Role of the bolus degree of structure on the protein digestibility during in vitro digestion of a pea protein fortified sponge-cake chewed by elderly

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Main Title: Role of the bolus degree of structure on the protein digestibility during *in vitro* digestion of a pea protein fortified sponge-cake chewed by elderly

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Abstract

This study investigated the digestibility of proteins in a pea protein fortified sponge-cake, as well as the impact of the degree of structure of the bolus produced by elderly subjects on the digestibility of proteins by combining e*x vivo* and *in vitro* approaches *via* the standardized protocol INFOGEST. The sponge-cakes were consumed by a group of 20 elderly subjects with contrasting physiology, their boli were recovered just before swallowing, and their apparent viscosity was measured to delineate the bolus degree of structure. According to this criterion, two pools were formed with boli from subjects selected at the extremes: low viscosity (LV) and high viscosity (HV), with apparent viscosity values (at $120s^{-1}$) of 124 ± 18 Pa·s and 208 ± 19 Pa·s, respectively. The sponge-cakes and the two pools underwent *in vitro* digestion. Protein hydrolysis kinetics was followed by measuring the released primary amino groups (NH_2) and by SDS-PAGE at different time points. For all samples, the representative bands of pea proteins disappear gradually during digestion, accompanied by the appearance of bands indicating the presence of proteins with M_W< 15kDa. In addition, the NH_2 concentrations increase over time and do not differ between sponge-cake and pea protein isolate. Moreover, the degree of structure of the food bolus has no significant effect on the concentration of NH_2 released. These results showed that pea proteins in a fortified

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sponge-cake are bioaccessible under standardized conditions, and that the degree of structure of the bolus did not influence protein digestibility for these foods.

Nomenclature

Average number of peptide bonds hydrolyzed (n)

Degree of hydrolysis (DH)

Difference in absorbance between hydrolyzed and unhydrolyzed sample at 340 nm (ΔAbs_{340})

Dilution factor (d)

Fortified sponge-cake (FSC)

High viscosity (HV)

Low viscosity (LV)

Median particle diameter (D_{50})

Molar extinction coefficient (ε)

Molecular weight (M_W)

O-phthaldialdehyde (OPA)

Posterior Functional Unit (PFU)

Pea protein isolates (PP)

Protein concentration in the sample (c)

Student-Neuman-Keuls test (SNK)

Time points after 15 min, 30 min, 60 min and 120 min of gastric *in vitro* digestion (t_{15}, t_{30}, t_{60}', t_{120} ,respectively.

Time point after 120 minutes of *in vitro* gastric digestion and 120 minutes of *in vitro* intestinal digestion ($t_{120'g+120'i}$)

Time point before *in vitro* digestion starts (t_0)

Total number of peptide bonds per protein molecule (N)

Water content (WC)

1. Introduction

While cereal proteins are very low in lysine and rich in methionine, those of legumes and pulses are deficient in methionine and high in lysine (Young & Pellett, 1994). Thanks to this complimentary aminoacid profile, the fortification of cereal foods with protein from pulses, such as peas, has become a promising way to meet the nutritional needs of the growing and ageing population.

The elderly, in particular, have higher protein needs than younger adults (Bauer et al., 2013; Deutz et al., 2014), and their oral physiology is commonly impaired (Peyron, Woda, Bourdiol, & Hennequin, 2017; Vandenberghe-Descamps et al., 2016). In cereal foods, these changes impact the food bolus properties during chewing, mainly the viscosity (Assad-Bustillos, Tournier, Septier, Della Valle, & Feron, 2019); which can be considered as a good indicator of the bolus degree of structure. The latter may affect, in turn, the protein digestibility and bioavailability (Rémond et al., 2007).

Briefly, the digestive process consists in transforming food into its basic components (aminoacids, free fatty acids, and monosaccharides) for their absorption in the intestine. Digestibility is defined as the quantity of a compound that is released from its matrix and becomes available for absorption in the gastrointestinal tract. Once this compound has been released, the proportion that is absorbed and actually metabolized represents its bioavailability (Galanakis, 2017).

Dangin, Boirie, Guillet, & Beaufrère (2002) have shown that the hydrolysis kinetics of proteins can influence their metabolism and bioavailability. They have proposed the concept of “fast” and “slow” proteins showing that, for young subjects, slowly digested proteins are more efficient than rapidly digested ones to improve postprandial protein gain. Conversely, a “fast” protein may be more beneficial than a “slow” one in elderly subjects, to limit body protein loss (Dangin et al., 2002), and overcome anabolic resistance (Dardevet et al., 2012). Such concept, analogous to the glycemic index for glucose and starchy foods, seems to be closely related to the protein structure and to its interaction with other components within the

food matrix. For this reason, any factor leading to protein structure modification, such as thermal processing, pH variations, or the interaction with other molecules during processing, can have an impact on the proteolysis kinetics.

The enzymatic hydrolysis and the digestibility of pea protein isolates have been previously investigated (Barać et al., 2011; Overduin, Guérin-Deremaux, Wils, & Lambers, 2015). However, the highly cross-linked structure of soft cereal foods that is formed during baking, involving different protein sources like wheat and egg (Wilderjans, Luyts, Goesaert, Brijs, & Delcour, 2010), generates concern about the digestibility of the added pea proteins for fortification purposes. Moreover, the solubility of pea proteins is limited near neutral pH values (Boye, Zare, & Pletch, 2010), commonly found in sponge-cake batters (Gularte, Gómez, & Rosell, 2012; Zhang et al., 2012). Unfortunately, the literature on this subject is lacking and to date there are very few studies that have investigated the *in vitro* enzymatic hydrolysis of pea proteins within a food matrix. One of them was carried out by Laguna, Picouet, Guàrdia, Renard, & Sarkar (2017) in fortified fruit purees; and, to the best of our knowledge, the only similar study that has been reported in cereal foods concerns gluten-free cakes (Gularte et al., 2012). Even if the principal focus of this study was starch hydrolysis, the authors also compared the protein digestibility, assessed as the pH decline, of cakes prepared with different pulse flours during *in vitro* enzymatic hydrolysis. The results showed that all of the studied pulse flours, including pea, showed high *in vitro* digestibility, as reflected by their rapid decline in pH, with the exception of bean. These results are encouraging; however, they must be confirmed in regular wheat containing cakes and by using assessment methods which are more representative of physiological conditions.

