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Human gastrointestinal conditions affect *in vitro* digestibility of peanut and bread proteins

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Abstract

As plant proteins are increasingly used as a source of amino acids in the diet, studies on *in vitro* digestion of plant proteins are key to understand the different factors affecting proteolysis, with the ultimate goal of optimising the nutritional composition/intake of plant protein-rich products. More realistic scenarios including the most likely food matrix and physiologically relevant gastrointestinal (GI) conditions should be considered when assessing the *in vitro* digestion of proteins. The research described here compares the extent of hydrolysis of proteins from peanuts and wheat bread, in particular the vicilin-like 7S globulin (Ara h 1) and gliadin, respectively, with three GI scenarios simulating either infant, early phase adult (fed state) or late phase adult (fasted state) conditions. The digestibility of these proteins, in isolation or when naturally present in the respective food matrix, has been evaluated with SDS-PAGE, LC-MS/MS and a spectrophotometric assay. Results from the food matrices showed lower extent of total protein GI digestion under simulated infant conditions, intermediate behaviour under fed state adult conditions and larger extent under fasted state adult conditions. This was also the case for isolated gliadin. However, isolated Ara h 1 only showed lower extent of proteolysis in the gastric phase under infant conditions, reaching a similar extent to both adult conditions over the course of the intestinal phase. The food matrix seems to have delayed the proteolysis. Choosing an appropriate GI scenario as well as the matrix of the end food product is paramount when assessing *in vitro* protein digestion.

Keywords: protein, *in vitro* digestion, infant, INFOGEST protocol, food matrix, processing, peanut, bread wheat, Ara h 1, gliadin

1. Introduction

Plant proteins have increasingly attracted attention as a protein supply in the diet due to the higher environmental sustainability linked to its production and transport.[1] However, the transition to dietary protein that is largely plant-based is not so straight-forward for nutritional reasons, due to generally lower digestibility compared to animal proteins. Thus, careful investigations need to be undertaken in order to guarantee a safe consumption of newly developed products. Some plant proteins have shown certain drawbacks as compared to animal proteins, which include a nutritionally incomplete amino acid profile, anti-nutritional factors (hindering proteolysis), and potential allergenicity.[2, 3] The former can be overcome, for instance, by combining proteins from complementary plant sources to meet the essential amino acid requirements. The lower protein digestibility can be compensated for by increasing the intake. However, this certainly requires gaining more understanding on the digestion process of plant proteins. Static *in vitro* digestion tests have been proposed to evaluate the gastrointestinal (GI) fate of proteins.[4, 5] The physiologically relevance of these are paramount to fairly simulate *in vivo* conditions for screening purposes. With this requirement in mind, a recent study compared the effect of the GI scenario on the *in vitro* digestion of animal proteins (dairy and egg source).[6] The results showed a clear correlation between the enzyme activity, defined by the enzyme concentration and pH, and the rate and extent of protein digestion. Namely, an infant GI scenario, with lower total enzyme activity, led to lower extent of protein digestion. An adult GI scenario, however, led to intermediate extent of proteolysis in fed state, whereas larger extent of hydrolysis was observed in fasted state.

Another important factor to be considered in the assessment of protein digestion is the food matrix. Although more precise information on the mechanisms of digestion can be gained from studying isolated proteins, the results may not be predictive of digestion in complex food matrices. Our previous study on animal proteins showed that even in the liquid state, the food matrix and processing may affect the digestibility of proteins when compared with the isolated counterpart.[6] Reynaud and co-workers have also evaluated the impact of the food matrix and processing on the *in vitro* digestion of plant proteins, although with a single GI scenario.[7] Processing may also affect the ultrastructure of the natural food matrix, as proteins are partially denatured and conformations modified, affecting the stability to digestion.[8] Therefore, the aim of the current investigation, as a follow-up study, is the comparison of the same GI scenarios considered previously,[6] i.e. infant, early phase adult (or fed state) and late phase adult (or fasted state), on the digestion of plant proteins from peanut and bread wheat, that are widely consumed and have known immunogenic potential. The effect of the food matrix, which is in solid state, and the effect of thermal processing, since this is widely applied before product consumption, are taken into account. For instance, peanuts are often consumed in western countries after roasting, and baking is inherent to bread manufacture.

Ara h 1 from peanut (*Arachis hypogaea*) is one of the main storage proteins (7S globulin) of the seed, is highly susceptible to digestion[9, 10] and is also known as a major allergen. Gliadin from bread wheat (*Triticum aestivum*) is a mixture of storage proteins known as prolamins, primarily insoluble in water, which along with the group of glutenins constitute the gluten proteins. These are involved in the pathogenesis of celiac disease. Gliadins have a high level of proline residues, which renders certain large protein fragments highly resistant to GI digestion.[11-14] However, these findings were the results of studies on isolated proteins, and the research on the respective food

matrices indicates some delaying effect on protein digestibility. Di Stasio et al. used the INFOGEST standardised protocol (corresponding to an early phase adult)[5] to assess the protein digestibility in raw and roasted peanuts.[15, 16] They pointed out that some proteins in the peanut matrix, such as Ara h 3, may be hydrolysed to a lower extent than when isolated, by comparing their results with previous results in the literature.[10] In addition, they showed that the thermal processing of the whole food matrix can have an opposite impact on the stability of proteins to digestion to that of thermal processing of isolated proteins.[16, 17] This is a consequence of interactions with other proteins and non-proteins components (e.g. polysaccharides, lipids) and has scarcely been explored. On the other hand, Smith and co-workers compared the *in vitro* digestion of wheat gliadin in the bread matrix with that of an isolated fraction.[18] Their findings highlight that the matrix and intrinsic baking reduced the gluten digestibility, in particular in the gastric phase. All these studies used a single model of *in vitro* digestion that would correspond to adult GI conditions, however, not all of them used a standardised protocol, making comparisons across studies difficult.

To our best knowledge this is the first time that the *in vitro* digestion of peanuts and wheat bread has been compared at the physiologically relevant conditions in infants and adults in two different states: fed versus fasted, and at the same time the impact of food matrix/processing assessed by qualitative comparison with the digestibility of isolated proteins. Considering the effect of the food matrix and more likely processing is a relevant approach because the protein aggregation state, the interaction of proteins with other proteins and non-protein components and the presence of protease inhibitors affect the accessibility of proteases to the protein substrate, thereby contributing to the bioaccessibility and hence to the bioavailability.[19] The current study has combined SDS-PAGE, LC-MS/MS and a spectrophotometric assay to show differences in protein digestibility across the different GI scenarios, either on isolated proteins or in the food matrix, highlighting the importance of multiple protocols to fully assess protein digestion.

