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Abbreviations

IFs: infant formulas

DH: hydrolysis degree

AAB: amino acid bioaccessibility

AA: amino acids

EAA: essential amino acids

DM: dry matter

w/w: weight/weight

a_w: water activity

Tg: glass transition temperature

v/v: volume/volume

OPA: o-phthaldialdehyde

SD: standard deviation

WMP: whole milk powder

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Abstract

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

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

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1. Introduction

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

Protein intake early in life is essential for the development of infants, affecting growth, body composition, neurodevelopment, appetite and hormonal regulation (Michaelsen & Greer, 2014). Protein requirements for infants are 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 point of view, human milk is the gold standard for the newborn, and breastfeeding is highly recommended for the first six months of life (Victora et al., 2016). However, for many reasons, mothers may be unable to provide human milk and a milk replacer formula can be used instead (Agostoni et al., 2008). According to the applicable European regulation, the sources of proteins allowed for IFs are either cow milk protein, goat milk protein, soy protein isolate or hydrolysed rice protein (European Union, 2016). IF should provide similar amounts of EAA, as close as possible to those found in human milk.

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

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

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

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

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

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

2. Materials and Methods

2.1. Chemicals

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

2.2. Model infant formula ingredients

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

2.3. Model infant formula processing

Skim cow milk powder, lactose, maltodextrin and the different protein concentrates (whey protein as the reference and potato, rice, pea or faba bean proteins as the plant protein sources) were solubilized in water at 20 w/w% DM (dry matter; 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

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

2.4. Infant formula characterization

2.4.1. Dry matter, ash and protein content

Total DM was determined gravimetrically after heating at 102 ± 2°C for 7 h, and ash content after incineration at 525 ± 25°C in a muffle furnace, both according to the methods of Schuck, Dolivet & Jeantet (2012).

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

2.4.2. Fat and free fat content

The total fat content was measured by Gerber's acid-butyrometric method after dissolution of proteins by the addition of 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-µm 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 ± 0.1°C using the Novasina aw-meter (Novasina, Switzerland).
204 In order to determine the glass transition temperature (Tg), the powders were first equilibrated in a 20% relative humidity
205 atmosphere using the SPSx-1µ Sorption Test System (ProUmid GmbH & Co. KG, August-Nagel-Str., Germany). The Tg
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 mm 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

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

2.4.6. Color

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

2.4.7. Rehydration properties

Dispersibility and solubility were determined according to Schuck et al. (2012). The dispersibility index is the amount of 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 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 soluble particles (i.e., remaining in the supernatant after centrifugation of 160 g for 5 min) after 13 g powder have been 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).

2.4.8. Viscosity

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

2.4.9. Trypsin inhibitor activity

The trypsin inhibitor activity of each protein source was assessed by measuring the enzymatic activity of a pancreatin (porcine pancreatin, P7545, Sigma-Aldrich, St. Quentin Fallavier, France) solution in the presence or absence of the 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 5 mM p-toluene-sulfonyl-L-arginine methyl ester (TAME) solution, 50 µL of pancreatin solution at 40 µg/ml and 50 µL of 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 path, and absorbance was measured at 247 nm for 10 min at 30-s intervals at 25°C. The activity is expressed in TAME units where one unit hydrolyzes 1 mmol of TAME per minute at 25°C.

2.5. *In vitro* digestion

The meals subjected to *in vitro* digestion were prepared by solubilizing model infant formula powders in water under 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 $$\% \text{ DH} = 100 \times (NH_{2(t)} - NH_{2(t0)}) / (NH_{2(tot)} \times F)$$

where $NH_{2(t)}$ is the amount of primary amines after t min digestion (expressed in mg of NH_2 per L of digesta), $NH_{2(t0)}$ is the amount of initial primary amines before digestion (meal + secretions) expressed in mg of NH_2 per L of meal diluted with the gastric secretions, $NH_{2(tot)}$ is the maximum amount of primary amines (after total acid hydrolysis of the meal), and F is 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 measurements were carried out in triplicate.

