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1 **Abbreviations**

2 IFs: infant formulas

3 DH: hydrolysis degree

4 AAB: amino acid bioaccessibility

5 AA: amino acids

6 EAA: essential amino acids

7 DM: dry matter

8 w/w: weight/weight

9 a_w : water activity

10 Tg: glass transition temperature

11 v/v: volume/volume

12 OPA: o-phthaldialdehyde

13 SD: standard deviation

14 WMP: whole milk powder

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33 ***In vitro* static digestion reveals how plant proteins modulate model infant formula digestibility**

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45

46 **Abstract**

47 Infant formulas (IFs) are the key nutritional source for infants who cannot be breastfed. There is currently a growing
48 interest in these sensitive products in order to control their quality and to design their composition with regard to
49 nutritional balance. In a context of sustainable development and increasing growth of the world population, it seems
50 essential to search for alternative to animal protein in food today. Plant proteins offer interesting nutritional and functional
51 benefits thanks to the latest improvement through research and development. In this context, five model IFs were
52 developed with identical composition, except that 50% of the proteins were either whey proteins in the “milk-reference
53 IF”, pea, faba bean, rice or potato proteins in the four “plant IFs” tested. The IFs were evaluated using an *in vitro* static
54 gastro-intestinal model simulating infant conditions. The protein hydrolysis degree (DH) and the amino acid
55 bioaccessibility (AAB) were used as indicators of protein digestibility. Results showed that both DH and AAB were very
56 similar between the milk-reference IF, pea and faba bean IFs, but significantly lower for the rice and potato IFs. This study
57 provides new insights into the impact of protein sources on IF digestibility.

58

59 **Keywords:** Infant formula; *In vitro* digestion; Plant protein; Protein digestibility

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61 **Declaration of interest:** Linda Le Roux and Raphaël Chacon are employees of Sill Dairy International. Other authors
62 have no conflicts of interest.

63

64 1. Introduction

65 The basic function of proteins in nutrition is to supply adequate amounts of essential amino acids (EAA) to meet the
66 metabolic needs. The quality of a protein depends on its AA composition (Friedman, 1996). The nutritional value of
67 proteins also depends on their origin since all are not equivalent with respect to their AA content and sensitivity to
68 technological processes, which can modify their accessibility to digestive enzymes (Friedman, 1996; Machado et al.,
69 2008). In addition to the AA content, the digestibility of proteins also need to be taken into account (WHO, FAO & UNU,
70 2007). While the overall concept of digestibility is simple, as the ratio of the difference of the ingested and excreted
71 nitrogen to the ingested nitrogen, its *in vivo* measurement is actually complicated and several criteria have been suggested
72 to estimate an approximation of this value (FAO, 2013).

73 Protein intake early in life is essential for the development of infants, affecting growth, body composition,
74 neurodevelopment, appetite and hormonal regulation (Michaelsen & Greer, 2014). Protein requirements for infants are
75 greater than for adults, with 1.5 vs. 0.8 g of protein per kg of body weight and per day (Heird, 2012). From a qualitative
76 point of view, human milk is the gold standard for the newborn, and breastfeeding is highly recommended for the first six
77 months of life (Victora et al., 2016). However, for many reasons, mothers may be unable to provide human milk and a
78 milk replacer formula can be used instead (Agostoni et al., 2008). According to the applicable European regulation, the
79 sources of proteins allowed for IFs are either cow milk protein, goat milk protein, soy protein isolate or hydrolysed rice
80 protein (European Union, 2016). IF should provide similar amounts of EAA, as close as possible to those found in human
81 milk.

82 Besides, the demand for animal proteins is expected to increase to about double the present consumption by 2050, driven
83 by population growth and by the emerging middle classes in developing countries (Egbert & Payne, 2009; FAO, 2006). It
84 seems essential to search for alternative protein sources that show nutritional quality close to animal proteins one. In that
85 respect, there is a growing interest in utilizing plant proteins as partial replacers of animal proteins in food (Ainis, Ersch, &
86 Ipsen, 2018). There are multiple reasons why plant proteins are still underutilized for human food. Their lower nutritional
87 values as compared with animal proteins (deficiency in one or more EAA; lower protein digestibility) (WHO, FAO, &
88 UNU, 2007), the difficulties in maximizing their physical functionality due to their large molecular weight and size and
89 poor solubility in water (Day, 2013), and the economic cost associated with isolation and recovery of protein fractions are
90 hurdles for their use in food (Day, 2013). Plant proteins also contain anti-nutritional factors such as phytic acid, trypsin
91 inhibitors or phenolic compounds that can lower the protein digestibility (Guillamón et al., 2008; Kalogeropoulos et al.,
92 2010). However, there has been considerable improvement through research and development to enhance both the
93 nutritional and functional properties of plant proteins. For instance, the use of specific technological treatments can remove

94 most of the anti-nutritional factors and thus improve biological value and digestibility of such proteins (Lajolo &
95 Genovese, 2002; Le Gall, Guéguen, Séve, & Quillien, 2005). While soy protein continues to dominate as an alternative
96 plant protein to replace animal-based protein, a range of new food products is starting to appear, which use other grains,
97 legumes and vegetables as sources of proteins (Asgar, Fazilah, Huda, Bhat, & Karim, 2010; Schmidt, Novales, Boué, &
98 Axelos, 2010; Schwartz et al., 2015).

99 Many research groups studied the digestibility of either human milk or IF based primarily on cow milk protein or soy
100 protein (Bourlieu et al., 2015; Chatterton, Rasmussen, Heegaard, Sørensen, & Petersen, 2004a, 2004b; El-Agamy, 2007;
101 Lonnerdal, 2014; Nguyen, Bhandari, Cichero, & Prakash, 2015; Sakai et al., 2000). Reche et al. (2010) studied hydrolyzed
102 rice protein-based IF. Maathuis, Havenaar, He & Bellmann (2017) as well as Hodgkinson et al. (2019) compared the
103 protein digestion of goat- and cow' milk-based IFs. Other authors studied the ability of using plant proteins in IFs, but the
104 majority concerned legume proteins only and some were focused on encapsulation capacity of probiotics in follow-on IFs
105 (for 6-12 months infants). Ulloa, Valencia & Garcia (1988) showed that chickpea protein was a potentially utilizable
106 product as a milk substitute for children with gastrointestinal problems and demonstrated its good nutritional values that
107 complied with the Codex Alimentarius Commission standards for IFs. Similarly, Malunga et al. (2014) designed,
108 formulated and determined the nutritional quality of chickpea-based infant follow-on formula that demonstrated to meet
109 the minimum nutrition requirements of EU regulation on infant follow-on formula. Kent & Doherty (2014) discussed the
110 use of pea protein as suitable for the microencapsulation of probiotics for follow-on IF application but did not mention its
111 nutritional benefits. Similarly, Khan, Korber, Low & Nickerson (2013) used legume protein isolates (chickpea, faba, lentil
112 and pea proteins) as capsule wall materials for probiotics delivery in food and demonstrated their good protection
113 capability and delivery of probiotics under simulated gastrointestinal conditions. Recently, a patent related the process to
114 develop IF based on potato protein, naturally hypoallergenic and suitable for infants with cow's milk protein allergy
115 [WO2018 115340 (A1)]. These relevant studies on the ability of using plant proteins in IFs need to be furthered and
116 completed with other protein sources that would be suitable to infant needs directly from birth.

117 **In this context, the aim of the project was to develop new model IFs in which whey proteins will be partially replaced by**
118 **plant protein sources. These new protein sources were not yet allowed according to the regulation but the aim of the**
119 **project was to investigate future possibilities in this field.** In the present study, different protein sources were selected
120 based on the following criteria: they should contain an EAA profile suited to infant needs (EU, 2016), should be
121 commercially available and should be **alternative protein sources** to animal or plant proteins already used in IFs (EU,
122 2016). **Four plant proteins, i.e., pea, faba bean, rice and potato were thus used to design four “plant IFs”. A reference whey**
123 **protein was used to prepare the “milk-reference IF”.** In this study, the following question was investigated: How plant

124 proteins modulate the digestibility of **model** IFs compared to a milk-reference **model** IF? To answer to this question, plant
125 protein-substituted IFs were produced at a pilot scale and tested using an *in vitro* static digestion model developed on the
126 basis of an extensive literature review of infant physiology (Ménard et al., 2018). First, physicochemical parameters of the
127 produced IF powders have been evaluated and compared to the milk-reference IF to assess the functional quality of these
128 new IFs. The digestibility of the IFs have been investigated by measuring trypsin inhibitor activity, protein hydrolysis
129 degree (DH) as well as bioaccessibility of EAA.

