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

Linda LE ROUX¹,², Raphaël CHACON¹, Didier DUPONT², Romain JEANTET³, Amélie DEGLAIRE³*, Françoise NAU²

¹Sill Dairy International, Raden, 29860 Plouvien, France.
²STLO, INRA, AGROCAMPUS OUEST, 35042 Rennes, France.

*Corresponding author: STLO, INRA, AGROCAMPUS OUEST, 35042 Rennes, France.

E-mail addresses: linda.le-roux@inra.fr (Linda LE ROUX), Raphael.Chacon@Sill.Fr (Raphaël CHACON), didier.dupont@inra.fr (Didier DUPONT), romain.jeantet@agrocampus-ouest.fr (Romain JEANTET), amelie.deglaire@agrocampus-ouest.fr (Amélie DEGLAIRE), francoise.nau@agrocampus-ouest.fr (Françoise NAU).

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

Declaration of interest: Linda Le Roux and Raphaël Chacon are employees of Sill Dairy International. Other authors have no conflicts of interest.
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
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 · 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 · 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 · 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
determined gravimetrically after evaporation of the solvent. Total and free fat analyses were carried out in duplicate (AFNOR, 1990).

2.4.3. Amino acid content

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

2.4.4. Water activity and glass transition temperature

Water activity (\(a_w\)) was measured at 25°C ± 0.1°C using the Novasina aw-meter (Novasina, Switzerland).

In order to determine the glass transition temperature (Tg), the powders were first equilibrated in a 20% relative humidity atmosphere using the SPSx-1µ Sorption Test System (ProUmid GmbH & Co. KG, August-Nagel-Str., Germany). The Tg was then determined at this constant sorption point by using a modulated temperature differential scanning calorimetry method according to Schuck et al. (2012).

2.4.5. Powder size distribution

The powder size distribution was determined using a laser scattering granulometer (Mastersizer, Malvern Instruments Ltd, Malvern, UK) with a 300-mm measurement cell (0.5-880 mm range). Powders were mixed with coarser powder (sucrose) 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
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 short, the oral phase was omitted. The rationale of the digestive parameters is detailed in Ménard et al. (2018).

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 dynamic digestion model DIDGI validated for IF digestion where the mean flow rate of secretions was fixed at 0.53 ml/min at the half-time gastric emptying of 78 min (Ménard et al., 2014). The gastric secretions were composed of 94 mM NaCl and 13 mM KCl. A quantity of 268 U of pepsin per mL of total gastric content was added to simulate the gastric phase, which lasted for 120 min. Gastric digestion was stopped by raising the pH to 7 with 1 M NaOH. To simulate the intestinal phase, the meal:total secretions (gastric and intestinal) ratio (v:v) was 39:61 (based on an overall mean secretion 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 intestinal secretions were composed of 164 mM NaCl, 10 mM KCl and 85 mM sodium bicarbonate, and adjusted to pH 7. Bovine bile extract was added to a final concentration of 3.1 mM of bile salts. The addition of pancreatin for a trypsin activity of 16 U/mL of intestinal content initiated the intestinal phase, which lasted for 120 min. Both gastric and intestinal phases were completed at 37°C in a water bath under magnetic stirring (300 rpm). For each IF, digestion was carried out in triplicate.

Aliquots were collected at 0, 1, 5, 60 and 120 min after the beginning of each digestive phase. Protease inhibitors were 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) per ml of intestinal digesta, before storage at -20°C until analysis. Each digested samples were sub-sampled to undertake the different analysis.

2.6. Digested sample analysis

2.6.1. Degree of hydrolysis (DH)

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

\[ \% \text{DH} = 100 \times \frac{(NH_{2\text{final}} - NH_{2\text{initial}})}{(NH_{2\text{initial}} \times F)} \]
where $\text{NH}_2(t)$ is the amount of primary amines after $t$ min digestion (expressed in mg of NH$_2$ per L of digesta), $\text{NH}_2(t_0)$ 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, $\text{NH}_2(t_{\text{tot}})$ is the maximum amount of primary amines (after total acid hydrolysis of the meal), and $F$ is the dilution factor to express $\text{NH}_2(t_{\text{tot}})$ 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 “fl.ld.fl” 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 trypsin 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
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 vitro digestion compared to the three other IFs (p<0.05).