2. Materials and methods

2.1 Preparation of isolated proteins and source of solid meals

The protein gliadin (GL) from wheat was purchased from Sigma-Aldrich (Cat. No. G3375, 87% purity) and used as received. Ara h 1 was purified (≥95% purity by SDS-PAGE) from raw red skin peanuts purchased in a local supermarket according to a previously published procedure,[9, 20] using a single step lectin affinity column (of ConA Sepharose). These isolated proteins were dispersed at a concentration of 5 mg/mL in Milli-Q® water, in order to be consistent with the initial test protein concentration used in the original protocol of the pepsin resistance test,[4] which is used herein as a gastric late phase adult model. Dry roasted peanuts and sliced white wheat bread were purchased in a local supermarket and used before the “best by” date.

2.2 In vitro digestion

All chemicals used were of analytical grade and purchased from Sigma-Aldrich. Milli-Q® water was used for the preparation of the simulated salivary (SSF), gastric (SGF) and intestinal fluids (SIF), and their electrolyte composition is specified in Table S1 (supplementary material). All of the protocols of *in vitro* digestion comprised a gastric and intestinal phase in sequence. In the case of the solid meals (i.e. peanuts and bread), a 2 min oral phase was preceding the gastric phase. For the oral

phase of bread, the enzyme α-amylase from human saliva (Cat. No. A1031) was included. In the gastric phase, the enzyme pepsin (Cat. No. P7012) from porcine origin was used. In the intestinal phase, the individual enzymes trypsin (Cat. No. T0303, porcine) and chymotrypsin (Cat. No. C4129, bovine) were used for the isolated proteins, whereas pancreatin from porcine pancreas (Cat. No. P7545, 8 x USP) was used for the solid meals and the amount added was based on the required trypsin activity in the final volume of the intestinal content. Their activities were determined as described in the electronic supplementary material of Brodkorb et al.[21] Individual bile salts (≥97%) sodium glycocholate (NaGC, Cat. No. G7132) and sodium glycochenodeoxycholate (NaGCDC, Cat. No. G0759) were used in equimolar ratio for the isolated proteins, whereas porcine bile extract (Cat. No. B8631) was used for the solid meals.

All *in vitro* digestion experiments were performed in 50 mL conical centrifuge tubes mounted horizontally in a shaking incubator at 37 °C and 100 rpm. The *in vitro* digestion of each isolated protein/solid meal was conducted in triplicate for each protocol. Control experiments for each *in vitro* digestion protocol were also performed by replacing the initial volume/weight of isolated protein/meal by Milli-Q® water.

2.2.1 Oral phase of solid meals

The simulated oral phase of the solid meals for the three protocols described below (infant, early phase adult and late phase adult) is that recommended in the INFOGEST harmonised protocol.[5] Briefly, peanuts and bread slices were ground with a mincer and grater, respectively, to provide an initial particle size similar to that obtained by chewing (~ 3 mm). The initial amount of ground solid meal used for each protocol (5 g for infant and early phase adult and 0.5 g for late phase adult) was mixed with SSF (Table S1 and Table 1) at a ratio meal to SSF of 50:50 (w/v) and the pH was set to 7. The oral bolus was then subjected to the gastric phase of each protocol.

2.2.2 Infant protocol

The infant static *in vitro* digestion protocol was originally intended for liquid food formulations and therefore only comprises a gastric and intestinal phase in sequence of 60 min each, as described by Menard and co-workers.[22] The protocol was adapted with the inclusion of an oral phase as in previous section for the digestion of the solid meals. Another adaptation was the replacement of bovine bile extract by either porcine bile extract in the digestion of meals, or an equimolar mixture of two purified bile salts (NaGC and NaGCDC) which represent the two major forms in human bile[23] in the digestion of isolated proteins.

Briefly, in the gastric phase, 5 mL of isolated protein (5 mg/mL) or 10 g of oral bolus from solid meal were mixed with SGF (Table S1 and Table 1) at a ratio protein solution or meal to SGF of 63:37 (v/v). The pH was set to 5.3. After gastric digestion, the pH was raised to 7 with 1 M NaOH in order to stop pepsin activity before intestinal digestion. In the intestinal phase, the gastric chyme was mixed with SIF (Table S1 and Table 1) at a ratio of gastric chyme to SIF of 62:38 (v/v) and adjusted to pH 6.6 with 1 M HCl.

2.2.3 Early phase adult protocol

The early phase adult static *in vitro* digestion protocol followed the INFOGEST international consensus[5] with the following adaptations: the oral phase was omitted for isolated proteins, the length of gastric and intestinal phases was 60 min each and an equimolar mixture of NaGC and

NaGCDC replaced the bile extract for the *in vitro* digestion of isolated proteins, in order to retain consistency with the infant protocol.

In the gastric phase, 5 mL of isolated protein (5 mg/mL) or 10 g of oral bolus from solid meal were mixed with SGF (Table S1 and Table 1) at a ratio protein solution or meal to SGF of 50:50 (v/v) and the pH was set to 3. In the intestinal phase, the gastric chyme was mixed with SIF (Table S1 and Table 1) at a ratio gastric chyme to SIF of 50:50 (v/v) and adjusted to pH 7 with 1 M NaOH.

2.2.4 Late phase adult protocol

The late phase adult static *in vitro* digestion protocol comprised a gastric phase of 60 min following the pepsin resistance test protocol as described in the literature.[4] In the case of solid meals, an oral phase as stated in 2.2.1 preceded the gastric phase. In the gastric phase, 0.5 mL of isolated protein (5 mg/mL) or 1 g of oral bolus from solid meal was mixed with SGF (Table S1 and Table 1) at a ratio protein solution or meal to SGF of 5:95 (v/v). The pH was set to 1.2. After gastric digestion, the gastric chyme was immediately subjected to the intestinal phase as in 2.2.3.

Table 1: Summary of the *in vitro* digestion protocols, including the enzyme activity (U/mL in the final volume of each phase) and the total concentration of bile salts (mM in the final intestinal volume).

	Infant	**Early phase adult**	**Late phase adult**
2 min of oral phase (only for solid meals)			
Salivary α-amylase	75	75	75
Oral pH	7	7	7
60 min of gastric phase			
Pepsin	268	2000	2500 (10 U/µg of test isolated protein)
Gastric pH	5.3	3	1.2
60 min of intestinal phase			
Trypsin (individual enzyme or in pancreatin)	16	100	100
Chymotrypsin (individual enzyme)	4	25	25
Bile salts	3.1	10	10
Intestinal pH	6.6	7	7

2.3 Sampling and pre-treatment

Aliquots of 200 µL were collected at 0.5, 2, 5, 10, 20, 30 and 60 min of both gastric and intestinal phase. Protease activity was immediately stopped by adding 5 µL of Pepstatin A (0.73 mM) to gastric samples, or 10 µL of Pefabloc® (0.1 M) to intestinal samples. All samples were frozen at -20 °C until further analysis.