2.6.2. Amino acid bioaccessibility

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

2.7. Statistical analysis

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

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

3. Results and discussion

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

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

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

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

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 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, regardless of the infant powder, except for the rice IF, which was 0.3 points below for nitrogen, 2 points below for protein and 3 points below for total fat. In fact, it was noticed that during process (from solubilisation step and particularly during the concentration step), rice protein based IF showed solubility limits with noticeable matter losses that might explain the lower protein and fat contents obtained compared to the other IFs.

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

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

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

Lastly, the colors of the five IFs powders were different from one another, particularly potato IF which was darker (lower L value) than the others. After dispersion in water and homogenization, the viscosity was significantly higher for the 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 concentrate to be dried influences the quality of the powder (bulk density, solubility, etc.) by varying the size of the spray droplets (Pierre Schuck, Méjean, Dolivet, Beaucher, & Famelart, 2005). For an optimal spray, the viscosity of the concentrate being dried for infant formula should be around 60 mPa.s (Vestergaard, 2004) and should not exceed 200 mPa.s to allow subsequent spray drying. This means that potato IF's viscosity was far too high to an optimal drying (more than 20 times than the recommendations), thus its parameters should be adjusted to improve powder quality.

To sum up, it seems possible to produce IFs in which cow milk proteins are partially replaced by plant proteins, without deviating too much from an exclusively reference-milk formula with regard to the key physicochemical criteria usually considered standard for a spray-dried powder IF. However, the rice IF showed technological and functional issues that would lead to lower production efficiency and would therefore not be an appropriate candidate to replace whey proteins in IFs. Moreover, potato IF showed an extremely high viscosity that should be optimized further in order to ensure optimal drying.

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

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

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

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

The activity of porcine trypsin did not significantly differ when measured in the presence of whey proteins (used in the milk-reference IF), pea, faba bean and rice protein concentrates, with comparable value to the control (105.1 ± 5.0 U/mg). On the contrary, it was significantly lower (24.1 ±2.8 U/mg) when measured in the presence of potato protein (Table 4). This result suggests that only the potato protein used in the present study contained porcine trypsin inhibitors.

However, porcine trypsin was used in the present test, and not human trypsin. Since inhibitors are specific to each enzyme, the present results do not offer evidence of the presence or absence of inhibitors of human digestive enzymes in the plant protein sources studied here. Actually, Feeney, Means and Bigler (1968) did not report any inhibition activity against human trypsin in potato protein, whereas bovine trypsin, and even more so, bovine chymotrypsin, were inhibited in the same conditions (porcine trypsin was not analyzed in that study). Moreover, a recent study explained that *in vitro* protein digestibility determined by porcine tryptic hydrolysis should be almost two times higher than the one determined by bovine or human tryptic hydrolysis (Deng, Gruppen & Wierenga, 2018). Thus, if low digestibility is reported in the presence of potato protein in this study, it could be explained by the results reported in Table 3, but it will not mean that the same results would be observed in the presence of human enzymes.

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

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

Then, as soon as the intestinal enzymes were added, proteolysis drastically increased for all formulas, except for that of potato (Fig. 2). For pea, faba bean, rice and potato IFs, proteolysis continued to increase before reaching a plateau at 60 min of intestinal digestion, whereas proteolysis continued to increase until the end of the intestinal phase (120 min) for the milk-reference IF. At the end of the intestinal digestion, DH ranged from 28.8 ± 3.3% to 51.4 ± 3.2% for potato and pea IFs, respectively. During the entire intestinal phase, the pea IF showed a DH higher than or similar to the milk-reference IF 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 bioaccessbility 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.

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

4. Conclusion

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

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

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

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

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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