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

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

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

Several studies highlight the resistance of cow milk whey proteins to gastric digestion whereas they are more extensively degraded during intestinal phase (Bouliue et al., 2015; Bouzerzour et al., 2012), which likely explains the high bioaccessibility of EAA observed in the milk-reference IF. Similarly, Nguyen et al. (2015) study the digestion of cow milk based IFs (with different casein to whey protein ratios) and soy IF using an in vitro static model (pH drop method) adapted to infant conditions. The authors showed that IF containing higher amount of caseins had a more rapid digestion compared to IF with more whey protein content after 2 hours of intestinal digestion. This suggests that in the small intestine proteases hydrolyse caseins quicker than whey proteins. This difference in digestibility can be related to the difference in the structure and composition of casein and whey proteins. Due to the high degree of phosphorylation, caseins have an
open structure (Holt, Carver, Ecroyd, & Thorn, 2013; Swaisgood, 1993) and are sensitive to proteolysis. However, the presence of phosphorylated peptides surviving casein digestion can also create specific areas that resist to proteolysis (Cattaneo, Stuknytė, Ferraretto, & De Noni, 2017), even during in vitro digestion with infant conditions (Dupont et al., 2009). In contrast, native whey proteins contain a high amount of cysteine that create disulphide bonds making whey proteins a compact structure that restricts the action of digestive proteases (Lacroix et al., 2006). At the same time, the effect of processing (heat-treatment) on whey proteins has been reported to enhance β-Lactoglobulin digestibility as the protein unfold due to heat treatment above 65°C and thus became more sensitive to proteolysis (Mandalari, Mackie, Rigby, Wickham, & Mills, 2009). Finally, the specificity of caseins and whey proteins as well as their modification occurring upon processing treatment are factors affecting their digestibility. In the present study, whey proteins might be partly denatured due to processing treatment and thus explained the higher amount of free amino acids released after the digestion of the reference infant formula. However, caseins are present in the same amount in each infant formulas but its interaction with the other proteins can be different and thus modify the sensitivity of each infant formula during digestion.

It is also well known that plant-based proteins are less digestible than animal proteins due to difference in terms of structure. In fact, the secondary structure of plant proteins is characterized by a high content in β-sheet conformation and a relatively low α-helix amount compared to that of animal proteins, it is particularly the case for legume proteins such as soy, pea and faba bean proteins (Carbonaro, Maselli, & Nucara, 2012). The high content in β-sheet conformation is related to its resistance to proteolysis in the gastrointestinal tract since hydrophobic β-sheet structure facilitates protein aggregation resulting in decreasing digestibility (Carbonaro et al., 2012; Nguyen et al., 2015). Moreover, heat treatment during processing has also been reported to cause β-sheet aggregation among molecules and have effect on the resistance to digestion of proteins (Carbonaro et al., 2012; Carbonaro, Maselli, & Nucara, 2015). Contrary to legume proteins, cow milk proteins present very little secondary structure and are mainly based on an association of β-sheet and α-helix structures only coming from whey proteins (Permyakov & Berliner, 2000). Since the IFs in the present study are all composed of a mix of cow milk proteins and either whey proteins in the milk-reference IF or plant proteins in the plant-based IFs, the impact of the secondary structure dominated by β-sheets on protein digestibility should be limited for the milk-reference IF, pea and faba bean IFs, and thus explain their relatively similar EAA bioaccessibility profile (Fig. 3). The lower proteolysis and EAA release measured for the rice IF in the present study, in comparison to the reference, pea and faba bean IFs, is in accordance with Gastanduy, Cordano & Graham (1990). These authors reported that the in vivo digestibility of IF based on high protein rice flour was lower than cow's milk-derived formulas, resulting in a low content 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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References


in cooked dry legumes usually consumed in the Mediterranean countries. Food Chemistry, 121(3), 682–690. https://doi.org/10.1016/j.foodchem.2010.01.005


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

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

<sup>*</sup>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).

