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

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Amelia Torcello-Gómez,^{a,*} Didier Dupont,^b Julien Jardin,^b Valérie Briard-Bion,^b Amélie Deglaire,^b
Kerstin Risse,^{a,1} Elodie Mechoulan,^{a,2} and Alan Mackie^{a,*}

^aSchool of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, UK

^bSTLO, INRAE, Institut Agro, 35042 Rennes, France

*Corresponding authors: m.a.torcellogomez@leeds.ac.uk (A. Torcello-Gómez),
a.r.mackie@leeds.ac.uk (A. Mackie)

¹Present address: Institute of Food Technology and Food Chemistry, Faculty III Process Sciences,
Technical University of Berlin, Koenigin-Luise-Str. 22, 14195 Berlin, Germany

²Present address: Institute of Technology, University of Angers, 4 Boulevard de Lavoisier, 49000
Angers, France

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

39 1. Introduction

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

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

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

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

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

108

109 2. Materials and methods

110 2.1 Preparation of isolated proteins and source of solid meals

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

119 2.2 *In vitro* digestion

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

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

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

140 2.2.1 Oral phase of solid meals

141 The simulated oral phase of the solid meals for the three protocols described below (infant, early
142 phase adult and late phase adult) is that recommended in the INFOGEST harmonised protocol.⁵
143 Briefly, peanuts and bread slices were ground with a mincer and grater, respectively, to provide an
144 initial particle size similar to that obtained by chewing (~ 3 mm). The initial amount of ground solid
145 meal used for each protocol (5 g for infant and early phase adult and 0.5 g for late phase adult)
146 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
147 to 7. The oral bolus was then subjected to the gastric phase of each protocol.

148 2.2.2 Infant protocol

149 The infant static *in vitro* digestion protocol was originally intended for liquid food formulations and
150 therefore only comprises a gastric and intestinal phase in sequence of 60 min each, as described
151 by Menard and co-workers.²² The protocol was adapted with the inclusion of an oral phase as in
152 previous section for the digestion of the solid meals. Another adaptation was the replacement of
153 bovine bile extract by either porcine bile extract in the digestion of meals, or an equimolar mixture
154 of two purified bile salts (NaGC and NaGCDC) which represent the two major forms in human
155 bile²³ in the digestion of isolated proteins.

156 Briefly, in the gastric phase, 5 mL of isolated protein (5 mg/mL) or 10 g of oral bolus from solid
157 meal were mixed with SGF (Table S1 and Table 1) at a ratio protein solution or meal to SGF of
158 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
159 order to stop pepsin activity before intestinal digestion. In the intestinal phase, the gastric chyme
160 was mixed with SIF (Table S1 and Table 1) at a ratio of gastric chyme to SIF of 62:38 (v/v) and
161 adjusted to pH 6.6 with 1 M HCl.

162 2.2.3 Early phase adult protocol

163 The early phase adult static *in vitro* digestion protocol followed the INFOGEST international
164 consensus⁵ with the following adaptations: the oral phase was omitted for isolated proteins, the
165 length of gastric and intestinal phases was 60 min each and an equimolar mixture of NaGC and

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

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

172 2.2.4 Late phase adult protocol

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

179

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

183

184 2.3 Sampling and pre-treatment

185 Aliquots of 200 μ L were collected at 0.5, 2, 5, 10, 20, 30 and 60 min of both gastric and intestinal
186 phase. Protease activity was immediately stopped by adding 5 μ L of Pepstatin A (0.73 mM) to
187 gastric samples, or 10 μ L of Pefabloc[®] (0.1 M) to intestinal samples. All samples were frozen at -20
188 $^{\circ}$ C until further analysis.

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

193 2.4 SDS-PAGE analysis of larger peptides (> 5 kDa)

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

204 2.5 LC-MS/MS analysis of smaller peptides (< 5 kDa)

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

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

215 2.6 OPA assay

216 The ortho-phthaldialdehyde (OPA) spectrophotometric assay was performed to quantify the
217 amount of NH₂ groups released during the proteolysis of both meals with the three *in vitro*
218 protocols. This is indicative of the hydrolysis of total protein. The procedure followed is described
219 in our previous study.⁶ Each measurement was conducted in triplicate. Data are presented as
220 mean values \pm standard deviation. Comparison among *in vitro* digestion protocols over time was
221 made with two-way ANOVA and post hoc Bonferroni multiple comparison test with a threshold for
222 significance $p \leq 0.05$.