*data from suppliers

NA: not available data

Table 2. Biochemical and physical composition of the five infant formulas (IFs). Data are means ± SD. Values with a different 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 ± 0.01^{a}	98.1 ± 0.1^{a}	96.9 ± 0.2^{a}	97.9 ± 0.1^{a}	97.5 ± 1.1^{a}
Ashes (w/w% DM)	1.7 ± 0.02^{a}	1.7 ± 0.02^{a}	1.8 ± 0.01^{a}	1.6 ± 0.01^{a}	1.6 ± 0.03^{a}
Total nitrogen (w/w% DM)	1.8 ± 0.03^{a}	1.8 ± 0.01^{a}	1.8 ± 0.01^{a}	1.5 ± 0.02^{b}	1.8 ± 0.01^{a}
Total protein (w/w% DM)	11.7 ± 0.2^{a}	10.8 ± 0.07^{b}	10.4 ± 0.07^{b}	8.7 ± 0.03^{c}	10.7 ± 0.04^{b}
Total fat (w/w% DM)	20.1 ± 0.1^{a}	20.1 ± 0.1^{a}	21.1 ± 0.1^{a}	17.2 ± 0.1^{b}	20.9 ± 1.4^{a}
Free fat (w/w% total fat)	8.1 ± 0.5^{c}	14.1 ± 0.01^{b}	5.2 ± 0.7^{c}	21.8 ± 3.4^{a}	6.2 ± 0.8^{c}
d(0.5) (μm)	34.9 ± 0.4^{a}	35.9 ± 0.6^{a}	37.2 ± 0.1^{a}	35.9 ± 0.6^{a}	36.0 ± 0.1^{a}
a_w	0.12 ± 0.01^{c}	0.11 ± 0.01^{c}	0.18 ± 0.01^{a}	0.16 ± 0.02^{a}	0.17 ± 0.09^{a}
Tg (°C)	47.0 ± 2.0^{a}	47.3 ± 3.1^{a}	50.9 ± 3.8^{a}	51.1 ± 3.9^{a}	50.7 ± 1.6^{a}
Solubility (%)	100.0 ± 0.1^{a}	96.0 ± 0.1^{b}	96.0 ± 0.1^{b}	93.5 ± 1.8^{c}	99.5 ± 0.4^{a}
Dispersibility (%)	88.3 ± 0.6^{c}	88.4 ± 0.2^{c}	90.7 ± 2.5^{b}	96.4 ± 0.7^{a}	85.4 ± 1.4^{d}
Viscosity (Pa.s)	0.03 ± 0.01^{b}	0.04 ± 0.02^{b}	0.15 ± 0.01^{b}	0.01 ± 0.01^{b}	5.4 ± 0.43^{a}
Color parameters					
L	75.9 ± 0.1^{a}	73.1 ± 0.3^{b}	73.2 ± 0.4^{b}	73.4 ± 0.9^{b}	66.4 ± 0.9^{c}
a	-3.1 ± 0.2^{c}	-2.3 ± 0.2^{b}	-3.8 ± 0.1^{c}	-2.0 ± 0.2^{b}	0.8 ± 0.3^{a}
b	9.8 ± 0.5^{d}	13.3 ± 0.5^{b}	15.9 ± 0.1^{a}	9.5 ± 0.6^{d}	11.0 ± 0.6^{a}

Table 3. Nutritional composition of the five infant formulas (IFs) compared to the European regulation. Data are means ± SD. Values 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 ± 1.7		
Protein (g / 100 kcal)	1.8-2.8[1]			2.4 ± 0.2		
Fat (g / 100 kcal)	4.4-6.0			4.4 ± 1.2		
Carbohydrates (g / 100 kcal)	9-14			12.8 ± 0.9		
EAA content in the IFs compared to the European regulation[2] (mg amino acid/100 kcal)						
Tyrosine	76^{d}	88.5 ± 3.1^{bc}	91.7 ± 3.4^{b}	86.6 ± 1.2^{c}	108.5 ± 4.1^{a}	116.2 ± 7.2^{a}
Lysine	113^{e}	227.6 ± 0.2^{a}	188.4 ± 6.7^{bc}	173.0 ± 0.5^{c}	156.4 ± 3.1^{d}	198.0 ± 3.8^{b}
Phenylalanine	83^{d}	102.0 ± 1.0^{c}	121.3 ± 3.6^{b}	108.3 ± 1.4^{c}	126.1 ± 0.2^{b}	136.9 ± 1.9^{a}
Leucine	166^{f}	270.4 ± 0.2^{a}	219.4 ± 2.6^{d}	205.2 ± 0.2^{e}	232.4 ± 2.6^{c}	254.8 ± 1.4^{b}
Isoleucine	90^{e}	150.9 ± 2.4^{a}	124.9 ± 7.5^{cd}	114.5 ± 1.0^{d}	124.6 ± 3.8^{c}	131.8 ± 1.2^{b}
Methionine	23^{d}	67.4 ± 1.4^{b}	60.2 ± 4.8^{b}	48.1 ± 3.8^{c}	77.0 ± 2.6^{ab}	81.8 ± 2.2^{a}
Valine	88^{d}	149.2 ± 0.7^{b}	139.5 ± 2.6^{bc}	127.8 ± 1.2^{c}	158.6 ± 0.7^{a}	144.1 ± 2.2^{b}
Histidine	40^{c}	56.5 ± 0.7^{b}	59.9 ± 6.7^{ab}	60.4 ± 2.6^{a}	62.1 ± 4.6^{a}	58.0 ± 0.5^{ab}
Threonine	77^{c}	144.4 ± 0.2^{a}	94.8 ± 4.1^{b}	103.2 ± 1.7^{b}	107.3 ± 0.7^{b}	148.9 ± 2.6^{a}