<table>
<thead>
<tr>
<th></th>
<th>Reference IF</th>
<th>Pea IF</th>
<th>Faba bean IF</th>
<th>Rice IF</th>
<th>Potato IF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total DM (w/w%)</td>
<td>98.3 ± 0.01a</td>
<td>98.1 ± 0.1a</td>
<td>96.9 ± 0.2a</td>
<td>97.9 ± 0.1a</td>
<td>97.5 ± 1.1a</td>
</tr>
<tr>
<td>Ashes (w/w% DM)</td>
<td>1.7 ± 0.02a</td>
<td>1.7 ± 0.02a</td>
<td>1.8 ± 0.01a</td>
<td>1.6 ± 0.01a</td>
<td>1.6 ± 0.03a</td>
</tr>
<tr>
<td>Total nitrogen (w/w% DM)</td>
<td>1.8 ± 0.03a</td>
<td>1.8 ± 0.01a</td>
<td>1.8 ± 0.001a</td>
<td>1.5 ± 0.02b</td>
<td>1.8 ± 0.01a</td>
</tr>
<tr>
<td>Total protein (w/w% DM)</td>
<td>11.7 ± 0.2a</td>
<td>10.8 ± 0.07b</td>
<td>10.4 ± 0.07b</td>
<td>8.7 ± 0.03b</td>
<td>10.7 ± 0.04b</td>
</tr>
<tr>
<td>Total fat (w/w% DM)</td>
<td>20.1 ± 0.1a</td>
<td>20.1 ± 0.1a</td>
<td>21.1 ± 0.1a</td>
<td>17.2 ± 0.1b</td>
<td>20.9 ± 1.4a</td>
</tr>
<tr>
<td>Free fat (w/w% total fat)</td>
<td>8.1 ± 0.5c</td>
<td>14.1 ± 0.01b</td>
<td>5.2 ± 0.7c</td>
<td>21.8 ± 3.4a</td>
<td>6.2 ± 0.8b</td>
</tr>
<tr>
<td>d(0.5) (μm)</td>
<td>34.9 ± 0.4a</td>
<td>35.9 ± 0.6a</td>
<td>37.2 ± 0.1a</td>
<td>35.9 ± 0.6a</td>
<td>36.0 ± 0.1a</td>
</tr>
<tr>
<td>(a_{w})</td>
<td>0.12 ± 0.01c</td>
<td>0.11 ± 0.01c</td>
<td>0.18 ± 0.01a</td>
<td>0.16 ± 0.02a</td>
<td>0.17 ± 0.09a</td>
</tr>
<tr>
<td>Tg (°C)</td>
<td>47.0 ± 2.0a</td>
<td>47.3 ± 3.1a</td>
<td>50.9 ± 3.8a</td>
<td>51.1 ± 3.9b</td>
<td>50.7 ± 1.6a</td>
</tr>
<tr>
<td>Solubility (%)</td>
<td>100.0 ± 0.1a</td>
<td>96.0 ± 0.1b</td>
<td>96.0 ± 0.1b</td>
<td>93.5 ± 1.8a</td>
<td>99.5 ± 0.4a</td>
</tr>
<tr>
<td>Dispersibility (%)</td>
<td>88.3 ± 0.6a</td>
<td>88.4 ± 0.2a</td>
<td>90.7 ± 2.5a</td>
<td>96.4 ± 0.7a</td>
<td>85.4 ± 1.4d</td>
</tr>
<tr>
<td>Viscosity (Pa.s)</td>
<td>0.03 ± 0.01b</td>
<td>0.04 ± 0.02b</td>
<td>0.15 ± 0.01b</td>
<td>0.01 ± 0.01b</td>
<td>5.4 ± 0.43b</td>
</tr>
<tr>
<td>Color parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>75.9 ± 0.1a</td>
<td>73.1 ± 0.3b</td>
<td>73.2 ± 0.4b</td>
<td>73.4 ± 0.9a</td>
<td>66.4 ± 0.9a</td>
</tr>
<tr>
<td>a</td>
<td>-3.1 ± 0.2c</td>
<td>-2.3 ± 0.2b</td>
<td>-3.8 ± 0.1c</td>
<td>-2.0 ± 0.2a</td>
<td>0.8 ± 0.3a</td>
</tr>
<tr>
<td>b</td>
<td>9.8 ± 0.5d</td>
<td>13.3 ± 0.5b</td>
<td>15.9 ± 0.1a</td>
<td>9.5 ± 0.6a</td>
<td>11.0 ± 0.6a</td>
</tr>
</tbody>
</table>
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).

