A.P.; Investigation: S.R., V.S.-L., and M.A.P.; Writing – Original Draft Preparation: S.R.; Writing – Review & Editing: S.R., M.G., L.A., P.T., A.V., V.S.-L., and M.A.P.; Visualisation: S.R., V.S.-L., and M.A.P.; Supervision: S.R., V.S.-L., and M.A.P.; Project Administration: V.S.-L. and M.A.P.; Funding Acquisition: V.S.-L. and M.A.P.

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.

Data availability

The authors do not have permission to share data.

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