Peanut digesta samples were defatted before submitting to SDS-PAGE analysis. Peanut digesta aliquots were mixed with hexane (1:1 v/v), vortexed for at least 1 min, then centrifuged at 10,000 x g for 10 min at 20 °C, and the top layer (containing mixture of hexane and lipids) carefully removed with a micropipette. The aqueous supernatant left was used for SDS-PAGE analysis.

2.4 SDS-PAGE analysis of larger peptides (> 5 kDa)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify intact protein and peptides greater than 5 kDa in the digested and undigested samples under reducing conditions with the three *in vitro* digestion protocols. The procedure followed is described in our previous study.[6] Wells were loaded with 1.5 µg of isolated protein or 42 µg of total protein in peanuts (assuming all is soluble), taking into account the protein to simulated GI fluid ratio in order to evaluate the sole impact of the proteolysis. In the case of gliadin and bread digesta, wells were loaded with the maximum amount allowed by the considered protocol of digestion taking into account the corresponding dilution ratio. This is because gliadin (either isolated or within the bread matrix) has poor solubility in water and it is also difficult to assess its solubility at each time point within the digesta. The SDS-PAGE was repeated at least in duplicate.

2.5 LC-MS/MS analysis of smaller peptides (< 5 kDa)

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used to identify peptides smaller than 5 kDa in the digested and undigested isolated protein samples with the three *in vitro* digestion protocols. Prior to mass spectrometry analysis, additional Pepstatin was added to all gastric samples. The gastric samples were diluted to the required protein concentration and filtered (0.45 µm filter) for the injection of 50 ng of protein (10 µL) into the spectrometer. For the intestinal samples, 10 µL were injected corresponding to 120 ng of protein (unfiltered) for the infant and early phase adult protocols and 6 ng of protein (filtered) for the late phase adult protocol.

For mass spectrometry analysis, the procedure followed is described in our previous study.[6] A statistical analysis of the identified peptides longer than 9 amino acids was performed.

2.6 OPA assay

The ortho-phthaldialdehyde (OPA) spectrophotometric assay was performed to quantify the amount of NH_2 groups released during the proteolysis of both meals with the three *in vitro* protocols. This is indicative of the hydrolysis of total protein. The procedure followed is described in our previous study.[6] Each measurement was conducted in triplicate. Data are presented as mean values ± standard deviation. Comparison among *in vitro* digestion protocols over time was made with two-way ANOVA and post hoc Bonferroni multiple comparison test with a threshold for significance $p \leq 0.05$.

3. Results and discussion

The aim of this study was to probe the effect of physiologically relevant GI scenarios on the digestibility of widely consumed plant proteins, Ara h 1 from peanut and gliadin from bread wheat. For this purpose, three *in vitro* protocols simulating digestion in infants and adults in fed (early phase) or fasted state (late phase), have been applied to the isolated plant proteins and respective food matrices, i.e. peanuts and bread.

3.1 In vitro digestion of isolated plant proteins

Figure 1A shows the SDS-PAGE of the undigested and digested Ara h 1 with the three *in vitro* models: infant at the top (Figure 1 A1, A2), early phase adult in the middle (Figure 1 A3, A4) and late phase adult at the bottom (Figure 1 A5, A6). Ara h 1 from peanut is a stable homotrimer in its native form with a molecular weight (Mw) of approximately 235 kDa. SDS-PAGE (Figure 1A) shows a major band of around 67 kDa, which corresponds to the monomeric form as a result of the denaturing conditions of the lithium dodecyl sulphate sample buffer used in the SDS-PAGE analysis,[10] and a minor band of ca. 33 kDa likely corresponding to a subunit.[20] The other minor band of around 130-150 kDa may correspond to a dimeric form, as reported elsewhere.[9]

Figure 1 (A1, A3, A5) shows that in general, Ara h 1 is rapidly hydrolysed under gastric conditions. The fast digestion of intact Ara h 1 in the early and late phase adult models is in agreement with the results reported by Eiwegger et al. and Fu et al., respectively, under similar conditions for each adult model.[9, 24] Nevertheless, the kinetics is slower in the infant model as compared to both adult models. Although, the disappearance of intact protein, which was determined from densitometry analysis (Figure S1 supplementary material), did not show statistically significant differences. However, it is worth noting that as soon as the gastric phase of the infant model started, extensive precipitation of Ara h 1 occurred and sedimented aggregates were observed until 60 min. This is likely due to the pH of 5.3, which is close to the isoelectric point of Ara h 1,[25] therefore, a reduced amount of protein would be available in solution for pepsin cleavage. This may actually explain the sudden decrease in band intensity corresponding to intact Ara h 1 after 30 s of infant gastric digestion (Figure 1 A1). Interestingly, this reduced Ara h 1 soluble fraction remains stable until min 10 of the infant gastric digestion. Afterwards, slightly smaller products became visible in the SDS-PAGE and only after 30-60 min is a faint smeared band (3-14 kDa) detected, although the intact protein is still visible. Previous results on *in vitro* gastric digestion of purified peanut allergens, showed that Ara h 1 was much more rapidly digested with a pepsin to test protein ratio similar to that used in the infant model (85 U/mg test protein).[10] The most plausible explanation for the different rate and extent of hydrolysis obtained in our study lies in the gastric pH, which is 5.3 versus 1.2 in the reported study. This pH of 5.3 is well above the optimum range for pepsin activity (pH 1.6-4).[26] On the other hand, the smaller Mw products that are already visible after 30 s of gastric digestion for both adult models are gradually hydrolysed (Figure 1 A3, A5), and intact protein is no longer detected, as shown previously.[9] Therefore, Ara h 1 seems to be more resistant to pepsin under infant gastric conditions and also non-digested protein remains in the aggregates after starting the intestinal phase.

In the intestinal phase of the infant model, the pH is raised to 6.6, which allows the re-solubilisation of Ara h 1 aggregates over the course of the intestinal digestion. The rapid digestion of the re-solubilised Ara h 1 may explain the appearance of hydrolysis products of larger molecular weight or increase in quantity (bands becoming more intense) over time (Figure 1 A2). This is supported by the absence of the intact protein throughout the intestinal phase. By the end of the intestinal phase, no bands were detected corresponding to hydrolysis products and the digesta

was completely clear in appearance, with no visible aggregates. This suggests a complete digestion of Ara h 1, as far as the SDS-PAGE allows detection. The same was seen for both adult models (Figure 1 A4, A6), considering that only small Mw products were detected at the end of the gastric phase, which rapidly disappeared upon starting the intestinal phase.