In this regard, it is known that the best way to study protein metabolism is to perform *in vivo* studies in either humans or animals (Shani-Levi et al., 2017); but given their high cost and ethical restrictions, an alternative approach is to assess digestibility and protein hydrolysis kinetics by *in vitro* digestion. These methods are rapid, less expensive, and less labor extensive and they allow the screening of various foods under standardized conditions. For these reasons, they are useful for detecting processing-induced changes in protein quality

and to investigate the effects of food composition and structure on digestion (Bohn et al., 2018). To this extent, an international consensus for a harmonized static protocol based on physiologically relevant conditions in humans has been recently achieved during the INFOGEST cost action (Minekus et al., 2014). Recently, Gao et al. (2019) used this protocol to study the influence of the degree of breakdown of artificial and real bread boli produced *ex vivo* from human mastication. They found that more disintegrated boli had a higher starch hydrolysis rate. These results suggest that the oral processing phase, which has been commonly overlooked in digestibility studies, may also have an influence in the hydrolysis kinetics of other nutrients, such as proteins. To date, there are no records of published studies that have investigated the effect of bolus degree of structure on protein digestibility. Recently we have studied in detail the oral processing of sponge-cake by elderly, and characterized precisely the state of the bolus by determining its water content, particle size distribution, and apparent viscosity at different stages of chewing (Assad-Bustillos et al, 2019a,b). From these results, it appeared that bolus apparent viscosity could be considered as a good indicator of the degree of structure of the bolus. Then we confirmed the relevance of these methods by studying the oral processing of sponge-cake enriched in proteins (Assad-Bustillos et al, 2019c).

In this context, the objectives of this paper were: i) to evaluate the digestibility of pea proteins in fortified sponge-cake via the *in vitro* digestion protocol INFOGEST; ii) to determine the impact of the degree of structure of the bolus, determined from its apparent viscosity, collected from healthy elderly subjects with contrasting physiology, on the digestibility of pea proteins, by using a combination of *ex vivo* and *in vitro* approaches.

2. Material and methods

2.1 Products and pea protein source

Pea protein fortified sponge-cakes (FSC) featuring the nutritional claim “High in protein” were provided by CERELAB® (France), as well as the pea protein isolates (PP) used for the fortification (NUTRALYS BF, Roquette, France). Their composition is detailed in Table 1.

[Table 1 about here]

2.2 Bolus collection and pooling

Mouthfuls of 20 cm^3 (≈5 g) of sponge-cake were consumed by 20 subjects aged 75 ± 5 years old with contrasting physiology: 10 (5F, 4M age = 72 ± 5) with a satisfactory dental status (DS), defined by the number of Posterior Functional Units (PFU’s≥7); and 10 (6F, 5M, age = 77 ± 4) with poor dental status (PFU’s ≤ 4) (Leake, Hawkins, & Locker, 1994).The participants were asked to expectorate the boli just before swallowing. Bolus apparent viscosity was measured by capillary rheometry, according to the procedure described by Assad-Bustillos, Tournier, Septier, et al. (2019b). Whereas bolus particle size at swallowing did not change significantly between subjects, whatever their dental status, apparent viscosity varied a lot and was therefore considered as an indicator of the degree of structure of the bolus (see also Appendix, Table A). Among the 20 subjects, the 4 ones whose boluses had the lower viscosity (η ($120s^{-1}$) ≈125 Pa.s) and the 4 subjects whose boluses led to the higher viscosity ((η ($120s^{-1}$) ≈208 Pa.s) were selected (Table 2). Since capillary rheometry is destructive, these subjects were asked to produce another set of boli by repeating the procedure. This second set of boli was frozen in liquid nitrogen right after their collection, and stored at -80°C until the day of analysis. The day of the experiment, these 2 x 4 boli were used to form two pools based on their degree of structure: low (LV) and high (HV) viscosity (Table 2). Pools were formed principally for two reasons: i) *in vitro* digestions require a large amount of bolus; ii) a better representativity is sought by including as many subjects as possible in each pool. The pools were formed by gently mixing the boli with a spatula, after thawing in a chamber at 4°C, in order to preserve their original structure. The water content (WC) of the pools, shown in Table 2, was determined by desiccation in an

oven at 130 °C during 24 h. The measurement was performed in triplicate. The study was approved by the local ethical committee (CPP N° IRB 2016-A00916-45).

[Table 2 about here]

2.3 In vitro digestion protocol

Fortified sponge-cakes (FSC) and bolus pools of high and low viscosities (HV and LV) underwent *in vitro* digestion by following the static standardized INFOGEST protocol (Minekus et al., 2014), starting at the oral phase in the case of sponge-cakes, and directly at the gastric phase in the case of bolus pools. The principal steps followed are summarized in Figure 1. In order to establish the proteolysis kinetics, one digestion was carried out per desired time of analysis (four time points during the gastric phase=$t_{15'}$, $t_{30'}$, $t_{60'}$ and $t_{120'}$; and one at the end of the intestinal phase= $t_{120'g+120'i}$), for a total of 5 simultaneous independent digestions per sample. All digestions were carried out by triplicate, including pea protein isolates (PP) as a positive control. A negative control was performed in order to verify that no hydrolysis took place in the absence of enzymes. The compositions of the simulated digestive fluids used are detailed in the original publication (Minekus et al., 2014). Only analytical-grade reagents and Milli-Q purified water were used for the preparation of solutions. All volumes were adjusted by using semi-automatic calibrated pipettes. Previous to the beginning of digestion, FSC was ground with a commercial food processor (Compact 3200, Magimix, France). The grinding time (5 min) was set in order to obtain a fine granulometry, close to that of real boli (median particle size D_{50}≈0.30, Table 2). The D_{50} of the grounded FSC was 0.44 ± 0.03 mm, and was measured by image analysis as described by Assad-Bustillos, Tournier, Feron, et al. (2019).