130 To our knowledge, this is the first time that **model IF** containing plant proteins other than soy and hydrolyzed rice have
131 been reported, designed and their behavior during digestion investigated.

132 2. Materials and Methods

133 2.1. Chemicals

134 Porcine pepsin (P7012; 2971 IU/mg), porcine pancreatin (P7545; 6.79 IU/mg), bovine bile extract (B8631; 3.1 mmol/g), as
135 well as the enzyme inhibitors pepstatin A (P5318) and pefabloc (76307) were all obtained from Sigma-Aldrich, St.
136 Quentin Fallavier, France. Enzyme activities were determined as described in the Electronic Supplementary Information of
137 (Brodkorb et al., 2019). All other chemicals were of standard analytical grade.

138 2.2. Model infant formula ingredients

139 Skim cow milk **powder** was purchased from Sill, Plouvien, France. Maltodextrin (Glucidex® Maltodextrin Premium 19)
140 was purchased from Roquette, Lestrem, France. Lactose, whey protein concentrate (Protarmor™80) and demineralized
141 whey protein concentrate (Lactarmor™ DM 90) were all purchased from Armor Protéines in Loudéac, Saint-Brice-en-
142 Coglès and Pontmain, France. Pea protein concentrate (*Pisum sativum*, Nutralys® XF) was purchased from Roquette
143 Frères, Vic-sur-Aisne, France. Faba bean protein concentrate (*Vicia faba*, Vitessence™ Pulse CT 3602) was purchased
144 from Ingredion, Hamburg, Germany. Rice protein concentrate (*Oriza sativa L.*, RicePro NG BIO) was purchased from
145 Seah International, Wimille, France. Potato protein isolate (*Solanum tuberosum*, Solanic®200) was purchased from Arles
146 Agroalimentaire, Rognac, France. An oil blend based on vegetable fat and adapted to IFs was purchased from Cargill
147 Refined Oils Europe, Izegem, Belgium. All nutritional composition of each ingredients are presented in Table 1.
148 **Moreover, the WPNi (whey protein nitrogen index) was determined from Schuck et al. (2012) method and was 7.5 g**
149 **nitrogen / kg of powder which corresponded to a “low heat powder”.**

150 2.3. Model infant formula processing

151 Skim cow milk **powder**, lactose, maltodextrin and the different protein concentrates (whey protein as the reference and
152 potato, rice, pea or faba bean proteins as the plant protein sources) were solubilized in water at 20 w/w% DM (dry matter;
153 w/w: weight/weight) at 45°C under stirring at 35 Hz for 1 h (Fig. 1). The protein concentrates represented 50 w/w% of the

154 total protein content of the formula whereas the others 50 w/w% came from skim cow milk proteins (all five infant
155 powders were iso-nitrogenous). Neither vitamins nor minerals were added since this study was primarily focused on
156 protein sources and explain the expression of “model infant formula” used in the present study. The solution was then
157 pasteurized at 80°C for 35 s. In this respect, it should be mentioned that this pasteurization treatment, here applied for pre-
158 heating infant formula before concentration and drying, is probably much lower than what would be performed at an
159 industrial scale where sterilization is usually applied to ensure the microbiological safety of the IFs (Kent et al., 2015;
160 Zhuang et al., 2019). Then, a concentration step was followed to approximately 45 w/w% DM in a single-stage evaporator
161 (GEA, St Quentin-en-Yvelines, France) with an evaporation capacity close to $70 \text{ kg} \cdot \text{h}^{-1}$ at 60°C. The oil blend was added
162 to the concentrate and was homogenized at 60°C and 8/2 MPa. Finally, the solution was spray-dried from 52 w/w% to 98
163 w/w% DM using a pilot-scale Niro Minor (GEA-PE, Saint Quentin en Yvelines, France) equipped with a bi-fluid nozzle
164 of fixed geometrical features (0.8 mm liquid orifice diameter; 3.4 mm (internal) and 4.8 mm (external) air orifice
165 diameters) run at fixed air pressure (0.15 MPa). The concentrates were sprayed at a flow rate of $65 \pm 2 \text{ ml} \cdot \text{min}$. The inlet
166 and outlet air temperatures were set at $175 \pm 5^\circ\text{C}$ and $75 \pm 5^\circ\text{C}$, respectively. The evaporation capacity was approximately
167 $3.25 \text{ kg} \cdot \text{h}^{-1}$. The resulting powders were finally stored in light proof plastic bags at 20°C during maximum 4 weeks
168 pending for characterizations.

169 2.4. Infant formula characterization

170 2.4.1. Dry matter, ash and protein content

171 Total DM was determined gravimetrically after heating at $102 \pm 2^\circ\text{C}$ for 7 h, and ash content after incineration at $525 \pm$
172 25°C in a muffle furnace, both according to the methods of Schuck, Dolivet & Jeantet (2012).

173 Total nitrogen content was determined according to the IDF, (2001a) using the Kjeldhal method. A nitrogen-to-protein
174 conversion factor of 6.38 was used for the cow milk based ingredients (Section 2.2) and for the reference cow milk protein
175 based IF (Mariotti, Tomé, & Mirand, 2008). For the IFs composed of 50% of cow milk proteins and 50% of plant proteins,
176 the conversion factors used were the average of the one of cow milk proteins (6.38) and those of plant proteins, that is to
177 say 5.40 for pea and faba bean proteins, 5.34 for rice proteins and 5.60 for potato proteins, respectively (Mariotti et al.,
178 2008). These factors were also used to evaluate protein content, respectively, in the plant based ingredients (Section 2.2).
179 All measurements were carried out in duplicate.

180 2.4.2. Fat and free fat content

181 The total fat content was measured by Gerber's acid-butyrometric method after dissolution of proteins by the addition of
182 sulfuric acid and of amyl alcohol to facilitate the separation of milk fat by centrifugation at 350 g. The free fat content was

183 determined gravimetrically after evaporation of the solvent. Total and free fat analyses were carried out in duplicate
184 (AFNOR, 1990).

185 2.4.3. Amino acid content

186 The total amino acid concentration was determined after total acid hydrolysis of the sample (2 mg protein) in 2 mL 6 N
187 HCl and at 110°C for 24 h. A replicate of each sample was oxidized beforehand by performic acid and incubated for 16 h
188 at 4°C to analyze the sulfur amino acids (methionine and cysteine). The hydrolyzed samples were dried at 40°C under
189 vacuum in a rotary evaporator before being re-dispersed in 2 mL deionized water, filtered using a 0.45- μ m Syringe Filter
190 (Restek, Bellefonte, PA, USA), and then diluted (1:3) in 0.2 M lithium citrate buffer, pH 2.2. Amino acids were then
191 analyzed by cation exchange chromatography using an Automatic Amino Acid Analyzer (Biochrom Ltd., Cambridge, UK)
192 equipped with a cation exchange column 200 mm x 4.6 mm with a sulfonated polystyrene resin, reticulated by
193 divinylbenzene and conditioned in lithium form, from Biochrom 30 (Serlabo technologies, Trappes, France). Samples
194 were eluted with a 0.2 M lithium citrate buffer, pH 2.2, at 0.42 mL/min with post-column derivatization with ninhydrine
195 (Ultra Ninhydrin Reagent Kit, Biochrom) according to the procedure used by Moore, Spackman & Stein (1958).
196 Absorbance was measured at 570 nm for all amino acids. Amino acid quantification was achieved by measurement of each
197 peak area and using an external calibration curve previously established with amino acid standards (A9906, Sigma-
198 Aldrich, St. Quentin Fallavier, France). **The determination of tryptophan was not possible using ionic chromatography, due**
199 **to its degradation following acid hydrolysis.** Chromatographic assay was not replicated on each sample, but since digestion
200 experiments were carried out in triplicate, three independent values of amino acid concentrations were available for each
201 product.