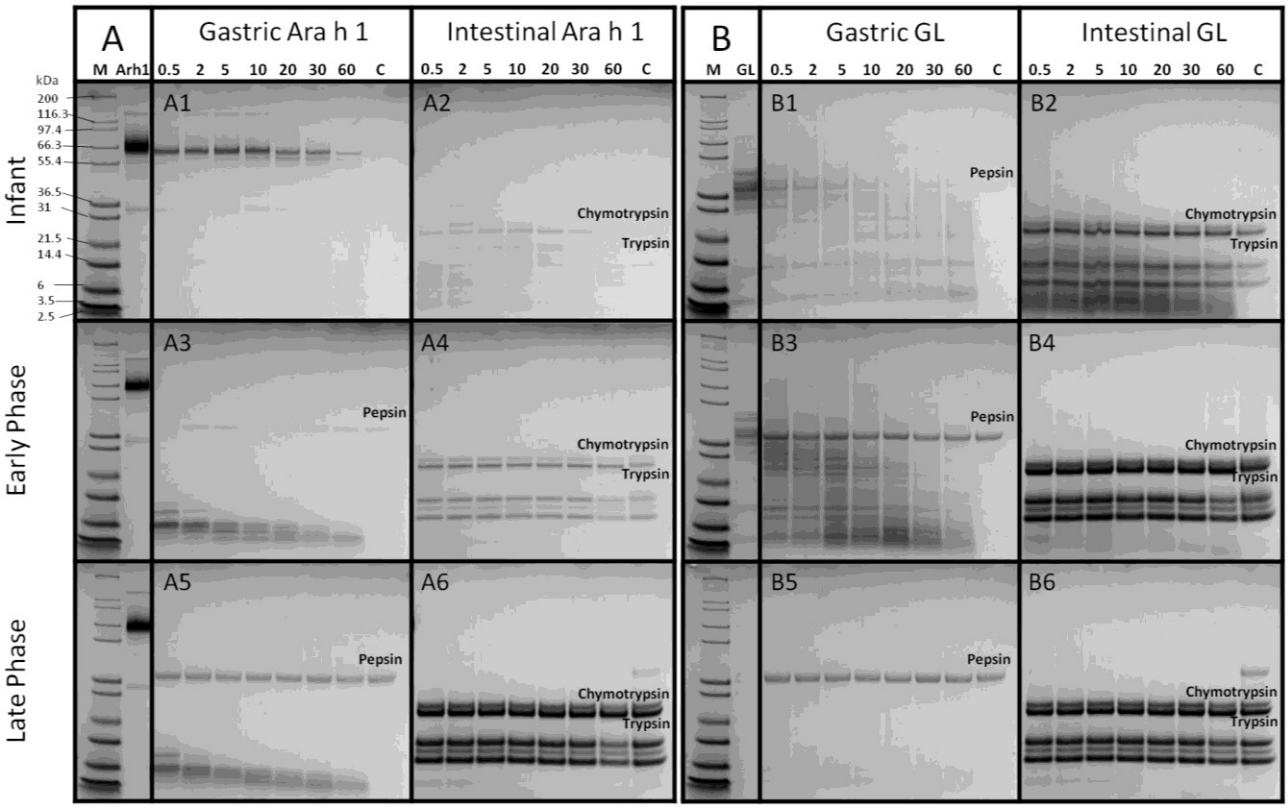

Figure 1: SDS-PAGE of the digesta of isolated Ara h 1 (A) and gliadin (B) with the infant, early phase adult and late phase adult models. The numbers at the top of the lanes represent the time in min of the gastric or intestinal phase. The M lane corresponds to the Mw marker. "Arh1" and "GL" lanes are the protein blank (undigested) and the C lane is the control of the digestive enzymes.

Results from LC-MS/MS analysis are shown as a box plot of the peptide Mw over time for the gastric and intestinal phase with the infant, early phase adult and late adult models (Figure 2) and the number of total unique peptide sequences identified in each scenario (Table 2). *In vitro* digestion of Ara h 1 with the three models led to the identification of 485 unique peptides in the gastric phase and 682 in the intestinal phase (Table 2). The median Mw values of peptides tend to decrease with time during the gastric phase with the early and late phase adult models (Figure 2a). This agrees with the decreasing Mw of hydrolysis products (< 6 kDa) observed by SDS-PAGE (Figure 1 A3, A5). The opposite behaviour is noted during the gastric phase of the infant model (Figure 2a). The median Mw of the peptides increased during the first 10 min and remained constant afterwards. This was supported by the SDS-PAGE results (Figure 1 A1) showing appearance of small Mw hydrolysis products (3.5-6 kDa) only from 20 min onwards. This may be related to the very slow digestion of aggregated Ara h 1 under the infant gastric pH conditions. Conversely, the trend of decreasing Mw observed in both adult models, also observed in Figure 1 (A3, A5), suggests a larger extent of digestion of Ara h 1 in the gastric phase. This is further supported by

294 the higher number of peptides identified in the gastric phase of the early and late phase adult
295 models, 472 and 405, respectively, as compared to the infant model, 100 (Table 2).

296 In the intestinal phase, the infant model led to larger Mw peptides with the median value
297 gradually decreasing over time and the final extent is comparable to both adult models (Figure 2a).
298 The infant model led to higher number of peptides identified in the intestinal phase (638) than in
299 the gastric phase (100) (Table 2). All these together agree with the fact that re-solubilised Ara h 1
300 was further digested by the end of the intestinal phase (Figure 1 A2). Thus, after 60 min of
301 intestinal digestion, Ara h 1 was almost completely digested to small peptides regardless of the *in*
302 *vitro* model, in agreement with the SDS-PAGE results. A time evolution of the median Mw of
303 peptides for the early and late phase adult models is not clear, but the values are in general
304 slightly lower for the early phase model. Nevertheless, 240 peptides slowly disappeared in the
305 early phase model and rapidly in the late phase model. The lower number of intestinal peptides
306 identified in the late phase adult model (Table 2) may be linked with the smaller amount injected
307 as a consequence of the high dilution of test protein inherent to this digestion protocol. Therefore,
308 direct comparisons of the number of peptides cannot be made between models in the intestinal
309 phase.