223

224 3. Results and discussion

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

230 3.1 *In vitro* digestion of isolated plant proteins

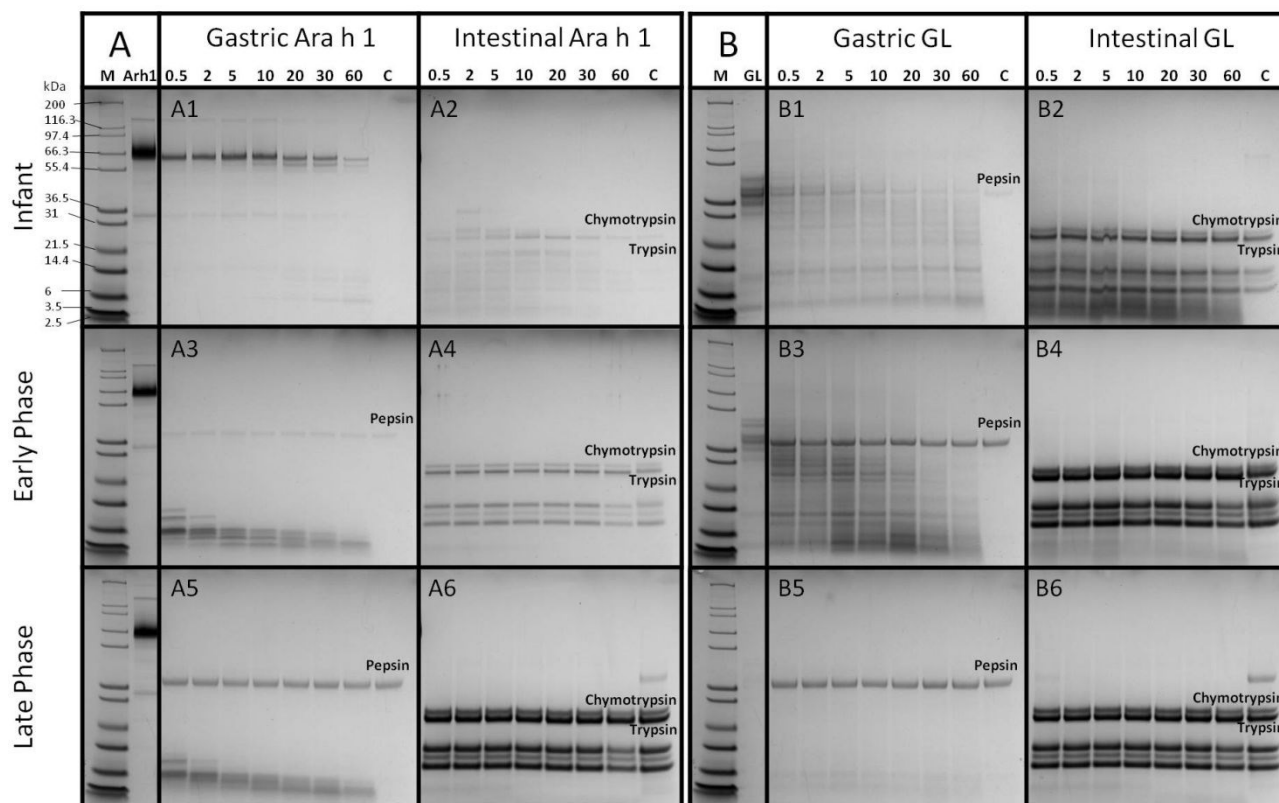
231 Figure 1A shows the SDS-PAGE of the undigested and digested Ara h 1 with the three *in vitro*
232 models: infant at the top (Figure 1 A1, A2), early phase adult in the middle (Figure 1 A3, A4) and
233 late phase adult at the bottom (Figure 1 A5, A6). Ara h 1 from peanut is a stable homotrimer in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