202 2.4.4. Water activity and glass transition temperature

203 Water activity (a_w) was measured at 25°C \pm 0.1°C using the Novasina aw-meter (Novasina, Switzerland).
204 In order to determine the glass transition temperature (T_g), the powders were first equilibrated in a 20% relative humidity
205 atmosphere using the SPSx-1 μ Sorption Test System (ProUmid GmbH & Co. KG, August-Nagel-Str., Germany). The T_g
206 was then determined at this constant sorption point by using a modulated temperature differential scanning calorimetry
207 method according to Schuck et al. (2012).

208 2.4.5. Powder size distribution

209 The powder size distribution was determined using a laser scattering granulometer (Mastersizer, Malvern Instruments Ltd,
210 Malvern, UK) with a 300-mm measurement cell (0.5-880 μ m range). Powders were mixed with coarser powder (sucrose)
211 in ratio 1:1, in order to avoid agglomeration, and dispersed with a dry sampling system. The refractive index of dried

212 particles was 1.45, and 30 kPa air pressure was used. The median diameter $d(0.5)$ was chosen to describe the particle size
213 distribution where $d(0.5)$ is the particle diameter below which 50% of the material volume exists.

214 2.4.6. Color

215 The color of the powders was measured using the CIELAB color space. Color is defined by the brightness L (from 0 to
216 100) and the chromaticity coordinates a^* (from green to red; -60 to $+60$) and b^* (from blue to yellow; -60 to $+60$). The
217 three parameters were obtained using a chromameter (Konica Minolta Photo Imaging France SAS, Roissy, France)
218 previously calibrated with a white reference plate.

219 2.4.7. Rehydration properties

220 Dispersibility and solubility were determined according to Schuck et al. (2012). The dispersibility index is the amount of
221 DM dispersed in water after 13 g powder have been added to 100 g water at 40°C under stirring with a spatula for 15 s. It
222 is expressed as the w/w% of matter that can pass through a 200- μm mesh size sieve. The solubility index is the v/v% of
223 soluble particles (i.e., remaining in the supernatant after centrifugation of 160 g for 5 min) after 13 g powder have been
224 added to 100 g water at 40°C and mixed in a blender for 90 s after adding two droplets of defoaming agent (octan-1-ol).

225 2.4.8. Viscosity

226 Apparent viscosity was measured using a controlled-stress rheometer (Rheometer, TA DHR2 Hybrid Instruments,
227 Crawley, UK), equipped with a coaxial cylinder geometry and a solvent trap. Temperature was controlled by a Peltier
228 apparatus ($\pm 0.1^{\circ}\text{C}$). Apparent viscosity was measured on homogenized samples at 45°C , corresponding to the process
229 temperature during the homogenization step. The shear rate was set at 1 to 1000 s^{-1} , under steady-state with the coaxial
230 cylinder with a bob diameter of 28 mm and bob length of 41.98 mm.

231 2.4.9. Trypsin inhibitor activity

232 The trypsin inhibitor activity of each protein source was assessed by measuring the enzymatic activity of a pancreatin
233 (porcine pancreatin, P7545, Sigma-Aldrich, St. Quentin Fallavier, France) solution in the presence or absence of the
234 different protein sources used in the present study. For each measurement, 2.6 mL of 0.2 M TRIS buffer pH 8.1, 300 μL of
235 5 mM p-toluene-sulfonyl-L-arginine methyl ester (TAME) solution, 50 μL of pancreatin solution at 40 $\mu\text{g}/\text{ml}$ and 50 μL of
236 protein solution at 1.6 g/100 ml (or 50 μL water for the blank) were introduced in a 4-mL quartz cell with a 1-cm light
237 path, and absorbance was measured at 247 nm for 10 min at 30-s intervals at 25°C . The activity is expressed in TAME
238 units where one unit hydrolyzes 1 mmol of TAME per minute at 25°C .

239 2.5. *In vitro* digestion

240 The meals subjected to *in vitro* digestion were prepared by solubilizing model infant formula powders in water under
241 stirring at 5 Hz for 1 h at 37°C . The *in vitro* digestion model used was set up in order to simulate infant digestion for the

242 full-term newborn at 28 days of life (Ménard et al., 2018). Since IFs are liquid and the time of residence in the mouth is
243 short, the oral phase was omitted. The rationale of the digestive parameters is detailed in Ménard et al. (2018).
244 Briefly, the gastric pH was initially set at 5.3 with 1 M HCl, with a meal:gastric secretion ratio (v:v) of 63:37 based on the
245 dynamic digestion model DIDGI validated for IF digestion where the mean flow rate of secretions was fixed at 0.53
246 ml/min at the half-time gastric emptying of 78 min (Ménard et al., 2014). The gastric secretions were composed of 94 mM
247 NaCl and 13 mM KCl. A quantity of 268 U of pepsin per mL of total gastric content was added to simulate the gastric
248 phase, which lasted for 120 min. Gastric digestion was stopped by raising the pH to 7 with 1 M NaOH. To simulate the
249 intestinal phase, the meal:total secretions (gastric and intestinal) ratio (v:v) was 39:61 (based on an overall mean secretion
250 flow rate of 0.85 ml/min at 78 min of digestion, Ménard et al. 2014) and the pH was adjusted to 6.6 with 1 M HCl. The
251 intestinal secretions were composed of 164 mM NaCl, 10 mM KCl and 85 mM sodium bicarbonate, and adjusted to pH 7.
252 Bovine bile extract was added to a final concentration of 3.1 mM of bile salts. The addition of pancreatin for a trypsin
253 activity of 16 U/mL of intestinal content initiated the intestinal phase, which lasted for 120 min. Both gastric and intestinal
254 phases were completed at 37°C in a water bath under magnetic stirring (300 rpm). For each IF, digestion was carried out in
255 triplicate.

256 Aliquots were collected at 0, 1, 5, 60 and 120 min after the beginning of each digestive phase. Protease inhibitors were
257 then immediately added, namely 10 µL of Pepstatin A (0.72 µM) per ml of gastric digesta or 50 µL of Pefabloc (0.1 M)
258 per ml of intestinal digesta, before storage at -20°C until analysis. Each digested samples were sub-sampled to undertake
259 the different analysis.

260 2.6. Digested sample analysis

261 2.6.1. Degree of hydrolysis (DH)

262 The DH was calculated from the measurement of primary amines released during the *in vitro* digestion. Primary amines
263 were measured in the soluble fraction of samples thawed, obtained after centrifugation for 20 min at 10,000 g and 4°C,
264 using the o-phthaldialdehyde (OPA) method according to Darrouzet-Nardi, Ladd & Weintraub, (2013). The OPA assays
265 were carried out by adding 50 µL of sample to 100 µL of OPA reagent in the wells of a flat-bottom 96-well microtiter
266 plate (Greiner Bio-One, Courtaboeuf, France). The absorbance was measured after exactly 10 min at 340 nm with a
267 Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A calibration curve was
268 prepared using methionine standard solutions (0 to 2 mM). The total free primary amines were determined in each meal
269 before digestion after total acid hydrolysis in 6 N HCl at 110°C for 24 h. The DH was calculated as follows:

$$270 \quad \% \text{ DH} = 100 \times (\text{NH}_{2(t)} - \text{NH}_{2(t0)}) / (\text{NH}_{2(\text{tot})} \times F)$$

271 where $NH_{2(t)}$ is the amount of primary amines after t min digestion (expressed in mg of NH_2 per L of digesta), $NH_{2(0)}$ is the
272 amount of initial primary amines before digestion (meal + secretions) expressed in mg of NH_2 per L of meal diluted with
273 the gastric secretions, $NH_{2(tot)}$ is the maximum amount of primary amines (after total acid hydrolysis of the meal), and F is
274 the dilution factor to express $NH_{2(tot)}$ in mg of NH_2 per L of digesta (F value depends on gastric or intestinal digesta). All
275 measurements were carried out in triplicate.