[1]1.8 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 of protein content when using soy protein isolate or hydrolysed proteins as protein source (EU, 2016). This reference range of values 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 requirements.

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

Table 4. Porcine trypsin activity (U/mg) measured in the presence of each protein source in solution and the substrate only (control). Data are means ± 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 ±5.0 [a]	108.5 ±0.9 [a]	108.6 ±0.5 [a]	109.2 ±3.4 [a]	107.4 ±1.1 [a]	24.1 ±2.8 [b]

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

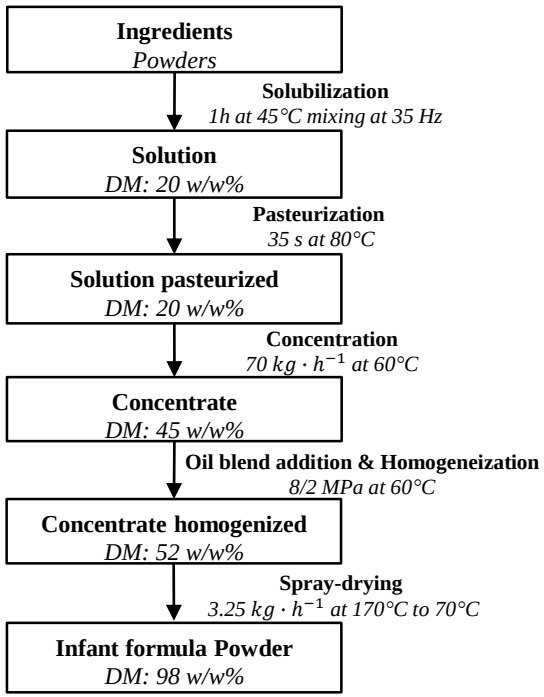

Ingredients
Powders
Solubilization
1h at 45°C mixing at 35 Hz
Solution
DM: 20 w/w%
Pasteurization
35 s at 80°C
Solution pasteurized
DM: 20 w/w%
Concentration
70 kg · h⁻¹ at 60°C
Concentrate
DM: 45 w/w%
Oil blend addition & Homogeneization
8/2 MPa at 60°C
Concentrate homogenized
DM: 52 w/w%
Spray-drying
3.25 kg · h⁻¹ at 170°C to 70°C
Infant formula Powder
DM: 98 w/w%