310

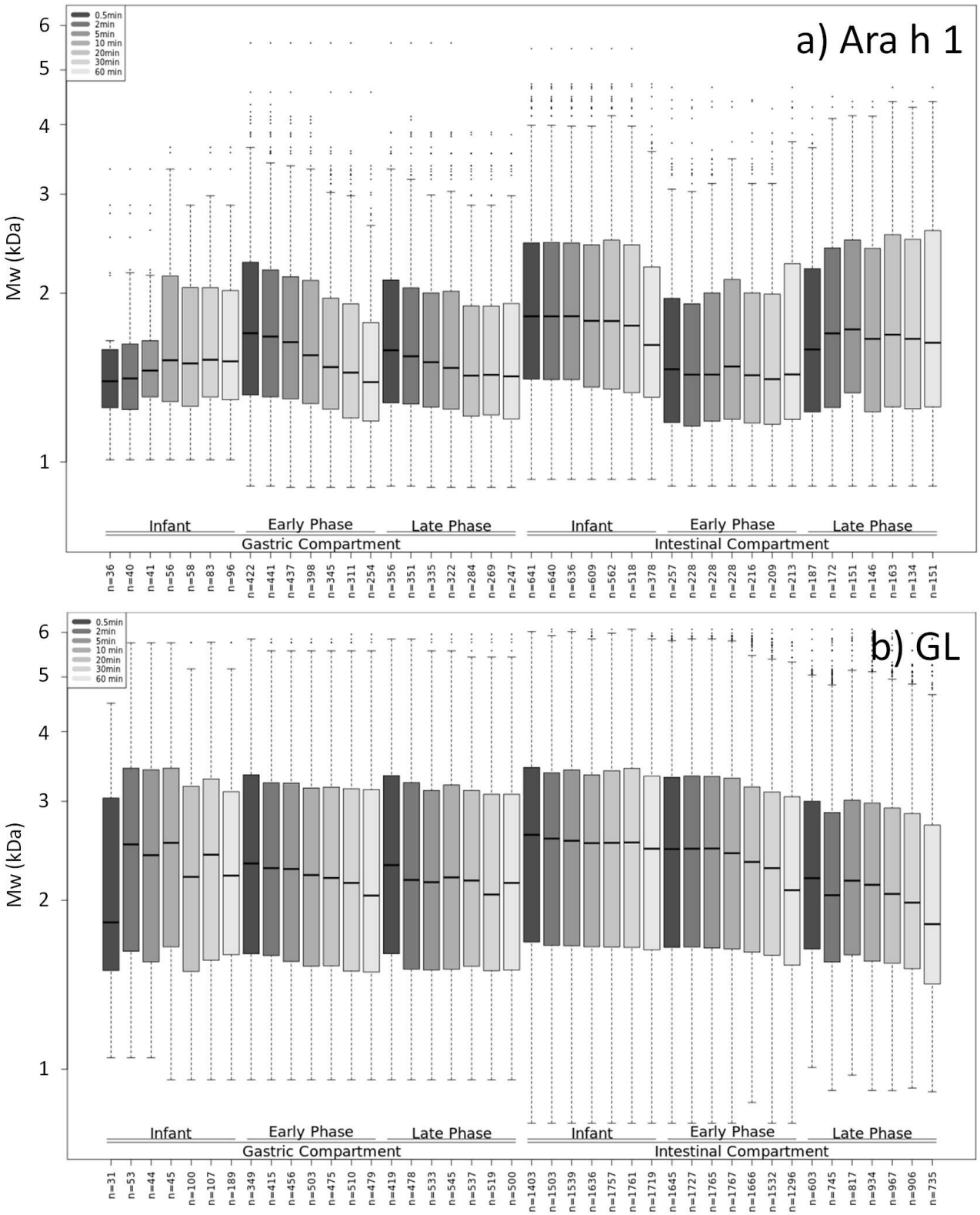

Figure 2: A box plot of the peptide molecular weight from Ara h 1 (a) and gliadin (b) after gastric and intestinal digestion with the three *in vitro* models. Numbers at the bottom are the number of unique peptide sequences identified at each time point.

Table 2: Summary of the total number of unique peptide sequences identified for each *in vitro* digestion model and with the three models together.

Protein	Compartment	Total from the three models	Infant	Early Phase	Late Phase
Ara h 1	Gastric	485	100	472	405
	Intestinal	682	638	339	261
Gliadin	Gastric	611	201	549	577
	Intestinal	2074	1941	1931	1217

Figure 1B displays the SDS-PAGE of the undigested and digested gliadin with the three *in vitro* models. Gliadin comprises monomeric proteins initially subdivided into *α/β*-, *γ*-, and *ω*-fractions, according to their electrophoretic profile at low pH.[27] They show as multiple bands at the Mw range of 35-45 kDa in SDS-PAGE (Figure 1B).[18] Due to their poor solubility in water, a minor soluble fraction would be at first accounted for, to be detected in SDS-PAGE, which may vary upon digestion by pepsin and trypsin/chymotrypsin. For this reason, the amount of protein loaded into SDS-PAGE for each model of digestion is the maximum allowed by the dilution of protein by simulated gastrointestinal fluid in the specific model, but keeping consistency among the protein blank, gastric and intestinal phases within each model. Therefore, the dilution factor of the gliadin blank goes from lowest in the infant model, to highest in the late phase adult model. In addition, densitometry analysis is not relevant in this case, since the extent of solubility may vary at each time point of digestion and the accuracy for the much diluted samples in the late phase adult model is compromised.

Figure 1 B1 shows that for the infant model, the soluble fraction of intact gliadin is partially hydrolysed soon after starting the gastric digestion (30 s). This is reflected in the decreased intensity of the group of bands corresponding to gliadins accompanied by the appearance of smeared bands of lower Mw (3-40 kDa) indicative of digestion products. By the end of the gastric phase, a relatively large fraction of insoluble gliadin was still present. At the beginning of the intestinal phase, the increased intensity of the bands corresponding to smaller Mw fragments of gliadin (Figure 1 B2), as compared to the end of the gastric phase, suggests that remaining insoluble gliadin was at least partially hydrolysed by trypsin and chymotrypsin. Over the course of the intestinal phase, these soluble protein fragments were gradually broken down into smaller ones, but could still be detected after 60 min of intestinal digestion (3-6 kDa). Despite some fraction of insoluble gliadin remaining after 60 min of intestinal digestion, no intact protein was visually detected in the SDS-PAGE for the soluble counterpart.

Figure 1 (B3, B4) displays the proteolysis of gliadin for the early phase adult model. As for the infant model, the soluble fraction of intact gliadin seems to be rapidly hydrolysed after 30 s of the gastric digestion (Figure 1 B3). Nevertheless, the presence of more intense bands corresponding to smaller fragments, as compared to the infant gastric model, suggests a more extensive digestion of the insoluble gliadin. In fact, the amount of precipitated solid at the end of the gastric phase for the early phase adult model seemed less than for the infant model. Smith and co-workers also observed a rapid gastric hydrolysis of gliadin fraction isolated from wheat under conditions of pepsin to test protein ratio similar to the infant model, but lower pH (2.5), which allows optimum

pepsin activity.[18] Namely, the gliadin fraction (35-45 kDa) was hydrolysed after 10 min of gastric digestion with a trace of smaller Mw fragments remaining afterwards and gradually breaking down into smaller fragments over the 60 min of the gastric phase. Subsequent intestinal digestion in the early phase adult model showed initially a smeared band of 3-6 kDa, which decreased progressively in Mw and in intensity throughout the duration of this phase (Figure 1 B4). No intact protein was visually detected on SDS-PAGE, and only a trace of precipitated gliadin remained. This supports a larger extent of gliadin digestion in the intestinal phase as compared to the infant model.

