



De la fabrication à la digestion in vitro de formules infantiles innovantes en partie composées de protéines végétales : une approche multi-échelle

Linda Le Roux

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Linda LE ROUX

**De la fabrication à la digestion *in vitro* de formules infantiles innovantes
en partie composées de protéines végétales : une approche multi-échelle**

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Abbreviations

AA	Amino acid	MGFM	Milk fat globule membrane
AAA	Aromatic amino acids (phenylalanine + tyrosine)	MFG	Milk fat globule
AAB	Amino acid bioaccessibility	N.A.	Not Available (data)
AAP	American Academic of Pediatrics	OPA	O-phthaldialdehyde
AAS	Amino acid score	PDCAAS	Protein Digestibility Corrected Amino Acid Score
AID	Apparent ileal digestibility	pI	Isoelectric point
ANF	Antinutritional factors	PIF	Pea infant formula
CI	Chemical index	PPC	Pea protein concentrate
CLSM	Confocal laser scanning microscopy	PPI	Potato protein isolate
DH	Degree of hydrolysis	RIF	Reference infant formula
DHA	Acid docosaheptaenoic	RPC	Rice protein concentrate
DI	Dispersibility index	SAA	Sulphuric amino acids (methionine + cysteine)
DIAAS	Digestible Indispensable Amino Acid Score	SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
DM	Dry matter	SEC	Size exclusion chromatography
EAA	Essential amino acid	SGF	Simulated gastric fluid
EC	European Commission	SI	Solubility index
ELISA	Enzyme-linked immunosorbent assay	SIF	Simulated intestinal fluid
EPSGHAN	European Society for Paediatric Gastroenterology Hepatology and Nutrition	Tg	Glass transition temperature
FIF	Faba bean infant formula	TFI	True faecal digestibility
FAO	Food and Agriculture Organization	TID	True ileal digestibility
FDA	Food and Drug Administration	Trp	Tryptophan
FPC	Faba bean protein concentrate	UNICEF	United Nations of International Children's Emergency Fund
His	Histidine	Val	Valine
IFs	Infant formulas	v/v	volume/volume
Ig	Immunoglobulin	WHO	World Health Organization
Ile	Isoleucine	WMP	Whole milk powder
LEAA	limiting essential amino acid	WPC	Whey protein concentrate
Leu	Leucine	w/w	weight/weight
Lys	Lysine		

General Introduction

Scientific and technico-economic context

Early protein intake in life is essential for the development of infants, as it affects growth, body composition, neurodevelopment, appetite and hormonal regulation (Michaelsen & Greer 2014). The average protein requirement ranges from 0.98 g/kg/day at 6 months to 0.70 g/kg/day at 10 years, with a small decline towards the adult value thereafter (Millward 2012; World Health Organization et al. 2007). From a qualitative point of view, human milk represents the gold standard for the newborn, and breastfeeding is highly recommended for the first 6 months of life (Victora et al. 2016). However, only 38% of infants aged 0 to 6 months are exclusively breastfed worldwide (Black et al. 2013; WHO & UNICEF 2017). When breastfeeding is not sufficient or not possible, it is important to have high quality infant formulas (IFs) available (Agostoni et al. 2008). The nutritional requirements of infants must be satisfied by supplying IF products until infants become accustomed to complementary food (European Union 2016). Traditionally IF is based on cow milk proteins with an adapted casein to whey proteins ratio by the addition of whey protein concentrate. According to the applicable European regulation, the other sources of proteins allowed for the 1st age IFs (0 to 6 months) are either goat's milk protein, soy protein isolate and hydrolysed rice protein (EU 2016).

Besides, the projection of a global human population of around 9.5 billion by 2050 indicates that the demand for animal protein will double during this period (Egbert & Payne 2009; FAO 2006). It seems therefore essential to search for alternative protein sources that show nutritional and functional quality close to animal proteins one in regard to sustainability and food security (Gilani et al. 2011). In that respect, there is a growing interest in utilizing plant proteins as partial replacers of animal proteins in food (Ainis et al. 2018). While soy protein continues to dominate as an alternative to animal protein, a range of new food products have been studied, which include other grains, legumes and vegetables as protein sources (Ainis et al. 2018; Alves & Tavares 2019; Chihi et al. 2016; Messin et al. 2017). However, the great challenge remains in the improvement of the nutritional and the functional properties of such proteins (Amagliani & Schmitt 2017; Messin et al. 2017). It is well known that plant proteins have lower nutritional quality than animal proteins as they are often deficient in one or more essential amino acids (EAA), and less digestible, particularly because of the presence of antinutritional factors (ANF). The use of specific technological treatments can inactivate or inhibit most of these ANF and thus improve biological value and digestibility of such proteins (Kalpanadevi & Mohan 2013; Le Gall et al. 2005; Rizzello et al. 2016; Soetan & Oyewole 2007; Xu & Chang 2008). Moreover, it is noteworthy that the use of plant proteins as ingredients is generally associated with poor water solubility and a very pronounced taste (Day 2013; Silva et al. 2019; Wouters et al. 2016).

The basic function of proteins in nutrition is to supply adequate amounts of nitrogen and essential amino acids (EAAs) to meet human metabolic needs (Boye et al. 2012; Munro 1964; Young et al. 1989). The quality of a protein depends on its amino acids (AAs) composition and its digestibility (Friedman 1996). Therefore, the nutritional value of proteins depends on their origin since they are not all equivalent with

respect to their AA content and accessibility to digestive enzymes, especially when submitted to technological processes, which can particularly modify their structure (Friedman 1996; Lonnerdal 2014; Machado et al. 2008). The protein content of reconstituted IFs is often higher (~1.5 g per 100 g) than human milk (~1.2 g per 100 g) to compensate the differences in protein quality and AA profiles found in IFs (Le Huërou-Luron et al. 2018). However, protein digestion and availability for absorption of EAAs from IFs by infants are still incompletely known. The FAO (2013) recommends to evaluate the protein quality based on the true protein digestibility in the small intestine (ileal digestibility) of each EAA. The true EAA bioavailability should preferably be determined in humans (FAO 2013). Digestion experiments in infants, however, have clinical and ethical drawbacks. Digestion experiments in young piglets as previously described (Bouzerzour et al. 2012) also have ethical constraints. An option is to use *in vitro* gastrointestinal static models simulating infant digestion (Chatterton et al. 2004; Ménard et al. 2018; Wada & Lonnerdal 2015a). A better alternative might be the use of dynamic *in vitro* gastrointestinal models simulating infant digestion (de Oliveira et al. 2016; Maathuis et al. 2017; Ménard et al. 2014; Passannanti et al. 2017; Shani-Levi et al. 2013a; Zhang et al. 2014).

Many research groups have been studying for long the digestibility of either human milk or IF based primarily on cow milk protein or soy protein (Bouzerzour et al. 2012; Chatterton et al. 2004; El-Agamy 2007; Lonnerdal 1994, 2014; Nguyen et al. 2015). Reche et al. (2010) studied hydrolyzed rice protein-based IF. Maathuis et al. (2017) as well as Hodgkinson et al. (2018, 2019) compared the protein digestion of goat milk- and cow milk-based IFs. Other authors studied the ability of using plant proteins in IFs, but the majority concerned follow-on formulas (6 to 12 months) using chickpea protein (Malunga et al. 2014; Ulloa et al. 1988). Some others were dedicated to the capacity of plant proteins, as for example pea protein (Kent & Doherty 2014) or different legume proteins (Khan et al. 2013) to encapsulate probiotics in follow-on IFs. Recently, a process for preparing a 1st age potato protein-based IF that is suitable for infants has been patented [WO2018 115340 (A1)]. These relevant studies need to be further extended to other plant protein sources that would be suitable to infant needs from birth, on a nutritional and a functional point of view.

IFs are mostly spray-dried to a powdered form. Spray drying is commonly used in the food industry to extend shelf life and aid handling of products as well as reducing storage and transportation costs (Blanchard et al. 2013; Chen & Patel 2008). The manufacture of powdered IFs usually includes the following unit operations: mixing, pasteurization, evaporation, homogenization, spray drying and conditioning. Pasteurization aims to ensure microbiological safety and evaporation is conducted prior to drying in order to limit energy costs and increase the overall productivity. During IF homogenization, the oil phase is stabilised by proteins to form an oil-in-water emulsion (Dickinson 2001). Homogenization decreases the size of fat globules for preventing subsequent phase separation and reinforcing oil encapsulation (Sun et al. 2018). The properties of IFs, for example colour, solubility and storage stability can be affected by the component interactions (Li et al. 2016), as well as by the unit operations, storage conditions or powder handling. Consequences of not controlling these parameters lead to quality impairments in IFs, such as free fat, flecking, Maillard reactions, lactose crystallization (i.e., caking) and poor solubility (Schuck et al. 2007). A greater understanding of the relation between composition, manufacturing conditions and product-process interactions is essential to solve the above stated issues.



Company context

This thesis project was a CIFRE collaboration between STLO INRA Agrocampus Ouest (Rennes, France) and the company SILL (Plouvien, France). This company, established in 1962, is specialized in four main activities, namely dairy products, fruit juices, soups and prepared foods. Recently, SILL became active in baby food area, particularly in vegetable and fruit jar products. This company aims today to expand its range of baby food products by entering in the production of IF powder. In this way, SILL would like to offer innovative IFs that meet the infant nutritional recommendations and that are environmental-friendly. Moreover, this project was driven by the lack of whey protein sourcing, which is one of the key ingredients usually used to develop IFs.

Main objective

In this context, the aim of the project was to develop 1st age IFs in which whey protein concentrate usually added to the skimmed cow milk and representing 50% of the total protein in IFs would be substituted by plant protein sources. Although these new protein sources are not yet allowed according to the applicable European regulation, the aim of the project was to investigate on it in order to pave the route to future innovation in this field. The influence of protein sources on the processing capability, the functional properties and the digestibility of these new IFs compared to a standard dairy protein IF has therefore been investigated.

Manuscript content

Before presenting the whole objective and the experimental design of this thesis project, a bibliography review is proposed as the first part of this manuscript in order to provide information on the protein nutritional quality, the interest of plant protein sources alternative to animal proteins, the infant nutritional requirements and digestive specificities and finally some aspects on the IF manufacturing.

Then, the material and methods of the experimental design implemented in this project will be described. The results will mainly be presented in the form of publications submitted or in preparation for submission to peer-reviewed publications. The first chapter will focus on the selection of the alternative protein sources mainly on a nutritional point of view. The second chapter will determine the physicochemical properties and the *in vitro* static digestibility of IFs manufactured at a pilot scale. The third chapter will deal with the scale-up of the IFs manufacturing and influence of protein source on physicochemical and microstructure properties of IFs. Lastly, the digestibility will be investigated in more physiological conditions to compare some relevant indicators of protein nutritional quality between new protein sources and dairy protein used to formulate IFs.

Finally, in a last part of the manuscript, a general discussion and perspectives will let to emphasize the main results of this project and the research topics to further and complete the present research work.

1. Bibliography review

Part 1. Protein nutritional quality

1.1.1. Protein and amino acid structures

Proteins are macromolecules essential to life. Indeed, the protein synthesis in the body leads to tissue repair, growth replacement or energy production. Proteins also have functions in cellular mobility (actine, myosin), in immunity defences (immunoglobulins), intercellular communication (hormones or neuro-mediator) and they have an important role in chemical reactions as enzymes. Proteins are composed of sequences of AA which are 20 (Figure 1), linked together by peptide bonds (CHNO).

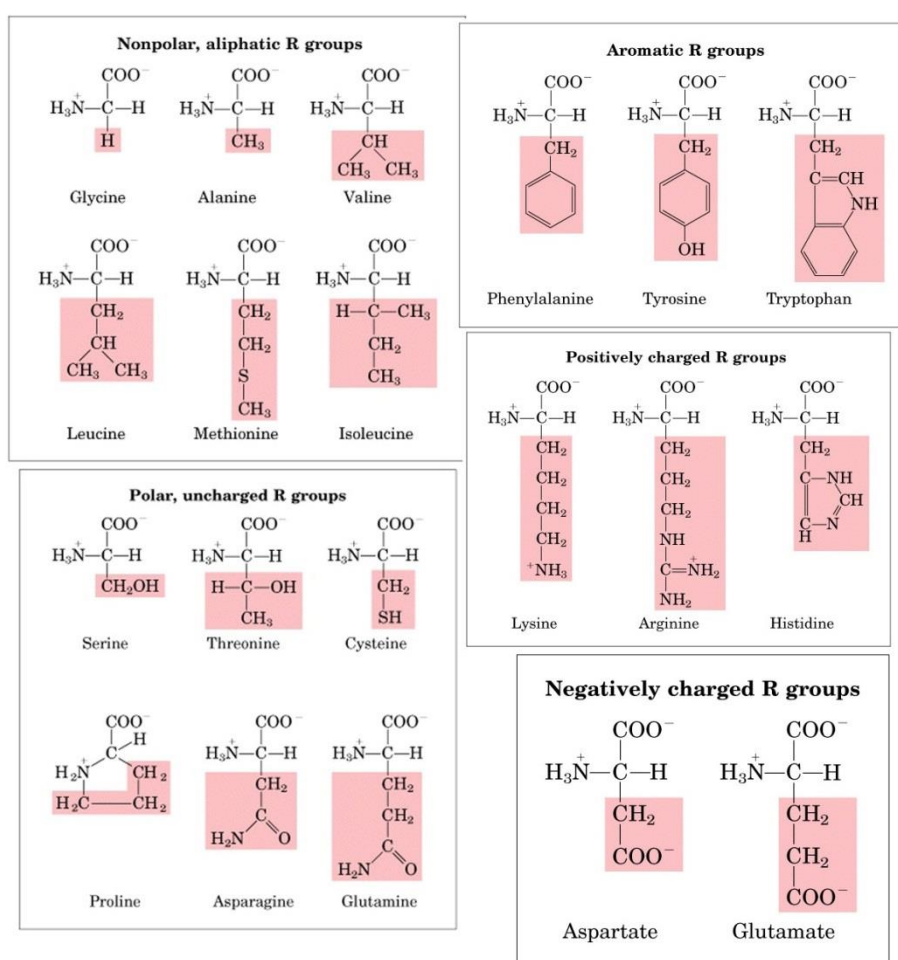


Figure 1. The twenty common amino acids. The side chains are pink colored.
(<https://amit1b.wordpress.com/the-molecules-of-life/about/amino-acids/>).

A protein is characterized by its AA sequence. This AA sequence defines the primary structure of the protein that corresponds to a single polypeptide chain. The secondary structure is determined by the folding of the polypeptide chain in regular structures into β sheet and α helix. The tertiary structure

corresponds to three-dimensional folding pattern of the protein due to side chain interactions. Finally, some proteins adopt a quaternary structure consisting of peptide chains gathered (Figure 2).

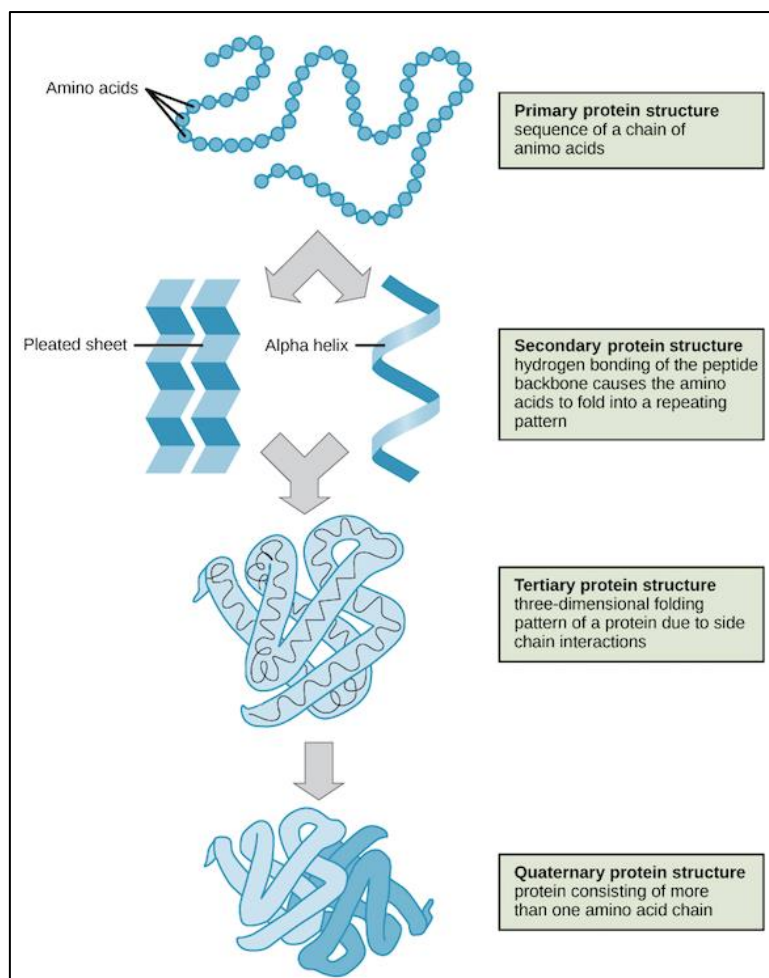


Figure 2. Primary, secondary, tertiary and quaternary structures of proteins. (Image modified from OpenStax Biology's modification of work by the National Human Genome Research Institute).

The structure of proteins can undergo reversible or irreversible modifications, in particular when heating which is a process often used to ensure the shelf life of foods. Sensitivity to heat treatments varies from one protein to another. Denaturation effects can create solubility modification due to exposure of hydrophilic or hydrophobic peptide sites, change of water retention capacity, modification of sensitivity to protease specific sites action, solution viscosity modification etc. The impact of processing treatments on protein functionality will be discussed in more details in Section 1.4.

Considering the world population growth, it is more important than ever to be able to define accurately the amount and quality of protein required to meet human nutritional needs (FAO 2013b). Protein quality evaluation is based on the capacity of food protein sources and diets to meet the protein and essential amino acids (EAA) requirements (Boye et al. 2012; Munro 1964; Young et al. 1989). Protein requirements correspond to intakes required to meet metabolic needs for amino acids (AA) and nitrogen for maintenance. They differ depending on the population age group with special needs for normal growth of infants and children, and on specific physiologic situations such as pregnancy and lactation in

women. The ability of a given protein to cover the nutritional needs depends both on its EAA content and on its digestibility and subsequent metabolism of the absorbed AA. The bioavailability (fraction of a nutrient that has been digested and absorbed and is available for the metabolic functions of the organism; Dupont & Nau 2019) of each EAA represents a major factor of nutritional quality of the different dietary proteins.

Thus, the only truly valid measures of protein quality for humans are those that can assess the efficiency of protein sources to provide normal growth in the respective targeted population. However, the assessment of protein quality in human population groups over the past decades has relied on indirect approaches involving *in vitro* assays (Brodkorb et al. 2019; Butts et al. 2012; Hur et al. 2011; McClements & Li 2010), and animal or human metabolic studies that can be commonly used to predict human protein and AA bioaccessibility (fraction of a nutrient that has been released in the gastrointestinal tract by the digestion process and is available for absorption; Dupont & Nau 2019). In order to ensure accuracy in those methods, some relevant parameters must be included to determine truly the quality of a protein, i.e. quantities of dietary EAA, digestibility of protein, and the AA bioavailability.

1.1.2. Protein and amino acids requirements

1.1.2.1. Protein consumption and requirements

Despite a considerable increase over the last 50 years (Figure 3), the world protein intake is characterized by a lot of disparities between countries as much by the quantity and the quality than by the nature of the proteins (plant or animal). Moreover, while the mean dietary protein requirement was estimated at 0.6 g of proteins/kg/day in 1989 (FAO et al. 1991), it was reevaluated in a large meta-analysis conducted in Rand et al. (2003), which concluded at a value 0.66 g of proteins /kg/day for dietary protein requirement and at 0.83 g/kg/day for dietary allowance or recommended intake, assumed to cover the needs of 97.5% of the population. The overall distribution of individual values from the meta-analysis conducted by Millward et al. (2012) and the values for the average, safe individual and population protein intake are shown in Figure 4. It should be noted that the protein requirements are higher for infants with 1.41 to 0.98 g/kg/day at 0 and 6 months, respectively (Millward 2012; WHO et al. 2007), and during pregnancy (0.5 to 7.3 g/kg/day) and breastfeeding (14.3 to 16.2 g/kg/day) (Millward 2012; WHO et al. 2007), as well as for the elderly (1 to 1.2 g/kg/day) (Bauer et al. 2013).