[Figure 1 about here]

2.3.1 Oral phase

Samples of ≈1 g of FSC or ≈0.2 g of PP were weighed in 15 mL Falcon tubes and 1 mL of simulated salivary fluid (SSF, pH=7) was added in order to respect the (1:1) proportions of the protocol, for a total volume of ≈2 mL. This quantity of PP led to similar overall protein content in the tube as for FSC. The water content of the artificial boli was determined as described for bolus pools and was found to be 72 ± 2 %, close to the average value reached for sponge cake boli at swallowing point (Assad-Bustillos et al., 2019b) . To begin the oral phase, human salivary α-amylase (Type IX-A, Sigma-Aldrich, USA) was added to achieve a concentration of 75 U/mL per tube. The tubes were held at 37° C for 2 minutes inside a shaking incubator (TH15, Edmund Bühler GmbH, Germany). To continue the gastric phase, the pH of the samples was lowered to 3, as described next.

2.3.2 Gastric phase

In samples issued from the oral phase (FSC and PP), the volume was doubled by adding 2 mL of simulated gastric fluid (SGF, pH=3), and 4N HCl, when necessary. Bolus pools (HV and LV), which directly integrated the gastric phase, were weighed at ≈1g of equivalent protein mass, taking into account their water content, in 15 mL Falcon tubes. Their pH was also adjusted to 3 by adding 2 mL of SGF and 4N HCl; as a result, their final volume (≈3 mL) was inferior to that of the samples issued from the oral phase (≈4 mL), but it was not adjusted, in order to preserve their initial structure. Pepsin from porcine gastric mucosa (Sigma-Aldrich, USA) was added to reach a concentration of 2000 U/mL in each tube. The tubes were kept at 37° C in the shaking incubator for 2 h. To stop the enzymatic reaction, pH was adjusted to 7 to pursue the intestinal phase.

2.3.3 Intestinal phase

For all samples issued from the gastric phase, simulated intestinal fluid (SIF, pH=7) was added in order to double their volume (4 mL for FSC and PP; 3 mL for bolus pools). When necessary, 2N NaOH was used to achieve the pH adjustment. Pancreatin from porcine pancreas (8XUSP, Sigma-Aldrich, USA) was added to reach a trypsin concentration of 100

U/mL in each tube. The trypsin activity of the pancreatin used in this experiment was previously determined as recommended in the INFOGEST supplementary material, and was found to be 80 U/mg. The tubes were placed inside the shaking incubator during 2 h at 37 °C.

2.3.4 Complete stop of the reaction and preparation for further analysis

According to the desired times of analysis ($t_{15'}$, $t_{30'}$, $t_{60'}$, $t_{120'}$ and $t_{120'g+120'i}$), the reaction in the corresponding tubes was definitely stopped by adding 250 µL of a 20% (w/v) Sodium-Dodecyl-Sulfate (SDS) solution (Bio-Rad, USA) as well as 180 µL of 99% β-mercaptoethanol (Sigma-Aldrich, USA). After this, all tubes were placed in a boiling water bath for 15 minutes, and then were centrifuged for 15 minutes at 10000 g. The supernatants, from now on referred as digesta, were recovered and kept at 4°C until further analysis.

2.4 Proteolysis kinetics follow up

The proteolysis kinetics of the collected digesta was determined i) qualitatively, by following the evolution of protein bands in gels obtained with Sodium-Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970) and ii) quantitatively, by measuring the amount of free primary amino groups (NH_2) released over time *via* the o-phthaldialdehyde (OPA) method (Church, Porter, Catignani, & Swaisgood, 1985).

2.4.1 Sodium-Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Previous to this analysis, gastric digesta were diluted (1:1) with Milli-Q purified water in order to reach the same final concentration as intestinal digesta. Following this, 1 mL of normalized digesta were mixed 1 mL of 2X Laemmli buffer sample (Bio-Rad, USA) in Eppendorf tubes. After being held in a boiling water bath during 10 minutes, 15 µL of each sample were carefully loaded in the wells of pre-cast polyacrylamide gradient gels (4-15% Mini-PROTEAN TGX 10 x 30 µL wells, Bio-Rad, USA). In each gel, one well was loaded with the unstained

molecular weight marker (14-116 kDa, Euromedex, France). The electrophoresis migration of the gels was carried out at 80 mA during 1h. Once the migration was completed, the gels were stained with Coomasie brilliant blue (G-250, Serva, Germany) prepared as described by Lawrence & Besir (2009), during 1 h, and then rinsed with distilled water. The gels were scanned using a flat-bed scanner (Image Scanner III, GE Healthcare, USA) and the images saved in TIFF format.

2.4.2 Release of primary amino groups (NH_2)

This method is based on the reaction of free amino primary groups (NH_2) with o-phthaldialdehyde (OPA) under reducing conditions to form an adduct that can be detected by spectrophotometry at 340 nm, and was first described by Church et al. (1985). The protocol used in this work was an adaptation from Bertrand-Harb, Nicolas, Dalgalarrondo, & Chobert (1993), as described next. The OPA reagent was prepared by weighing 40 mg of OPA (Sigma-Aldrich, USA) in a 50 mL Falcon tube containing 1 mL of pure methanol (Fisher Scientific, USA); the mix was vortexed until OPA was fully dissolved. Then, 25 mL of a 0.1 M $Na_2B_4O_7$ buffer (pH=9.3) were added, followed by 2.5 mL of a 10% (w/v) SDS solution (Bio-Rad, USA) and 100 µL of 99% β-mercaptoethanol (Sigma-Aldrich, USA). Finally, Milli-Q water was added to attain 50 mL (*q.s.).* The reagent was prepared as close as possible to the moment of use and covered with aluminum foil to protect it from light. Additionally, a standard curve of L-leucine (Sigma-Aldrich, USA) was prepared in a 50 mM Na_2HPO_4 buffer (pH = 7.8), with concentrations ranging from 0 to 4 mM. The OPA reaction was carried out in a 96 well non-binding microplate with flat bottom (Fisher Scientific, USA). Each well was loaded with 100 µL of OPA reagent with a multi-channel micropipette, followed by 10µL of the standard leucine solutions or the digesta, previously diluted by a factor of 10, so that their concentrations could be found within the standard curve range. The plate was covered, and the absorbance at 340 nm was read in a microplate spectrophotometer (Epoch, Biotek Instruments, USA) immediately after. A blank was made with the OPA reagent solution and distilled water. The reading was repeated twice.