Figure 1 (B5, B6) shows the SDS-PAGE of the digesta of the late phase adult model. As anticipated, the large dilution of this digestion protocol does not allow the proper detection of intact gliadin, although a faint smeared band between 3 and 6 kDa can be visualised throughout the gastric phase (Figure 1 B5) and first 5 min of the intestinal phase (Figure 1 B6). This along with the comparison with the results of the early phase adult model and the fact that insoluble fraction of gliadin was not observed by naked eye by the end of the intestinal phase, suggests that the largest extent of gliadin proteolysis takes place under conditions of the late phase adult model.

In vitro digestion of gliadin with the three models led to the identification of 611 unique peptides in the gastric phase and 2074 in the intestinal phase (Table 2). In the gastric phase, peptides released by the infant model exhibited a slightly higher median Mw than those obtained with both adult models (Figure 2b). There is a tendency of the median Mw to decrease over time, although the behaviour is more variable for the infant model. This may be related to fluctuations in the soluble part over the course of gastric digestion. The higher number of unique peptides identified in the gastric phase of both adult models, 549 and 577, as compared to 201 in the infant model, further supports the larger extent of digestion under adult conditions. This positive correlation between the extent of digestion and number of identified peptides in the gastric phase was also observed in the previous study on digestion of dairy and egg proteins.[6] During intestinal digestion, the peptides median Mw decreased with time, the lower median Mw peptides being identified in the late phase adult model, followed by intermediate Mw peptides in the early phase adult model and higher median Mw peptides found in the infant model. This confirms the largest extent of digestion in the late phase adult model.

3.2 In vitro digestion of roasted peanuts and white wheat bread

The results of *in vitro* digestion of roasted peanuts with the three models are presented in Figure 3A. Besides Ara h 1, other proteins can be identified in the lane corresponding to the peanut blank. The SDS-PAGE pattern of the peanut blank is very similar to that of crude raw peanut protein extract reported elsewhere.[10] The major band migrating around 25 kDa likely corresponds to the basic subunit of Ara h 3, whereas the major band within the range of 42-45 kDa likely corresponds to the acidic subunit of Ara h 3 under reducing conditions. The allergen Ara h 3 belongs to the 11S storage globulin family. A minor double band at 17-20 kDa is likely the contribution from two isoforms of Ara h 2 and the minor band at approximately 15 kDa may correspond to Ara h 6. Both allergens belong to the 2S albumin family.

In general, the gastric phase of peanut (Figure 3 A1, A3, A5) shows the appearance of proteolysis products smaller than 10 kDa and the increase in their intensity over time. The intensity of these products is only slightly evident at the end of the gastric phase for the infant model (Figure 3 A1).

The increasing concentration of hydrolysis products of certain Mw in the absence of detectable intact protein or protein fragments of larger Mw is because the non-digested ground peanut is initially in the solid state and thus insoluble in the aqueous phase. It seems that as soon as the proteins are released from the peanut matrix due to enzymatic action of pepsin, these are immediately cleaved giving rise to smaller Mw products, which increase in concentration as pepsinolysis proceeds. Between the two adult models, there are slight differences in the pattern of SDS-PAGE. Namely, a light band of around 35 kDa is persistent throughout the gastric phase of the early phase model (Figure 3 A3), whereas its intensity is appreciably lower in the late phase model (Figure 3 A5). In addition, a light smeared band covering the range of 10-20 kDa is initially observed for the early phase adult model, which gradually disappears during the first 5 min of gastric digestion and is absent in the late phase adult model. This suggests a slightly faster hydrolysis in the latter. The proteolysis seems even slower and to a lower extent in the infant model. In addition to the much later appearance of small Mw products as pointed out above, faint bands appeared in the last 30 min of gastric digestion at molecular weights corresponding to acidic (42-45 kDa) and basic (25 kDa) subunits of Ara h 3. Interestingly, these bands also appeared at the beginning of the gastric phase for the early phase adult model, which gradually vanished over the first 5-10 min, and were not detected at all in the late phase adult model. It is not surprising that Ara h 3 is detected in the digesta samples, despite the low concentration of soluble protein released from the peanut matrix, since it is the most abundant protein in peanut kernels.[28] Ara h 3 has been reported to be as rapidly hydrolysed by pepsin as Ara h 1 when isolated from the peanut matrix.[10] Ara h 1 is the second most abundant protein in peanut kernel although its extractability is reduced by roasting.[28] The intact protein Ara h 1 was not detected in the aqueous phase of the digesta by SDS-PAGE in any of the *in vitro* models. The relatively lower concentration released in the aqueous phase, as compared to Ara h 3, and rapid proteolysis as observed when isolated, may explain the present results.

Figure 3 (A2, A4, A6) shows the results of the intestinal phase. Besides the bands corresponding to enzymes in pancreatin (see control lane), two additional bands can be detected at approximately 25 kDa and 12-14 kDa, respectively, with increasing intensity over the course of the intestinal digestion, for the infant and early phase adult model (Figure 3 A2, A4). The intensity of these bands is lower in the latter. These bands were observed previously under reducing conditions in the soluble fraction of roasted peanut protein extract after trypsin digestion for 15 h.[29] They were also detected in the digesta of raw peanuts after GI digestion under early phase adult conditions (INFOGEST harmonised protocol).[30] These may correspond to large fragments of Ara h 3 (~ 25 kDa) and of Ara h 3, Ara h 2 and Ara h 6 (12-14 kDa), in accordance with previous identifications based on LC-MS/MS analysis of the tryptic peptides arising from the digestion-resistant bands.[15] These resistant protein fragments were also identified by SDS-PAGE in the digesta of roasted peanuts under the same GI conditions, although with lower intensity suggesting a more extensive digestion in the roasted peanuts.[16] Thus, multiple structural modifications of proteins due to thermal treatment of the food matrix can have an impact on their stability to digestion. The reason for the increasing band intensity over time is that solid fragments of peanut remained at the end of the gastric phase and subsequent intestinal digestion continued releasing intact protein and proteolysis products in the aqueous phase of the digesta. The fact that the intensity of the bands is lower in the early phase adult model correlates with the lower amount of peanut solids observed in this model. This suggests greater digestibility since the initial amount of meal was the same (5 g) in both infant and early phase adult models. Intact Ara h 1 was not visible throughout the

intestinal phase for any of the *in vitro* models. The persistence of stable large fragments of Ara h 3 and the absence of Ara h 1 are in agreement with the results reported previously for raw and roasted peanuts digested under the early phase adult conditions.[15, 16] These results contrast with the high susceptibility shown by Ara h 3 to pepsin when isolated from the food matrix.[10] Thus, the peanut matrix may delay or impair the digestibility of proteins in the presence of other components such as lipids and polysaccharides. The plant cell wall structure may also play an important role in protein retention in a similar way as cell wall encapsulation in almonds limits lipid bioaccessibility.[31, 32]