239 Figure 1 (A1, A3, A5) shows that in general, Ara h 1 is rapidly hydrolysed under gastric conditions.
240 The fast digestion of intact Ara h 1 in the early and late phase adult models is in agreement with
241 the results reported by Eiwegger et al. and Fu et al., respectively, under similar conditions for each
242 adult model.^{9, 24} Nevertheless, the kinetics is slower in the infant model as compared to both adult
243 models. Although, the disappearance of intact protein, which was determined from densitometry
244 analysis (Figure S1 supplementary material), did not show statistically significant differences.
245 However, it is worth noting that as soon as the gastric phase of the infant model started, extensive
246 precipitation of Ara h 1 occurred and sedimented aggregates were observed until 60 min. This is
247 likely due to the pH of 5.3, which is close to the isoelectric point of Ara h 1,²⁵ therefore, a reduced
248 amount of protein would be available in solution for pepsin cleavage. This may actually explain the
249 sudden decrease in band intensity corresponding to intact Ara h 1 after 30 s of infant gastric
250 digestion (Figure 1 A1). Interestingly, this reduced Ara h 1 soluble fraction remains stable until min
251 10 of the infant gastric digestion. Afterwards, slightly smaller products became visible in the SDS-
252 PAGE and only after 30-60 min is a faint smeared band (3-14 kDa) detected, although the intact
253 protein is still visible. Previous results on *in vitro* gastric digestion of purified peanut allergens,
254 showed that Ara h 1 was much more rapidly digested with a pepsin to test protein ratio similar to
255 that used in the infant model (85 U/mg test protein).¹⁰ The most plausible explanation for the
256 different rate and extent of hydrolysis obtained in our study lies in the gastric pH, which is 5.3
257 versus 1.2 in the reported study. This pH of 5.3 is well above the optimum range for pepsin activity
258 (pH 1.6-4).²⁶ On the other hand, the smaller Mw products that are already visible after 30 s of
259 gastric digestion for both adult models are gradually hydrolysed (Figure 1 A3, A5), and intact
260 protein is no longer detected, as shown previously.⁹ Therefore, Ara h 1 seems to be more resistant
261 to pepsin under infant gastric conditions and also non-digested protein remains in the aggregates
262 after starting the intestinal phase.