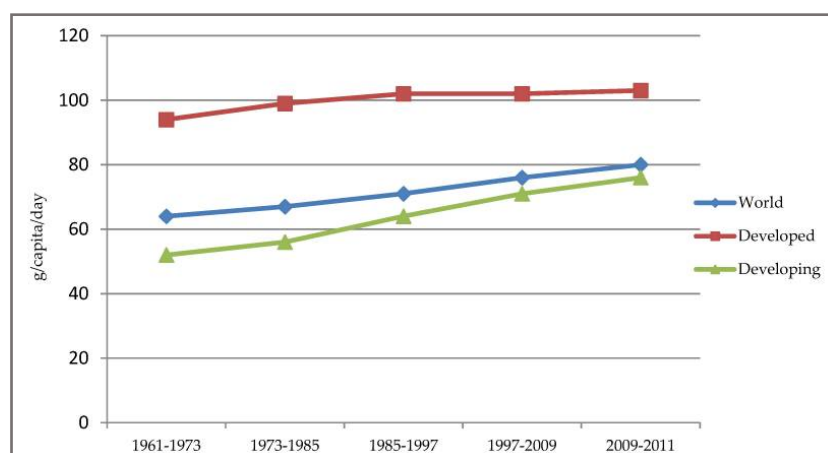


Figure 3. Evolution in protein consumption per capita (g/capita/day). (<http://faostat3.fao.org/>).

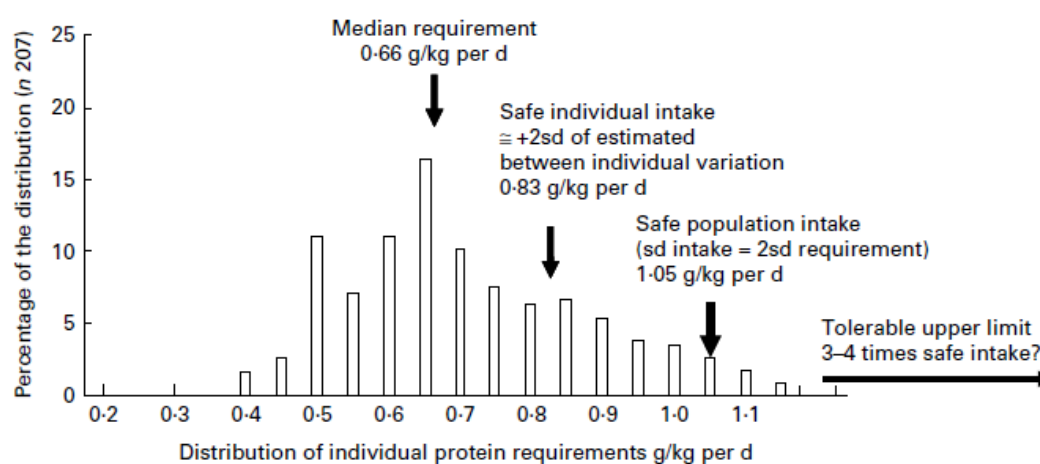


Figure 4. Distribution of the individual protein requirements identified in the meta analysis of nitrogen balance studies. (Millward 2012).

1.1.2.2. Amino acids requirements

Protein hydrolysis in AAs allows permanent reconstruction of body proteins from food AAs (25%) or endogenous AAs. Some AAs are called “essential” (Table 1) because they cannot be synthesized by the human metabolism (or cannot be synthesized in sufficient quantity) and must therefore be provided by an exogenous source, i.e. food. EAA have a specific chemical structure that cannot be synthesized by mammal enzymes. Thus, all hydrophobic and aromatic AAs are essential. Tyrosine and cysteine can be synthesized endogenously but in insufficient quantity; moreover, they need other EAAs (phenylalanine and methionine) to be synthesized. In both cases, these AAs are called conditionally essential (corresponded to a specific physiological condition). In the present study, only the 9 EAAs (except tryptophan that is lost during analysis) have been considered and then analysed, in addition with cysteine and tyrosine as these two conditionally-EAAs are often considered with methionine and phenylalanine to express sulphur-containing AAs (SAA) and aromatic AAs (AAA).

Table 1. Essential vs non-essential amino acids for humans.

Essential	Conditionally essential ¹	Non-essential
Histidine	Arginine	Alanine
Isoleucine	Cysteine	Asparagine
Leucine	Glutamine	Aspartate
Lysine	Glycine	Glutamate
Methionine	Proline	Serine
Phenylalanine	Tyrosine	
Threonine		
Tryptophan		
Valine		

¹required to some degree in young and/or for specific physiological conditions

Based on the nitrogen balance method, the (FAO et al. 1991), dietary requirements for total EAA amounted to 93.5 mg/ kg / day for a healthy adult. To determine these AA needs, the nitrogen balance method was used and defined as the minimum AA intake permitting to balance the nitrogen needs, the other AAs being provided in sufficient quantity. However, methodological advances, based on the use of stable isotopic methods, have allowed the demonstration that the recommended need from (FAO et al. 1991) could not maintained the AA homeostasis. As a result, new estimations of dietary amino acid requirements were reported for adult human in (WHO et al. 2007) and were of 184 mg/kg/day for the total EAA, i.e. two times higher than the values reported in 1991. The details of the requirement for each AA are given in Table 2.

Table 2. Amino acid scoring patterns for infants, children, adolescents and adults. (Amended values from the report World Health Organization 2007).

			His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
Tissue amino acid pattern (mg/g protein) ¹			27	35	75	73	35	73	42	12	49
Maintenance amino acid pattern (mg/g protein) ²			15	30	59	45	22	38	23	6	39
Protein requirements (g/kg/d)											
Age (yr)	Maintenance	Growth ³	amino acid requirements (mg/kg/d) ⁴								
0.5	0.66	0.46	22	36	73	63	31	59	35	9.5	48
1-2	0.66	0.20	15	27	54	44	22	40	24	6	36
3-10	0.66	0.07	12	22	44	35	17	30	18	4.8	29
11-14	0.66	0.07	12	22	44	35	17	30	18	4.8	29
15-18	0.66	0.04	11	21	42	33	16	28	17	4.4	28
>18	0.66	0.00	10	20	39	30	15	25	15	4.0	26
scoring pattern mg/g protein requirement ⁵											
0.5			20	32	66	57	27	52	31	8.5	43
1-2			18	31	63	52	25	46	27	7	41
3-10			16	30	61	48	23	41	25	6.6	40
11-14			16	30	61	48	23	41	25	6.6	40
15-18			16	30	60	47	23	40	24	6.3	40
>18			15	30	59	45	22	38	23	6.0	39

His, histidine; Ile, isoleucine; Leu, leucine; SAA, sulphur amino acids; AAA, aromatic amino acids, Thr, threonine, Trp, tryptophan; Val, valine

¹ Amino acid composition of whole-body protein.

² Adult maintenance pattern.

³ Calculated as average values for the age range: growth adjusted for protein utilization of 58%.

⁴ Sum of amino acids contained in the dietary requirement for maintenance (maintenance protein x the adult scoring pattern) and growth (tissue deposition adjusted for a 58% dietary efficiency of utilization x the tissue pattern).

⁵ Amino acid requirements/protein requirements for the selected age groups. Note that these values, some of which are slightly amended from the 2007 report, are the correctly calculated values. In the published report, the value for the SAA requirement for children aged 3-10 is incorrect (18mg/kg/d) as are the SAA patterns for infants preschool and school children up to 10, (28, 26 and 24 mg/g protein).

1.1.3. Protein quality evaluation: a long history

Evaluation of protein quality aims to assess the contribution of dietary protein in satisfying the metabolic requirement for N and AA (WHO et al. 2007). The different methods to evaluate the nutritional quality of proteins have been developed in parallel to the ones concerning the determination of the nitrogen and AA requirements. For a long time, protein quality evaluation was based on the impact of the protein source on the young rat growth allowing the measurement of the protein efficiency ratio or net protein ratio. These criteria have been criticized mainly because of discrepancies between the human and the rat for their AA requirements, in particular for sulphur-containing AA (National Research Council, 1978). Other approaches have thus been proposed.

The following method proposed for evaluating the nutritional quality of proteins was the **PDCAAS (Protein Digestibility Corrected Amino Acid Score)**, as recommended by the joint FAO/WHO Expert Consultation in 1989 (FAO et al. 1991) and further supported in 2007 (WHO et al. 2007). The three fundamental points of this approach are the protein digestibility, the reference EAA profile based on the human EAA requirement, and the ability of the protein to cover this EAA profile. As mentioned, protein digestibility is a primary determinant of the nitrogen bioavailability. Therefore, it is important for evaluating the nutritional quality of food proteins. The reference EAA profile relies on the notion of an « ideal » AA composition of a body protein based on the EAA requirement for human. It is put in regards to the EAA content of the dietary protein, resulting in the calculation of a chemical index (CI) or **chemical score**, such as follows:

$$CI = \frac{\text{mg of EAA in 1 g of dietary protein}}{\text{mg of EAA in 1 g of the reference pattern}} \quad (1)$$

The reference pattern or scoring pattern is proposed for six age groups such as infants, preschool children (1-2 y), 3-10 y children, up to adults (Table 2). It was first proposed to use the preschool children pattern to calculate the CI for adult. CI is calculated for all EAAs of the dietary protein, and the lowest CI, corresponding to the limiting EAA (LEAA), is considered for **PDCAAS calculation**, which is conducted as follows:

$$PDCAAS = (CI \text{ of LEAA in 1 g of dietary protein} \times \text{true faecal protein digestibility}) \times 100 \quad (2)$$

where the digestibility should be assessed in the growing rat. The PDCAAS score should be truncated to 100 % (or 1).

The PDCAAS method has now been in use for more than 20 years and has proved to be of considerable value in practice. However, it has been criticized on several counts, such as reported by (Rutherfurd et al. 2015):

- The PDCAAS uses faecal rather than ileal estimates of protein digestibility, yet **ileal digestibility** estimates are more accurate, as discussed by Moughan (2003) and Schaafsma, (2005), whereas faecal digestibility estimates are confounded by the metabolic activity of the microbiota of the hindgut (Rowan et al. 1994).
- Protein digestibility values are less accurate than individual **AA digestibility** values (Schaafsma 2000, 2005).

- Protein ingredient sources that have PDCAAS values >1 (or 100%), but that have been **truncated to 1**, cannot be adequately ranked as sources of the first-limiting amino acid (Gilani et al. 2008; Schaafsma 2000, 2005).
- The use of the **AA requirements of the preschool child** to estimate PDCAAS values for all humans and the accuracy of those requirement estimates have been questioned (Millward et al. 1989; Young & Borgonha 2000).

Taking these criticisms into account, the FAO (2013) has proposed that the PDCAAS be replaced by the **digestible indispensable amino acid score (DIAAS)**. The DIAAS is based on the determination of the true digestibility of individual AA at the end of the small intestine (ileum), and the pig has been recognized as a better model than rat for estimating protein and AA digestibility in foods for human. Moreover, DIAAS uses new reference protein patterns based on the pattern for the 3 to 10 years old children. DIAAS (%) is thus defined as:

$$\text{DIAAS} = (\text{CI of LEAA in 1 g of the dietary protein} \times \text{True ileal digestibility of the limiting EAA}) \times 100 \quad (3)$$

DIAAS should not be truncated above 1 or 100%.

Both ileal and faecal AA or N digestibility approaches may be subjected to important limitations, but ileal digestibility better reflects the amounts of AA released from the dietary protein by the action of the body enzymes and then absorbed through the intestinal epithelium. Digestibility should be based on the true ileal digestibility of each AA preferably determined in humans, but if not possible, in growing pigs or in growing rats in that order. Recommended AA scoring patterns (i.e. amino acid pattern of the reference protein) to be used for calculating DIAAS are as indicated in Table 3:

- Infants (birth to 6 months), pattern of breast milk.
- Young children (6 months to 3 y), pattern for the 0.5 y old infant.
- Older children, adolescents and adults, pattern for the 3 to 10 y old child.

Table 3. Recommended amino acid scoring patterns for infants, children and older children adolescents and adults. (FAO, 2013).

Age Group	His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
<i>scoring pattern mg/g protein requirement</i>									
Infant (birth to 6 months) ¹	21	55	96	69	33	94	44	17	55
Child (6 months to 3 year) ²	20	32	66	57	27	52	31	8.5	43
Older child, adolescent, adult ³	16	30	61	48	23	41	25	6.6	40

¹ Infant is based on the gross amino acid content of human milk from Table 4.

² Child group is from the 6 month (0.5 y) values from Table 3.

³ Older child, adolescent, adult group is from the 3-10 y values from Table 3.

Part 2. Alternative protein sources: what prospects?

1.2.1. Animal vs plant proteins

The demand for animal protein is expected to double by 2050, driven by population growth and by the emerging middle classes in developing countries (FAO 2006). Moreover, animal-based diet requires a significantly greater amount of environmental resources per calorie compared to a more grain-based diet (2 to 15 kg of plant foods are needed to produce 1 kg of meat; Aiking et al. 2011), as well as inefficient use of land and emission of gases (McMichael et al. 2007). In this context, it seems essential to search for alternative protein sources that show nutritional quality close to animal proteins one. In fact, there is a growing interest in utilizing plant proteins as partial replacers of animal proteins in food today (Ainis et al. 2018; Day 2013). Plant protein market represented 9.8 billion euros worldwide in 2018 and is expected to increase of 9% by 2023 (Technavio 2018). In 2018, North America was the largest geographical segment of the market studied and accounted for a share of around 38.6% of the market (Mordor Intelligence 2019).

In the context of human protein nutrition, the most important plant groups are cereal grains (wheat, rice, rye...) and food legumes (bean, pea, lentil...), including oil-seed legumes (soy, lupin...), either consumed as part of grain components (e.g. flours milled from grains), or as enriched protein ingredients as co-products of oil extraction or starch production (e.g. soy and gluten proteins). Protein content varies in large proportions from one plant source to another: between 8 and 12 % in cereals, between 20 and 25 % in dry legumes and up to 45% in oil-seed legumes like soy or lupin (Guéguen 1996; Popineau Y. 1985). Leaves contain between 15 and 20 % protein for example for alfalfa (Colas et al. 2013), but protein content is much lower in tubers, around 4 % in potato (Ralet & Guéguen 1999).

There are multiple reasons why plant proteins are still underutilized for human food. Their nutritional value is low as compared with animal proteins (deficiency in one or more EAAs and lower protein digestibility). It is difficult to maximize their techno-functionality due to their large molecular weight and size and poor solubility in water. The economic cost associated with isolation and recovery of protein fractions is also high. It is noteworthy that the use of plant proteins as ingredients is often associated with poor water solubility and a very pronounced taste (Day 2013; Silva et al. 2019; Wouters et al. 2016). Moreover, plant proteins also contain anti-nutritional factors (ANF) such as phytic acid, trypsin inhibitors or phenolic compounds that can lower the protein digestibility (Sarwar et al. 2012a).

However, there has been considerable progress through research and development to improve both the nutritional and functional properties of plant proteins. For instance, specific technological treatments enable to inactivate or inhibit most of the ANF (Kalpanadevi & Mohan 2013; Le Gall et al. 2005; Rizzello et al. 2016; Soetan & Oyewole 2007; Xu & Chang 2008) and thus improve biological value and digestibility of plant proteins. Soy protein serves as an excellent example of how scientific research can increase the nutritional value of proteins from plant sources. It also demonstrates how technological innovations can add value and diversify the use of plant proteins into a wide variety of food products (Childs et al. 2007; Friedman & Brandon 2001; Yeu et al. 2008). While soy protein serves as a reference as an alternative plant protein to replace animal protein, a range of new food products is starting to

appear, which use other grains, legumes and vegetables as sources of proteins (Asgar et al. 2010). In fact, the production of analogues for animal food products has accelerated in recent years, with some of the most promising alternatives based on proteins from plant sources, such as soybean and peas; the dairy-substitutes market has especially expanded. In fact, plant protein-based substitutes for meat and dairy products can deliver equivalent quality at lower costs, while fulfilling the world's priority of reducing greenhouse gas emissions and limiting destruction of forest land (Dijkstra et al. 2006; Linnemann & Dijkstra 2002).

As mentioned above, the nutritive values of various food proteins are to a large extent determined by the concentration and availability of the individual EAAs and total nitrogen (Boye et al. 2012; Young & Pellet 1994). Most animal proteins provide these EAAs in balanced proportions, but many plant proteins provide sub-optimal proportions. One of the EAAs, lysine, is at a lower concentration in most plant proteins compared with animal proteins, in particular for proteins from cereal sources (Young & Pellet, 1994; Millward, 1999). In addition, the sulphur containing AAs (methionine and cysteine) are also relatively lower in legumes compared with proteins of animal origin such as milk, egg and meat. However, the AAs content is only one of the factors determining the overall nutritional quality of dietary proteins. Other factors, such as the protein digestibility and the AAs bioavailability, can also affect the utilization of proteins by humans.

In general, the digestibility of plant proteins in their natural form is lower than the proteins from animal sources (WHO/FAO/UNU, 2007). However, plant proteins are often consumed after undergoing some processing to enhance their palatability, acceptance, but also to improve the digestibility of proteins. For instance, the ANF present in large amounts in plant proteins can be partially or completely inactivated or inhibited by heat treatment ANF (Kalpanadevi & Mohan 2013; Le Gall et al. 2005; Rizzello et al. 2016; Soetan & Oyewole 2007; Xu & Chang 2008).

Besides, one strategy to overcome the unbalanced AAs profile and low nutritional quality of individual plant proteins is to combine several plant protein sources. Thus, proteins from oilseeds or legumes that are low in sulphur-containing AAs can be used effectively in combination with most of the proteins from cereal grains which are deficient in lysine (Fenn et al. 2010; Repetsky & Klein 1982; Young & Pellet 1994). This association enables the complementation of limiting AAs and effectively meet recommended human nutritional requirements (Young & Pellet 1994).

Thus, the nutritional and functional properties of proteins depend on their origin as well as technological process. As mentioned, one recent challenge is to associate different sources of protein so as to formulate new ingredients or food products (Ainis et al. 2018; Alves & Tavares 2019). Therefore, a better understanding of the interactions between plant proteins and other plant or animal protein sources and their assemblies is expected.

1.2.2. Pea, faba bean, rice and potato: four promising plant proteins

1.2.2.2. Pea protein

Pea (*Pisum sativum* L.) is exploited extensively as an important source of commercial proteins. One of the reasons why pea is more commonly used instead of other legumes for commercial protein fractions

is that it can be grown extensively all over the world and the hull is easily removed. Global pea production shows a continuous increase for the last 30 years (McKay et al. 2003), due to significantly high demands on plant proteins and relatively low cost of pea production. The global market for pea protein is expected to reach 34.8 million US dollars by 2020 (Grand Review Research 2019).

Peas contain high levels of proteins and carbohydrates, relatively high concentrations of insoluble dietary fibres and low concentrations of lipids. Importantly, pea protein is a good source of EAAs when compared with the FAO reference standard pattern of AAs (Sirtori et al. 2012). Compared to cereal proteins, pea protein contains high levels of lysine, leucine and phenylalanine, but relatively low in sulphur-containing AAs (methionine and cysteine) (Pownall et al. 2010). Compared to soy or animal proteins, pea protein is characterized by its high digestibility, and relatively low negative health controversies (Babault et al. 2015; Banaszek et al. 2019; Yang et al. 2012a). Pea protein has been widely used as a substitute for soybean or animal proteins in various functional applications (Aluko et al. 2009; Barac et al. 2010; Maninder et al. 2007; Sandberg 2011; Wang et al. 2003).

Solubility of pea protein isolate strongly depends on pH with a minimum solubility between pH 5 and 6, which may diminish its subsequent functional properties (Adebiyi & Aluko 2011). Moreover, pea protein tends to form highly viscous solutions under high concentration. Chemical and enzymatic treatments of pea protein have been employed to overcome this viscosity issue as well as to improve its functional properties (Bajaj et al. 2016). Pea protein has also been used as emulsifier in spray-dried emulsions for the microencapsulation of oil (Gharsallaoui et al. 2009). Pea protein loses its net negative charge during acidification process to reach neutral charge around pH 5.2-6.1, its isoelectric point. Therefore, pea protein quickly aggregates and is subject to sedimentation in products that are acidified and heated (Lan et al. 2018).

1.2.2.3. Faba bean protein

Faba beans (*Vicia faba* L.) are widely cultivated and extensively grown in different parts of the world. They contain up to 30% of crude protein, approximately 50% of carbohydrate and no more than 15% of crude lipid (Macarulla et al. 2001). Due to their great resistance to low temperatures, they are, among leguminous plants, the best adapted to colder climates such as the Northern parts of Europe, and represent a source of energy, protein, folic acid, niacin, vitamin C, magnesium, potassium, iron and dietary fiber (Giménez et al. 2013).

Faba bean has a valuable content of both protein and energy (Crépon et al. 2010). Due to the high levels of lysine in their protein, they are an adequate complement to the protein of cereals (Chillo et al., 2008). Faba bean has also a great potential in the snack food industry (Smith & Hardacre 2011). The use of faba bean flour for partial or total replacement of soybean meal (Azaza et al. 2009) or of wheat flour in the preparation of wheat-based foods such as bakery and pasta has been studied recently (Petitot et al. 2010). It has been mentioned that their addition to wheat dough does not affect their sensory characteristics (Giménez et al. 2013; Petitot et al. 2010). However, faba bean need to be avoided from people with glucose 6-phosphate dehydrogenase (or G6PD) enzyme deficiency which may develop severe reaction named “favism” (Luzzatto 2001). Favism manifests itself after ingestion of foodstuffs consisting or containing the beans of the leguminous plant *Vicia faba* due to the presence of glucosides,

resulting in destruction of red cells. Favism is more common and more life-threatening in children (usually boys) than in adults. From the public health point of view, it has been proven that favism can be largely prevented by screening for G6PD deficiency and by education through mass media.