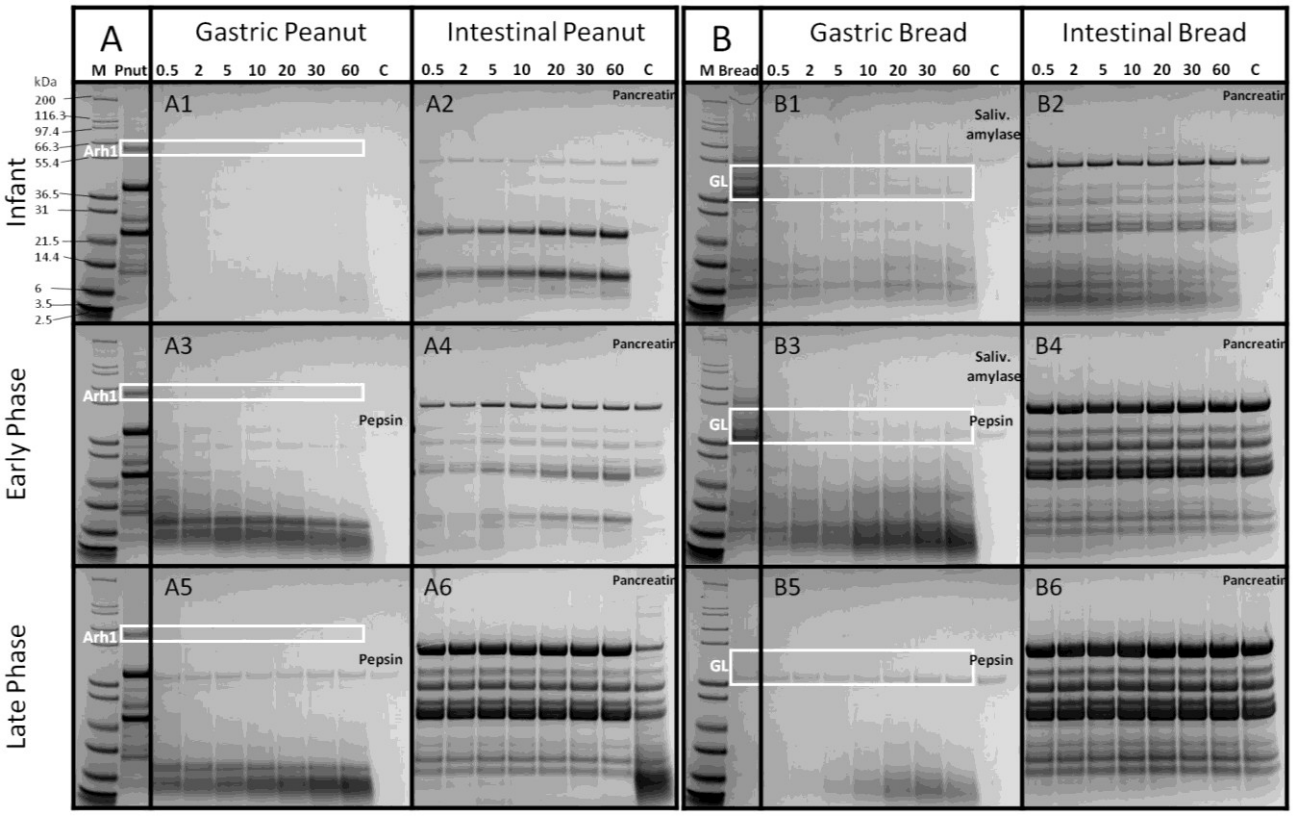

Figure 3: SDS-PAGE of the digesta of roasted peanuts (A) and white wheat bread (B) with the infant, early phase adult and late phase adult models. The numbers at the top of the lanes represent the time in min of the gastric or intestinal phase. The M lane corresponds to the Mw marker. The "Pnut" and "Bread" lanes correspond to the meal blank (undigested) and the C lane is the control of the digestive enzymes.

The OPA assay was performed on the digested peanut samples in order to quantify the primary amine groups released over time (normalised per mg of initial total protein before digestion) during the gastric and intestinal phase as indicative of total protein hydrolysis (Figure 4a and 4b). These levels are given in units of number of moles instead of molar concentration to account for the different volumes or dilution factors in each *in vitro* digestion model. In general, there is an increase of the primary amine groups as the pepsinolysis and pancreatic digestion proceeded. However, the rate and extent of total protein digestion differ across the three *in vitro* scenarios. Figure 4a shows the largest extent of gastric digestion under late phase adult conditions, followed by early phase adult and infant model with the lowest extent, in agreement with SDS-PAGE

results. In the intestinal scenario, however, there are no significant differences in the final extent of digestion across the three models, although larger amounts of primary amine groups are quantified for both adult models.