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

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

273



274

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

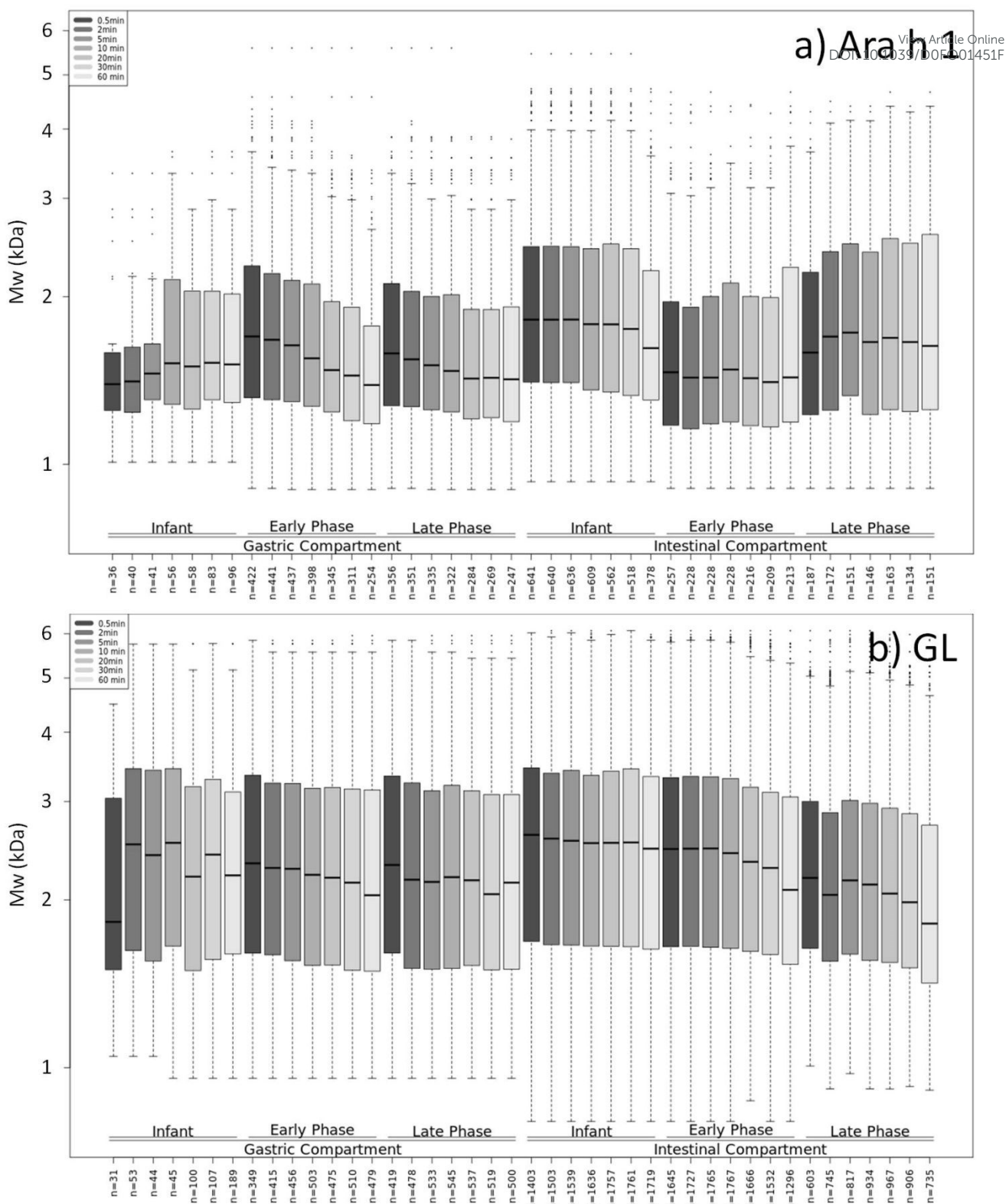
279

280 Results from LC-MS/MS analysis are shown as a box plot of the peptide Mw over time for the
 281 gastric and intestinal phase with the infant, early phase adult and late adult models (Figure 2) and
 282 the number of total unique peptide sequences identified in each scenario (Table 2). *In vitro*
 283 digestion of Ara h 1 with the three models led to the identification of 485 unique peptides in the
 284 gastric phase and 682 in the intestinal phase (Table 2). The median Mw values of peptides tend to
 285 decrease with time during the gastric phase with the early and late phase adult models (Figure 2a).
 286 This agrees with the decreasing Mw of hydrolysis products (< 6 kDa) observed by SDS-PAGE (Figure
 287 1 A3, A5). The opposite behaviour is noted during the gastric phase of the infant model (Figure
 288 2a). The median Mw of the peptides increased during the first 10 min and remained constant
 289 afterwards. This was supported by the SDS-PAGE results (Figure 1 A1) showing appearance of
 290 small Mw hydrolysis products (3.5-6 kDa) only from 20 min onwards. This may be related to the
 291 very slow digestion of aggregated Ara h 1 under the infant gastric pH conditions. Conversely, the
 292 trend of decreasing Mw observed in both adult models, also observed in Figure 1 (A3, A5),
 293 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). View Article Online
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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



a) Ara h 1

b) GL

311

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

315

316 **Table 2:** Summary of the total number of unique peptide sequences identified for each *in vitro*
 317 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

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318

319

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

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

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

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

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

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

382

383 3.2 *In vitro* digestion of roasted peanuts and white wheat bread

384 The results of *in vitro* digestion of roasted peanuts with the three models are presented in Figure
385 3A. Besides Ara h 1, other proteins can be identified in the lane corresponding to the peanut
386 blank. The SDS-PAGE pattern of the peanut blank is very similar to that of crude raw peanut
387 protein extract reported elsewhere.¹⁰ The major band migrating around 25 kDa likely corresponds
388 to the basic subunit of Ara h 3, whereas the major band within the range of 42-45 kDa likely
389 corresponds to the acidic subunit of Ara h 3 under reducing conditions. The allergen Ara h 3
390 belongs to the 11S storage globulin family. A minor double band at 17-20 kDa is likely the
391 contribution from two isoforms of Ara h 2 and the minor band at approximately 15 kDa may
392 correspond to Ara h 6. Both allergens belong to the 2S albumin family.