Moreover, the preparation of faba bean concentrate or isolate (and other protein isolates) is performed in order to obtain products with some improved functional characteristics and reduced levels of ANF. It was also mentioned that faba bean isolate prepared by spray drying appeared less damaging than freeze-drying (Cepeda et al. 1998). Finally, faba bean is found the most soluble in basic environment, around pH 9.0 (Multari et al. 2015).

1.2.2.4. Rice protein

Rice (*Oryza sativa* L.) represents one of the leading food crops in the world. It is cultivated today in more than 100 countries, on all continents. It is the staple food for over half the world's population, mainly in Asian countries, where it provides a considerable proportion of the protein intake (Muthayya et al. 2014).

Rice has the lowest protein content of all the major cereals (Shih 2003), at 7-9% by weight, but the digestibility and biological value of rice protein have been reported to be higher than those of the other major cereals (i.e., wheat, corn and barley) (Eggum et al. 1989). Therefore, rice represents an interesting source of proteins for the development of protein-enriched ingredients. Also, rice is generally regarded as a hypoallergenic food (Helm & Burks 1996), being one of the first foods to be introduced into the diet of infants (under hydrolyzed form), and being used in most elimination diets for food allergy diagnostic programs in children and adults (Gastanduy et al. 1990). Therefore, hydrolyzed rice protein IF represent a suitable alternative for infant allergic to cow milk (Agostoni et al. 2007; Fiocchi et al. 2006; Reche et al. 2010). Moreover, several studies suggest that rice proteins have anti-oxidative (Yang et al. 2012b; Zhao et al. 2012), anti-hypertensive (Li et al. 2007), anticancer (Kannan et al. 2008, 2010) and anti-obesity effects (Yang et al. 2011, 2012b).

Besides, the relatively low protein content in rice and the low solubility of rice proteins in water, make it sometimes difficult to incorporate it into formulated foods (Shih 2003). Despite this, some awareness of the nutritional and health properties of rice protein has been increasing in recent years. Currently, alkaline, enzymatic and physical treatments for the extraction of proteins from rice flour or rice bran are being studied and applied industrially (Fabian & Ju 2011; Shih 2003). In this way, rice proteins are used as value-added ingredients in nutritional products, including sport nutrition supplements, as an alternative to the more commonly used milk and soy proteins, and as previously mentioned for infant formulas (Agostoni et al. 2007; Fiocchi et al. 2006; Reche et al. 2010).

1.2.2.5. Potato proteins

The white potato (*Solanum tuberosum*) is the fourth leading food crop in the world after wheat, rice and maize. Traditionally viewed as a source of starch, the potato is also a source of protein in human diet. Despite its relatively low protein content (1–2% on fresh weight basis) (Camire et al. 2009), the contribution of the potato to protein intake in our diet is significant, given the amount of potato consumed.

The potato has greater dry matter and protein per unit growing area compared with the cereals (Bamberg & Rio 2005). Moreover, potato protein has excellent biological value of 90–100 compared with whole

egg (100), soybean (84), and beans (73) (Buckenhuskies 2005). Potato protein has a balanced composition among individual EAAs to meet the nutritional needs of infants, small children and adults (Lopez de Romana et al. 1981; Woolfe 1987). In addition, allergic reactions to potato proteins have shown less than 5% allergenicity compared to 15% and 9% for eggs and cow milk, respectively (Majamaa et al. 2001). Given the agricultural availability, high content and balanced composition of EAAs, and low allergenicity, potato protein can be a valuable source of protein for human nutrition. However, strong protease inhibitors are present in potato tubers (Bryant et al. 1976; Mareš et al. 1989).

Potato proteins are shown to be highly soluble with an increased solubility from pH 4 to 8 (Jackman & Yada 1988) and seem to exhibit good emulsifying (Holm & Eriksen 1980) and foaming (Knorr 1980) properties. Recently, a large scale process has been developed to isolate proteins from raw potato tubers in a minimally processed (native form). The process involves chromatography and ultrafiltration techniques, and does not rely upon any chemical processing that would alter the protein (Giuseppin et al., 2008; Phillips & Williams, 2011). Potato protein isolates are now commercially available as ingredients (<http://www.solanic.eu>) and can be used in a wide range of food applications including meat-free analogues, gluten-free bakery products or dairy-free products. Potato protein isoaltes have also shown a relatively good digestibility *in vitro* and *in vivo* for some of the purified protein fractions (He et al. 2013a) .

1.2.3. Functional properties of proteins

Besides nutritional functions, proteins also have physical functional roles in food preparation, processing, storage and consumption which contribute to the quality and organoleptic attributes of food products. The most important functional properties of protein in foods include solubility, thickening effect, gelling, emulsifying, and foaming abilities. These properties relate to the way in which proteins interact with macromolecules (carbohydrates, lipids and proteins) and microcomponents (gases, salts, volatiles and water), as well as the molecular size, the structure (primary, secondary and tertiary), and the global charge of the protein. The environment of the protein during process will change protein structure and thus affects its functional properties and consequently its nutritional functions.

Plant proteins were first classified by (Osborne 1924) on the basis of their solubility. The four major classes of plant proteins that are since known as “Osborne fractions” are: albumins, globulins, prolamins and glutelins. Albumins are soluble in water and coagulable by heat, whereas globulins are insoluble in water but soluble in saline solutions. Prolamins are insoluble in either water or saline solutions but extractable in concentrated aqueous alcohol solutions (i.e. 60-70% v/v). Glutelins are not soluble in neutral aqueous solutions, saline or alcohol but may be extractable in dilute aqueous acid or alkali solutions. Most of plant extracts contain these four protein classes, but in highly variable proportions.

1.2.3.1. Structural specificities of proteins and sensitivity to proteases

A number of plant proteins present high resistance to proteolysis in the gastrointestinal tract because of specific structural properties. As concerns legume proteins, their structural stability has been reported to affect not only *in vivo* digestibility and availability of EAA, but also the production of bioactive sequences. Examples of three-dimensional structure of legume proteins, in comparison with that of well-

characterized proteins from animal sources (myoglobin, bovine serum albumin and β -lactoglobulin) are presented in Figure 5. In comparison with proteins of animal origin, it is evident that proteins from legume seeds are characterized by a high content in β -sheet conformation and a relatively low amount in α -helix, a feature that is shared by other plant proteins, notably those from cereal grains (Carbonaro et al. 2012). It can be observed that in β -lactoglobulin from cow milk, the presence of a predominantly β -sheet structure is possibly related to its resistance to denaturation during gastrointestinal digestion (Sawyer & Holt 1993).

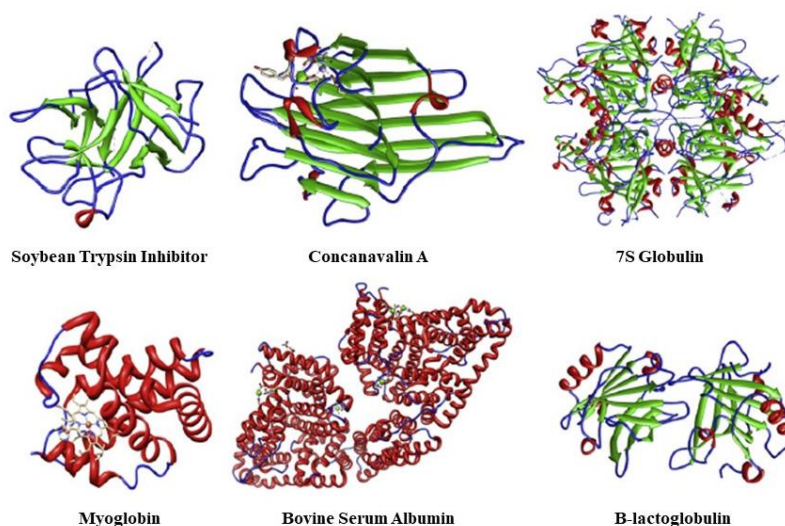


Figure 5. 3D structure of legume proteins in comparison with animal proteins as rendered by the Chimera software, available at <http://cgl.ucsfzsu/chimera/>. (Carbonaro et al. 2015).

The protein fraction of plant foods with a high cysteine content, the “albumin fraction” according to classical Osborne classification, has been found to be quite resistant to heat denaturation and proteolytic – trypsin, chymotrypsin and pepsin– digestion. Stability is conferred by the presence of a high number of disulfide bonds contained in low molecular weight (MW) proteins (Carbonaro et al. 2015).

1.2.3.2. Solubility

Protein solubility is often a prerequisite for other functional properties such as emulsification and foaming. Protein solubility can be affected by pH, ionic strength, type of solvent and temperature. Since proteins are least soluble at their isoelectric point, a common method used to isolate the most soluble plant proteins (largely, albumins and/or globulins) is based on this isoelectric point principle. Proteins are first solubilised using acid, alkali or solvent (with or without salt) away from their isoelectric point, and then precipitated out by adjusting the pH of the protein extract to the target isoelectric point.

The isolated proteins have good protein solubility at neutral pH. However, for most plant proteins, particularly cereal proteins that contain high levels of prolamins and glutelins, solubility at neutral pH is extremely low due to their low contents of charged AA residues.

1.2.3.3. Emulsification

The amphiphilic nature of some proteins (mainly animal proteins) allows them to be adsorbed at oil/water and air/water interfaces and stabilize food emulsions and foams (Damodaran 2006; Dickinson 2010).

The ability of a protein to function as a food emulsifier is governed by its structure and properties at colloidal interfaces.

Recent studies have rendered some new insights into the structure of soy proteins (either individual or as a mixture) adsorbed at emulsion interfaces. β -Conglycinin from soy proteins has better emulsifying properties compared with glycinin, due to its smaller molecular mass, higher structural flexibility (more easy to unfold) (Chove et al. 2001). Similar conclusions have also been reported for pea proteins. Vicilin from pea protein was found to be more surface active and led to better emulsifying properties compared with legumin (Dagorn-Scaviner et al. 1987). The vicilin/legumin ratio was found to influence the efficiency of pea isolates as emulsifying agents.

The ability of plant proteins to stabilize emulsions is highly influenced by pH, ionic environment, variation in processing pre-treatment of the proteins and thermal processing of emulsion-based foods. Moreover, plant proteins are generally supposed forming a relatively thicker interfacial layer at oil/water interfaces, compared with dairy proteins, due to their much larger molecular size and structural constraint by disulphide crosslinks (Wong et al. 2012). The weak protein interactions once they are adsorbed at interfaces, and the thickness of protein films would explain the higher stability of emulsions stabilised by some of the plant proteins compared with dairy proteins (Day et al. 2009; Keerati-u-rai & Corredig 2009).

1.2.3.4. Gelation

The aggregation phenomenon, which is the first step for protein gelation, is commonly obtained by heating globular proteins above their denaturation temperature. This applies to a variety of globular proteins either coming from animal or plant sources. Upon denaturation, the proteins unfold, expose reactive groups and hydrophobic regions previously buried in the core of the globular structure, and then aggregate by forming hydrophobic interactions, hydrogen bonds and thiol/disulfide exchanges (Durand et al. 2002; Nicolai et al. 2011). How the aggregation of globular proteins takes place, and the characteristics of the resulting aggregates and gels, depends on the equilibrium between repulsive and attractive interactions between heat denatured proteins. For example, a high level of electrostatic repulsions (low ionic strength and pH far from the isoelectric point) is required to prevent rapid and random protein aggregation, and instead to favour ordered linear polymers (Doi 1993).

Table 5 summarizes the physicochemical properties of pea, faba bean, rice, potato and cow milk proteins. Pea and faba bean proteins, which both belong to legumes, have a majority of globulin proteins (legumin, vicilin and convicilin), and molecular size ranging from 37 to 67 kDa. The pI are quite similar between pea and faba bean proteins, ranging from 5.2 to 6.1 for the major proteins of pea and 5.5 to 5.8 for faba bean major proteins. Rice has a majority of glutelin proteins with a molecular size of 56 kDa and pI ranging from 4.6 to 4.8 for rice glutelin. Potato has a majority of albumin proteins (patatin) with molecular size of 42 kDa, and a pI of 5.1-5.4. Cow milk contains a majority of caseins (80%) and whey proteins (20%) with molecular size of 19-25 kDa and pI of 4.2-5.1 for the major casein proteins; 14 to 88 kDa for whey proteins and a pI of 5.1-5.2 for the major whey proteins. These differences in physicochemical properties can easily explain the different functional properties of these proteins, and their specific behaviour during mechanical or heat treatments as well as through simulating digestion process.

Table 5. Physicochemical properties of cow milk, pea, faba bean, rice and potato proteins.

Protein	Scientific name	Protein Family	Protein Name	Proportion (%)	Molecular Size (kDa)	AA number	PI*	Uniprot code	References
P E A	<i>Pisum sativum</i>	Globulin	Legumin (11S)	30-40	56-58	503-517	6.06-6.11	P02857 P15838	Amagliani & Schmitt (2017); Guéguen et al. (2016)
			Vicilin (7S)	15-20	46-52	410-459	5.33-5.39	P13918 P02854	
			Convicilin (7S)	15-20	42-67	386-571	5.23-6.11	P13915 P13919	
		Albumin	Albumin (2S)	15-25	14	130	5.6-6.89	P62929 P62931	
F A B A B E A N	<i>Vicia faba L.</i>	Globulin	Legumin (11S)	30-35	37-54	329-484	5.46-5.78	P05190 P16080 P16079	Carbonaro et al. (2005); Crépon et al. (2010); Vioque et al. (2012)
			Vicilin (7S)	15-20	53	463	5.76	P08438	
			Convicilin (7S)	15-20	55-57	477-497	5.84	BOBCL7 BOBCL8	
		Albumin	Albumin (2S)	15-25	N.A	N.A	N.A		
R I C E	<i>Oriza sativa L.</i>	Albumin	Albumin	2-6	44	425	5.11	Q7X9A8	Amagliani et al. (2017); Ju et al. (2001)
		Globulin	Globulin	12	46-52	436-470	4.84-6.28	Q852L2 Q6ZLB0	
		Glutelin	Glutelin	80	53-56	480-496	4.66-4.84	Q6ZLB0 Q10NQ3	
		Prolamin	Prolamin	4	46	436	4.84-5.11	Q6ZLB0 Q7X9A8	
P O T A T O	<i>Solanum tuberosum</i>	Albumin	Patatin	50-60	42	387	5.05-5.36	P15478 P15477	Camire et al. (2009); Ralet & Guéguen (2000)
		Globulin	Globulin	25-26	35	306	N.A		
		Prolamin	Prolamin	2-4	15-40	130-360	4.23-6.52	M1CDL3 M0ZXN3 A4UV13	
		Glutelin	Glutelin	9	N.A	N.A	N.A		
C O W M I L K	Bovin	Caseins and casein micelles (80 %)	αs1-casein	38	23.6	199	4.2-4.8		Cheftel & Lorient (1982) Dalglish & Corredig (2012) Modler (1985)
			α s2-casein	11	25.3	207	N.A		
			κ -casein	14	19.0	169	4.1-5.8		
			β-casein	36	24.0	209	4.6-5.1		
		Whey proteins (20%)	β-lactoglobulin	44	18.4	162	5.2		

α-lactalbumin	20	14.2	123	5.1
Serum-albumin	4	69.0	609	4.8
Lactoferrin	1.6	88.0	698	N.A
Immunoglobulines	11	160-960	N.A	4.6-6.0
Caseino-macro-peptids	20	8.0	64	N.A

*calculated from the amino acid sequence of the protein using the Compute pI/MW tool of ExPASy.

Part 3. Infant nutritional needs

Infants are the people under the age of 12 months and infant formula (IF) is the product presented as human milk substitute, which satisfies the nutritional requirements of infants (European Union 2016). It should nevertheless be pointed out that human milk is the best food for adequate growth and development of infants as it contains a balance of essential nutrients and specific bioactive components such as growth factors, immune factors, enzymes etc. that are explicitly available only in mothers' milk (Michaelsen & Greer 2014). Therefore, IF forms a substitute only when human milk is inadequate or unavailable for any reason (Agostoni et al. 2008). Ideally, both breast-fed and formula-fed infants should show similar growth and development patterns (Lonnerdal 2014). To achieve this goal, IFs are formulated in such a way as to mimic the composition in macronutrients (protein, fat, and carbohydrates) and micronutrients (vitamins and minerals) of human milk (Lonnerdal 2014). Alongside this, there is a need to study the digestibility of various ingredients supplemented in IF to better understand the degradation mechanism of these components as well as the bioaccessibility of the digested nutrients in the gastrointestinal tract.

1.3.1. Infant nutritional requirements

1.3.1.1. Recommendations and reality

As mentioned above, human milk is the gold standard for infants as it satisfies all the specific needs during the first six months of life. Many national and international organizations as the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN), the World Health Organization (WHO), the Food and Agriculture Organization (FAO) and the United Nations International Children's Emergency Fund (UNICEF) agree to promote breastfeeding during the first six months of life, followed by breastfeeding combined with food supplement until the age of 2 years to provide an optimal growth of the child (Agostoni et al. 2009; WHO & UNICEF 2003).

However, breastfeeding remains far below the recommendations. In fact, only 38% of infants aged 0 to 6 months are exclusively breastfed worldwide (Black et al. 2013; WHO 2013; WHO & UNICEF 2014) and a lot of disparities exist between countries (Figure 6) (Victora et al. 2016). For instance, in France, 70% of infants are breastfed from birth, but only 39% are still breastfed at 3 months (with 10% exclusively breastfed), 19 to 23% at 6 months and 5 to 9% at 1 year (Figure 7) (Chouraqui et al. 2018; Wagner et al. 2015).

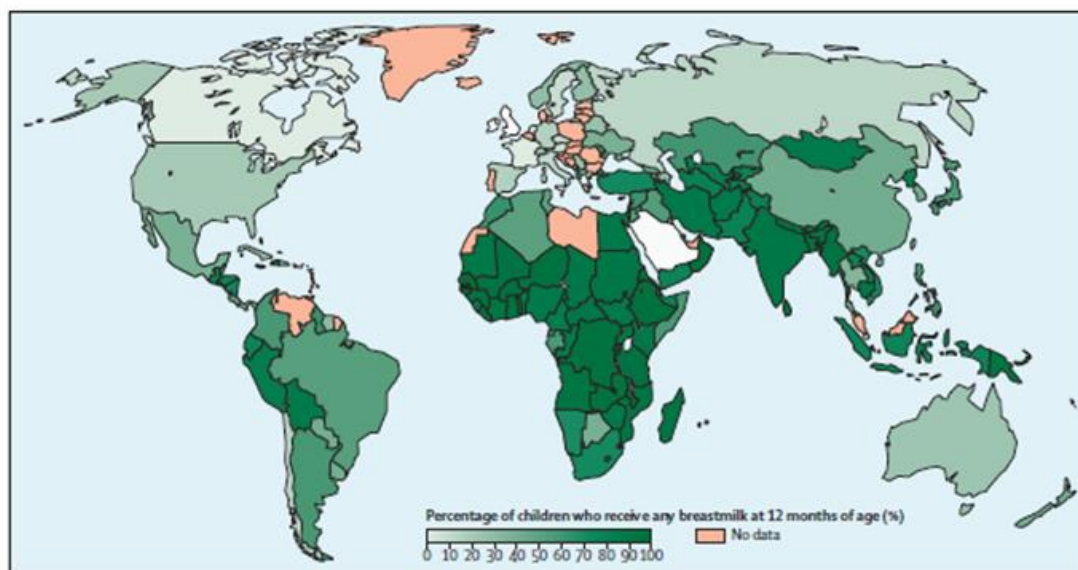


Figure 6. Global distribution of breastfeeding at 12 months of age after birth (data are from 153 countries between 1995 and 2013). (Victora et al. 2016).