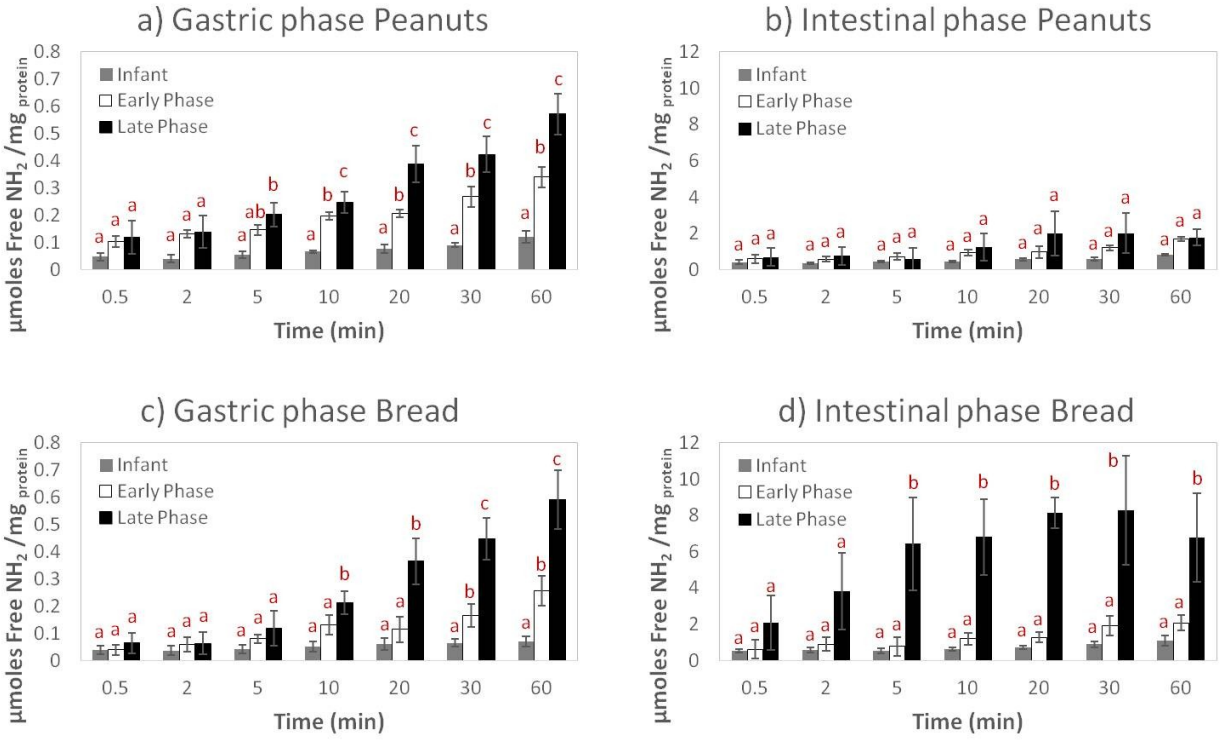

Figure 4: Levels of primary amine groups per mass of initial total protein during gastric and intestinal digestion of roasted peanuts and white wheat bread with the three models. The values were corrected for the level of primary amine groups present in the control of digestive enzymes. Different letters mean significant differences ($p \leq 0.05$) between models over time.

Figure 3B displays the corresponding SDS-PAGE of white bread digested *in vitro* with the three models. Bands corresponding to gliadin have been identified within the Mw range of 35-45 kDa in the bread blank, which may co-migrate with low-molecular weight glutenin subunits.[33] The bands at around 60 kDa and 14-16 kDa could correspond to the albumins/globulins protein families of *β*-amylase and α-amylase/trypsin inhibitors, respectively, and the faint band at around 100 kDa likely corresponds to high-molecular weight glutenin subunits.[33] The faint single band at approximately 9 kDa could correspond to non-specific lipid transfer protein (LTP) (allergen Tri a 14),[34] one of the metabolic proteins (albumins and globulins). Salivary amylase, which was included in the oral phase of bread, can also be seen in the control lane of the gastric phase for the infant and early phase adult model (Figure 3 B1, B3), because the gastric dilution factor with regards to the oral phase (x 1.59 and x 2, respectively) is lower as compared to that in the late phase adult model (x 20). It appears at a Mw of 56 kDa approximately.

There is no visible trace of intact seed storage proteins (high- and low-molecular weight glutenin subunits, and gliadin) in the aqueous phase of gastric digesta for both adult models (Figure 3 B3, B5). For the infant model, there is a light smeared band corresponding to gliadin and possible low-

molecular weight glutenin subunits throughout the gastric phase, indicating protein resistance to digestion under the milder infant conditions (Figure 3 B1). In general, bands with Mw < 14 kDa became more intense over the course of gastric digestion for all the *in vitro* digestion models, suggesting accumulation of proteolytic products.[33] Minor bands also gradually appeared in the gastric phase between molecular weights of 14 and 35 kDa for infant and early phase adult model, and they seemed absent in the late phase adult model, suggesting greater extent of proteolysis. The band at 9 kDa in undigested bread is present throughout the gastric phase of the infant and early phase adult model and difficult to detect in the late phase adult model because of the high dilution of this digestion protocol. This band is likely to correspond to LTP which has been reported to be highly resistant to simulated GI digestion either when isolated or in the food matrix.[34, 35] The smeared band that appeared at approximately 20 kDa and remained until the end of the gastric phase of the early phase adult model (Figure 3 B3) may correspond to stable fragments of gliadins and low-molecular weight glutenins that accumulated over time.[18] This band was not clearly detected at the end of the gastric phase of the isolated gliadin (Figure 1 B3), suggesting a lower extent of digestion in the bread matrix.

The digestion products observed at the end of the gastric phase gradually disappeared over the course of the intestinal phase for the infant model (Figure 3 B2). Although, remaining protein fragments of Mw up to 14 kDa were still visible at 60 min of the intestinal phase. In contrast, these products immediately disappeared after starting the intestinal phase in both adult models (Figure 3 B4, B6), leaving no detectable trace by SDS-PAGE by the end of the intestinal phase. This corroborates once more the observed trend of faster and larger extent of digestion in both adult models. OPA assay results (Figure 4c and 4d) confirmed this trend of total protein digestion in white wheat bread in both gastric and intestinal phases. Namely, lower extent of protein digestion in the infant model, followed by the early phase adult and late phase adult models ($p < 0.05$ at 60 min in both gastric and intestinal phases).

The resistance of bread proteins to digestion under the infant GI conditions may be an interrelated factor between the lower content of proteases and that of pancreatic amylase. Smith and co-workers showed that the digestion of a bread matrix is a synergistic process, where the proteolysis of the gluten network enhances the hydrolysis of the starch granules embedded in it and vice versa.[18] Only in the infant model is there a trace of small Mw protein fragments (< 14 kDa) remaining at the end of the intestinal phase (Figure 3 B2), which contrasts with the pattern observed for isolated gliadin in Figure 1 B2 (< 6 kDa). However, one cannot discern that these peptides in bread digesta come exclusively from gliadin, but likely from other gluten and wheat proteins. It has been reported that the bread matrix can reduce the digestion of gluten proteins ascribable to the combined processing-induced changes of baking and the smaller surface area to volume ratio in the bolus as compared to isolated fractions.[18]

4. Conclusions

The final extent of total protein digestion in both food matrices (peanut and bread) in the gastric and intestinal compartments is affected by the GI scenario. The extent of proteolysis is lower under simulated infant conditions and higher under late phase adult conditions. This is also true for isolated gliadin. The extent of digestion of isolated Ara h 1 is lower in the gastric phase under infant conditions, however, it matches that under both adult conditions at the end of the intestinal

phase. The low levels of both proteases and pancreatic amylase in infants may compromise to a larger extent the protein digestibility in starch-rich products, as a consequence of the synergistic effect of protein and starch digestion.[18]

Regarding the effect of the food matrix on protein digestibility, some delay effect can be inferred in the digestion of peanuts. Intact Ara h 1 seemed absent throughout the gastric and intestinal phase of roasted peanuts, which is supported by the rapid GI hydrolysis observed when isolated. However, the presence of persistent Ara h 3 by the end of the intestinal phase, being as labile as Ara h 1 when isolated under GI conditions, may suggest that the peanut matrix has certain encapsulation effect, retarding proteolysis of certain proteins. Regarding gliadin, the retarding effect of the bread matrix on its hydrolysis may only be visible in the gastric phase of the infant model, suggested by the detection of trace amounts of intact protein after 60 min.

The inclusion of brush border enzymes in the digestion protocols in future approaches may help elucidate if these findings are still true in a more realistic scenario.

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Conflicts of interest

There are no conflicts of interest to declare.

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