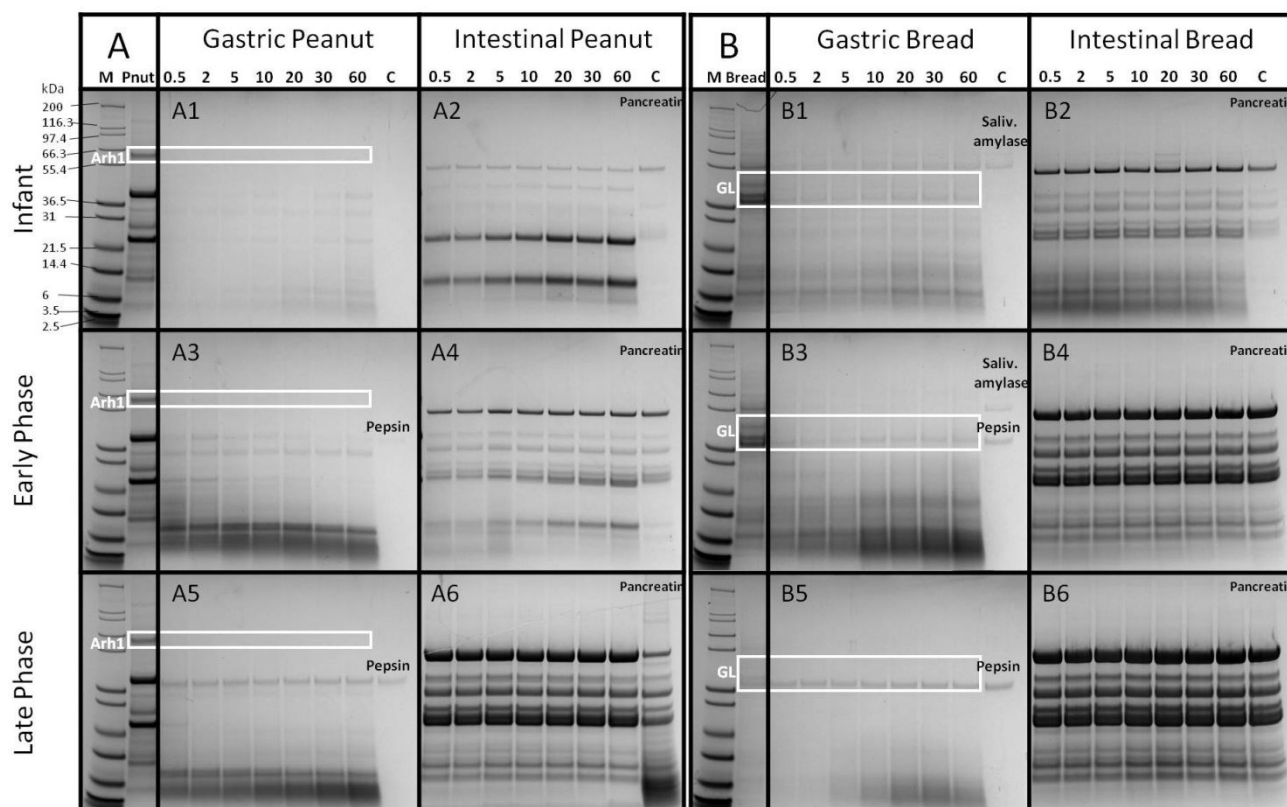
393 In general, the gastric phase of peanut (Figure 3 A1, A3, A5) shows the appearance of proteolysis
394 products smaller than 10 kDa and the increase in their intensity over time. The intensity of these
395 products is only slightly evident at the end of the gastric phase for the infant model (Figure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