Fig. 1

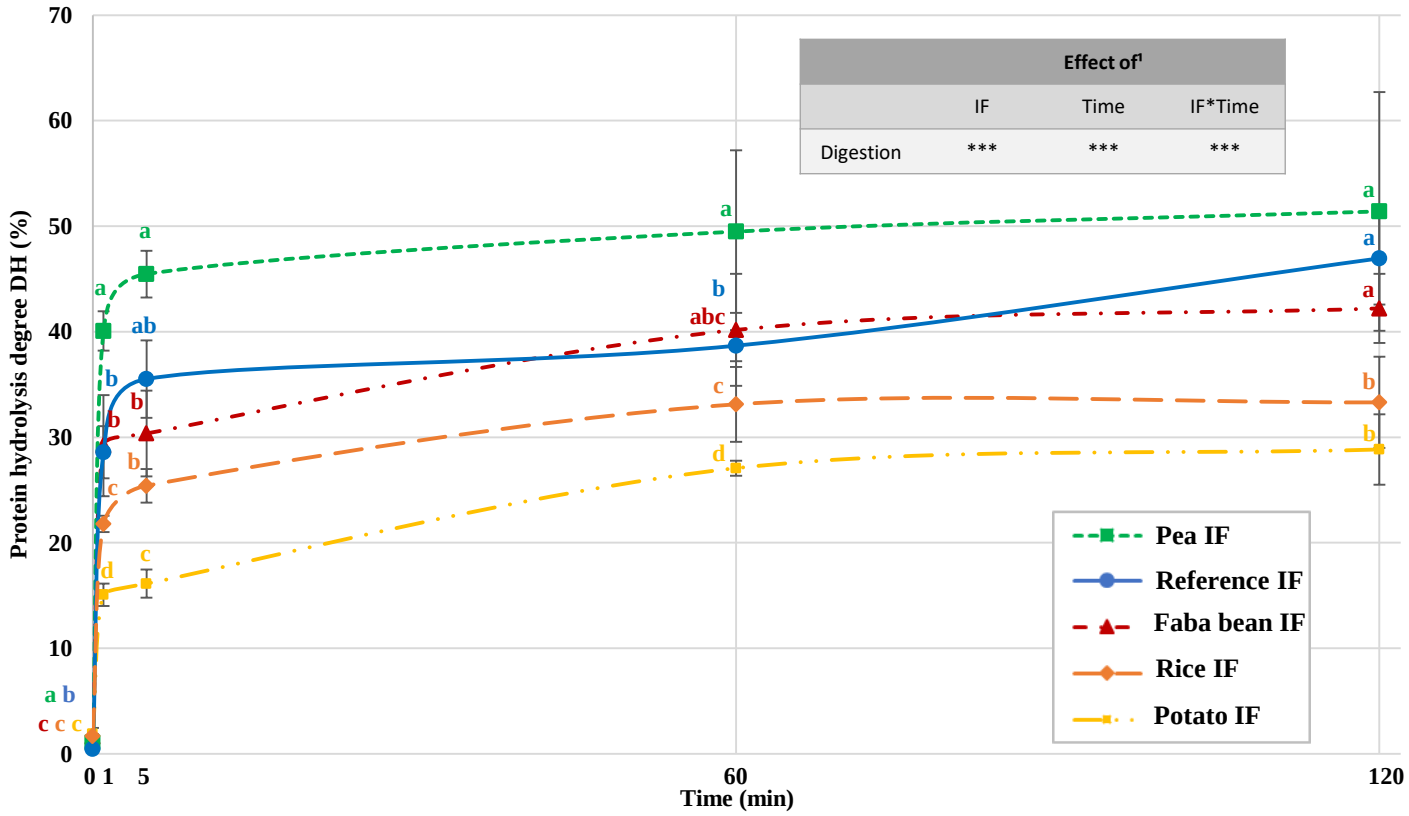

Effect of[1]
IF
Time
IF*Time
Digestion
***
***
***
Protein hydrolysis degree DH (%)
Time (min)
0
10
20
30
40
50
60
70
0 1 5
60
120
Pea IF
Reference IF
Faba bean IF
Rice IF
Potato IF