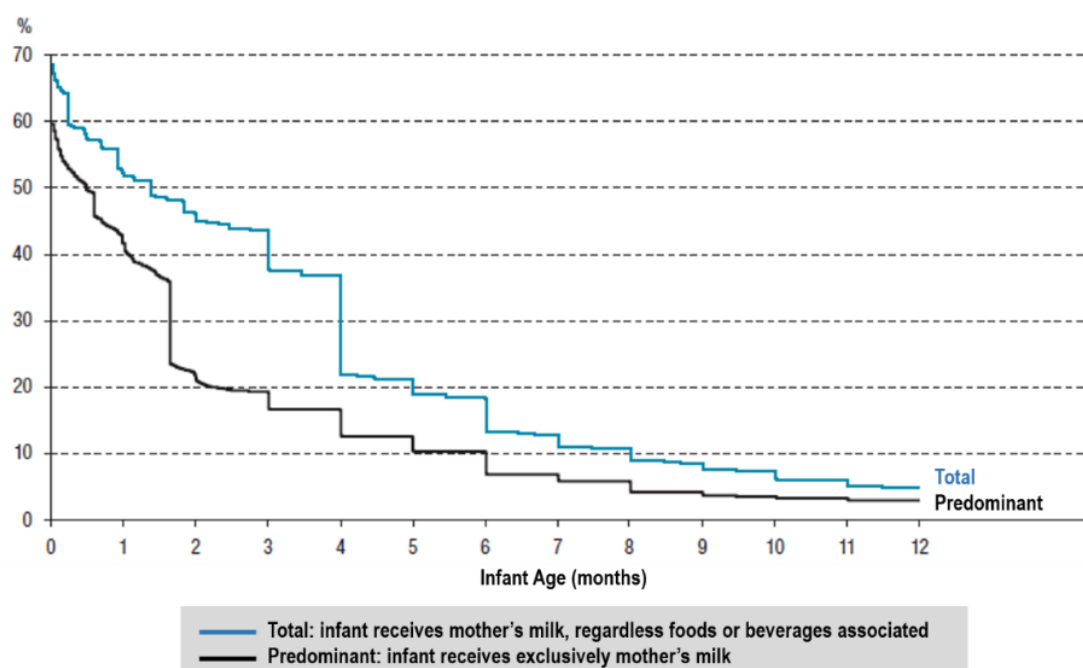


Figure 7. Prevalence of breastfeeding from birth to 12 months in France. Elfe study 2011. (Wagner et al. 2015).

Exclusively breastfeeding during the first six months of life is one of the main challenge in nutrition by 2025 (WHO & UNICEF 2003; WHO & UNICEF 2014). However, when mothers may not or do not wish to breastfeed their children, the only alternatives are the IFs (Agostoni et al. 2008). In order to develop more biomimetic IFs, it is thus essential to understand the specificities of human milk composition and structure.

1.3.1.2. Composition and structure of macronutrients in human milk

Human breast milk is generally and universally recognized as the optimal choice for nutrition during the first year of life. It contains many different macronutrients (proteins, lipids, carbohydrates), micronutrients (vitamins and minerals), water and biologically active molecules, and its composition highly varies throughout the lactation cycle (Figure 8), throughout the day, with maternal diet and length of gestation, and is believed to match with the infant needs (Andreas et al. 2015; Gidrewicz & Fenton 2014). Human milk also contains several bioactive factors which exert antimicrobial, immunomodulatory and digestive functions, and compensate for immaturities in the neonatal immune and digestive systems (Andreas et al. 2015; Donovan 2016; Labbok et al. 2004; Victora et al. 2016). Moreover, human milk plays an important role in the development of the intestinal microbiota of the newborn, particularly thanks to pre- and probiotics components (such as oligosaccharides and bacteria) (Wang et al. 2016).

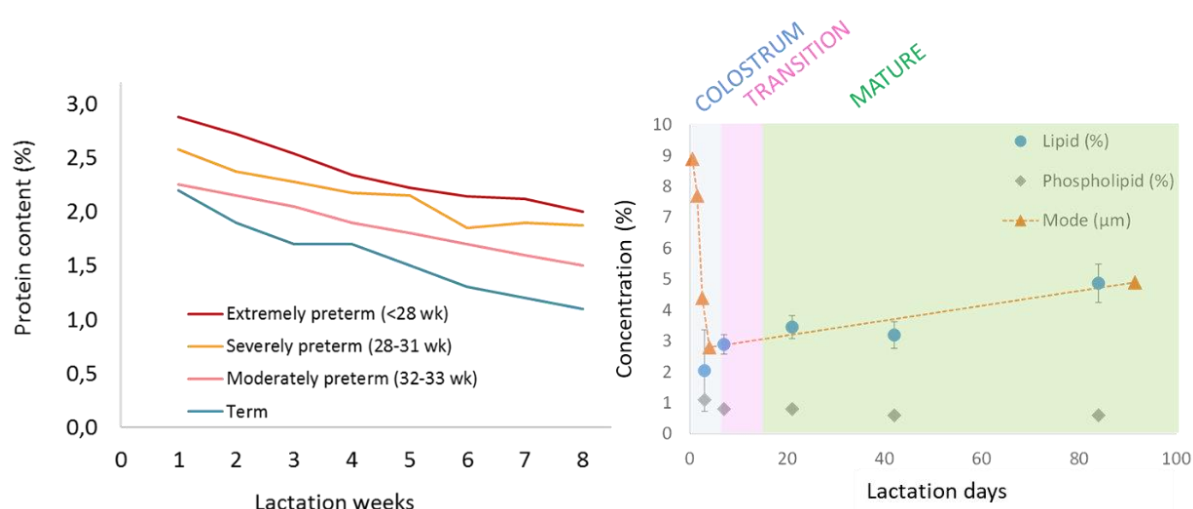


Figure 8. Protein and lipid changes over lactation.

(Protein content: adapted from (Bauer & Gerss 2011); Total lipid, cholesterol and phospholipid contents: adapted from Jensen 1999; Mode diameter: adapted from Michalski et al. 2005).

In terms of structure, human milk is a natural oil-in-water emulsion composed by native milk fat globules (MFG) dispersed in an aqueous phase containing a colloidal suspension of caseins and soluble components, such as whey proteins, carbohydrates and non-micellar mineral fraction. The nutritional composition of human milk in terms of lipids and carbohydrates will be shortly presented to finally focus on proteins, which is in the heart of this PhD project. Vitamins and minerals will not be discussed in the present manuscript.

CARBOHYDRATES

Lactose, a disaccharide of glucose bound to galactose, is the main carbohydrate in human milk and is also the macronutrient with the most stable concentration during lactation period (67-87 g/L) (Nommsen et al. 1991). Human milk also contains a large amount of oligosaccharides (HMO, Human Milk Oligosaccharides) as well as glycolipids and glycoproteins. HMO are complex carbohydrates formed by three to ten monosaccharides (Andreas et al. 2015) and are present in concentration from 5 to 20 g/L in term milk with more than 200 forms. Not digested by the infant, HMO have been largely studied for

acting as prebiotics and playing an essential role in modulating the microbiota and preventing infections (Wang et al. 2016).

LIPIDS

The fat in human milk represents 40 to 55 % of its total energy (Koletzko et al. 2001). Lipids from human milk provide to the infants essential fatty acids (FA), phospholipids, liposoluble vitamins which act as precursor for hormones and eicosanoids (long chain polyunsaturated fatty acids, *i.e.*, arachidonic acid) synthesis (Jensen 1999). Fat play many different functions: energetic, structural thanks to their integration in cells membrane, and functional. Fat is the macronutrient that varies the most during human lactation (Figure 8) (Michalski et al. 2005). Milk Fat Globule (MFG) presents complex physicochemical structure with polydispersed sizes ranging from 0.1 to 10 μm (Figure 9) in mature human milk (4 μm on average) (Bourlieu et al. 2015; Michalski 2009)(Bourlieu & Michalski, 2015). Triglycerides (TG) are exclusively located in the core of MFG, which is surrounded by a three-layer biological membrane consisting of a phospholipid/cholesterol monolayer with incorporated proteins (Figure 9). These lipid droplets are secreted from the lactocytes by fusion with the apical plasma membrane, after being surrounded by secretory vesicles (Figure 10) (Hernell et al. 2016).

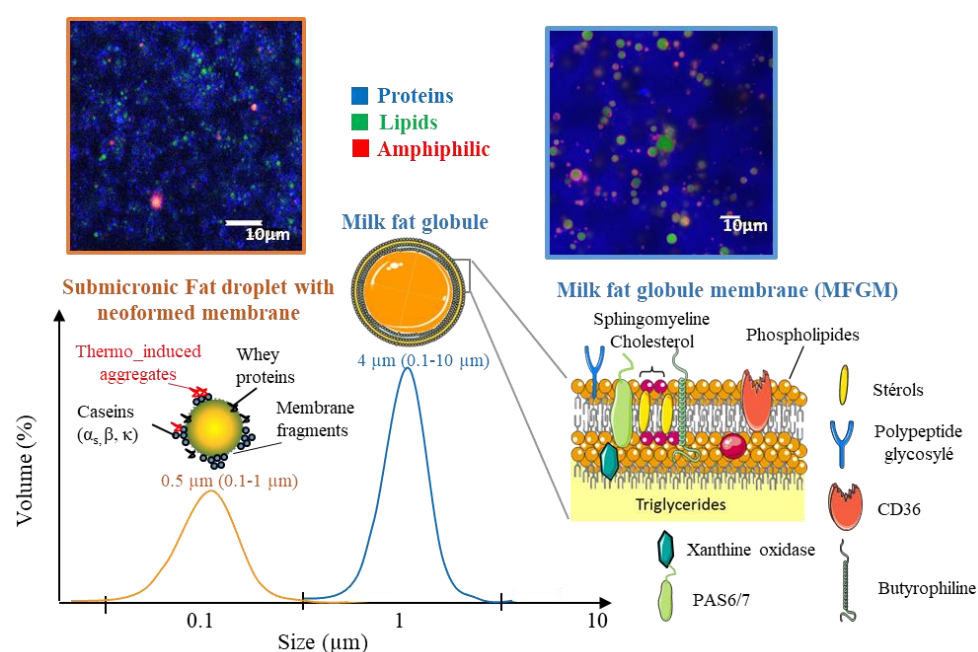


Figure 9. Comparison between fat structure in human milk (MFG) and infant formula (submicronic fat droplet).
(Adapted from Bourlieu et al. 2015; Michalski 2009).

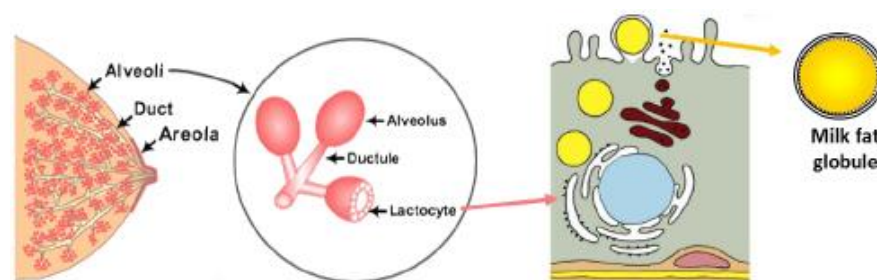


Figure 10. Secretion process of human milk fat globule. (Hernell et al. 2016).

PROTEINS

Proteins in human milk is decreasing between the beginning and the end of the lactation, going from 18-24 g/L to 15-20 g/L after 3-4 months and 8-14 g/L at 6 months age for a term infant (Figure 8) (Bauer & Gerss 2011). Protein content is remarkably higher in immature milk for preterm infants (Figure 8), reaching up to 30 g/L in an extremely preterm infant (birth before 28 weeks) (Bauer & Gerss 2011). Proteins in human milk are composed of around 40 % caseins (mainly β -, then κ - and α s1) and 60% whey proteins (mainly α -lactalbumin, lactoferrin, immunoglobulins and serum albumin) (Lönnerdal 2003). However, the ratio between whey proteins and caseins varies, from 80:20 in the early lactation to 50:50 at 6 months of lactation. The main difference compared to cow milk proteins is the absence of α s2-casein and β -lactoglobuline in human milk (this will be discussed hereafter, see Table 7). Table 6 shows protein fractions in mature term human milk. The AA profile is entirely consistent with the infant needs.

Table 6. Protein composition of term mature human milk^a.

Fraction	Term HM (g/L)
Whey proteins	5.3-6.6
<i>α-lactalbumin</i>	2.5
<i>Lactoferrin</i>	1.5
<i>Immunoglobulins</i>	1.2
<i>Serum albumin^b</i>	0.4
<i>Osteopontin</i>	0.138
<i>Lactoperoxidase</i>	0.005
Caseins	3.5-4.4
<i>β-casein</i>	2.7
<i>κ-casein</i>	0.9
<i>αs1-casein</i>	0.6
MFGM proteins	0.05-0.1

^aSummarized from (Chatterton et al. 2013)

^bData from (Jensen 1995)

MFGM, milk fat globule membrane

Whey proteins

The high content of whey proteins in human milk may have major relevance in infant nutrition, since several whey proteins have important physiological functions and are often able to bind vitamins, minerals and hormones (Chatterton et al. 2004; Lonnerdal 1989). Below are described the three major whey proteins from HM.

(a) α -lactalbumin is the main whey protein in human milk and accounts for 41% of whey and 17-28% of total protein. It is a calcium-binding protein but it can also bind iron and zinc, and hence plays a role on the absorption of these minerals (Wada & Lönnerdal 2015a). Despite high resistance to digestion due to its stable structure when bound to calcium (Chatterton et al. 2013), α -lactalbumin hydrolysis releases bioactive peptides (Wada & Lönnerdal 2015a), that have been shown to have antibacterial and immuno-stimulatory properties, as well as to encourage the growth of bifidobacteria (Lonnerdal 2016).

(b) Lactoferrin is the second highest whey protein in human milk with an average amount of 1.4 g/L (Ferrer et al. 2000; Mao et al. 2017). It is a ferric iron-binding glycoprotein reported to resist the

gastrointestinal digestion and thus to exert several bioactivities. For these reasons, lactoferrin has been extensively studied over the last years (Donovan 2016; García-Montoya et al. 2012; Lonnerdal 2014). Several activities have been attributed to lactoferrin, such as antimicrobial, immunomodulatory, anti-inflammatory, anticarcinogenic, enzymatic and gene regulating activities (García-Montoya et al. 2012; Labbok et al. 2004).

(c) Immunoglobulins in human milk are secreted as sIgA, IgG1, IgG2 and IgM, in which sIgA makes up the largest proportion with over 90%, at around 1-2 g/L. The highest concentration of sIgA is found in human colostrum with 9 g/L (Lonnerdal 2014). Human colostrum contains approximately 100-fold higher concentration of immunoglobulins than cow milk. Immunoglobulins play an important part in protecting the newborn against infections from intestinal tract diseases (Feng et al. 2017; Uruakpa et al. 2002).

Caseins

The key caseins present in human milk are β - and κ -caseins, while α s1-casein is present in low concentrations (Lonnerdal 2016). They are associated and linked together in the micelles with mineral ions (mainly calcium, phosphorus and magnesium), and are reported to exert protective functions, i.e. immuno-modulatory and anti-inflammatory activities.

(a) β -casein is a highly phosphorylated protein that has been associated to antithrombotic, and antihypertensive activities (Holt et al. 2013; Swaisgood 1993). When being broken down in the gastrointestinal tract, smaller casein phosphopeptides are formed which facilitate calcium and zinc absorption (Lonnerdal 2014).

(b) κ -casein is heavily glycosylated and is present in smaller amounts than β -casein in human milk. This casein is supposed to inhibit bacterial adhesion and to exert immunomodulatory activity (Lonnerdal 2016).

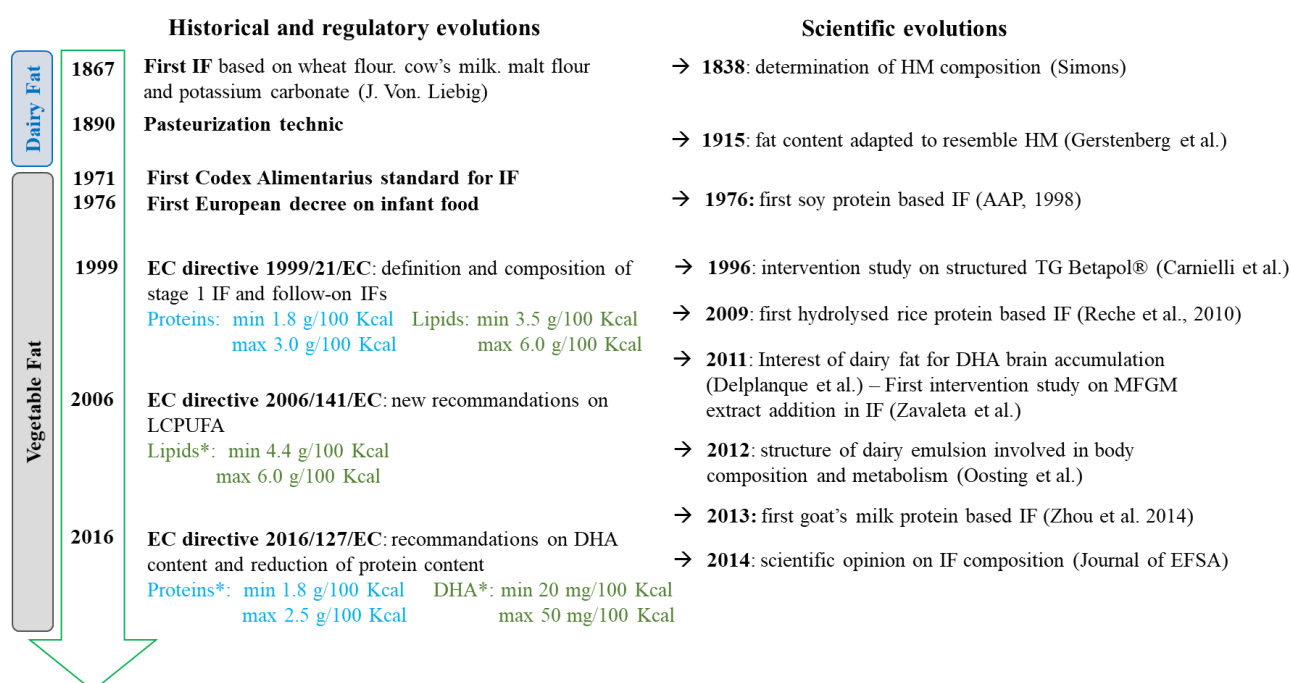
1.3.1.3. Regulation and composition of infant formulas

HISTORY AND REGULATION EVOLUTIONS

IFs are by definition foods intended for particular nutritional use by infants during the first months of life and satisfying by themselves the nutritional requirements of such infants until the introduction of appropriate complementary feeding (EU 2016).

Human milk substitutes existed before the modern age of formulas. Because some infants could not be fed by their mothers, humans adopted two methods for substitute feedings. The most obvious was the utilization of a surrogate mother (e.g., wet nurse), who would feed the child human milk. The alternative was to feed the child milk obtained from another mammal. The most frequently used sources were cow, sheep and goat (Fomon 1993). It has very recently been demonstrated that infants from prehistory were already fed with milk products derived from ruminants (Dunne et al. 2019). Until the end of the nineteenth century, the use of a wet nurse was by far the safest way to feed infants who could not be breastfed. As general sanitation measures improved during the latter part of the nineteenth century, and as differences in composition between human milk and that of other mammals were defined, feeding animal milk

In 20th century, successive improvements in scientific and technological advances emerged (improvement of safety conditions, pasteurization, glass baby bottle with rubber teat and a better understanding of human milk composition and infant nutritional needs) and provide better quality infant products (Bourlieu et al. 2017; Lonnerdal & Hernell 2016). In order to assure this point and also to provide appropriate intake of nutrients during infancy, different authorities worldwide are responsible for determining regulations and guidelines for IF: the US Food and Drug Administration (FDA), the European Commission (EC), and the ESPGHAN, as reviewed in (Zou et al. 2016). In 1961, the first whey-dominant formula was launched, and in 1972 came the first Codex Alimentarius standard for IFs. Since 1976, these authorities establish the minimum and maximum limitations for energy, macro- and micronutrient contents and ingredients allowed in IF in order to meet the infant nutritional needs (Figure 11). IF composition is therefore strictly regulated.



*Proteins and lipids contents corresponded to 1st age IF (0-6 months) recommendations with proteins based on cow milk source.

In order to adapt gradually to the needs of growing infants, the composition of IFs must vary according to the age of the infant. There are currently three different formulas (EU 2016):

- The first age IF is designed for infants from birth to 6 months age;
- The second age IF or follow-on IF is progressively introduced in transition period and dietary diversification from 6 months to 12-18 months infants;
- The third age IF or growing-up IF is also introduced in dietary diversification period for babies from 12 to 36 months.

According to (Lonnerdal & Hernell 2016), staging of IFs should be more specific and suited to infant changing needs during infancy. In fact, staging of term IFs would allow consideration of higher protein, energy, and DHA needs during the first months of life and lower requirements later during infancy, and, conversely, less iron during early life and more during late infancy. Although such staging would need clinical studies for evaluation of the concept, it seems reasonable to suggest at least three kinds of IFs: one for early infancy (0–2 or 3 months, stage 1), another one for 3 to 6 months of age (stage 2), and a third one after the first half of infancy (6–12 months) when the diet is becoming diversified (stage 3) (Figure 12). Hypothetically, even more stages may be considered, if this could be solved practically.