2.5 Data treatment and statistical analysis

The degree of hydrolysis (DH) of the samples was estimated according to the method described by Spellman, McEvoy, O'Cuinn, & FitzGerald (2003), by following the relations:

$$DH\ (\%) = 100 \times \frac{n}{N} \quad (1)$$

$$n = \frac{\Delta Abs_{340} \times MW \times d}{\varepsilon c} \quad (2)$$

Where:

n is the average number of peptide bonds hydrolyzed (defined in 2);

N is the total number of peptide bonds per protein molecule;

ΔAbs_{340} is the difference between the absorbance of the sample and the absorbance of the unhydrolyzed sample at 340 nm;

M_W is the molecular mass of the test protein (Da);

d is the dilution factor;

ε is the molar extinction coefficient at 340 nm;

and c is the protein concentration ($g \cdot L^{-1}$),

We assumed that $M_W \approx 54000$ Da, based on an estimation that considered the average ratio of vicilin/legumin to be 3.15 in pea proteins from different genotypes (Tzitzikas, Vincken, de Groot, Gruppen, & Visser, 2006); and N≈461 based on the length sequences of these proteins obtained from the Uniprot database (https://www.uniprot.org/). The ε coefficient is estimated to be 6000 $mol^{-1} \cdot cm^{-1}$ for a wide variety of aminoacids, according to Church et al. (1985). The protein concentration was estimated based on the proportion of PP (at 80% purity) contained in the unhydrolyzed sample (either the fortified sponge-cake or the isolates).

For statistical treatment, repeated measures Analyses of Variance (r-ANOVA) were carried out to investigate the differences in protein digestibility, represented by the amount of primary

amino groups (NH_2) and the Degree of Hydrolysis (DH) at each time point (repeated factor), between fortified sponge-cake and the pea protein isolates alone to determine the effect of the food matrix. The effect of the degree of structure of the bolus was also investigated by comparing the released NH_2 of high vs. low bolus viscosity pools at each time point (repeated factor). A one-way ANOVA was also performed to investigate the differences in the final degree of hydrolysis (DH) between all the samples. When significant effects were found, Student-Newman-Keuls (SNK) test was applied for post-hoc comparisons. For all treatments, a significance level of $\alpha=0.05$ was used, and the analyses were performed with the XLSTAT software (v.2016 18.06, Addinsoft, USA).

3. Results and Discussion

3.1 Qualitative assessment of proteolysis kinetics

The pea protein isolates (PP) gel pattern (Fig. 2 A) shows at t=0 the representative bands of the three main globulins present in pea proteins: legumin (MW ≈20 and ≈40 kDa), vicilin (MW ≈28-47 kDa), and convicilin (MW 70≈80 kDa) (Barać et al., 2011; Tzitzikas et al., 2006). These bands disappear gradually as digestion progresses, accompanied by the increasing appearance of bands with low molecular weight (MW <15 kDa). This indicates the proteins are well hydrolyzed into peptides. It can be seen that most of the vicilin and legumin bands disappear since the first 15 minutes of the gastric digestion. Conversely, the convicilin bands seem to resist gastric digestion and disappear only after the intestinal phase. This kind of resistance could be explained by the low hydrophobicity of pea globulins at their native state (Gueguen, 1989), which makes them inaccessible to pepsin. The same trend has been encountered by (Laguna et al., 2017), who observed persistence of vicilin and convicilin bands during the gastric phase for more than 120 minutes in pea protein fortified fruit puree. The fortified sponge-cake (FSC) gel pattern (Fig. 2 B) also shows the characteristic pea protein bands at t=0. As expected, this gel also contains new bands that were not visible in the PP gels that correspond to the wheat and egg protein fractions. For example, the band at

≈116 kDa could represent high molecular weight glutenin subunits, since the reducing conditions under which protein extraction was carried out (by adding β-mercaptoehtanol) allow this fraction to be visible in the gels by removing disulfide bonds (Shewry, Tatham, Forde, Kreis, & Miflin, 1986). Contrary to what is observed for PP, vicilin bands seem to resist the gastric digestion and disappear only at the end of the intestinal phase. The persistence of vicilin bands after heat treatment had been previously reported (Laguna et al., 2017), but they disappeared almost instantaneously after applying a high pressure treatment, causing the denaturation of the proteins which resisted thermal treatment. In fact, it is generally agreed that protein denaturation improves the enzymatic hydrolysis of aminoacids (Fennema, 2017). This is due to the binding of the polypeptide chain to the specific stereochemistry of the protease active site, which occurs more easily when the protein is at the unfolded state (Fontana, Polverino de Laureto, De Filippis, Scaramella, & Zambonin, 1997). On the other hand, it is known that a prolonged exposure to heat and / or to high temperatures can promote the formation of large protein aggregates that can actually be deleterious to digestibility (De Zorzi, Curioni, Simonato, Giannattasio, & Pasini, 2007; Habiba, 2002). In our case, we could hypothesize that this resistance to gastric digestion could be due to the presence of pea protein aggregates formed during baking, where cakes undergo temperatures of 200°C at the surface and 100°C at the core (Sommier, Dumoulin, Douiri, & Chipeaux, 2012). This process leads to the thermal denaturation of the pea globulins and the concomitant generation of high molecular weight (MW>700 kDa) aggregates that may not be accessible by enzymes (Mession, Sok, Assifaoui, & Saurel, 2013). Another possibility is a “matrix” effect, which may hinder the accessibility to pea proteins. This hypothesis states that the highly cross-linked protein network formed by the wheat and egg proteins during baking (Dewaest et al., 2017; Wilderjans, Luyts, Brijs, & Delcour, 2013) could prevent enzymes from accessing pea proteins, and thus explaining the observed delay in proteolysis.