276 2.6.2. Amino acid bioaccessibility

277 The amino acid bioaccessibility was determined as the percentage of free amino acids at the final digestion time based on
278 the total amino acids in the meal. Free amino acids were determined in samples thawed and previously deproteinized by
279 precipitation with sulfosalicylic acid and centrifugation (5,000 g, 15 min, 4°C). Total amino acids were determined after
280 acid hydrolysis in 6 N HCl at 110°C for 24 h. *In vitro* digested samples and acid hydrolyzed samples were analyzed for
281 amino acid content, as described in Section 2.4.3.

282 2.7. Statistical analysis

283 Statistical analyses were conducted with the use of R version 3.5.2 (The R Foundation, 2014). Regarding the degree of
284 hydrolysis, since the residues of a linear model with two factors (meal and digestion time) were found to be non-normal
285 (using the Kolmogorov-Smirnov test (“lillie.test” from the “nortest” package) (Fernandez, 1992)), a nonparametric
286 analysis for repeated measurements was conducted taking the type of meal and the digestion time (and their interaction)
287 into account with the “fl.lf.f1” function of the package “nparLD” (Noguchi, Gel, Brunner & Konietzschke, 2012). In the
288 event of a significant treatment effect, the function “npar.t.test” or “nparcomp” of the R package “nparcomp”
289 (Konietzschke, Hothorn & Brunner, 2012) was used each time. In the event of a significant interaction effect, a linear mixed
290 effect model with a random intercept on experiments to take account of repeated measurements was performed and
291 followed by the “diffsmeans” of the “lmerTest” package (Kuznetsova, Brockhoff & Christensen, 2017).

292 Regarding the physico-chemical composition of the different IFs, the amino acid profile and the final amino acid
293 bioaccessibility of the IFs, a one-way ANOVA (“anova.lme” function from the “nlme” package) was conducted with meal
294 as the factor, after verifying that the residues of this model were normal with the Kolmogorov-Smirnov test (“lillie.test”
295 from the “nortest” package) (Fernandez, 1992). A post-hoc test (“LSD.test” of the “agricolae” package) was conducted
296 when the differences were significant ($p < 0.05$). Results are expressed as means \pm SDs.

297 3. Results and discussion

298 **As mentioned in the introduction section**, the aim of this study was to assess the possibility of substituting a fraction of
299 cow milk proteins in model IFs with alternative plant protein sources. Four plant proteins, *i.e.*, pea, faba bean, rice and
300 potato were used to design four “plant IFs”. A reference whey protein was used to prepare the “milk-reference IF”. The

301 five **model** IFs were characterized for their biochemical and physical properties before being digested using an *in vitro*
302 static model adapted to infant physiological conditions and evaluated on the nutritional composition, trypsin inhibitor
303 activity, kinetics of proteolysis as well as the EAA bioaccessibility.

304 3.1. The physicochemical properties of plant protein IFs are close the milk reference IF

305 Since data are missing in the literature in terms of biochemical and physical composition of IFs, the values of a 26% fat
306 whole milk powder (WMP) (as described in Shuck et al. (2012)) were used for comparative purposes in the discussion
307 below about the main physicochemical characteristics of the five IFs prepared in this study (Table 2).

308 For all the infant powders, the DM and ash contents were equal to 97.7 ± 0.5 w/w% and 1.7 ± 0.1 w/w%, respectively. The
309 nitrogen, protein and fat contents were equal to 1.8 ± 0.04 w/w% , 10.9 ± 0.6 w/w% and 20.1 ± 0.1 w/w%, respectively,
310 regardless of the infant powder, except for the rice IF, which was 0.3 points below for nitrogen, 2 points below for protein
311 and 3 points below for total fat. In fact, it was noticed that during process (from solubilisation step and particularly during
312 the concentration step), rice protein based IF showed solubility limits with noticeable matter losses that might explain the
313 lower protein and fat contents obtained compared to the other IFs.

314 Free fat content differed between the five IFs, ranging from 5.2 ± 0.7 w/w% to 21.8 ± 3.4 w/w% free fat for faba bean IF
315 and rice IF, respectively. IFs generally contain a relatively large amount of unsaturated and, consequently, oxidizable fatty
316 acids. Hence, it is essential to control lipid stability and encapsulation during storage to ensure their nutritional value and
317 safety (Nasirpour, Scher, & Desobry, 2006). The free fat content should normally remain below 5% for a 26% fat WMP
318 (Vignolles, Jeantet, Lopez, & Schuck, 2007). The free fat of dried milk was considered as surface fat on the powder
319 particles, and the specific surface area of powders is closely related to particle size (Buma, 1971). In the present study, four
320 of the five IFs contained more than 5% free fat, which may be partly explained by the smaller particle size (median
321 diameter of 38.3 ± 3.7 μm) of the powders produced with the pilot spray dryer, in comparison to an industrial powder
322 (median diameter of 60 to 120 μm). This probably led to a higher surface exchange, less fat retained in the particles and,
323 consequently, more free fat released (Buma, 1971). It is suggested that some processing parameters (nozzle size and spray
324 pressure) may influence the free-fat content of spray-dried whole milk (Buma, 1971). Moreover, free fat phenomenon also
325 depends on the emulsifying capacity of the proteins to stabilize the oil droplets by adsorption at the oil-water interface
326 (Damodaran, 1994). (Cao, Wen, Li, & Gu, 2009) reported that the emulsification capacity of rice proteins was minimal at
327 pH 5 and increased significantly while increasing alkalinity or acidity, with a maximum emulsifying volumes of 43 % at
328 pH 11. In the present study, the pH during process was between 6.2-6.8 and 6.8 in the rehydrated IF powders that could
329 explain why rice IF showed the highest free fat value, since its emulsifying capacity was not optimal in these conditions.

330 Spray-drying, storage and quality of milk powder are significantly dependent on both the glass transition temperature (T_g)
331 and the water activity (a_w) (Schuck et al., 2007). The mean water activity (a_w) was 0.15 ± 0.03 , i.e., slightly lower than
332 the optimal value of 0.2 as defined by Efstathiou, Feuardent, Méjean & Schuck (2002) with regard to dry product
333 preservation given that there was a significant difference between the reference and pea IFs, on the one hand, and the faba
334 bean, rice and potato IFs, on the other. Thus, the shelf life and long-term quality of IFs as prepared in the present study
335 could be compromised, notably since lipid oxidation is likely to be favored at a low water activity value (Efstathiou et al.,
336 2002). The glass transition temperature (T_g) values of all the powders were not significantly different, with $49.4 \pm 2.1^\circ\text{C}$
337 as the mean inflexion of the T_g value at 0.2 water activity, regardless of the protein source. For a regular WMP, T_g is
338 usually in the range $42 \pm 2^\circ\text{C}$ at 0.2 water activity (Schuck et al., 2012), i.e., slightly lower than the T_g values measured
339 for the infant powders prepared in this study. This means that these IFs powders could tolerate higher storage temperatures
340 without the risk of powder quality alterations, e.g., caking or stickiness (Pierre Schuck et al., 2007).

341 The dispersibility of the powders ranged between $85.4 \pm 0.6\%$ and $96.4 \pm 0.7\%$ for the potato and rice IFs, respectively.
342 Except for the reference and pea IFs, which had similar dispersibility values, all IFs were significantly different from each
343 other with respect to this criterion. Dispersibility is the capacity of wet aggregates to uniformly disperse in contact with
344 water. WMP is considered dispersible if the dispersibility index is higher than 85% (Schuck et al., 2012). Hence, all the
345 powders prepared in this study could be considered as dispersible. Moreover, the reference and potato IFs were almost
346 100% soluble, pea and faba bean IFs had a solubility of around 96%, and the lowest solubility was measured for the rice IF
347 with 93.5%, keeping in mind that the insoluble part of the rice IF had been lost during processing, as previously
348 mentioned. It has been proved that rice proteins displayed minimum solubility in water in the pH range 4-5, while
349 solubility increased with increasing alkalinity or acidity (Cao et al., 2009; Chittapalo & Noomhorm, 2009; Khan et al.,
350 2013; Romero et al., 2012; Shih & Daigle, 2000; Zhao et al., 2012, 2013). As mentioned, the pH during process was
351 between 6.2-6.8 and around 6.8 in the rehydrated IF powder, closer to neutral than acidic conditions, it can easily explain
352 the solubility limitations observed for rice protein both during process and in the final product. In overall, the solubility
353 represents the loss of granular structure when the powder is solubilized in water. WMP is considered soluble when the
354 solubility index is above $89.5 \pm 2.2\%$ (Schuck et al., 2012). Hence, all the powders produced seemed to be soluble using
355 this evaluating method. However, it is important to mention that powder rehydration kinetics is dependent not only on
356 composition and structure of powders but also on the environmental conditions experienced during process. Thus, powder
357 dissolution could be even enhanced through process parameters improvement such as speed and temperature of mixing
358 (Jeantet, Schuck, Six, Andre, & Delaplace, 2010).