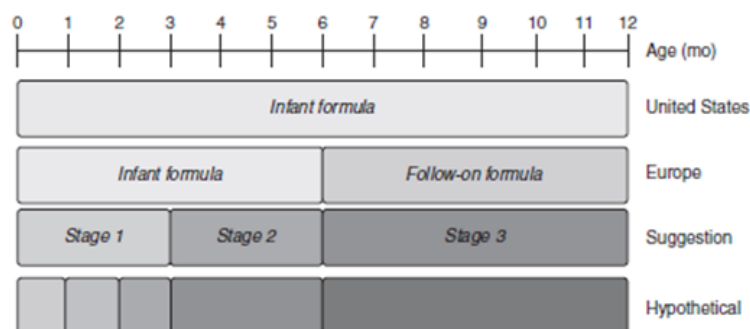


Figure 12. Staging of infant formula, present situation and suggested changes. (Lonnerdal & Hernell, 2016).

Most IFs are formulated with cow milk. However, the use of cow milk may be limited in some cases, such as lactose intolerance, allergy to cow milk proteins and the 'nutritional imbalance' of several nutrients. There are also several types of preparations (first age and/or second age) for special medical purposes to meet the special requirements of some infants (Maldonado et al. 1998):

- **Hypoallergenic formula** prepared from partially hydrolysed proteins to prevent infants with a high risk of cow milk protein allergy having an allergic reaction. However, several studies have shown contradictory findings on the efficacy of hydrolyzed formulas in preventing allergic diseases in infants (Alexander & Cabana 2010; Boyle et al. 2016; Greer et al. 2008; Hays & Wood 2005; Vandenplas et al. 2014).
- **Soy-based formula**, prepared with soy protein isolates, for infants with lactose intolerance and/or galactosemia (Heyman 2006; Thompson & Kharb 2007). However, a soy-based formula is not recommended for infants with cow milk protein allergy because in 10-14% of the cases, these patients also suffer from soy protein allergy (Agostoni et al. 2006; Bhatia et al. 2008).
- **Elemental formula**, using free amino acids instead of proteins or peptides, for infants who are allergic to hydrolysed cow milk protein or soy protein.
- **Anti-regurgitation formula** prepared with starches, carob seed flour, or other thickeners for infants prone to gastroesophageal reflux.
- **Lactose free formula** for infants who are lactose intolerant due to congenital or temporary lactase deficiency (Heyman 2006). Soy-based formulas are one example of lactose free formula, but other lactose free formulas exist.

Lastly, special formulas such as **transit formula enriched with lactose to prevent constipation, and nutrient-dense formulas enhanced in proteins, fats, minerals and vitamins for premature infants or low-birth-weight infants** also exist (Benson & Masor 1994).

The following is an outline of first age IFs (0-6 months).

The current European regulations and directives are n°2013/609 (which amends 2006/141 directive) and n°2016/127 from the European Commission (EU 2013, 2016). This last regulation mainly indicates a reduction of protein content to prevent from potential “metabolic stress” such as obesity (Lonnerdal 2016), the addition of hydrolysed proteins from demineralized cow whey proteins as a new protein source, and the addition of DHA is become mandatory to ensure a proper neurodevelopment of infants (Delplanque et al. 2011). **This last regulation also mentions that new ingredients may be added to IFs if they are suitable and safe according to scientific evidence.** The latest directive (2016/127/EC) should be applicable from February 2020 except for the hydrolysed protein based IFs that would be applicable from February 2021.

IF COMPOSITION AND STRUCTURE

IFs are manufactured milk-based formulas in powder or liquid form, mostly composed of skimmed cow milk added with cow whey protein concentrate, vegetable oils and other purified compounds such as specific fatty acids and micronutrients. The IFs result from successive technological operations that would be described in Section 1.4. IFs can be divided in three groups considering the protein source: IFs based on cow milk proteins (the most commonly used IF) or goat's milk proteins, IFs based on soy protein isolates and IFs based on hydrolysed proteins (which can be from cow milk, soy or rice protein sources).

Considering the most common IFs, it is evident that cow milk needs some modifications in order to cover infant nutritional needs. Table 7 shows the average composition of mature human milk as compared to cow milk and IF. Human milk contains less protein, less ash and more lactose compared to cow milk. In cow milk, the ratio casein/whey proteins is much higher than in human milk (80:20 and 40:60 w/w, respectively). As mentioned, contrary to cow milk, α s2-casein is absent in human milk (Chatterton et al., 2013). In addition, the major whey protein is β -lactoglobulin (50 % of total whey proteins), which is absent in human milk. β -lactoglobulin is thought of as an allergen (Wal 2004), and the disulphide (S-S) bonds in its structure may be responsible for its allergenicity (Matsumoto 2011). Therefore, removing β -lactoglobulin from cow's whey or using hydrolysed whey were suggested in order to make IFs closer to human milk (Eugenia Lucena et al. 2006). Inversely, α -lactalbumin is the main whey protein in human milk but accounts for only 3-3.5% of total proteins in cow milk (Heine et al. 1991). Because in human milk, α -lactalbumin accounts for 63.2% of total EAA with a high content of lysine and cysteine and a remarkably high content of tryptophan (5.9% of total amino acids), the problem with IF based on cow milk is the low level of tryptophan and cysteine (Heine et al., 1991). This is the reason why the protein content in IF is adjusted to 11-21 g of protein/L compared 9-17 g of protein/L in human milk, to compensate for the difference in EAA between human and cow milks (Davis et al. 2008; Elgar et al. 2016). Similarly, because lactoferrin content in cow milk is very low (0.1 to 0.8 g/L vs 2 to 6 g/L in mature human milk) (Rai et al. 2014), lactoferrin was the first supplement added to IF in 1986 (Ben 2008).

However, due to the high cost of this ingredient and the difficulty in preserving the bioactive function of lactoferrin during IF production, the addition of lactoferrin in commercial IFs are still limited (O'Callaghan et al. 2011).

With regard to the lipid fraction, its concentration, types of molecules (about 98% of triglycerides) and organization in milk fat globules are similar in human and cow milks (Jensen 1999); but, their fatty acids composition is very different. Notably, cow milk contains less long-chain polyunsaturated fatty acids and more short-chain fatty acids than human milk. However, the fat in most IFs currently used is a mixture of vegetable oils and thus has a much less complex composition than human milk fat. In fact, IF containing dairy fats were widely used in the first part of the 20th century before decreasing (Figure 11) and are still used in some parts of the world. In IF based on vegetable fat, the fat globule is usually smaller than in human milk, and is composed of submicronic droplets (0.3 – 0.8 μm) stabilized by a neoformed membrane mainly based on milk proteins (Figure 9). Non-dairy tensioactives (mainly vegetable or functionalized lecithins or esters of partial glycerides) are usually added to help stabilizing the emulsion (Bourlieu et al. 2015; Zou et al. 2016).

Table 7. Nutritional composition of mature human milk, cow milk and infant formula (cow milk protein based). (Le Huërou-Luron et al. 2018).

/ 100 ml	Human milk	Cow milk	Infant formula ^a
Energy (Kcal)	65–70 (57–83)	67.4 (65–71.2)	60–70
Proteins (g)	1.20 (0.9–1.7)	3.25 (3.1–3.8)	1.1–2.1
Caseins (g)	0.37 (0.32–0.42)	2.51 (2.46–2.80)	0.4–0.8
α-Caseins (%)	11.8 (11.1–12.5)	48.5 (48.3–48.5)	
β-Casein (%)	64.8 (62.5–66.7)	35.8 (35.8–37.9)	
κ-Casein (%)	23.5 (22.2–25.0)	12.7 (12.7–13.8)	
Size of micelles (nm)	64	182	
Whey proteins (g)	0.76 (0.68–0.83)	0.57 (0.55–0.70)	0.6–1.3
β-lactoglobulin (%)	Absent	20.1 (18.4–20.1)	
α-lactalbumin (%)	42.4 (30.3–45.4)	53.6 (52.9–53.6)	
Immunoglobulins (%)	18.2 (15.1–19.7)	11.7 (10.1–11.7)	
Serum albumin (%)	7.6 (4.5–9.1)	6.2 (5.5–7.7)	
Lactoferrin (%)	30.3	8.4	
Lysozyme (%)	1.66	Traces	
Caseins/Whey proteins	30–40/60–70	80/20	40/60
Fat (g)	3.2–3.6 (1.2–5.2)	3.6 (1.2–7.9)	2.6–4
Triglycerides (%)	98.1–98.8	97.0	
Phospholipids (%)	0.26–0.8	1.5	≤ 7
Cholesterol (mg/100 mL)	10–20	1.0–3.3	Absent
% Fatty acids relative to total fatty acids			
Butyric (C4:0)	0.1	1.4 (1.4–3.3)	nq ^b
Caproic (C6:0)	0.2	2.1 (1.6–2.2)	0.1
Caprylic (C8:0)	0.3 (0.1–0.3)	1.7 (1.3–1.8)	1.0–1.5
Capric (C10:0)	2.0 (1.1–2.1)	3.5 (3.0–3.6)	0.9–1.3
Lauric (C12:0)	6.8 (3.1–7.2)	3.9 (3.1–4.0)	7.8–11.5
Myristic (C14:0)	10.4 (5.1–10.9)	12.6 (13.0–14.2)	4.0–5.5
Palmitic (C16:0)	28.1 (20.2–29.6)	29.5 (24.0–42.7)	18.2–25.4
Palmitoleic (C16:1)	3.5 (3.5–5.7)	1.7	0.1–0.2
Stearic (C18:0)	6.9 (6.0–8.6)	13.3 (5.7–13.7)	3.5–4.0
Oleic (C18:1)	33.6 (33.3–46.4)	26.3 (16.7–27.1)	28.4–40.8
n-6 PUFA			
Linoleic (C18:2)	17 (6.0–24)	2.9 (1.6–3.0)	13.3–18.5
Arachidonic (C20:4)	0.5 (0.25–0.75)		Added:0.2–0.6 / not added: nq
n-3 PUFA			
α-linolenic (C18:3)	1.7 (1.0–3.4)	1.1 (0.5–1.8)	1.6–2.4
Docosahexaenoic (C22:6)	0.32 (0.10–0.60)		Added:0.2–0.3 / not added: nq
Carbohydrates (g)	7.4–7.8 (6.0–9.6)	4.5	5.4–9.5
Lactose (%)	85	100	47–100
Oligosaccharides (%)	0.5–2.0	Traces	
Maltodextrin (g)	Absent	Absent	1.1–2.6
Minerals (g)	0.21	0.90	0.25–0.50
Na (mg)	16	48	12–42
Ca (mg)	33	125	30–98
K (mg)	16.5	100	36–112
Fe (mg)	0.05	0.03	0.2–0.9
Mg (mg)	5	10	
P (mg)	20	84	

Mean value and, between brackets minimum–maximum values, reported in literature.

^aLipid composition provided in this table is that of an infant formula based of a blend of plant oils including palm oil and coconut oil, as an example of infant formula made with plant oils as lipid source.

^bNq, non quantifiable amount (<0.05%).

The energy and protein recommendations according to the current European regulation are presented in Table 8. It is noticed that protein from plant protein source (i.e., soy isolate protein) should be provided in higher amount than animal protein sources (cow and goat milks). In fact, this recommendation should compensate the limiting AA in soy protein isolate such as methionine and its less digestibility compared to animal proteins.

Table 8. Energy and protein recommendations for infant formulas divided in three groups: infant formulas based on cow's or goat's milk (2.1), soy protein isolate (2.2) and hydrolyzed rice protein (2.3). (EU 2016).

1. ÉNERGIE	
Minimum	Maximum
250 kJ/100 ml	293 kJ/100 ml
(60 kcal/100 ml)	(70 kcal/100 ml)
2. PROTÉINES	
Teneur en protéines = teneur en azote × 6,25	
2.1. Préparations pour nourrissons à base de protéines de lait de vache ou de lait de chèvre	
Minimum	Maximum
0,43 g/100 kJ	0,6 g/100 kJ
(1,8 g/100 kcal)	(2,5 g/100 kcal)
2.2. Préparations pour nourrissons à base d'isolats de protéines de soja, seuls ou mélangés avec des protéines de lait de vache ou de lait de chèvre	
Minimum	Maximum
0,54 g/100 kJ	0,67 g/100 kJ
(2,25 g/100 kcal)	(2,8 g/100 kcal)
Seuls les isolats de protéines de soja sont employés pour la fabrication de ces préparations pour nourrissons.	
2.3. Préparations pour nourrissons à base d'hydrolysats de protéines	
Minimum	Maximum
0,44 g/100 kJ	0,67 g/100 kJ
(1,86 g/100 kcal)	(2,8 g/100 kcal)

As mentioned in section 1.1, in order to meet the requirements in terms of protein for infants, one point is considering the protein content, but another key point is to provide the EAA in the proper concentration to cover the infant nutritional needs. IFs should contain at least all the EAA present in the reference protein that is human milk protein. The EAA and semi-essential AA recommended from the latest European regulation are presented in Table 9; it corresponds to the minimum amount of AA to provide to cover infant needs.

Table 9. Minimum essential and semi-essential AA recommendations for infant formula based on cow's milk or goat's milk or soy protein isolate, only or in mix with cow's or goat's milk proteins (EU 2016).

	Pour 100 kJ (*)	Pour 100 kcal
Cystéine	9	38
Histidine	10	40
Isoleucine	22	90
Leucine	40	166
Lysine	27	113
Méthionine	5	23
Phénylalanine	20	83
Thréonine	18	77
Tryptophane	8	32
Tyrosine	18	76
Valine	21	88

(*) 1 kJ = 0,239 kcal.

FUTURE TRENDS IN IF COMPOSITION

Despite the great advances in the optimization of IF composition, IF cannot provide all the numerous immune protective and bioactive factors present in human milk. Therefore, the addition of bioactive components in IFs and their structure optimization have been considered by many authors and industries (Gallier et al. 2015; Lonnerdal 2016; Zou et al. 2016).

Notably, the addition of specific fatty acids, lactoferrin, nucleotides, EAAs, oligosaccharides, prebiotics, probiotics, and MFG membrane fragments have become popular, as well as the modification of the triglycerides structure of fats and oils to mimic human milk triglycerides. Specialized formulas for specific needs with new protein sources have also been developed (Bourlieu et al. 2015; Gallier et al. 2015; Lonnerdal 2014; Mudd et al. 2016; Zou et al. 2016). In this way, scientific and technological advances are pushing forwards new fields of innovations in infant nutrition (Figure 13).

Besides, most clinical trials (27%) carried out on IFs, focused on protein composition with particularly high number of trials addressing cow milk allergy and assessing the safety of extensively hydrolysed IF or IF based on alternative source of proteins (Bourlieu et al. 2017). Other important fields of research concern prebiotics (oligosaccharides from human milk notably) which promote the development of bifidogenic microbiota for infants. Clinical trials targeting lipids in the IF represent 14% of the total trials. Moreover, more than 2000 patents about IF are registered in open database (espacenet patent). Among the most recent patents, several patents target IF supplementation with bioactive components such as oligosaccharides (US2016278421 (A1), US2016278414 (A1)), bovine colostrum component (MX2015017115 (A)), modified sweet whey (US2016310557), structured lipid (WO2016 176987) or technological processes to produce IF with large fat globules (US2016219911, NZ631411, US2016278413, NZ627997, US2016295895, US2016302611). Recently, a process to prepare a new

IF based on potato protein has been patented. This IF is claimed to be naturally hypoallergenic and thus suitable for infants with cow's milk protein allergy [WO2018 115340 (A1)]. Although great improvements in the fields of IF composition, data on the structure of human milk throughout lactation and on the digestive behavior of specific fractions of human milk lipids and proteins are still needed to build a strong basis for the optimization of IFs.

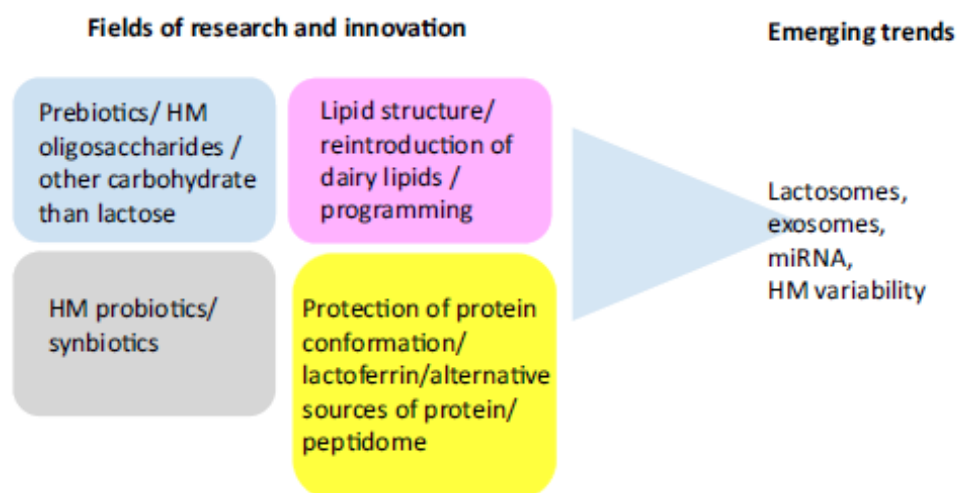


Figure13. Summary of fields of innovation based on recent analysis of clinical trials, WOS (web of science) and patent database about infant formulas. (Bourlieu et al. 2017).

1.3.2. Infant digestive system

The digestion process comprises chemical, mechanical and enzymatic degradations of foods and involves successive steps (oral, gastric and intestinal phases). The digestive conditions and efficacy depend on many factors, notably on the physiological stage (infant, adult, elderly) and on the characteristics of the food ingested. Although the development of the digestive functions occurs early during the uterine life, newborn infants (< 28 days of life) and infants up to six months are recognized to have an immature digestive system compared to older infants (> 6 months). The underdeveloped digestive functions of newborn infants can have an impact on their ability to digest and absorb nutrients, compromising their adequate growth and development (Bourlieu et al. 2014; Poquet & Wooster 2016).

This section aims to present the key parameters of the digestion of milk-based diet by newborn infants, including physiological characteristics and enzymatic processes. We will focus mainly on the gastroduodenal digestion of proteins.

1.3.2.1. Anatomy and functions

The main characteristics of the developing digestive system of the infant are summarized in Figure 14. Digestive immaturity at birth is mainly linked to secretory functions, motility and enzymes. Infants are fed exclusively on liquid milk-based diet during the first months of life (human milk or IF). Digestion process in infants who exclusively consume liquid milk does not happen at oral phase due to the very short transit time through mouth, pharynx and oesophagus (10-15 seconds) (Arvedson et al. 2002),

although the swallowed saliva can interact with the meal during the gastric phase (glycoprotein and salivary amylase mainly) (Bourlieu et al. 2014).

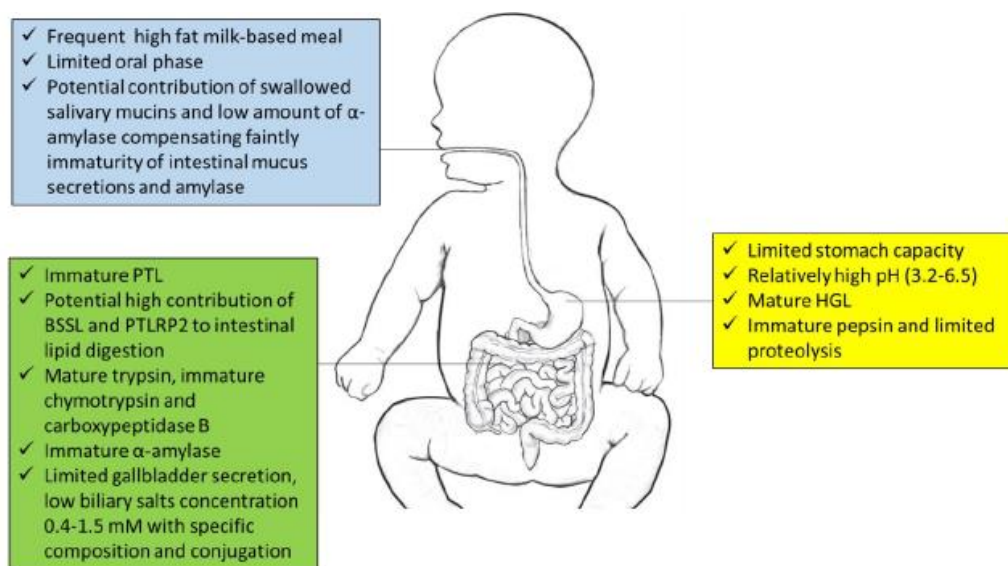


Figure. 14. Summary of the developing digestive physiology in the infant.

BSSL, bile salt-stimulated lipase; HGL, human gastric lipase; PTL, human pancreatic triglyceride lipase; PTLRP2, pancreatic triglyceride lipase-related protein 2. (Adapted from: Shani-Levi et al. 2017).