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>European regulation</th>
<th>Reference IF</th>
<th>Pea IF</th>
<th>Faba bean IF</th>
<th>Rice IF</th>
<th>Potato IF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal / 100 ml)</td>
<td>60-70</td>
<td>66.5 ± 1.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (g / 100 kcal)</td>
<td>1.8-2.8&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2.4 ± 0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat (g / 100 kcal)</td>
<td>4.4-6.0</td>
<td>4.4 ± 1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrates (g / 100 kcal)</td>
<td>9-14</td>
<td>12.8 ± 0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EAA content in the IFs compared to the European regulation<sup>2</sup> (mg amino acid/100 kcal)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>European regulation</th>
<th>Reference IF</th>
<th>Pea IF</th>
<th>Faba bean IF</th>
<th>Rice IF</th>
<th>Potato IF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>76&lt;sup&gt;d&lt;/sup&gt;</td>
<td>88.5 ± 3.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>91.7 ± 3.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>86.6 ± 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>108.5 ± 4.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>116.2 ± 7.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lysine</td>
<td>113&lt;sup&gt;e&lt;/sup&gt;</td>
<td>227.6 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>188.4 ± 6.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>173.0 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>156.4 ± 3.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>198.0 ± 3.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>83&lt;sup&gt;d&lt;/sup&gt;</td>
<td>102.0 ± 1.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>121.3 ± 3.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>108.3 ± 1.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>126.1 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>136.9 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Leucine</td>
<td>166&lt;sup&gt;f&lt;/sup&gt;</td>
<td>270.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>219.4 ± 2.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>205.2 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>232.4 ± 2.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>254.8 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>90&lt;sup&gt;e&lt;/sup&gt;</td>
<td>150.9 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>124.9 ± 7.5&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>114.5 ± 1.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>124.6 ± 3.8&lt;sup&gt;ce&lt;/sup&gt;</td>
<td>131.8 ± 1.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methionine</td>
<td>23&lt;sup&gt;d&lt;/sup&gt;</td>
<td>67.4 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.2 ± 4.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.1 ± 3.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>77.0 ± 2.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>81.8 ± 2.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Valine</td>
<td>88&lt;sup&gt;d&lt;/sup&gt;</td>
<td>149.2 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>139.5 ± 2.6&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>127.8 ± 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>158.6 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>144.1 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Histidine</td>
<td>40&lt;sup&gt;e&lt;/sup&gt;</td>
<td>56.5 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>59.9 ± 6.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>60.4 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.1 ± 4.6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>58.0 ± 0.5&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Threonine</td>
<td>77&lt;sup&gt;c&lt;/sup&gt;</td>
<td>144.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>94.8 ± 4.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>103.2 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>107.3 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>148.9 ± 2.6&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>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.

<sup>2</sup>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).

<table>
<thead>
<tr>
<th>Protein Source</th>
<th>Control</th>
<th>Reference¹</th>
<th>Pea</th>
<th>Faba bean</th>
<th>Rice</th>
<th>Potato</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin activity (U/mg)</td>
<td>105.1 ±5.0ᵃ</td>
<td>108.5 ±0.9ᵃ</td>
<td>108.6 ±0.5ᵃ</td>
<td>109.2 ±3.4ᵃ</td>
<td>107.4 ±1.1ᵃ</td>
<td>24.1 ±2.8ᵇ</td>
</tr>
</tbody>
</table>

¹The 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 & Homogenization
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
Protein hydrolysis degree (DH) (%)

Effect of [IF] Brazilian Pea IF, Reference IF, Faba bean IF, Rice IF, Potato IF

Digestion

Effect of

IF  Time  IF*Time

Digestion  ***  ***  ***

Fig. 2
Fig. 3