359 Lastly, the colors of the five IFs powders were different from one another, particularly potato IF which was darker (lower
360 L value) than the others. After dispersion in water and homogenization, the viscosity was significantly higher for the
361 potato IF with 5.5 Pa.s, compared to the others whose viscosity ranged from 0.01 to 0.15 Pa.s. The viscosity of a
362 concentrate to be dried influences the quality of the powder (bulk density, solubility, etc.) by varying the size of the spray
363 droplets (Pierre Schuck, Méjean, Dolivet, Beaucher, & Famelart, 2005). For an optimal spray, the viscosity of the
364 concentrate being dried for infant formula should be around 60 mPa.s (Vestergaard, 2004) and should not exceed 200
365 mPa.s to allow subsequent spray drying. This means that potato IF's viscosity was far too high to an optimal drying (more
366 than 20 times than the recommendations), thus its parameters should be adjusted to improve powder quality.
367 To sum up, it seems possible to produce IFs in which cow milk proteins are partially replaced by plant proteins, without
368 deviating too much from an exclusively reference-milk formula with regard to the key physicochemical criteria usually
369 considered standard for a spray-dried powder IF. However, the rice IF showed technological and functional issues that
370 would lead to lower production efficiency and would therefore not be an appropriate candidate to replace whey proteins in
371 IFs. Moreover, potato IF showed an extremely high viscosity that should be optimized further in order to ensure optimal
372 drying.

373 3.2. Plant protein sources are able to cover the minimum regulatory nutritional needs

374 As mentioned above, one of the criteria to ensure the nutritional quality of the modified IFs is to cover the nutritional
375 needs of infants. Consequently, the energy for 100 ml of IF, as well as the protein, fat and carbohydrate contents for 100
376 kcal of IF were all in agreement with the European regulation (EU, 2016) (Table 3). Similarly, the EAA content was
377 measured in the five IFs on the basis of a constant protein quantity fixed at 2.4 g protein/100 kcal of IF, and were all in
378 agreement with the European regulation (EU, 2016) with significantly higher EAA content than the standard protein.
379 However, the EAA tryptophan could not be quantified with the method used for AA analysis (section 2.4.3). Since
380 tryptophan has a paramount role in infant nutrition (Heine et al. 1999), it would be therefore necessary to determine further
381 its content in the innovative IFs designed in this study in order to confirm its agreement with the European regulation
382 requirements (EU, 2016).

383 3.3. Three of the four plant proteins do not inhibit porcine trypsin

384 Another key criterion for the nutritional quality of the alternative protein sources is the absence of anti-nutritional factors
385 and, especially, the absence of inhibitors of digestive enzymes. Despite the fact that it was impossible to address this issue
386 for all of the digestive enzymes, it was dealt with by measuring trypsin inhibition. Indeed, plant protein extracts are known
387 to contain trypsin inhibitors, which could be a risk for human nutrition and, even more, for infant nutrition (Sarwar, Wu &
388 Cockell, 2012).

389 The activity of porcine trypsin did not significantly differ when measured in the presence of whey proteins (used in the
390 milk-reference IF), pea, faba bean and rice protein concentrates, with comparable value to the control (105.1 ± 5.0 U/mg).
391 On the contrary, it was significantly lower (24.1 ± 2.8 U/mg) when measured in the presence of potato protein (Table 4).
392 This result suggests that only the potato protein used in the present study contained porcine trypsin inhibitors.
393 However, porcine trypsin was used in the present test, and not human trypsin. Since inhibitors are specific to each enzyme,
394 the present results do not offer evidence of the presence or absence of inhibitors of human digestive enzymes in the plant
395 protein sources studied here. Actually, Feeney, Means and Bigler (1968) did not report any inhibition activity against
396 human trypsin in potato protein, whereas bovine trypsin, and even more so, bovine chymotrypsin, were inhibited in the
397 same conditions (porcine trypsin was not analyzed in that study). Moreover, a recent study explained that *in vitro* protein
398 digestibility determined by porcine tryptic hydrolysis should be almost two times higher than the one determined by
399 bovine or human tryptic hydrolysis (Deng, Gruppen & Wierenga, 2018). Thus, if low digestibility is reported in the
400 presence of potato protein in this study, it could be explained by the results reported in Table 3, but it will not mean that
401 the same results would be observed in the presence of human enzymes.

402 3.4. Pea and faba bean IFs are equivalent to the milk-reference IF with respect to *in vitro* proteolysis

403 The kinetics of proteolysis was determined from the quantification of the free primary amines detected in the soluble
404 fraction of the digested IFs divided by the free primary amines measured in the IF after total acidic hydrolysis
405 (corresponded to the maximum hydrolysis rate). The degree of hydrolysis (DH) is defined as the proportion of cleaved
406 peptide bonds in a protein (Rutherford, 2010). During the gastric phase of *in vitro* digestion, the proteolysis was very
407 limited (DH < 2% at the end of the gastric phase and corresponding to the time 0 min on Fig. 2). Low proteolysis during
408 gastric digestion is explained by a reduced pepsin secretion coupled with a higher gastric pH (pH 5.3 used to simulate the
409 gastric compartment in the present study vs pH 2 for pepsin optimal activity) in infant's stomach (Agunod, Yamaguchi,
410 Lopez, Luby, & Glass, 1969; Davidson & Lönnerdal, 1987; Henderson, Hamosh, Armand, Mehta, & Hamosh, 2001;
411 Johnson, 2014).

412 Then, as soon as the intestinal enzymes were added, proteolysis drastically increased for all formulas, except for that of
413 potato (Fig. 2). For pea, faba bean, rice and potato IFs, proteolysis continued to increase before reaching a plateau at 60
414 min of intestinal digestion, whereas proteolysis continued to increase until the end of the intestinal phase (120 min) for the
415 milk-reference IF. At the end of the intestinal digestion, DH ranged from $28.8 \pm 3.3\%$ to $51.4 \pm 3.2\%$ for potato and pea
416 IFs, respectively. During the entire intestinal phase, the pea IF showed a DH higher than or similar to the milk-reference IF
417 and significantly higher than the rice and the potato IFs. However, proteolysis was equal ($p > 0.05$) for the reference, pea

418 and faba bean IFs at the end of the intestinal digestion. In contrast, rice and potato IFs were less hydrolyzed at the end of *in*
419 *vitro* digestion compared to the three other IFs ($p < 0.05$).

420 The present results are comparable to those reported by He, Spelbrink, Witteman and Giuseppin (2013) who studied potato
421 protein (the same source as the one used in the present study) in solution in comparison to different reference proteins
422 (whey, soy and pea). These authors studied the *in vitro* digestibility with a static model at the adult stage and showed that,
423 at the end of digestion, whey proteins had the highest DH value (60%), whereas the proteolysis of potato, soy, and pea
424 proteins were similarly lower (30% DH value). Proteolysis is expected to be limited under infant conditions compared to
425 adult conditions since enzyme concentrations are much lower in the infant model (eight times less pepsin units/g of
426 proteins). Moreover, as mentioned, in the infant model compared to the adult one, the pH value is higher in the gastric
427 phase (pH 5.3 vs. pH 3). However, we assume that the classification of the protein DH should be the same for infant and
428 adult models, which is not the case for the pea protein IF that showed the same DH as whey protein IF and is significantly
429 higher than potato protein IF in He et al. (2013). This difference observed for pea IF could be explained by the sensitivity
430 of pea protein to the different process steps occurred in the present study (pasteurization, concentration, homogenization
431 and spray-drying) that can improve its digestibility by unfolding the protein and allowing greater access of gastrointestinal
432 enzymes for hydrolysis (Ma, Boye, & Hu, 2017).