The stomach is the first main digestive compartment in the infant. This compartment stores, mixes, initiates the hydrolysis and regulates the emptying of milk bolus from the stomach into the small intestine. Gastric juice, secreted by the epithelial cells of the gastric mucosa, contains mainly enzymes (pepsins, human gastric lipase), hydrochloric acid, sodium bicarbonate as well as mucins that form a protective barrier for the gastric mucosa. The chyme (meal and gastric secretions) progressively arrives into the small intestine, which is separated into three parts: duodenum, jejunum and ileum. During the neonatal period, digestive immaturity at the intestinal phase is more marked than that at the gastric phase (Figure 15). However, most of the hydrolysis and absorption of nutrients take place in duodenum and jejunum. The next step, the large intestine is mostly in charge of the absorption of water and electrolytes.

Other accessory organs are crucial to the digestive process, i.e. pancreas (production of the alkaline pancreatic secretion containing lipases, proteases and amylase), liver (production of bile, metabolism of lipids and proteins, storage of glycogen and vitamins) and gallbladder (storage and release of bile toward the duodenum) (Berseth 2006).

Figure 15 illustrates the difference in maturity of the main enzymes acting in gastric and intestinal digestion from 10 weeks of gestation to 24 months after birth. In overall, in the stomach of a newborn, the acidification capacity is limited (pH of 3.2-6.5 compared to 1.5-1.8 in adult stomach, Bourlieu et al. 2014; Shani-Levi et al. 2013). The pepsin is immature before the age of 2 years, whereas the gastric lipase is already mature from birth. In the intestine, the pH is quite similar to the adult one (Kaye 2011), the adverse phenomena are observed compared to the stomach where the main protein enzyme, trypsin, is mature but the main pancreatic lipase (PTL) is immature (although, other forms of lipase,

namely PLRP2 and BSSL, will compensate the immaturity of PTL until the age of 2). Moreover, the efficacy of the enzymatic hydrolysis and of absorption are affected by several factors such as pH, lumen mineral content (e.g. Ca^{2+} precipitation of fatty acids), and the presence of biosurfactants (e.g. the role of bile salts and phospholipids in fatty acids absorption) will change through digestion due to gastric or pancreatic secretions (Poquet & Wooster 2016).

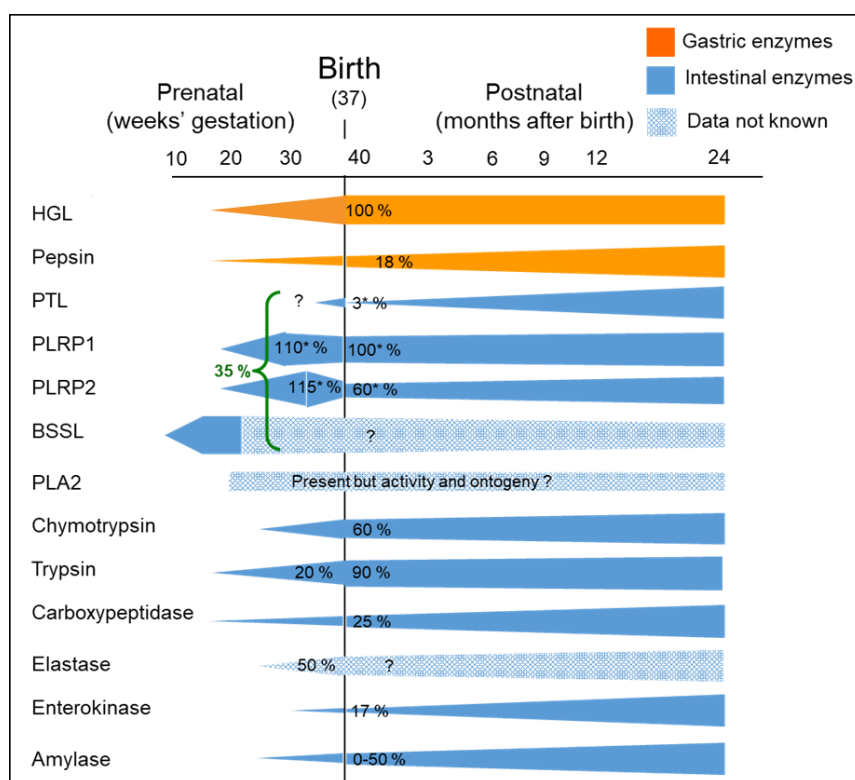


Figure 15. Ontogeny, levels of activity of the main digestive enzymes. Figure refers to the values at birth or at a given postnatal age for term infants. (Bourlieu et al. 2014).

1.3.2.2. Digestion of lipids in infants

The major difference in lipid digestion and absorption between infants and adults is the lipid intake per kilogram of bodyweight, which is much higher (three to five times) in infants than in adults (Andersson et al. 2011). Also, the activity of digestive lipases varies between infant's stomach and intestine during the first months of life (Figure 15).

GASTRIC LIPOLYSIS

Gastric lipolysis plays a more important role in fat digestion in infants than in adults. Enzyme gastric lipase digests the milk fat in the infant diet. Gastric lipase is active over a wide range of pH (1.5-7.0) and does not require bile salts for activation (Hamosh 1996; Ville et al. 2002). Thus, gastric lipase is able to act properly in the infant stomach. Besides, fatty acids produced in gastric phase encourages the activity of pancreatic lipase due to the better interface between fat globules and the aqueous environment (Bernbäck et al. 1989). Thus, fat hydrolysis in the stomach may quantitatively be more important for infants than in adults (Carey et al. 1983; Hamosh et al. 1981). Thus, the high level of gastric lipase may compensate for the low amount of pancreatic lipases and explains why infants can consume a high

amount of fat (Armand et al. 1996; Hamosh 2006). Moreover, Armand et al (1996) observed rapid gastric lipolysis of human milk compared to IF due to the significant amount of lipase present in human milk.

INTESTINAL LIPOLYSIS

Pancreatic triglyceride lipase (PTL), pancreatic lipase-related to protein 2 (PLRP 2), and bile salt-stimulated lipase (BSSL) are the main lipases involved in the intestinal digestion of lipids. Pancreatic lipase-related to protein 1 (PLRP 1) was detected in the small intestine of newborns, but has no lipase activity (Berton et al. 2009). Lipids need to be emulsified by bile salts first to enable hydrolysis by pancreatic lipases. The activity of pancreatic lipases and the concentration of bile salts in infants are very low (Lebenthal et al. 1983) compared to adults. While in adults, PTL is the main lipolytic enzyme in the small intestine, PLRP2 and BSSL are predominant during lipid digestion in infants (Andersson et al. 2011). It is believed that breastfed infants are able to digest lipids in the small intestine better than formula-fed infants because of significant activity of BSSL present in mothers' milk (Hamosh 1996).

1.3.2.3. Digestion of proteins in infants

Proteases are classified as endo- or exopeptidases. Endopeptidases cleave peptide bonds at the middle of the protein sequence, releasing large or less peptide fragments that are subsequently hydrolyzed to small peptides. Exopeptidases cleave the peptide bond at the N-terminal (aminopeptidases) or C-terminal (carboxypeptidases) releasing the terminal AA one by one. The combined action of both kinds of proteases enables the degradation of proteins into peptides and free AA (Rassin & Shattuck 2006).

GASTRIC PROTEOLYSIS

Proteolysis in the stomach is due to pepsin, an endopeptidase activated from pepsinogen by selective cleavage of a small basic peptide. The secretion of pepsinogen in newborn infants is reported to be lower than in children and adults, reaching adult levels by only two years of age (Figure 15) (Henderson et al. 2001). Furthermore, the relatively high gastric pH in newborn (i.e. pH 6-5 during the first hour of digestion) is far from the optimum pH for pepsin activity, i.e. pH 2. The difference in acidification capacity between infants and adults is illustrated in Figure 16. Thus, the combination of these two factors (reduced pepsin secretion and acidification) explains the limited proteolysis reported during the gastric phase in infants compared with adults (Chatterton et al. 2004; Henderson et al. 2001). The major gastric proteolysis products are polypeptides with N-terminal AA including phenylalanine and leucine, as well as small amounts of oligopeptides and AA. Gastric kinetics of proteolysis plays a major role determining in which state proteins arrive in the small intestine and then influences the intestinal kinetics of proteolysis, absorption and bioactivity functions.

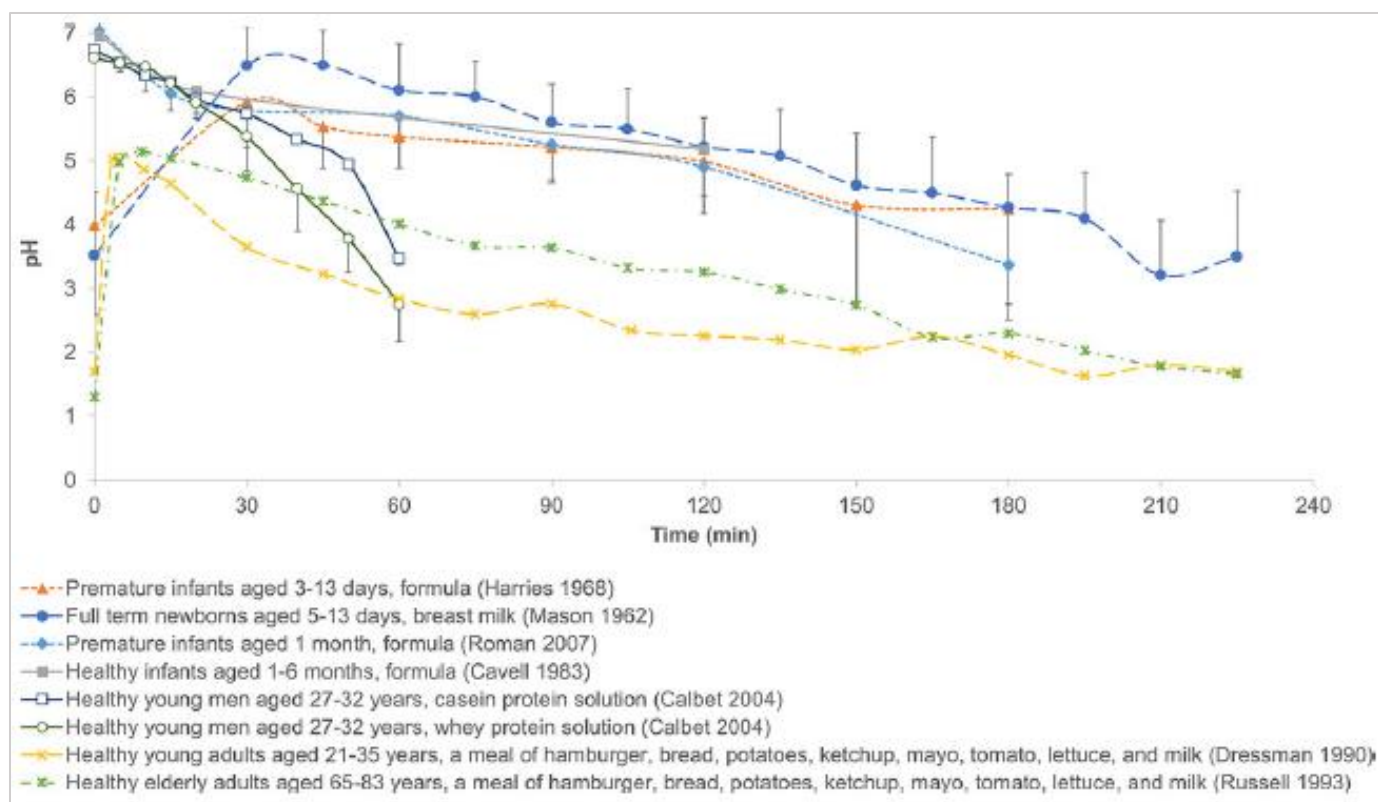


Figure 16. Postprandial gastric pH of healthy infants and adults. (Gan et al. 2018).

INTESTINAL PROTEOLYSIS

In the intestinal phase, pancreatic proteases are secreted as inactive zymogens and are activated by trypsin. Trypsin is activated from trypsinogen by intestinal enterokinase, secreted from intestinal epithelial cells in response to food stimulation (Bourlieu et al. 2014; Dallas et al. 2012). *In vivo* data present high variability in the proteolytic activity in the intestine, with the ontogeny of trypsin and chymotrypsin in one-month old preterms corresponding to around 90% and 60% of the level found in adults, respectively (Figure 15) (Bourlieu et al. 2014). Trypsin cleaves peptides at the carboxyl side of lysine and arginine, while chymotrypsin cleaves at the carboxyl side of tyrosine, tryptophan or phenylalanine. Complementing their action, carboxypeptidase B cleaves the basic AA arginine and lysine from the C-terminal (Dallas et al. 2012). Other peptidases in the intestinal brush border (e.g. γ -glutamyltranspeptidase, oligoaminopeptidase, dipeptidylaminopeptidase IV) may help to complete the hydrolysis of peptides into AA, even in premature infants. The peptides and free AA generated are absorbed by carrier-mediated mechanisms across the basolateral membrane into the portal blood, by intracellular vesicle mediated transport system (transcytosis) or by paracellular passive diffusion (Wada & Lönnerdal 2014).

1.3.3. Different *in vitro* models to study infant protein digestion

The use of *in vitro* models to simulate digestion through the gastrointestinal tract has become widely more popular than obtaining data from *in vivo* experiments due to no ethical restrictions, better control, higher repeatability, lower cost, less time requirements and high-throughput potential (Bohn et al. 2018; Bouzerzour et al. 2012). The *in vitro* models help observe the digestibility, structural changes, and the

release of nutrients under simulated gastrointestinal digestion (Brodkorb et al. 2019; Gouseti et al. 2019; Hur et al. 2011). The growing concern about building *in vitro* models based on specific infant physiology has been highlighted in recent reviews. Bourlieu et al. (2014) reviewed studies that characterized gastroduodenal conditions in full-term and preterm infants from birth to six months. Poquet & Wooster (2016) analyzed characteristics of infant physiology that are related to lipid digestion. In terms of protein digestion, the immaturity of the infant digestive system (Figure 15) affects the efficacy of protein digestion and absorption, leading to specific issues in this population such as allergy or inflammation. Regarding the relationship of diet to infant health, infants specific research models are needed to understand protein digestion in more detail.

1.3.3.1. *Static and semi-dynamic vs dynamic models*

To study infant protein digestion, *in vitro* models should mimic conditions of the infant gastrointestinal tract that are relevant to protein digestion. Major adjustments include higher gastric pH, reduced enzyme concentration, and longer transit time compared to adult models (Bourlieu et al. 2014). *In vitro* digestion simulating infant conditions can be static (Chatterton et al. 2004; Ménard et al. 2018; Nguyen et al. 2015; Wada & Lönnerdal 2015a,b), semi-dynamic (Amara et al. 2014; Bourlieu et al. 2015) or dynamic (de Oliveira et al. 2016; Maathuis et al. 2017; Ménard et al. 2014; Passannanti et al. 2017; Shani-Levi et al. 2013; Zhang et al. 2014).

IN VITRO STATIC AND SEMI-DYNAMIC MODELS

Static models are widely used as they are quite easy to set up and do not require specific tools. These models consist of a succession of bioreactors recreating the environment that the food will meet when entering the different compartments of the digestive tract. In each compartment (mouth, stomach, duodenum), a single set of parameters is set up at the start of the digestion (pH, meal dilution, enzyme concentration, mixing), without any intervention during the digestion time (no pH adjustment, no flow of enzymes and no emptying). Figure 17 presents gastric and intestinal conditions of the *in vitro* digestion model proposed by Ménard et al. (2018), for infant digestion compared to adult. These systems can be used for the study of digestion of purified molecules (i.e. to assess the potential allergenicity of proteins) but also for screening different food matrices, when a choice has to be made among a large number of formulations before studying digestion on a limited number of matrices (Ménard & Dupont 2014).

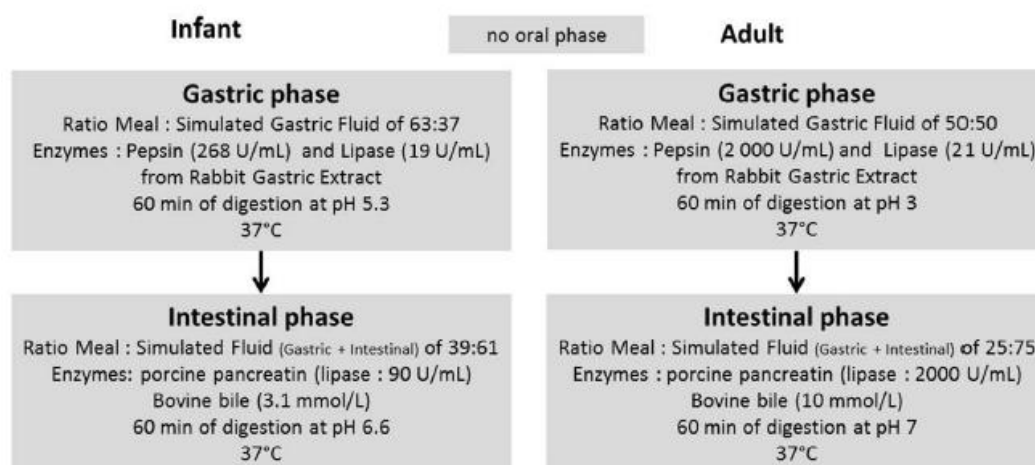


Figure 17. Infant and adult digestive conditions used in the static *in vitro* digestion models.

(Ménard et al. 2018).

In the semi-dynamic model, a pH-stat device can be programmed to establish subsequent stage of pH, which is adjusted by the addition of simulated gastric fluid, resulting in variation of the pH, enzymes and the dilution of the meal by gastric secretions. In such a model, for example, the fatty acids released can be directly titrated by NaOH; however, there is no emptying and no flux among the compartments (Bourlieu et al., 2015).

IN VITRO DYNAMIC MODELS

Since static and semi-dynamic models are overly simplified in their representation of the digestive tract, the direct applicability of findings to the digestive process is limited. Recently developed, gastrointestinal dynamic models have incorporated a more realistic representation of the digestive tract, since they simulate the dynamic changes of pH, enzymatic flow and activity, emptying rate, and flux between gastric and intestinal phases. These dynamic models allow the collection of samples from different compartments over the digestion process, providing data in terms of kinetics of disintegration and hydrolysis. Different dynamic models have been developed to mimic one or several compartments (de Oliveira et al. 2016; Kong & Singh 2010; Ménard et al. 2014; Minekus et al. 1999; Molly et al. 1993; Passannanti et al. 2017; Shani-Levi et al. 2013; Zhang et al. 2014).

The most popular gastrointestinal dynamic model is the TIM-1 (TNO gastrointestinal model 1), which includes stomach and the three parts of the small intestine (duodenum, jejunum, ileum) (Minekus et al., 1995). Controlled by a computer, this model takes into account parameters such as human temperature, gastric pH decrease, digestive secretions, emptying rates, peristalsis movements and nutrient absorption in the intestine by a dialysis system (Guerra et al. 2012). Blanquet et al. (2004) applied the TIM-1 model to study the behavior of oral drug dosage under *in vitro* infant digestion. More recently, a TIM-2 model was developed from TIM1, which can additionally mimic the microbiota (Yoo & Chen 2006). Another complete *in vitro* dynamic model and adapted to older infants is the Model of Infant Digestive Appartus (M.I.D.A) which reproduced the physiology of the gastrointestinal tract of a six-month-old infant (Passannanti et al. 2017). It consisted of four consecutive compartments simulating the esophagus, the stomach, the pyloric sphincter and the intestine.

A more simplified gastrointestinal dynamic system, DIDGI® (INRA, France) contains two successive compartments simulating the stomach and the small intestine, controlled by the STORM® software (Figure 18). It simulates the flows of ingested food and among compartments, digestive secretions, the gastric pH decrease, emptying rates, and temperature (Ménard et al. 2014). However, this model does not mimic absorption and the continuous mixing applied in order to avoid phase separation does not simulate peristaltic movements, and could artificially modulate the structures formed during digestion. This model was validated to simulate infant digestion on piglets (Ménard et al. 2014).

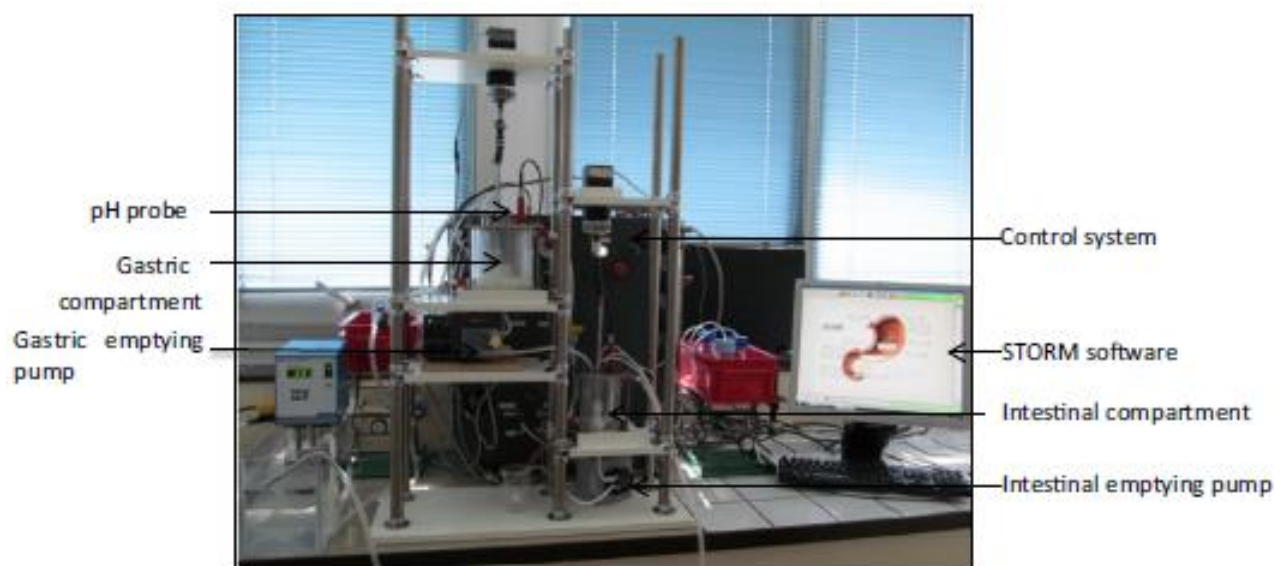


Figure 18. The *in vitro* gastro-intestinal dynamic digestion system (DIDGI). (Ménard et al. 2014).