396 The increasing concentration of hydrolysis products of certain Mw in the absence of detectable
397 intact protein or protein fragments of larger Mw is because the non-digested ground peanut is
398 initially in the solid state and thus insoluble in the aqueous phase. It seems that as soon as the
399 proteins are released from the peanut matrix due to enzymatic action of pepsin, these are
400 immediately cleaved giving rise to smaller Mw products, which increase in concentration as
401 pepsinolysis proceeds. Between the two adult models, there are slight differences in the pattern
402 of SDS-PAGE. Namely, a light band of around 35 kDa is persistent throughout the gastric phase of
403 the early phase model (Figure 3 A3), whereas its intensity is appreciably lower in the late phase
404 model (Figure 3 A5). In addition, a light smeared band covering the range of 10-20 kDa is initially
405 observed for the early phase adult model, which gradually disappears during the first 5 min of
406 gastric digestion and is absent in the late phase adult model. This suggests a slightly faster
407 hydrolysis in the latter. The proteolysis seems even slower and to a lower extent in the infant
408 model. In addition to the much later appearance of small Mw products as pointed out above, faint
409 bands appeared in the last 30 min of gastric digestion at molecular weights corresponding to acidic
410 (42-45 kDa) and basic (25 kDa) subunits of Ara h 3. Interestingly, these bands also appeared at the
411 beginning of the gastric phase for the early phase adult model, which gradually vanished over the
412 first 5-10 min, and were not detected at all in the late phase adult model. It is not surprising that
413 Ara h 3 is detected in the digesta samples, despite the low concentration of soluble protein
414 released from the peanut matrix, since it is the most abundant protein in peanut kernels.²⁸ Ara h 3
415 has been reported to be as rapidly hydrolysed by pepsin as Ara h 1 when isolated from the peanut
416 matrix.¹⁰ Ara h 1 is the second most abundant protein in peanut kernel although its extractability is
417 reduced by roasting.²⁸ The intact protein Ara h 1 was not detected in the aqueous phase of the
418 digesta by SDS-PAGE in any of the *in vitro* models. The relatively lower concentration released in
419 the aqueous phase, as compared to Ara h 3, and rapid proteolysis as observed when isolated, may
420 explain the present results.

421 Figure 3 (A2, A4, A6) shows the results of the intestinal phase. Besides the bands corresponding to
422 enzymes in pancreatin (see control lane), two additional bands can be detected at approximately
423 25 kDa and 12-14 kDa, respectively, with increasing intensity over the course of the intestinal
424 digestion, for the infant and early phase adult model (Figure 3 A2, A4). The intensity of these
425 bands is lower in the latter. These bands were observed previously under reducing conditions in
426 the soluble fraction of roasted peanut protein extract after trypsin digestion for 15 h.²⁹ They were
427 also detected in the digesta of raw peanuts after GI digestion under early phase adult conditions
428 (INFOGEST harmonised protocol).³⁰ These may correspond to large fragments of Ara h 3 (~ 25 kDa)
429 and of Ara h 3, Ara h 2 and Ara h 6 (12-14 kDa), in accordance with previous identifications based
430 on LC-MS/MS analysis of the tryptic peptides arising from the digestion-resistant bands.¹⁵ These
431 resistant protein fragments were also identified by SDS-PAGE in the digesta of roasted peanuts
432 under the same GI conditions, although with lower intensity suggesting a more extensive digestion
433 in the roasted peanuts.¹⁶ Thus, multiple structural modifications of proteins due to thermal
434 treatment of the food matrix can have an impact on their stability to digestion. The reason for the
435 increasing band intensity over time is that solid fragments of peanut remained at the end of the
436 gastric phase and subsequent intestinal digestion continued releasing intact protein and
437 proteolysis products in the aqueous phase of the digesta. The fact that the intensity of the bands is
438 lower in the early phase adult model correlates with the lower amount of peanut solids observed
439 in this model. This suggests greater digestibility since the initial amount of meal was the same (5 g)
440 in both infant and early phase adult models. Intact Ara h 1 was not visible throughout the