433 3.5. Pea and faba bean IFs are equivalent to the milk-reference IF with respect to *in vitro* essential amino acid 434 bioaccessibility

435 The overall trend showed similar EAA bioaccessibility for the milk-reference, pea and faba bean IFs which were all
436 significantly higher than those found for the potato IFs (Fig. 3). Rice IF showed an intermediate profile with significantly
437 lower EAA released for leucine, isoleucine, lysine, phenylalanine, valine, threonine and tyrosine compared to the milk-
438 reference, pea and faba bean IFs. These results are in accordance with the proteolysis degrees reported above (Section 3.4)
439 where lower DH values were found for rice and potato IFs compared to the three other IFs.

440 Several studies highlight the resistance of cow milk whey proteins to gastric digestion whereas they are more extensively
441 degraded during intestinal phase (Bourlieu et al., 2015; Bouzerzour et al., 2012), which likely explains the high
442 bioaccessibility of EAA observed in the milk-reference IF. Similarly, Nguyen et al. (2015) study the digestion of cow milk
443 based IFs (with different casein to whey protein ratios) and soy IF using an *in vitro* static model (pH drop method) adapted
444 to infant conditions. The authors showed that IF containing higher amount of caseins had a more rapid digestion compared
445 to IF with more whey protein content after 2 hours of intestinal digestion. This suggests that in the small intestine
446 proteases hydrolyse caseins quicker than whey proteins. This difference in digestibility can be related to the difference in
447 the structure and composition of casein and whey proteins. Due to the high degree of phosphorylation, caseins have an

448 open structure (Holt, Carver, Ecroyd, & Thorn, 2013; Swaisgood, 1993) and are sensitive to proteolysis. However, the
449 presence of phosphorylated peptides surviving casein digestion can also create specific areas that resist to proteolysis
450 (Cattaneo, Stuknytė, Ferraretto, & De Noni, 2017), even during *in vitro* digestion with infant conditions (Dupont et al.,
451 2009). In contrast, native whey proteins contain a high amount of cysteine that create disulphide bonds making whey
452 proteins a compact structure that restricts the action of digestive proteases (Lacroix et al., 2006). At the same time, the
453 effect of processing (heat-treatment) on whey proteins has been reported to enhance β -Lactoglobuline digestibility as the
454 protein unfold due to heat treatment above 65°C and thus became more sensitive to proteolysis (Mandalari, Mackie,
455 Rigby, Wickham, & Mills, 2009). Finally, the specificity of caseins and whey proteins as well as their modification
456 occurring upon processing treatment are factors affecting their digestibility. In the present study, whey proteins might be
457 partly denatured due to processing treatment and thus explained the higher amount of free amino acids released after the
458 digestion of the reference infant formula. However, caseins are present in the same amount in each infant formulas but its
459 interaction with the other proteins can be different and thus modify the sensitivity of each infant formula during digestion.
460 It is also well known that plant-based proteins are less digestible than animal proteins due to difference in terms of
461 structure. In fact, the secondary structure of plant proteins is characterized by a high content in β -sheet conformation and a
462 relatively low α -helix amount compared to that of animal proteins, it is particularly the case for legume proteins such as
463 soy, pea and faba bean proteins (Carbonaro, Maselli, & Nucara, 2012). The high content in β -sheet conformation is related
464 to its resistance to proteolysis in the gastrointestinal tract since hydrophobic β -sheet structure facilitates protein
465 aggregation resulting in decreasing digestibility (Carbonaro et al., 2012; Nguyen et al., 2015). Moreover, heat treatment
466 during processing has also been reported to cause β -sheet aggregation among molecules and have effect on the resistance
467 to digestion of proteins (Carbonaro et al., 2012; Carbonaro, Maselli, & Nucara, 2015). Contrary to legume proteins, cow
468 milk proteins present very little secondary structure and are mainly based on an association of β -sheet and α -helix
469 structures only coming from whey proteins (Permyakov & Berliner, 2000). Since the IFs in the present study are all
470 composed of a mix of cow milk proteins and either whey proteins in the milk-reference IF or plant proteins in the plant-
471 based IFs, the impact of the secondary structure dominated by β -sheets on protein digestibility should be limited for the
472 milk-reference IF, pea and faba bean IFs, and thus explain their relatively similar EAA bioaccessibility profile (Fig. 3). The
473 lower proteolysis and EAA release measured for the rice IF in the present study, in comparison to the reference, pea and
474 faba bean IFs, is in accordance with Gastanduy, Cordano & Graham (1990). These authors reported that the *in vivo*
475 digestibility of IF based on high protein rice flour was lower than cow's milk-derived formulas, resulting in a low content
476 of plasma AAs.

477 Lastly, despite the fact that potato protein has a balanced composition of EAA to meet the nutritional requirements of
478 infants (Table 3), the present study highlighted a very low level of EAA released under *in vitro* digestion conditions. In
479 accordance with the present results, He et al. (2013) reported a limited postprandial plasma levels of AAs for potato
480 protein which was at least two times lower than for whey protein. This lower EAA release could also be explained by the
481 high trypsin inhibitor activity found in potato protein (Table 4).

482 4. Conclusion

483 This is the first time that **model** IFs, containing plant proteins other than soy and hydrolyzed rice, have been reported,
484 designed and their behaviour during digestion investigated.

485 In the present study, the feasibility of producing plant protein-based IFs close to a milk-reference IF in terms of physico-
486 chemical and functional properties was demonstrated. Only the rice protein source showed solubility limits that negatively
487 impacted IF production of this protein source was also limited. Moreover, potato IF showed an extremely high viscosity
488 that would not be optimal for the drying, thus should be adjusted to ensure a better powder quality. Further experiments at
489 a semi-industrial scale will make it possible to confirm these results in a more representative way.

490 In terms of nutritional quality, the *in vitro* static digestion model made it possible to compare the five IFs taking most of
491 the immaturity specificities of infant digestion into account. The type of protein sources tested in the present study had an
492 impact on the degree of protein hydrolysis and on the EAA bioaccessibility, which together account for digestibility. The
493 pea IF showed similar and even higher *in vitro* digestibility than the milk-reference formula; the faba bean IF was also
494 very close to the reference with respect to this criterion. However, the rice IF, and even more so, the potato IF showed
495 lower *in vitro* digestibility. Consequently, rice and potato proteins would not be appropriate candidates to partially replace
496 whey proteins in IFs from a nutritional point of view.

497 However, one should keep in mind that enzymes from different species behave differently and thus, such hypothesis on *in*
498 *vitro* digestibility value of IFs studied with porcine enzymes have to be furthered with *in vivo* data closer to infant
499 physiological conditions. Moreover, both the gastric emptying and the continuous secretion of digestive enzymes and
500 hydrochloric acid were not simulated in the present *in vitro* static conditions. For those reasons and because *in vivo*
501 experiments are difficult to perform (ethical, financial and time-consuming reasons) *in vitro* digestion experiments within
502 dynamic conditions will be conducted with the milk-reference, pea and faba bean IFs in order to even more accurately
503 reproduce infant physiological conditions and to confirm that it seems possible to produce plant protein based IFs on a
504 functional and a nutritional points of view close to a milk-reference IF.

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734 Table 1. Nutritional composition of the ingredients for infant formula development (DM: dry matter)

Ingredient name	Skim Milk	Lactose	Maltodextrin	Lactarmor™ DM 90	Protarmor™ 80	Nutralys® XF	Vitessence™ Pulse	RicePro NG BIO	Solanic® 200	Oil blend
Dry matter (%)	96.1	96.2	95.0	95.1	94.2	94.7	95.2	97.2	96.3	99.9
Protein (w/w% DM)	35.1	0.01	0.04	12.0	81.4	71.7	60.9	71.8	84.2	NA
Fat (w/w% DM)	1.1*	NA	NA	1.0*	7.0*	NA	4*	8*	NA	91.1*
Carbohydrates (w/w% DM)	54.5*	96.0*	94.9*	81.6*	4.2*	NA	24*	NA	3.8*	NA
Ashes (w/w% DM)	5.5*	0.2*	0.03*	0.4*	1.6*	5*	NA	6*	NA	NA

735 *data from suppliers
 736 NA: not available data
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763 Table 2. Biochemical and physical composition of the five infant formulas (IFs). Data are means \pm SD. Values with a different
 764 superscript letter for each characteristic and between the five IFs are significantly different ($p < 0.05$).