[Figure 2 about here]

Contrary to PP and FSC which contain artificial saliva, gel patterns from the bolus pools (Fig. 2 C and D) show a more streaked appearance at t=0; this is likely due to the presence of a large variety of proteins contained in real human saliva (Humphrey & Williamson, 2001). Despite this, the principal pea protein bands can still be recognized, and these tend to disappear as the digestion progresses, accompanied by an increase of low MW fractions. Like previously encountered in FSC, it seems that the vicilin bands (≈45 kDa) resist the gastric digestion, and paradoxically appear stronger at t_{120} than t_{60}. This may be due to an increase of solubility of the pea proteins as digestion progresses. Nevertheless, these bands disappear after the intestinal phase. Finally, these gel patterns suggest there is little difference between bolus pools with low (Fig. 2 C) and (Fig. 2 D) high viscosity; however this can only be confirmed by a quantitative analysis. For this reason, the quantification of the release of primary amino groups in the digesta was carried out and will be discussed in the following section.

3.2 Quantitative assessment of proteolysis kinetics

The proteolysis of pea protein isolates (PP) and fortified sponge-cake (FSC), observed for from the SDS-PAGE gel patterns, is confirmed by the increasing concentration of primary amino group (NH_2) over time (Figure 3 A). Significant ($p<0.05$) differences between PP and fortified FSC are found at t_0, $t_{15'}$, $t_{30'}$ and $t_{60'}$, where the NH_2 concentration is higher for PP (Further detail of the repeated measures ANOVA parameters obtained is given in the *Appendix*, tables B and C), except at t_0. This is coherent with what is observed in the gels, where protein bands disappear first for PP in comparison to FSC during the gastric phase. However, at the end of the gastric phase, the NH_2 concentration is no longer different between the two samples, indicating that the same level of proteolysis is achieved in both cases. Moreover, no significant differences were observed at the end of the intestinal phase. This also means that pancreatin is more effective in the hydrolysis of proteins than pepsin. Similar results were obtained by Pasini, Simonato, Giannattasio, Peruffo, & Curioni (2001), who observed a decrease in the *in vitro* digestibility of wheat proteins of bread crumb when

compared to the unheated dough during the gastric phase (with pepsin); however, after the addition of pancreatic enzymes and by the end of intestinal digestion, the digestibility of bread crumb and dough turned out to be similar. As previously discussed, this is likely caused by the heavy cross-linking of the matrix, which delays the proteolysis during the early phases of gastric digestion. This effect is well illustrated by the degree of hydrolysis (DH) profile of both samples (Fig. 3 C). The DH gives information on the actual number of peptide bonds cleaved during the reaction, so it is a useful way of monitoring the extent of protein degradation. The final DH attained values are comparable to those obtained by Spellman et al. (2003) for whey protein isolates (17%) by using the same quantification method. Accordingly, Lorieau et al. (2018) reported DH values that range between 15 to 40% in cheese matrices with different structures formed by different processing conditions.

Regarding the bolus degree of structure, there are no differences in the NH_2 concentration between the low (LV) and high viscosity (HV) groups (Fig. 3 B). This is coherent with the SDS gel patterns, which show an increase of the low MW fractions, and confirmed by the almost identical DH profile (Fig. 3 D) of both samples. The only significant ($p=0.05$) difference between HV and LV is found at t120', where the NH_2 concentration of HV is slightly superior to LV. Moreover, a correlation analysis between DH and bolus properties (viscosity, WC and median particle size) was performed for each step of the digestion and no significant results were obtained whatever the correlation (see table E in the *appendix* section). The absence of difference between these two conditions is a promising result since it means that proteins in a fortified sponge-cake remain bioaccessible regardless of the degree of structure of the bolus produced by elderly with contrasting physiology. However, when interpreting our results, it is important to keep in mind that INFOGEST is a standardized protocol that is relevant in adult physiological conditions, and therefore does not take into account the changes in the gastro-intestinal tract that overcome with ageing. Some of these changes include a general decrease in enzyme secretion (except for α-amylase), a higher pH gradient, lower gastrointestinal contractions and slower transit passage rates (Rémond et al.,

2015; Shani-Levi et al., 2017). Further research is needed in order to confirm the results obtained in this study by taking into account these aspects.

[Figure 3 about here]

Finally, it can be seen that the DH of bolus pools reached at the end of digestion are not significantly different between them, but they are slightly superior (p=0.04) to the DH obtained by the FSC and PP samples (Fig. 4 D and Table D in appendix section). This could be attributed to the differences in the median particle size (D_{50}) between the real (≈0.30 mm) vs. artificial (≈0.45 mm) boli, as it is hypothesized a smaller particle size could enhance enzymatic hydrolysis analogous to what is seen for starch hydrolysis (Gao et al., 2019). However, in the same study, the starch digestion level was higher for the artificial as compared to the real boli, being attributed to the their differences in hydration level, which were ≈ 20% higher in artificial boli. For that reason, the hydration level (1:1) recommended in the INFOGEST protocol was judged as inappropriate since it does not represent real saliva hydration conditions. In our case, artificial boli had a ≈ 10% higher water content (WC) than real boli; yet, our results suggest that protein hydrolysis is not affected, or at least not enhanced, by a higher bolus hydration level. In a recent study, Freitas, Le Feunteun, Panouillé, & Souchon (2017) suggested that salivary α-amylase could play an important role in the digestion of proteins in bread due to their close interaction with starch within the food matrix. This was also suggested by Pentikäinen et al. (2014), who underlined the effect of salivary amylase on starch hydrolysis of chewed wheat and rye breads. Therefore, another explanation of the higher DH of proteins observed in real boli could rely in the activity of α-amylase, which could have been higher than in artificial boli. In order to clarify this, future studies that combine *ex vivo* boli and *in vitro* digestion approaches should include a control of this variable.