Fig. 2

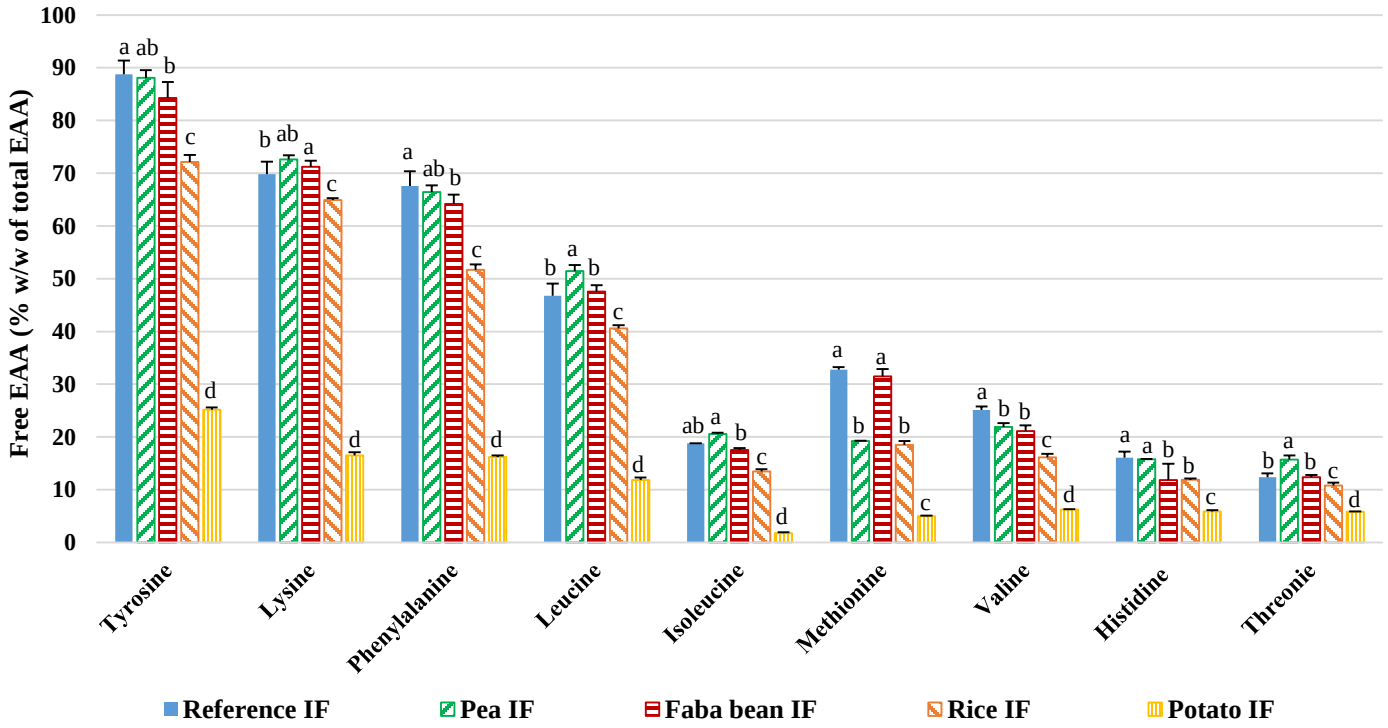
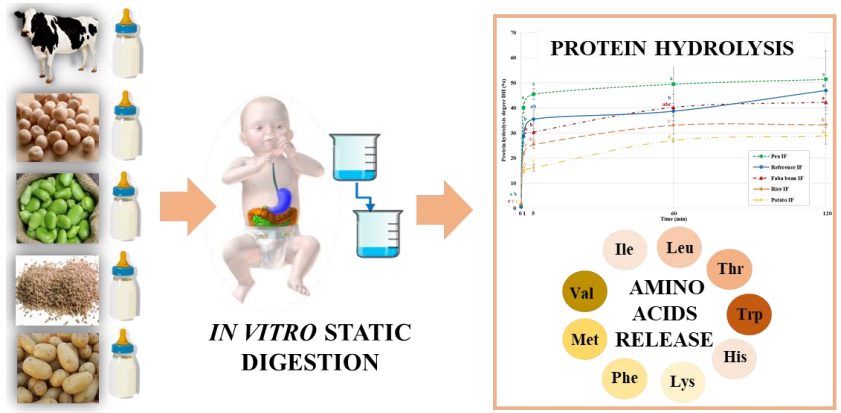

Free EAA (% w/w of total EAA)
100
90
80
70
60
50
40
30
20
10
0
Tyrosine
Lysine
Phenylalanine
Leucine
Isoleucine
Methionine
Valine
Histidine
Threonie
Reference IF
Pea IF
Faba bean IF
Rice IF
Potato IF

Fig. 3


IN VITRO STATIC DIGESTION
PROTEIN HYDROLYSIS
Ile
Leu
Thr
Val
AMINO ACIDS RELEASE
Trp
Met
His
Phe
Lys