OTHER APPROACHES TO STUDY IN VITRO DIGESTION

Another approach combining *in vitro* models and human intestinal cells in culture has been proposed, integrating active and facilitated transport processes and brush border enzyme activities (Abrahamse et al. 2012; Poquet & Wooster 2016). Notably, Caco-2 cells have been used to investigate the intestinal absorption and lipid metabolism of lipolysis products in the intestinal epithelium (Andersson et al. 2011; Lipkie et al. 2014; Vors et al. 2012). A model combining human intestinal organoids and human pluripotent stem cells has been developed by Workman et al. (2017), in order to create an enteric nervous system activity and to study motility disorders of the human gastrointestinal tract. Microfluidics devices can also be used to simulate digestion such as the lipid droplet microfluidic digestion system recently developed by Marze et al. (2014) that showed equivalent results to those obtained from *in vitro* static digestion of emulsions. Recently, a “near-real” dynamic *in vitro* human stomach system has been advanced made up with silicone and manufactured with 3D-printing technology (Wang et al. 2019). This simulated stomach has similar morphology, dimension and wrinkled inner structure to that present *in vivo* and was able to generate consistent gastric emptying ratios of both solid and liquid food fractions (Wang et al. 2019).

1.3.3.2. Setup of *in vitro* models adapted to infant digestion

Developing relevant *in vitro* models of digestion relies on the availability of *in vivo* data about the digestive conditions in the course of the meal digestion. Anatomical and physiological parameters determining these conditions depend on age, meal, fed conditions and other specific characteristics of the target to simulate (e.g. preterm *versus* term newborns) (Bourlieu et al. 2014). Previous reviews have compiled the physiologic data available in literature about infant digestive conditions (Poquet & Wooster 2016; Bourlieu et al. 2014; Abrahamse et al. 2012). Notably, Bourlieu et al. (2014) underlined the immaturity in the digestive conditions in terms of enzymatic parameters (type of enzymes and level of activity) and the specificity of non-enzymatic parameters (milk-based diet, frequency of feeding, bile salts concentrations).

IN VITRO STATIC MODEL ADAPTED TO INFANT CONDITIONS

The parameters used on *in vitro* models for digestion differ from one study to another, which makes comparison between data problematic. Moreover, the enzymatic parameters are scarcely expressed in international units (i.e. international units/mL of gastro or intestinal content) and the enzyme characterization and activity are most of the time omitted in the digestion protocol description. Furthermore, duration of digestion and the meal proportion in the total volume vary widely (Brodkorb et al. 2019). To face this challenge, the INFOGEST network took up the issue and proposed to the scientific community in the field of digestion a consensus protocol for mimicking digestion at the adult stage with an *in vitro* static model (Brodkorb et al. 2019; Minekus et al. 2014). Focusing on infant stage, a review has highlighted the variability in the parameters used to mimic infant digestion (Shani-Levi et al. 2017). Lastly, a physiologically relevant harmonized model of *in vitro* static digestion at the infant stage (28 days of life, gestation age from 38 to 42 weeks) has been recently proposed by Ménard et al. (2018), based on parameters relying on *in vivo* data published in the literature (Bourlieu et al. 2014; de Oliveira et al. 2016). In comparison with the adult international consensus model (Minekus et al. 2014; Brodkorb et al. 2019), this infant model is characterized by the omission of the oral phase, because of the very short residence time in the mouth for liquid products, and by specific digestive parameters summarized in Figure 17.

Gastric phase

Parameters (meal to secretions ratio, pH) were determined based on the infant gastric conditions occurring at the emptying half-time, assumed to be more representative than the final time point. As described by Bourlieu et al. (2014), a gastric emptying half-time of 78 min has been reported for IF. The meal to secretions ratio was based on the simulation of secretion flows in the dynamic digestion model DIDGI® validated for infant formula digestion (Ménard et al. 2014), in which the mean flow rate of secretions was fixed at 0.53 mL/min. At 78 min after the start of meal ingestion, the ratio (v/v) of meal to secretion was 63 to 37. Compilation of data measuring gastric pH in infants allowed the determination of a linear regression describing the gastric acidification curve:

$$\text{pH} = -0.015 \cdot \text{time (min)} + \text{pH (meal)} \quad (4)$$

Considering the gastric emptying half-time of 78 min and a pH of meal (IF) of 6.52, gastric pH in the static model was set up at 5.3.

The measurement of postprandial enzyme activities in infant gastric aspirates resulted in average values of 63 U of pepsin and 4.5 U of lipase per mL of gastric content and per kg of body weight of infant (Armand et al. 1996; Roman et al. 2007). Thus, considering the mean body weight of a one-month old infant of 4.25 kg (de Oliveira et al. 2016), enzyme activities were set up at 268 U/mL of gastric content for pepsin and 19 U/mL of gastric content for lipase. Pepsin and gastric lipase were added as rabbit gastric extract (RGE). Rabbit gastric lipase presents 85% of sequence homology as compared to the human one (Roussel et al. 1999). The added amount of RGE covered 100% of the pepsin activity and 110% of the lipase activity required (21 U/mL). Gastric fluid composition was based on a study on 30 full-term infants reported by Hyde (1968). The simulated gastric fluid (SGF) was composed of sodium chloride and potassium chloride fixed at 94 and 13 mM, respectively, and adjusted to pH 5.3 with HCl 1 M. After 60 min of gastric digestion, the pH was increased to 7 by addition of NaOH 1 M in order to stop gastric enzyme activities before further intestinal digestion.

Intestinal phase

The vial containing the 60 min gastric phase was adjusted to the intestinal pH of 6.6 using HCl 1 M. As for the gastric phase, the meal to secretions ratio for the intestinal phase was determined at 78 min of digestion using the simulation of secretion flows (bile, pancreatin and sodium bicarbonate) in the dynamic digestion model DIDGI® (Ménard et al. 2014), in which the overall mean secretion flow rate was 0.85 mL/min. Thus, at 78 min of digestion, the ratio (v/v) meal to total secretion (gastric and intestinal) for the intestinal phase was 39 to 61. More precisely, the total volume of the intestinal phase was composed of 39% of meal, 23% of gastric secretion and 38% of intestinal secretion.

The composition of simulated intestinal fluid (SIF) was determined based on the characterization of duodenal fluid of 1-week-old full-term infants (Zoppi et al. 1973): 164 mM sodium chloride, 10 mM potassium chloride and 85 mM sodium bicarbonate, pH 7. Calcium chloride was added separately before the beginning of the intestinal phase at a concentration of 3 mM within the volume of the intestinal fluid (Zoppi et al. 1973). Bovine bile extract was added to a final content of 3.1 mM bile salts, which corresponds to the average postprandial value determined in duodenal aspirates of eight 2 week-old infants (Signer et al. 1974). The added amount of pancreatin covered the intestinal lipase activity required, i.e. 90 U/mL of intestinal content (Norman et al. 1972), and the trypsin activity needed, i.e. 16 U/mL of intestinal content, which was consistent with previously reviewed *in vivo* data (Poquet & Wooster, 2016).

As already mentioned, *in vitro* static models are simple, easy to use but are not extremely physiologically relevant since, for obvious reasons, it is difficult to recreate the complexity of the gastrointestinal tract in a series of beakers. Indeed, these static models do not recreate the transient biochemical conditions and the flow of the food through the different compartments occurring in the real digestive system (Guerra et al. 2012). Therefore, these models have to be considered essentially as screening techniques, for when a high number of foods are submitted to digestion in order to identify, for example, key parameters that may affect the hydrolysis of food macronutrients.

IN VITRO DYNAMIC MODEL ADAPTED TO INFANT CONDITIONS

In order to get closer to the physiological reality of the gastrointestinal tract, several different *in vitro* dynamic digestion systems have been developed (Guerra et al. 2012). These models are much more sophisticated than the static ones and include regulation of the pH, dynamic flows of food and concentration of digestive enzymes in the different compartments.

In particular, a relevant dynamic model adapted to infant conditions has been proposed by Ménard et al. (2014) and has been validated from *in vivo* data collected from piglets. Parameters for gastric and intestinal phases were chosen to closely mimic the digestive conditions of term newborns fed human milk at the postnatal age of four weeks. The *in vitro* dynamic system was controlled by the STORM® software, which allows regulating and monitoring the digestive parameters (Guillemin et al. 2010). The pH acidification in the gastric compartment followed a linear regression, from the initial pH set at the meal pH. Fasted conditions were based on 2 mL of simulated gastric fluid (SGF) adjusted at pH 3.1. In the intestinal compartment, the pH was constant and fixed at 6.2. The transit time in the stomach and in the intestine followed an exponential pattern fitted by the mathematical model described by (Elashoff et al. 1982; Ewer et al. 1994). Gastric emptying was monitored by applying a half-time ($t_{1/2}$) of 78 min and a β parameter of 1.2. Gastric porcine pepsin amount was determined from data obtained in preterm newborns (Armand et al. 1996; Roman et al. 2007), assuming that pepsin activity increases with body weight, which is linked to the digestive maturity. The mean body weight of term newborns at four weeks of age was considered 4.25 kg (WHO 2007). Bovine bile and porcine pancreatin extracts were added to reach the average values reported for term newborns in postprandial intestinal conditions, previously mentioned in the description on the *in vitro* static model (Ménard et al. 2018).

A number of other recent works proposed different *in vitro* models adapted to infant conditions to study protein digestion, summarized in Table 10. It may be noted that the extent of adjustments vary considerably from one research group to another. Additionally, the composition and the amount of food material, as well as the techniques to evaluate the digestibility differ between studies. All these differences lead to difficulties in comparing results of protein digestion between the research groups and reveal how varying conditions can affect protein digestibility.

Table 10. Recent studies on infant protein digestion using *in vitro* models. (Adapted from Gan et al. 2018).

Model	Food	Analytical techniques	Adaptations for infants	Reference
Static infant gastric digestion model (with porcine pepsin or human neonatal gastric juice)	Whey protein concentrate, infant formula	SDS-PAGE, HPLC, N-terminal sequencing Western blot	(1) Human neonatal gastric fluid for <i>in vitro</i> digestion; (2) pH 6.5, 5.0, 4.0, 3.5, 3.0 and 2.0; (3) 1hr incubation.	Chatterton et al. (2014)
Static infant gastrointestinal model	Human milk with and without pasteurization (62.5 °C for 30 min)	LC-MS/MS	(1) Gastric pH 4.0; (2) 15 min for gastric digestion at 37°C; (3) Intestinal pH 7.0; (4) 5 min at 37°C for intestinal digestion.	Wada & Lönnerdal (2015b)
Static infant gastrointestinal model	Standard formula, extensively hydrolyzed casein formula, partially	LC-MS/MS	(1) Gastric pH 4.0; (2) 15 min for gastric digestion at 37°C; (3) Intestinal pH 7.0; (4) 5 min at 37°C for intestinal digestion.	Wada & Lönnerdal (2015a)

	hydrolyzed whey protein formula			
Static infant gastrointestinal model	Human milk, infant formula	LC-MS/MS	(1) Gastric pH 3.5; (2) 30 min at 37°C for gastric digestion; (3) Intestinal pH 7.0; (4) 60 min at 37°C for intestinal digestion.	Hernández-Ledesma et al. (2007)
Static infant gastrointestinal model	Human milk, infant formula	LC-MS/MS, ELISA	(1) Gastric pH 4.0; (2) 100 U pepsin/mg protein; (3) gastric phase lasted 120 min at 37°C; (4) Intestinal pH 6.5; (5) 5 mg pancreatin and 30 mg bile salt extract/mL sample for duodenal digestion; (6) intestinal phase lasted 120 min at 37°C.	Su et al. (2017)
Static infant gastroduodenal model (with enzymes and surfactants)	Milk protein solutions, Maillard reaction products, food matrices (milk and yogurt) with different processing	SDS-PAGE, ELISA, immunoblotting, RP-HPLC, LC-MS/MS	(1) Gastric pH 3.0 instead of 2.5; (2) 22.75 U pepsin/mg substrate (8-fold reduction compared to adult model); (3) Duodenal pH 6.5; (4) 1 mM duodenal bile salt (4-fold reduction), 3.45 U trypsin/mg substrate (10-fold reduction), 0.04 U chymotrypsin/mg; substrate (10-fold reduction).	Dupont et al. (2009, 2010) Moscovici et al. (2014)
Semi-dynamic infant gastric model	Model formulas with different processing	Chemical characterization for lipids and proteins, structural characterization for emulsions	Three subsequent stages: 0–60 min at pH6, 60–120 min at pH5, 120–180 min at pH 4.	Bourlieu et al. (2015)
pH static and dynamic gastric models for infants and adults	Milk protein solutions, protein emulsions	SDS-PAGE, light scattering and fluorescence microscopy	(1) gastric digestion time 240 min (120 min for adults); (2) pH gradients from 6.5 to 3.5 (4.5 to 1.5 for adults) in dynamic models, and pH 2.5 in static models; (3) 210 U pepsin/mg protein (240 U for adults).	Shani-Levi et al. (2013)
Static gastric model	Infant formulas	SDS-PAGE, immunoblotting	(1) Porcine pepsin 4.0 µg/mL (2) pH adjusted to 1.5, 2.0, 2.5, 3.0 or 4.5 (3) Gastric digestion lasted 30, 60 or 120 min at 37°C in a shaking water bath	Sakai et al. (2000)
Dynamic gastric model mimicking infants aged 9–12 months	Human milk	SDS-PAGE, LC-MS/MS, immunoblotting	(1) pH gradients from 7 to 2 over 60 min; (2) enzymatic content; (3) contraction; (4) half gastric emptying at 48 min.	Zhang et al. (2014)
Static bench-top gastroduodenal model	Cow milk protein infant formula and soy protein infant formula		(1) gastric pH of 4.0 at 37°C; (2) meal:gastric fluid ratio is 2:1 (v:v); (3) 22.75 U/mg pepsin to total protein ; (4) gastric phase lasted for 60 min; (5) intestinal pH 8.0±0.03; (6) 2 mM bile salts and 3.45 U/mg trypsin and 0.04 U/mg α-chymotrypsin; (7) duodenal phase lasted for 60 min.	Nguyen et al. (2015)
Dynamic infant (28 days) gastrointestinal model (DIDGI) validated with in vivo data (piglet model)	Infant formula adapted to piglet	SDS-PAGE, ELISA	(1) gastric pH followed acidification curve: $Y = -0.011 \cdot t + 5.4$ (t: time after ingestion in min); (2) half gastric emptying at 70 min (and Elashoff fitting $\beta=1.23$); (3) 1250 U/mL pepsin and 60 U/mL gastric lipase; (4) intestinal pH of 6.5; (5) half intestinal emptying at 200 min (and Elashoff fitting $\beta=2.2$); (6) 10% pancreatin and 1% bile; (7) temperature was set at 37°C.	Ménard et al. (2014)
Static infant (28 days) gastrointestinal model	Infant formula (0–6 months)	Particle size distribution, CLSM	(1) gastric pH of 5.3; (2) meal:gastric fluid ratio is 63:37 (v:v); (3) 268 U/mL pepsin and 19 U/mL lipase from rabbit;	Ménard et al. (2018)

		microscopy, SDS-Page, NMR	(4) gastric phase lasted 60 min at 37°C; (5) intestinal pH of 6.6; (6) meal:gastric+intestinal fluid ratio is 39:61 (v:v); (7) porcine pancreatin with 90 U/mL lipase activity; (8) intestinal phase lasted 60 min at 37°C.	
Dynamic infant (28 days) gastrointestinal model (DIDGI)	Pasteurized and raw human milk	SDS-PAGE, Amino acid analysis, CLSM microscopy	(1) gastric pH followed acidification curve: $Y = -0.0155 \cdot t + \text{milk pH}$ (t: time after ingestion in min); (2) half gastric emptying at 47 min (Elashoff fitting $\beta=0.9$); (3) 268 U/mL pepsin and 19.2 U/mL gastric lipase; (4) intestinal pH of 6.2; (5) half intestinal emptying at 200 min (Elashoff fitting $\beta=2.2$); (6) 90 U/mL pancreatic lipase and 3.1 mmol/L bile salts; (7) temperature was set at 37°C.	de Oliveira et al. (2016)
Static gastric digestion simulating infant (6 months) and young child (1-3 years) conditions	Cow and goat milk infant formulas	SDS-PAGE, immunoblotting, RP-HPLC, peptides composition	(1) Gastric pH at 5.0 (infant conditions) or pH 3.0 (young child conditions) (2) 30 U/mg of milk (infant) or 180 U/mg of milk (young child) (3) Gastric digestion lasted 60 min at 37°C in orbital shaking water bath at 120 S.min-1	Hodgkinson et al. (2018, 2019)
Dynamic gastrointestinal model (tiny-TIM) for 1 to 6 months infants	Goat milk infant formula, Cow milk infant formula and Human milk	Total nitrogen, α -amino nitrogen, Amino acid analysis	(1) gastric pH-curve from pH 6.7 to 4.8 from 0 to 30 min, to pH 3.8 from 30 to 60 minutes, and to pH 3.2 from 60 to 120 minutes; (2) half gastric emptying at 60 min (and Elashoff fitting $\beta=1.5$); (3) 268 U/mL pepsin and 19.2 U/mL gastric lipase; (4) intestinal pH 6.5 ± 0.3 ; (5) digestion lasted 240 min at 37°C.	Maathuis et al. (2017)
Static infant gastrointestinal model	Goat milk colostrum and mature goat milk	Zeta potential, particles size, protein profile and peptides composition	(1) gastric and intestinal pH 6.5 (2) porcine gastric mucosa pepsin at 22.75 U mg ⁻¹ of protein (3) 180 min for the gastric phase and 60 min for the intestinal phase, both under continuous shaking at 250 rpm min ⁻¹ and 37°C (4) 3.45 U mg ⁻¹ of trypsin and 0.04 U mg ⁻¹ of α -chymotrypsin.	Sun et al. (2019)

1.3.4. Other models to study infant digestion

Human clinical trials remain undoubtedly the best approach to study food digestion (Deglaire et al. 2009). However, getting ethical agreement for performing such experiments on humans is difficult, especially when invasive approaches are needed to collect effluents. Furthermore, it is almost impossible for ethical reasons to work on healthy specific populations like neonates, exceptions apart (de Oliveira et al. 2016). However, for those reasons, animal models have been widely used. Among the different animal models available, pig is regarded as the best model to mimic the upper part (stomach and small intestine) of the human gastrointestinal tract (Deglaire & Moughan 2012; Rowan et al. 1994). Pigs can be either slaughtered at specific time points after food ingestion, or equipped with cannulas and catheters to follow the kinetics of digestion (Barbé et al. 2013). When focusing on infant digestion, the use of piglets remains the best strategy to understand what happens in the neonate's gastrointestinal tract (Bouzerzour et al. 2012; Lemaire et al. 2018). This model was specifically used to understand the

digestion of IF at the beginning of life (Moughan et al. 1991). However, for this specific physiological stage, cannulas have limits since small animals cannot be fitted with this equipment, which justifies the use of conventional animal assays in a restricted number of cases.

Figure 19 summarizes the differences in terms of systems, advantages and challenges between *in vitro* and *in vivo* approaches to study infant protein digestion.