Conclusion

We have found that pea proteins in fortified sponge-cakes, holding the claim “high in protein”, are bioaccessible under standardized conditions. Also, in the same conditions, the degree of

structure of the bolus resulting from the mastication of elderly subjects has no influence on the digestibility of the proteins, at least within the range of viscosity studied, which corresponds to realistic orders of magnitude of a bolus produced by healthy elderly subjects with contrasting physiology. It was seen that is possible to combine *ex vivo* and *in vitro* methods, particularly the INFOGEST method, to assess protein digestibility and follow the kinetics of protein hydrolysis of cereal foods. As perspectives, it would be interesting to adapt the protocol to the physiological digestive conditions of the elderly and to evaluate in these conditions bolus structural changes during digestion including oral processing step. This approach will allow identifying the contribution in term of structural changes of each step of digestion (from the original food put in the mouth to intestinal step) in protein digestibility for this type of cereal food matrix.

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Ethical statement

Conflict of Interest: The authors declare that they do not have any conflict of interest.

Ethical Review: The study was approved by the local ethical committee (CPP N° IRB 2016-A00916-45) and the French National Agency of Drugs and Safety (ANSM).

Informed Consent: Written informed consent was obtained from all study participants.

Author Contributions

ABM, DVG and GF developed the study design, conducted data collection and analysis, and drafted the manuscript. PJ, RH and CY conducted experimental procedures;, collaborated in data analysis and provided manuscript revisions.

References

Assad-Bustillos, M., Tournier, C., Feron, G., Guessasma, S., Reguerre, A. L., & Della Valle, G. (2019a). Fragmentation of two soft cereal products during oral processing in the elderly: Impact of product properties and oral health status. *Food Hydrocolloids*, *91*(January), 153–165. https://doi.org/10.1016/j.foodhyd.2019.01.009

Assad-Bustillos, M., Tournier, C., Septier, C., Della Valle, G., & Feron, G. (2019b). Relationships of oral comfort perception and bolus properties in the elderly with salivary flow rate and oral health status for two soft cereal foods. *Food Research International*, *118*(September), 13–21. https://doi.org/10.1016/j.foodres.2017.11.057

Assad-Bustillos, M., Tournier, C., Septier, C., Della Valle, G., & Feron, G. (2019c). Oral processing and comfort perception of soft cereal foods fortified with pulse proteins in the elderly with different oral health status. *Submitted to Food & Function*

Barać, M., Čabrilo, S., Pešić, M., Stanojević, S., Pavlićević, M., Maćej, O., & Ristić, N. (2011). Functional Properties of Pea (Pisum sativum, L.) Protein Isolates Modified with Chymosin. *International Journal of Molecular Sciences*, *12*(12), 8372–8387. https://doi.org/10.3390/ijms12128372

Bauer, J., Biolo, G., Cederholm, T., Cesari, M., Cruz-Jentoft, A. J., Morley, J. E., … Boirie, Y. (2013). Evidence-Based Recommendations for Optimal Dietary Protein Intake in Older People: A Position Paper From the PROT-AGE Study Group. *Journal of the American Medical Directors Association*, *14*(8), 542–559. https://doi.org/10.1016/j.jamda.2013.05.021

Bertrand-Harb, C., Nicolas, M.-G., Dalgalarrondo, M., & Chobert, J. M. (1993). Determination of alkylation degree by three colorimetric methods and amino acid analysis: A comparative study. *Sciences Des Aliments*, *13*(3), 577–584.

Bohn, T., Carriere, F., Day, L., Deglaire, A., Egger, L., Freitas, D., … Dupont, D. (2018).

Correlation between in vitro and in vivo data on food digestion. What can we predict with static in vitro digestion models? *Critical Reviews in Food Science and Nutrition*, *58*(13), 2239–2261. https://doi.org/10.1080/10408398.2017.1315362

Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International*, *43*(2), 414–431. https://doi.org/10.1016/j.foodres.2009.09.003

Church, F. C., Porter, D. H., Catignani, G. L., & Swaisgood, H. E. (1985). An o-phthalaldehyde spectrophotometric assay for proteinases. *Analytical Biochemistry*, *146*(2), 343–348.

Dangin, M., Boirie, Y., Guillet, C., & Beaufrère, B. (2002). Influence of the Protein Digestion Rate on Protein Turnover in Young and Elderly Subjects. *The Journal of Nutrition*, *132*(10), 3228S-3233S. https://doi.org/10.1093/jn/131.10.3228S

Dardevet, D., Remond, D., Peyron, MA, Papet, I., Savary-Auzeloux, I., Mosoni, L. (2012). Muscle Wasting and Resistance of Muscle Anabolism: The "Anabolic Threshold Concept" for Adapted Nutritional Strategies during Sarcopenia. *Scientific World Journal.*, Article 269531. DOI: 10.1100/2012/269531

De Zorzi, M., Curioni, A., Simonato, B., Giannattasio, M., & Pasini, G. (2007). Effect of pasta drying temperature on gastrointestinal digestibility and allergenicity of durum wheat proteins. *Food Chemistry*, *104*(1), 353–363. https://doi.org/10.1016/J.FOODCHEM.2006.11.057

Deutz, N. E. P., Bauer, J. M., Barazzoni, R., Biolo, G., Boirie, Y., Bosy-Westphal, A., … Calder, P. C. (2014). Protein intake and exercise for optimal muscle function with aging: Recommendations from the ESPEN Expert Group. *Clinical Nutrition*, *33*(6), 929–936. https://doi.org/10.1016/J.CLNU.2014.04.007

Dewaest, M., Villemejane, C., Berland, S., Michon, C., Verel, A., & Morel, M.-H. (2017).

Changes in protein size distribution during wheat flour cake processing. *LWT - Food Science and Technology*, *79*, 333–341. https://doi.org/10.1016/j.lwt.2017.01.036

Fennema, O. (2017). *Fennema's Food Chemistry*. (S. Damodaran & K. L. Parkin, Eds.) (5th ed.). CRC Press.