	Reference IF	Pea IF	Faba bean IF	Rice IF	Potato IF
Total DM (w/w%)	98.3 \pm 0.01 ^a	98.1 \pm 0.1 ^a	96.9 \pm 0.2 ^a	97.9 \pm 0.1 ^a	97.5 \pm 1.1 ^a
Ashes (w/w% DM)	1.7 \pm 0.02 ^a	1.7 \pm 0.02 ^a	1.8 \pm 0.01 ^a	1.6 \pm 0.01 ^a	1.6 \pm 0.03 ^a
Total nitrogen (w/w% DM)	1.8 \pm 0.03 ^a	1.8 \pm 0.01 ^a	1.8 \pm 0.01 ^a	1.5 \pm 0.02 ^b	1.8 \pm 0.01 ^a
Total protein (w/w% DM)	11.7 \pm 0.2 ^a	10.8 \pm 0.07 ^b	10.4 \pm 0.07 ^b	8.7 \pm 0.03 ^c	10.7 \pm 0.04 ^b
Total fat (w/w% DM)	20.1 \pm 0.1 ^a	20.1 \pm 0.1 ^a	21.1 \pm 0.1 ^a	17.2 \pm 0.1 ^b	20.9 \pm 1.4 ^a
Free fat (w/w% total fat)	8.1 \pm 0.5 ^c	14.1 \pm 0.01 ^b	5.2 \pm 0.7 ^c	21.8 \pm 3.4 ^a	6.2 \pm 0.8 ^c
d(0.5) (μ m)	34.9 \pm 0.4 ^a	35.9 \pm 0.6 ^a	37.2 \pm 0.1 ^a	35.9 \pm 0.6 ^a	36.0 \pm 0.1 ^a
a_w	0.12 \pm 0.01 ^c	0.11 \pm 0.01 ^c	0.18 \pm 0.01 ^a	0.16 \pm 0.02 ^a	0.17 \pm 0.09 ^a
Tg ($^{\circ}$ C)	47.0 \pm 2.0 ^a	47.3 \pm 3.1 ^a	50.9 \pm 3.8 ^a	51.1 \pm 3.9 ^a	50.7 \pm 1.6 ^a
Solubility (%)	100.0 \pm 0.1 ^a	96.0 \pm 0.1 ^b	96.0 \pm 0.1 ^b	93.5 \pm 1.8 ^c	99.5 \pm 0.4 ^a
Dispersibility (%)	88.3 \pm 0.6 ^c	88.4 \pm 0.2 ^c	90.7 \pm 2.5 ^b	96.4 \pm 0.7 ^a	85.4 \pm 1.4 ^d
Viscosity (Pa.s)	0.03 \pm 0.01 ^b	0.04 \pm 0.02 ^b	0.15 \pm 0.01 ^b	0.01 \pm 0.01 ^b	5.4 \pm 0.43 ^a
Color parameters					
L	75.9 \pm 0.1 ^a	73.1 \pm 0.3 ^b	73.2 \pm 0.4 ^b	73.4 \pm 0.9 ^b	66.4 \pm 0.9 ^c
a	-3.1 \pm 0.2 ^c	-2.3 \pm 0.2 ^b	-3.8 \pm 0.1 ^c	-2.0 \pm 0.2 ^b	0.8 \pm 0.3 ^a
b	9.8 \pm 0.5 ^d	13.3 \pm 0.5 ^b	15.9 \pm 0.1 ^a	9.5 \pm 0.6 ^d	11.0 \pm 0.6 ^a

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786 Table 3. Nutritional composition of the five infant formulas (IFs) compared to the European regulation. Data are means \pm SD. Values
 787 with a different superscript letter for each essential amino acid (EAA) are significantly different ($p < 0.05$).

	European regulation	Reference IF	Pea IF	Faba bean IF	Rice IF	Potato IF
Energy (kcal / 100 ml)	60-70			66.5 \pm 1.7		
Protein (g / 100 kcal)	1.8-2.8 ¹			2.4 \pm 0.2		
Fat (g / 100 kcal)	4.4-6.0			4.4 \pm 1.2		
Carbohydrates (g / 100 kcal)	9-14			12.8 \pm 0.9		
EAA content in the IFs compared to the European regulation ² (mg amino acid/100 kcal)						
Tyrosine	76 ^d	88.5 \pm 3.1 ^{bc}	91.7 \pm 3.4 ^b	86.6 \pm 1.2 ^c	108.5 \pm 4.1 ^a	116.2 \pm 7.2 ^a
Lysine	113 ^c	227.6 \pm 0.2 ^a	188.4 \pm 6.7 ^{bc}	173.0 \pm 0.5 ^c	156.4 \pm 3.1 ^d	198.0 \pm 3.8 ^b
Phenylalanine	83 ^d	102.0 \pm 1.0 ^c	121.3 \pm 3.6 ^b	108.3 \pm 1.4 ^c	126.1 \pm 0.2 ^b	136.9 \pm 1.9 ^a
Leucine	166 ^f	270.4 \pm 0.2 ^a	219.4 \pm 2.6 ^d	205.2 \pm 0.2 ^c	232.4 \pm 2.6 ^c	254.8 \pm 1.4 ^b
Isoleucine	90 ^e	150.9 \pm 2.4 ^a	124.9 \pm 7.5 ^{cd}	114.5 \pm 1.0 ^d	124.6 \pm 3.8 ^c	131.8 \pm 1.2 ^b
Methionine	23 ^d	67.4 \pm 1.4 ^b	60.2 \pm 4.8 ^b	48.1 \pm 3.8 ^c	77.0 \pm 2.6 ^{ab}	81.8 \pm 2.2 ^a
Valine	88 ^d	149.2 \pm 0.7 ^b	139.5 \pm 2.6 ^{bc}	127.8 \pm 1.2 ^c	158.6 \pm 0.7 ^a	144.1 \pm 2.2 ^b
Histidine	40 ^c	56.5 \pm 0.7 ^b	59.9 \pm 6.7 ^{ab}	60.4 \pm 2.6 ^a	62.1 \pm 4.6 ^a	58.0 \pm 0.5 ^{ab}
Threonine	77 ^c	144.4 \pm 0.2 ^a	94.8 \pm 4.1 ^b	103.2 \pm 1.7 ^b	107.3 \pm 0.7 ^b	148.9 \pm 2.6 ^a

788 ¹1.8 g corresponded to the minimum value of protein content when using cow's or goat's milk proteins and 2.8 g is the maximum value
 789 of protein content when using soy protein isolate or hydrolysed proteins as protein source (EU, 2016). This reference range of values
 790 was chosen since the IFs in the present study were based on a mix of cow's milk and plant proteins and thus should meet both
 791 requirements.

792 ²The EAA composition of the European regulation corresponds to the minimum amount to meet the requirements for IFs based on cow's
 793 or goat's milk proteins and soy protein isolates alone or mixed with cow's or goat's milk proteins (EU, 2016)

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817 Table 4. Porcine trypsin activity (U/mg) measured in the presence of each protein source in solution and the substrate only (control).
818 Data are means \pm SD. Values with a different superscript letter are significantly different ($p < 0.05$).