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

449



450

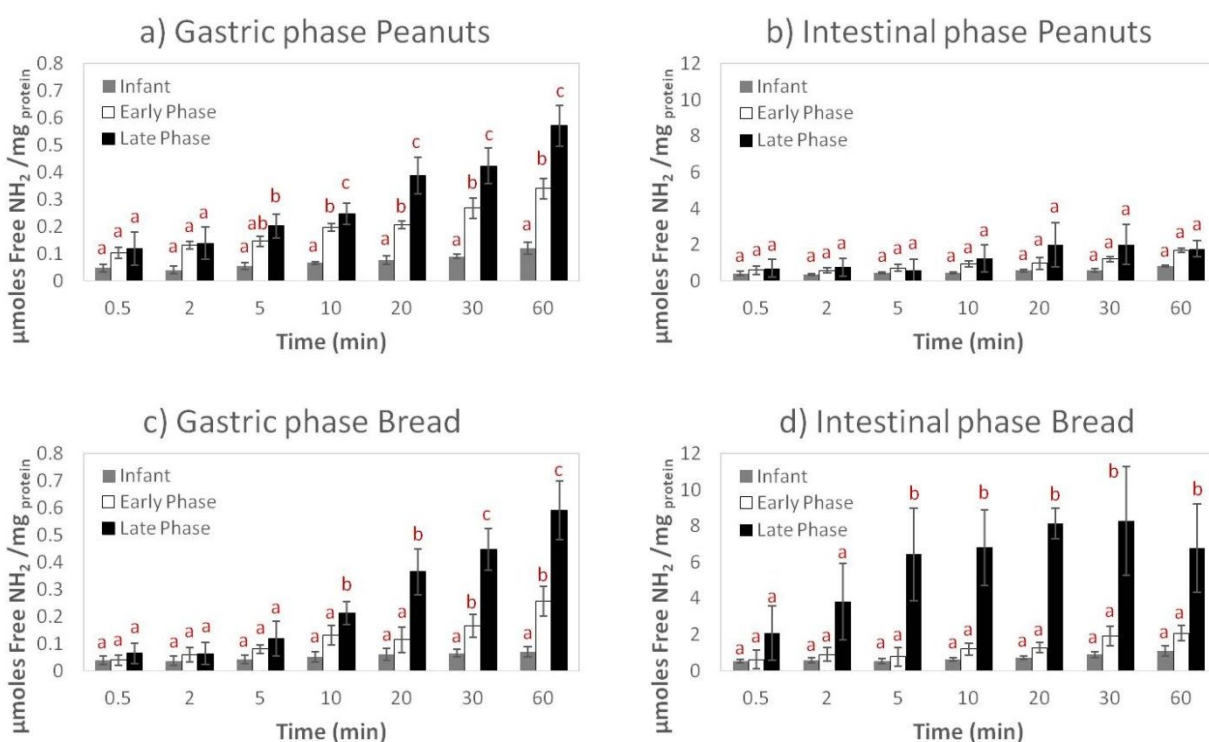
451 **Figure 3:** SDS-PAGE of the digesta of roasted peanuts (A) and white wheat bread (B) with the
 452 infant, early phase adult and late phase adult models. The numbers at the top of the lanes
 453 represent the time in min of the gastric or intestinal phase. The M lane corresponds to the Mw
 454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

456

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

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

469



470

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

475

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

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

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

506 The digestion products observed at the end of the gastric phase gradually disappeared over the
507 course of the intestinal phase for the infant model (Figure 3 B2). Although, remaining protein
508 fragments of Mw up to 14 kDa were still visible at 60 min of the intestinal phase. In contrast, these
509 products immediately disappeared after starting the intestinal phase in both adult models (Figure
510 3 B4, B6), leaving no detectable trace by SDS-PAGE by the end of the intestinal phase. This
511 corroborates once more the observed trend of faster and larger extent of digestion in both adult
512 models. OPA assay results (Figure 4c and 4d) confirmed this trend of total protein digestion in
513 white wheat bread in both gastric and intestinal phases. Namely, lower extent of protein digestion
514 in the infant model, followed by the early phase adult and late phase adult models ($p < 0.05$ at 60
515 min in both gastric and intestinal phases).

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

527

528 4. Conclusions

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

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

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

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

547

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554

555 Conflicts of interest

556 There are no conflicts of interest to declare.

557

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Peanut and wheat proteins either isolated or within the food matrix were subjected to different static *in vitro* digestion models (infant, fed and fasted adult). Proteolysis differed across models.

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