Fontana, A., Polverino de Laureto, P., De Filippis, V., Scaramella, E., & Zambonin, M. (1997). Probing the partly folded states of proteins by limited proteolysis. *Folding and Design*, *2*(2), R17–R26. https://doi.org/10.1016/S1359-0278(97)00010-2

Freitas, D., Le Feunteun, S., Panouillé, M., & Souchon, I. (2017). The important role of salivary α-amylase in gastric digestion of wheat bread starch. *Food & Function*. https://doi.org/10.1039/C7FO01484H

Galanakis, C. (2017). What is the Difference Between Bioavailability Bioaccessibility and Bioactivity of Food Components? | SciTech Connect. Retrieved January 9, 2018, from http://scitechconnect.elsevier.com/bioavailability-bioaccessibility-bioactivity-food-components/

Gao, J., Lin, S., Jin, X., Wang, Y., Yin, J., Dong, Z., & Zhou, W. (2019). In vitro digestion of bread: How is it influenced by the bolus characteristics? *Journal of Texture Studies*. https://doi.org/10.1111/jtxs.12391

Gueguen, J. (1989). Relation between Conformation and Surface Hydrophobicity of Pea (Pisum sativum L.) Globulins. *Journal of Agricultural and Food Chemistry*, *37*(5), 1236–1241. https://doi.org/10.1021/jf00089a008

Gularte, M. A., Gómez, M., & Rosell, C. M. (2012). Impact of Legume Flours on Quality and In Vitro Digestibility of Starch and Protein from Gluten-Free Cakes. *Food and Bioprocess Technology*, *5*(8), 3142–3150. https://doi.org/10.1007/s11947-011-0642-3

Habiba, R. . (2002). Changes in anti-nutrients, protein solubility, digestibility, and HCl-extractability of ash and phosphorus in vegetable peas as affected by cooking methods.

Food Chemistry, *77*(2), 187–192. https://doi.org/10.1016/S0308-8146(01)00335-1

Humphrey, S. P., & Williamson, R. T. (2001). A review of saliva: Normal composition, flow, and function. *The Journal of Prosthetic Dentistry*, *85*(2), 162–169. https://doi.org/10.1067/mpr.2001.113778

Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, *227*(5259), 680–685. https://doi.org/10.1038/227680a0

Laguna, L., Picouet, P., Guàrdia, M. D., Renard, C. M. G. C., & Sarkar, A. (2017). In vitro gastrointestinal digestion of pea protein isolate as a function of pH, food matrices, autoclaving, high-pressure and re-heat treatments. *LWT - Food Science and Technology*, *84*, 511–519. https://doi.org/10.1016/j.lwt.2017.06.021

Lawrence, A.-M., & Besir, H. (2009). Staining of Proteins in Gels with Coomassie G-250 without Organic Solvent and Acetic Acid. *Journal of Visualized Experiments*, (30). https://doi.org/10.3791/1350

Leake, J. L., Hawkins, R., & Locker, D. (1994). Social and functional impact of reduced posterior dental units in older adults. *Journal of Oral Rehabilitation*, *21*(1), 1–10. https://doi.org/10.1111/j.1365-2842.1994.tb01119.x

Lorieau, L., Halabi, A., Ligneul, A., Hazart, E., Dupont, D., & Floury, J. (2018). Impact of the dairy product structure and protein nature on the proteolysis and amino acid bioaccessiblity during in vitro digestion. *Food Hydrocolloids*, *82*, 399–411. https://doi.org/10.1016/j.foodhyd.2018.04.019

Mession, J. L., Sok, N., Assifaoui, A., & Saurel, R. (2013). Thermal denaturation of pea globulins (Pisum sativum L.) - Molecular interactions leading to heat-induced protein aggregation. *Journal of Agricultural and Food Chemistry*, *61*(6), 1196–1204. https://doi.org/10.1021/jf303739n

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., … Brodkorb, A.

(2014). A standardised static in vitro digestion method suitable for food – an international consensus. *Food & Function*, *5*(6), 1113. https://doi.org/10.1039/c3fo60702j

Overduin, J., Guérin-Deremaux, L., Wils, D., & Lambers, T. T. (2015). NUTRALYS ® pea protein: characterization of in vitro gastric digestion and in vivo gastrointestinal peptide responses relevant to satiety. *Food & Nutrition Research*, *59*(1), 25622. https://doi.org/10.3402/fnr.v59.25622

Pasini, G., Simonato, B., Giannattasio, M., Peruffo, A. D. B., & Curioni, A. (2001). Modifications of Wheat Flour Proteins during in Vitro Digestion of Bread Dough, Crumb, and Crust: An Electrophoretic and Immunological Study. *Journal of Agricultural and Food Chemistry*, *49*(5), 2254–2261. https://doi.org/10.1021/jf0014260

Pentikäinen, S., Sozer, N., Närväinen, J., Ylätalo, S., Teppola, P., Jurvelin, J., … Poutanen, K. (2014). Effects of wheat and rye bread structure on mastication process and bolus properties. *Food Research International*, *66*, 356–364. https://doi.org/10.1016/j.foodres.2014.09.034

Peyron, M. A., Woda, A., Bourdiol, P., & Hennequin, M. (2017). Age-related changes in mastication. *Journal of Oral Rehabilitation*, *44*(4), 299–312. https://doi.org/10.1111/joor.12478

Rémond, D., Machebeuf, M., Yven, C., Buffière, C., Mioche, L., Mosoni, L., & Mirand, P. P. (2007). Postprandial whole-body protein metabolism after a meat meal is influenced by chewing efficiency in elderly subjects. *American Journal of Clinical Nutrition*, *85*(5), 1286–1292. https://doi.org/10.1093/ajcn/85.5.1286

Rémond, D., Shahar, D. R., Gille, D., Pinto, P., Kachal, J., Peyron, M.-A., … Vergères, G. (2015). Understanding the gastrointestinal tract of the elderly to develop dietary solutions that prevent malnutrition. *Oncotarget*, *6*(16), 13858–13898.

https://doi.org/10.18632/oncotarget.4030

Shani-Levi, C., Alvito, P., Andrés, A., Assunção, R., Barberá, R., Blanquet-Diot, S., … Lesmes, U. (2017). Extending in vitro digestion models to specific human populations: Perspectives, practical tools and bio-relevant information. *Trends in Food Science & Technology*, *60*, 52–63. https://doi.org/10.1016/j.tifs.2016.10.017