	Control	Reference ¹	Pea	Faba bean	Rice	Potato
Trypsin activity (U/mg)	105.1 \pm 5.0 ^a	108.5 \pm 0.9 ^a	108.6 \pm 0.5 ^a	109.2 \pm 3.4 ^a	107.4 \pm 1.1 ^a	24.1 \pm 2.8 ^b

819 ¹The reference protein corresponded to whey protein in the milk-reference IF of the present study

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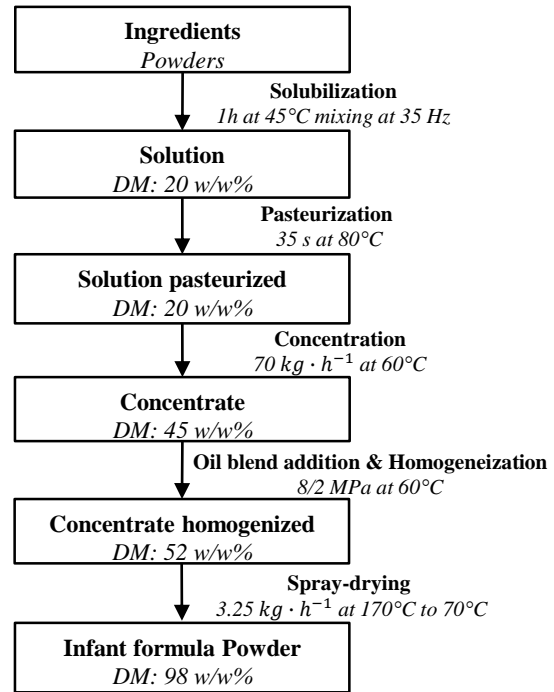


Fig. 1

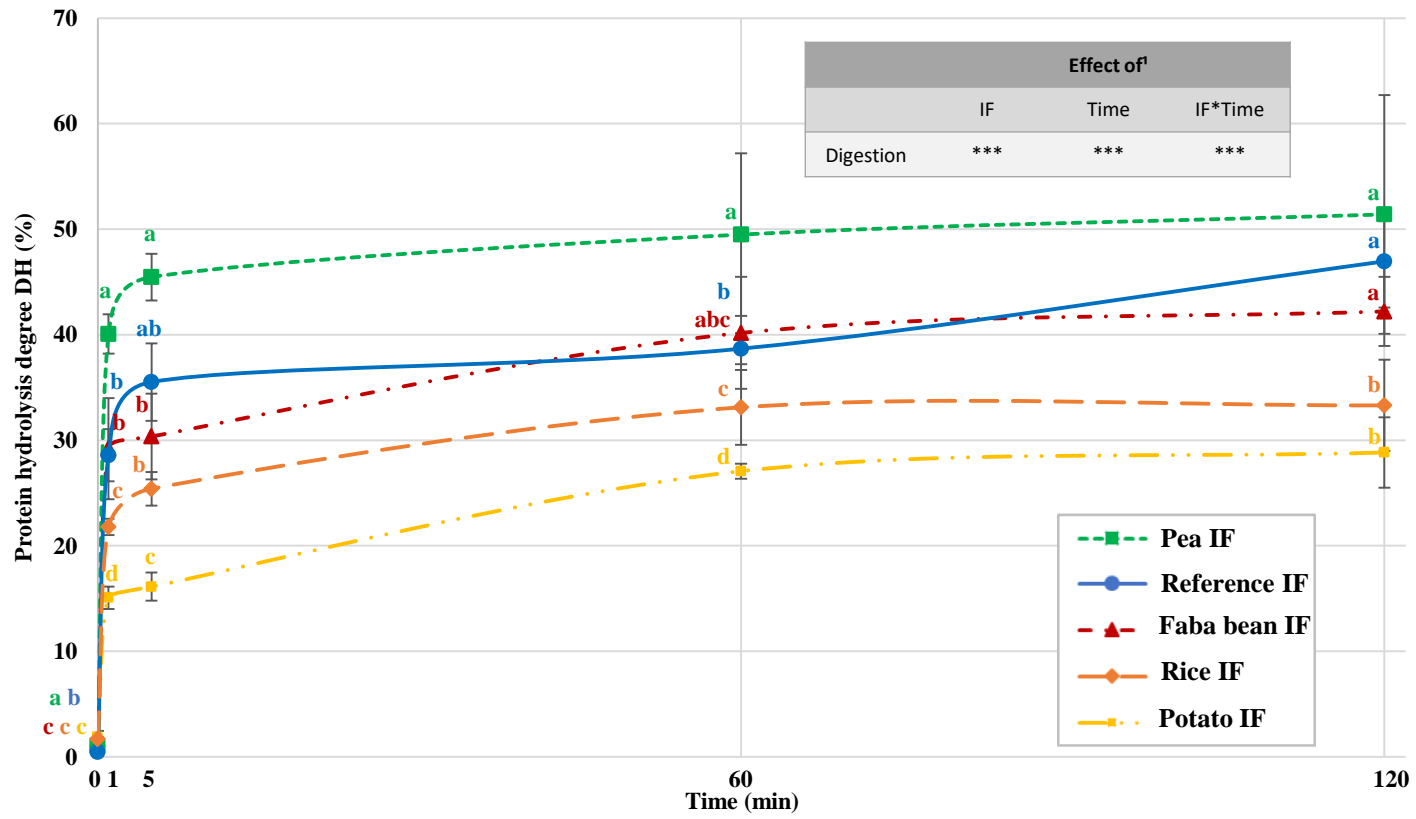


Fig. 2

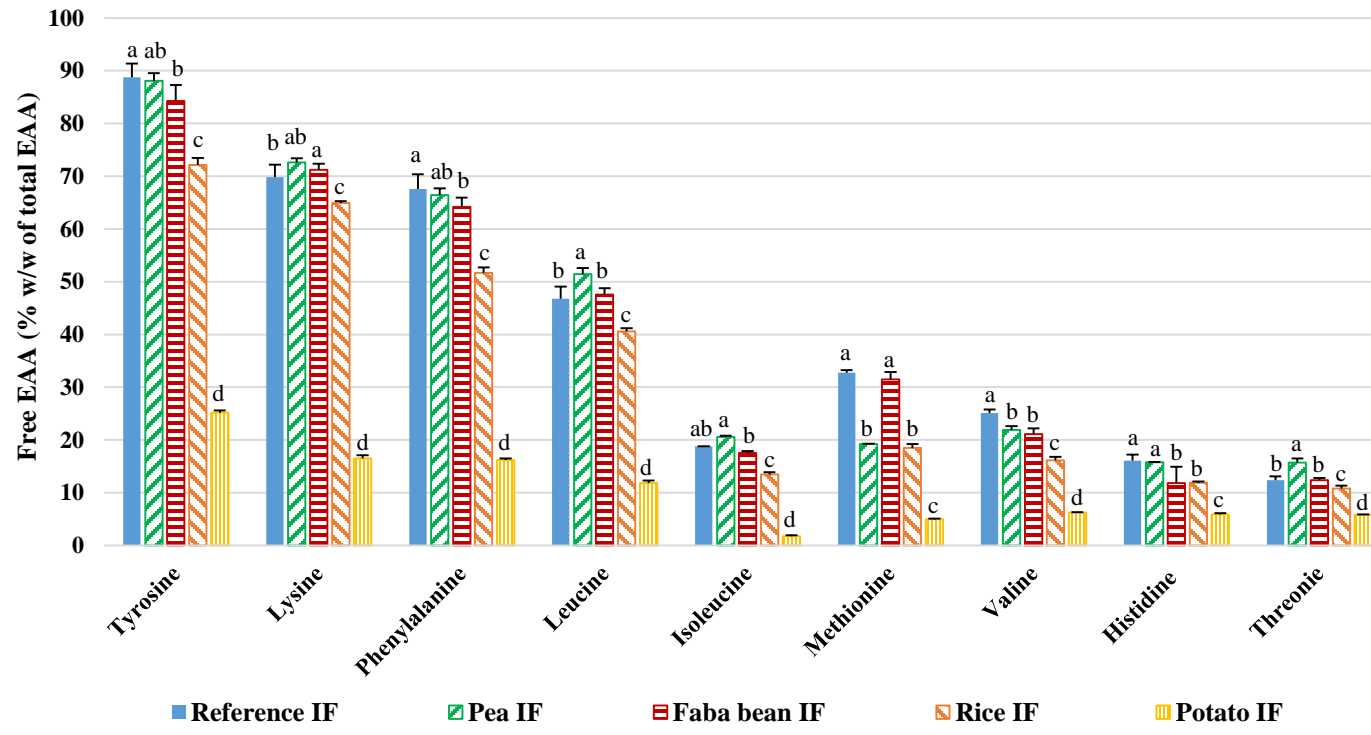


Fig. 3