Shewry, P. R., Tatham, A. S., Forde, J., Kreis, M., & Miflin, B. J. (1986). The classification and nomenclature of wheat gluten proteins: A reassessment. *Journal of Cereal Science*, *4*(2), 97–106. https://doi.org/10.1016/S0733-5210(86)80012-1

Sommier, A., Dumoulin, E., Douiri, I., & Chipeaux, C. (2012). Characterization of sponge cake baking in an instrumented pilot oven. *International Journal of Food Studies*, *1*(October), 144–158. https://doi.org/10.7455/ijfs/1.2.2012.a5

Spellman, D., McEvoy, E., O'Cuinn, G., & FitzGerald, R. J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal*, *13*(6), 447–453. https://doi.org/10.1016/S0958-6946(03)00053-0

Tzitzikas, E. N., Vincken, J.-P., de Groot, J., Gruppen, H., & Visser, R. G. F. (2006). Genetic Variation in Pea Seed Globulin Composition. *Journal of Agricultural and Food Chemistry*, *54*(2), 425–433. https://doi.org/10.1021/jf0519008

Vandenberghe-Descamps, M., Labouré, H., Prot, A., Septier, C., Tournier, C., Feron, G., & Sulmont-Rossé, C. (2016). Salivary Flow Decreases in Healthy Elderly People Independently of Dental Status and Drug Intake. *Journal of Texture Studies*, *47*(4), 353–360. https://doi.org/10.1111/jtxs.12191

Wilderjans, E., Luyts, A., Brijs, K., & Delcour, J. A. (2013). Ingredient functionality in batter type cake making. *Trends in Food Science & Technology*, *30*(1), 6–15. https://doi.org/10.1016/j.tifs.2013.01.001

Wilderjans, E., Luyts, A., Goesaert, H., Brijs, K., & Delcour, J. A. (2010). A model approach to starch and protein functionality in a pound cake system. *Food Chemistry*, *120*(1), 44–51. https://doi.org/10.1016/J.FOODCHEM.2009.09.067

Young, V. R., & Pellett, P. L. (1994). Plant proteins in relation to human protein and amino acid nutrition. *The American Journal of Clinical Nutrition*, *59*(5), 1203S-1212S. https://doi.org/10.1093/ajcn/59.5.1203S

Zhang, Y.-Y., Song, Y., Hu, X.-S., Liao, X.-J., Ni, Y.-Y., & Li, Q.-H. (2012). Effects of sugars in batter formula and baking conditions on 5-hydroxymethylfurfural and furfural formation in sponge cake models. *Food Research International*, *49*(1), 439–445. https://doi.org/10.1016/J.FOODRES.2012.07.012

Tables

	Fortified sponge-cake	Pea protein isolates§
Energy (kcal/100 g)	257†	395
Proteins (g/100 g)	13 †	80
Fat (g/100 g)	5 †	6
Carbohydrates (g/100 g)	40†	3
Others (g/100 g)	0.5†	4
Water content (g/100 g)	30 ± 2‡	7

†Values were determined by a certified laboratory (Eurofins, France). ‡Values reported are the mean (n=5) ± standard deviation of measures performed in our laboratory. §Values reported by the manufacturer extracted from the technical sheet.

Table 1. Composition (wet basis) of the fortified sponge-cakes and the pea protein isolates used in their formulation.

Pool 1 Low viscosity (LV)						**Pool 2** High viscosity (HV)					
Subject	DS[1]	Stimulated Salivary Flow (mL/min)	Bolus viscosity at 120 s^{-1} (Pa.s)	Bolus median particle size (D_{50})	WC (%)	Subject	DS	Stimulated Salivary Flow (mL/min)	Bolus viscosity at 120 s^{-1} (Pa.s)	Bolus median particle size (D_{50})	WC (%)
S065	S	2.6	129	0.19	62.9	S006	S	1.3	211	0.28	47.8
S096	S	3.2	145	0.26	60.8	S003	S	1.5	191	0.29	49.3
S164	P	2.2	102	0.26	62.7	S121	P	0.9	196	0.25	50.4
S167	P	2.9	119	0.39	61.8	S017	P	1.5	234	0.29	49.7
		2.7 ± 0.4* **	**124 ± 18*** **	**0.24 ± 0.12**	**62.05±1*** **			**1.3 ± 0.4**	**208 ± 18**	**0.28 ± 0.02**	**49.3±1**

[1] DS (Dental Status), S=Satisfying, P=Poor

Table 2. Physical characteristics of the bolus pools and physiology of the subjects who originated them. Means ± SD are highlighted in bold. *** mean statistical difference (p<0,001) between LV and HV for the corresponding parameter

Figure Legends

Figure 1. Summary of the principal steps followed during the *in vitro* digestion based on the INFOGEST protocol for the different studied samples: Fortified sponge-cake (FSC), Pea protein isolates (PP), and bolus pools with High (HV) and low (LV) viscosities. SSF= Simulated salivary fluid; SGF= Simulated gastric fluid; SIF=Simulated intestinal fluid.

Figure 2. SDS-PAGE gels showing the protein gel patterns of the digesta at different times before (t0) and during the gastric (t15', t30', t60', t120') and intestinal (t120'g+120'i) phases. (A) Pea protein isolates; (B) Fortified Sponge-cake; (C) Low viscosity bolus pool; (D) High viscosity bolus pool.

Figure 3. Evolution of the (A,B) total NH_2 concentration of the digesta before digestion (t0), during the gastric (t15', t30', t60', t120') and after the intestinal (t120'g+120'i) phases; and the (C,D) degree of hydrolysis (DH) expressed as the percentage of NH_2 cleaved during digestion.(A,C) show protein isolates (PP) and fortified sponge cake (FSC); (B,D) show low (LV) and high viscosity (HV) bolus pools. Different letters mean significant differences ($p<0.05$) at every hydrolysis time. *$p<0.01$; **$p<0.001$; ***$p<0.0001$.