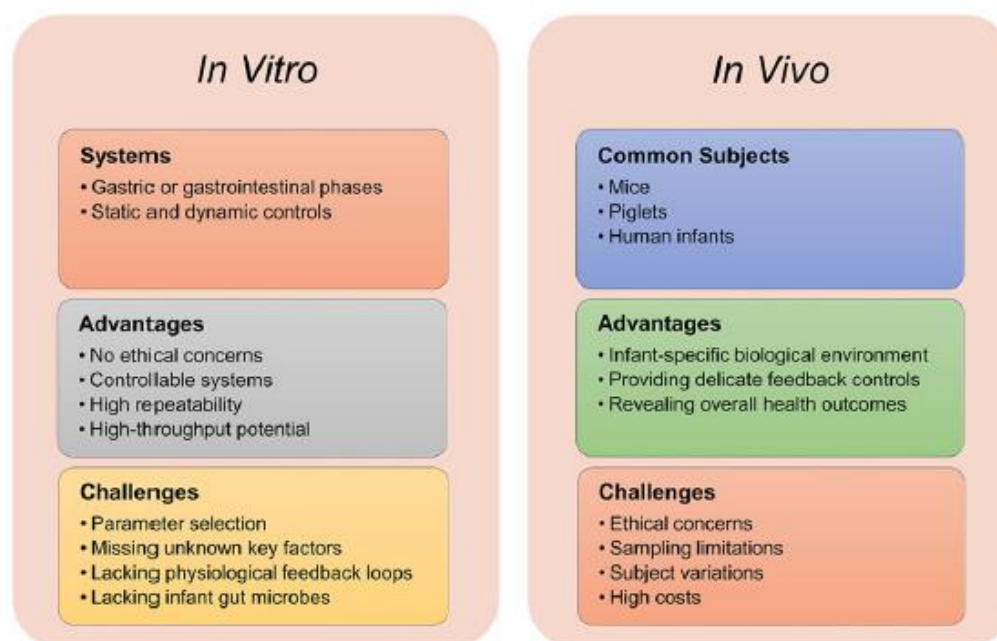


Figure 19. Comparison of *in vitro* and *in vivo* approaches of studies on infant protein digestion.
(Gan et al. 2018).

1.3.5. Impact of protein source on infant digestion

Protein intake early in life is essential for infant health, affecting growth, body composition, neurodevelopment, appetite and hormonal regulation (Michaelsen & Greer 2014). The susceptibility of proteins to digestion depends on specific structure characteristics (Macierzanka et al. 2009; Zhang et al. 2014). Factors like phosphorylation, size, charge, tertiary structure, AA content and glycosylation are suggested to influence degradation of proteins (Dallas et al. 2012).

In terms of structure, the folded structure of a protein influences its digestibility (Duodu et al. 2002). Hydrogen bonds, and even more disulfide bonds or non-disulfide crosslinking stabilize proteins and can create a compactly folded tertiary structure, making it difficult for enzymes to access cleavable peptide bonds. For that reason, breaking disulfide bonds in soybean and sorghum proteins increases their *in vitro* digestibility (Boonvisut & Whitaker 1976; Hamaker et al. 1987). Post-translational modifications might block proteases from binding onto their substrates and inhibit the enzymatic activity. Thus, dephosphorylation of bovine milk proteins increases protein digestibility in an infant *in vitro* model (Liu et al. 2016). Moreover, resistance to digestion is a characteristic of food allergens (Breiteneder & Mills 2005). This molecular property allows the intact proteins or their large fragments containing epitopes to reach the gut and trigger adverse immune responses in high-risk populations (Sicherer & Sampson

2010). Cow milk protein is the most common protein source for IFs, and cow milk protein allergy is the most prevalent food allergy in early childhood (Host 2002). Partially or extensively hydrolyzed IFs have been developed to reduce allergenicity and recommended for high risk infants who are not exclusively breastfed (Alexander & Cabana 2010; Hays & Wood 2005; Vandenplas et al. 2014). However, systematic reviews and meta-analyses on clinical studies have shown contradictory findings on the efficacy of hydrolyzed formulas in preventing allergic diseases in infants (Alexander & Cabana 2010; Boyle et al. 2016; Greer et al. 2008; Hays & Wood 2005; Vandenplas et al. 2014).

1.3.5.1. Cow milk vs human milk proteins

Caseins and whey proteins differ in structure and thus in sensitivity to digestion. The ratio caseins to whey proteins in IF influence the gastric emptying and the protein hydrolysis rate (Boirie et al. 1997; Meyer et al. 2015). It has been demonstrated that diets with higher casein content had a slower gastric emptying than diets with higher whey protein content (Brun et al. 2012). In infants aged between 3 months to 1 year, the gastric emptying is faster after feeding with human milk or whey protein dominant IF than with caseins dominant IF (Billeaud 1990). These differences in gastric emptying rate are mainly explained by the coagulation capacity of caseins. In fact, the higher the casein content is in a diet, the bigger and stronger the curd is in the stomach, and thus slowing down the gastric emptying.

However, caseins are sensitive to proteolysis, whereas whey proteins are more resistant to gastric digestion and can sometimes remain intact in the intestine (Inglingstad et al. 2010). The intestinal juice in the infants aged between 5 days to 19 months has a greater capacity to hydrolyse caseins than whey proteins (Jakobsson et al. 1982). Similarly, in piglets' model fed with their mothers' milk, the ileal digestibility of AA from caseins was greater than that of AA from whey proteins (Mavromichalis et al. 2001). Nevertheless, rats and piglets fed diets enriched in whey proteins showed higher weight gain, mineral absorption and protein content in blood than those fed casein enriched diets (Dael et al. 2005).

1.3.5.2. Goat milk proteins

Goat milk provides an alternative protein source for the manufacture of milk formula for infants and young children and are currently allowed in the European regulation (EU 2016). It has been shown that goat IFs provide growth and nutritional outcomes in infants similar to that of standard whey-dominant cow milk-based IF (Xu et al. 2015; Zhou et al. 2014). While the protein α 1-casein is the dominant casein in cow milk, with 12–15 g/L, goat milk contains variable levels of this protein dependent on genotype, ranging from 0.9 to 7 g/L (Marletta et al. 2007). Because of its lower α 1-casein content, goat milk results in a finer, softer curd compared with cow milk (Haenlein 2004); it may also contribute to a reduced allergenic potential for goat milk (Hodgkinson et al. 2012; Lara-Villoslada et al. 2004).

In vitro gastric digestion under infant and child conditions of cow and goat milk based IFs showed a more extensive hydrolysis of caseins from goat milk formula compared to cow milk formula after 60 min of gastric digestion. This could be explained by the different pH at which caseins in goat milk begin to coagulate (pH 4.1) compared to caseins from cow milk (pH 4.6) (Recio et al. 1997). Maathuis et al. (2017) used a dynamic *in vitro* gastrointestinal model (tiny-TIM) to compare the kinetics of protein digestion of a goat milk IF compared to a cow milk IF and human milk under infant conditions. These authors showed that the true ileal protein digestibility of goat and cow milk IFs was similar to that of

human milk. Protein hydrolysis of cow milk IF was delayed compared to goat milk IF (in accordance with Hodgkinson et al. 2018) and to human milk. Finally, DIAAS of goat and cow milk IFs for infants did not differ to that of human milk. Rutherford et al. (2006) investigated *in vivo* digestion using piglets to compare goat and cow milk IFs. These authors concluded similar true ileal AA digestibility for almost all AAs and similar protein quality between the goat and cow milk IFs. Finally, Sun et al. (2019) analysed the digestion of goat milk colostrum and goat mature milk through *in vitro* gastrointestinal model and showed differences in digestion properties between the two milks mainly in duodenal phase. Both milks differ in surface charges, particle size distribution, protein hydrolysis degree and peptide composition.

1.3.5.3. Soy proteins

As mentioned, soy-based IFs are used for babies suffering from galactosemia or lactose intolerance (Heyman, 2006; Thompson & Kharb 2007). Nowadays, soy based formulas have become prevalent, accounting for approximately 25% of IFs sold in the United States and 13% in New Zealand (Agostoni et al. 2006; Lonnerdal 1994), but not many studies have investigated the digestibility of soy based IFs. Yet, pre-treatments of soy protein isolates with proteases are suggested to improve soy protein digestibility (El-Agamy 2007; Li et al. 2013). Indeed, soy proteins are not easy to digest due to specific structure features and consequences of the heat treatments needed to inactivate or inhibit the ANF (proteases inhibitors, tannins or phytates) present in legumes such as soy (Gatel 1994; Liener 1994). Then, heat processing promotes aggregation of β -sheet structures in soy proteins that enhances its resistance to digestion (Carbonaro et al. 2012).

Other significant concern has been raised relating to the effect of phytoestrogenic isoflavone content in soy based IF on nutritional adequacy and endocrine development during infancy and later life. Soy products also contain phytoestrogens (isoflavones) that may act as weak hormonal disruptors due to their functional similarity to mammalian estrogens. However, there has been no conclusive evidence that soy based formula affect human development, reproduction or endocrine function (Sinai et al. 2019; Testa et al. 2018).

1.3.5.4. Rice hydrolysed proteins

Guidelines for the dietary management of infants diagnosed with allergy to cow milk protein recommend substitution of the cow milk IFs by extensively hydrolysed formulas based on cow milk, modified soy protein formulas and, in certain cases, amino acid-based formulas. Generally, extensively hydrolysed cow milk formulas have a bitter taste, and this poor palatability can be a cause of refusal by infants. As a result, attention has been given to providing further options for feeding infants allergic to cow milk protein, in particular the use of hydrolysed formulas based on rice protein supplemented with lysine and threonine to achieve an AA profile similar to that of human milk (Koletzko et al. 2005).

Two studies (Fiocchi et al. 2003, 2006) have shown that infants with allergy to cow milk protein as well as other food allergies have good tolerance to hydrolysed rice protein formulas. Furthermore, the nutritional adequacy of such IFs was shown in healthy infants who demonstrated normal growth parameters when fed a partially hydrolysed rice protein formula in a double blind, randomized trial (Lasekan et al. 2006). Reche et al. (2010) compared the clinical tolerance of a hydrolysed rice protein formula and its efficacy in feeding infants diagnosed with cow milk protein allergy, with an extensively

hydrolysed cow milk protein formula. It was concluded that the hydrolysed rice protein formula was well tolerated by infants with moderate to severe symptoms of cow milk protein allergy. Children receiving this formula showed similar growth and development of clinical tolerance to those receiving an extensively hydrolysed cow milk protein formula. In accordance with current guidelines, this hydrolysed rice protein formula was tolerated by more than 90% of children with cow milk protein allergy and therefore could provide an adequate and safe alternative to cow milk protein-hydrolysed formulas for these infants.

1.3.6. Alternative proteins for infant nutrition

As mentioned in Section 1.2, there is a growing interest for the use of alternative protein sources with nutritional quality close to animal proteins, mainly for sustainable development concerns. From a nutritional point of view, with proper combination, plant proteins can supply sufficient amounts of EAA for human requirements. However, plant proteins often need thermal or mechanical treatments, and AA supplementation if used alone. Then, mixing multiple plant proteins, or plant and animal proteins can be a means of overcoming AA deficiencies or anti-nutritional factors that can impair the protein digestibility. For instance, pea protein has been successfully used as replacements for whey and casein in a number of products. Importantly, pea protein is a good source of EAA (apart from cysteine and methionine) when compared to the FAO reference standard pattern of AA (Sirtori et al. 2012). (Berrazaga et al. 2019) demonstrated that dairy gel fortified with faba bean proteins resulted in improved protein efficiency and protein retention as well as muscle mass gain in young rats. Moreover, plant proteins are deemed to be less allergenic in comparison to animal proteins (Nesterenko 2012).

Today, only soy proteins and hydrolysed rice proteins are accepted as plant protein sources in the formulation of 1st age IFs (0-6 months) regarding the latest European regulation (EU 2016) in alternative to human milk, cow and goat milk IFs. Some 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 (6-12 months). Ulloa et al. (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 et al. (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. As previously mentioned in section 1.3.1.3., a process for preparing a 1st age IF based on potato protein that is suitable for infants with cow's milk protein allergy, has been patented [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 nutritional requirements directly from birth.

Part 4. IF manufacturing

The global dairy sector is currently going through change. The dairy price index shows prices 26% below its peak from February 2014 (FAO, 2018). The demand for milk products from China is beginning to slow, trade sanctions on Russia and the end of “milk quotas” within the European Union (EU) has caused a period of excess supply and low prices (Deloitte Development 2017). Despite this, the dairy sector is expanding and projected to grow at a rate of 1.8% per year over the next 10 years, to 13.2 million tons of powdered milk estimated by 2024 (Research and Markets 2019). This increase is mainly due to rising urbanization and growing incomes in emerging markets (Deloitte Development 2015).

The development of milk powders has revolutionized the dairy industry and allowed for a highly nutritional foodstuff to be exported safely around the world. Milk contains 85–90% water; it is reduced by removing the water and can reduce the milk weight to 12%, thus allowed for cheaper and easier transport. IF powder represents 40% of the entire baby food market worldwide, 33% of the IF market being shared by Europe and North America, and 53% by Asia. Global IF powder production was evaluated at 500 000 tons in 2007 increasing to 2.7 million tons in 2017 (Bonke 2016; Chen 2018).

1.4.1. Ingredients and Process

IFs are presented in a sterilized liquid form or in a powder form. Liquid IFs are more convenient to use and can avoid errors in the reconstitution of powdered formula. Powdered IFs are much easier to transport and store. Two different processes can be used to manufacture powdered IFs: the dry mix process and the wet mix with spray drying process. Sometimes a combined process is used, in which some of the ingredients are processed using wet mixing, in order to produce a base powder to which the rest of the ingredients are added using dry blending (Proudy et al. 2008; Spreer & Mixa 1998). The following sections will focus on the production of powdered IFs using the wet mix with spray drying process.

1.4.1.1. The wet mix process

The typical wet mix process involved to obtain IF is presented in Figure 20. Ingredients are first mixed together in a liquid form. After pasteurization, homogenization and evaporation, the concentrated emulsion is dehydrated by spray drying to produce a powdered IF. A well-controlled spray drying process can provide good solubility of the powder during reconstitution. However, this process needs greater investment and higher energy consumption compared with the dry mix process, and the production costs are therefore higher. Moreover, it also needs greater understanding of the operational parameters, especially for the spray drying process. An inappropriate drying operation may lead to non-compliant product quality, economic and substantial losses (Schuck 2002).

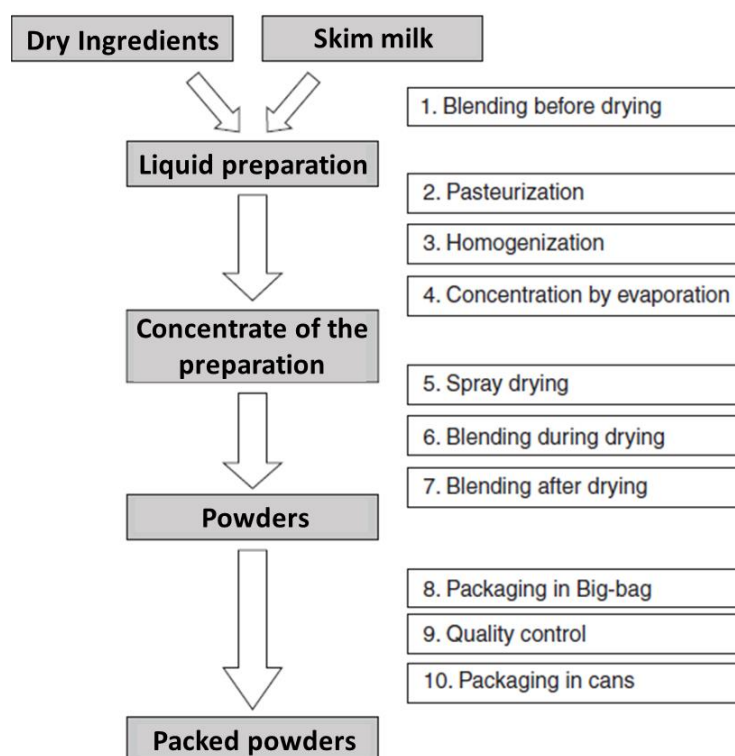


Figure 20. The wet mix and spray drying process for the manufacture of powdered infant formulas.
(Blanchard et al. 2013).

1.4.1.2. Blending before drying

As previously mentioned, the composition of IFs is designed to be roughly similar to a human mother's milk composition at approximately 1 to 3 months postpartum. The most commonly used IFs are based on cow milk and are enriched with whey proteins to rebalance the casein:whey protein ratio and to meet the proper EAA profile as found in human milk. IFs also contain a blend of vegetable oils (e.g. palm oil, coconut oil, rapeseed oil and sunflower oil) or mixtures of vegetable oils with cow milk fat, with the intention of mimicking the fatty acids composition of human milk. Lactose as a carbohydrate source, vitamins, minerals and other ingredients depending on the manufacturer (e.g. prebiotics) are also included in formulations. Emulsifiers and stabilizers are commonly used to prevent the separation of the oil phase from water in the reconstituted IF. They include citric acid esters of monoglycerides and diglycerides, lecithins (from vegetable sources such as soya, or from fish and krill), gums and maltodextrins.

For most cow milk-based IFs, the majority of the dry ingredients are dissolved in water or in skim milk to form a liquid preparation. Because of their heat sensitivity, the fat and vitamins are generally mixed with the liquid preparation after pasteurization and before homogenization. The pH of the mix can be adjusted by using potassium hydroxide/sodium hydroxide or citric acid (Montagne et al. 2009). The liquid preparation is then pasteurized and homogenized to obtain a stable and uniform liquid preparation.

Some other ingredients can also be added subsequently at different stages according to their characteristics.

1.4.1.3. Pasteurization

A pasteurization step is conducted in order to ensure the safety and to extend shelf life of the IFs. Pasteurisation is a process that consists of heating substances over 60°C during an appropriate time, with the purpose of killing harmful organisms such as bacteria, viruses, protozoa, moulds and yeasts. The process was named after its inventor, French scientist Louis Pasteur. Unlike sterilisation, pasteurisation is not intended to kill all micro-organisms and make the food sterile. Instead, pasteurisation aims to achieve a given 'log reduction' in the number of viable organisms, reducing their number so that they are unlikely to cause disease (assuming the pasteurised product is refrigerated and consumed before its expiration date) (Stehlik 2008). In IF processing, pasteurization consists of specific temperature/time combination depending on the dry matter of the fluid considered: for example, 72°C/15s for the liquid milk or demineralised whey, 90°C/1-3min for a liquid premix at 15% dry matter and/or 72°C/2min for a concentrate at 50% dry matter.

1.4.1.4. Homogenization

The objective of homogenization is to create a stable oil-in-water emulsion by mixing the blend of fats and the other components, especially proteins and emulsifiers (Dickinson 2001). The pressure applied upon homogenization (typically 10-20 MPa) is adjusted to form small size droplets (*i.e.* mean diameters: 0.1-1 µm) in order to ensure the physical stability of the processed emulsion during process, and after hydration of the powder in the baby bottle (Sun et al. 2018).

Consequently, the lipid droplets found in the reconstituted IFs have significantly smaller size than human milk fat globules (4-5 µm in human milk; Figure 9). Therefore, the surface exchange between fat droplets and aqueous phase is much higher in IFs (about 20-40 m²/g fat) compared with human milk (2 m²/g fat) (Lopez et al. 2015). (Dickinson 2001).

1.4.1.5. Evaporation

Evaporation is a key processing step for the removal of water, as it requires less energy than spray drying. Indeed, about 91% of the water is evaporated at this stage, although representing only 40% of the overall energy of IF processing. Evaporation is systematically carried out in falling film vacuum evaporators. The reduced pressure allows boiling at a lower temperature and thus protects the ingredients from heat damage (Pisecky 1997). To concentrate IFs prior to drying, continuous multiple-effect evaporators, usually of the tubular variety, are used (Caric 1993). In order to minimize the energy cost, the existing evaporators have either six or seven effects, or includes thermal vapor recompression, or one or two effects with mechanical vapor recompression.

It is important to mention that the viscosity of the concentrate to be dried also influences the quality of the powder (bulk density, solubility, etc.) by varying the size of the spray droplets (Schuck et al. 2005). For an optimal spray, the viscosity of the concentrate being dried should not exceed 200 mPa.s (Vestergaard 2004).

1.4.1.6. Spray drying

After the evaporation and homogenization steps, the concentrated liquid preparation is dehydrated by spray drying to produce a powdered IF. The spray drying process is the most widely used technique for

the manufacture of powdered IFs (O'Callaghan & Wallingford 2002) as this technique allows better preservation of the functional and nutritional properties of the IFs compared to roller dried products (Zink 2003).

During spray drying, the liquid product is sprayed out in the form of fine droplets into a chamber in which hot air circulates. The resulting huge increase of the surface area between liquid and air permits rapid moisture and heat transfers. For instance, spraying 1 liter of liquid in droplets of mean diameter of 50 μm results in the increase of the air/liquid interface total area up to 120 m^2 (Spreer & Mixa 1998).

A typical drying system consists of (Figure 21):

- (1) One or several high pressure nozzles or a rotary atomizer/wheel for the atomization of the concentrate.
- (2) A drying chamber/tower.
- (3) An integrated fluid bed for secondary drying (not shown on Figure 21).
- (4) An external or integrated fluid bed dryer for cooling of the powder (Montagne et al. 2009).
- (5) One or several cyclone(s) and/or bag filter(s) which provide an air/powder separation system (GEA NIRO 2013; Vestergaard 2004).

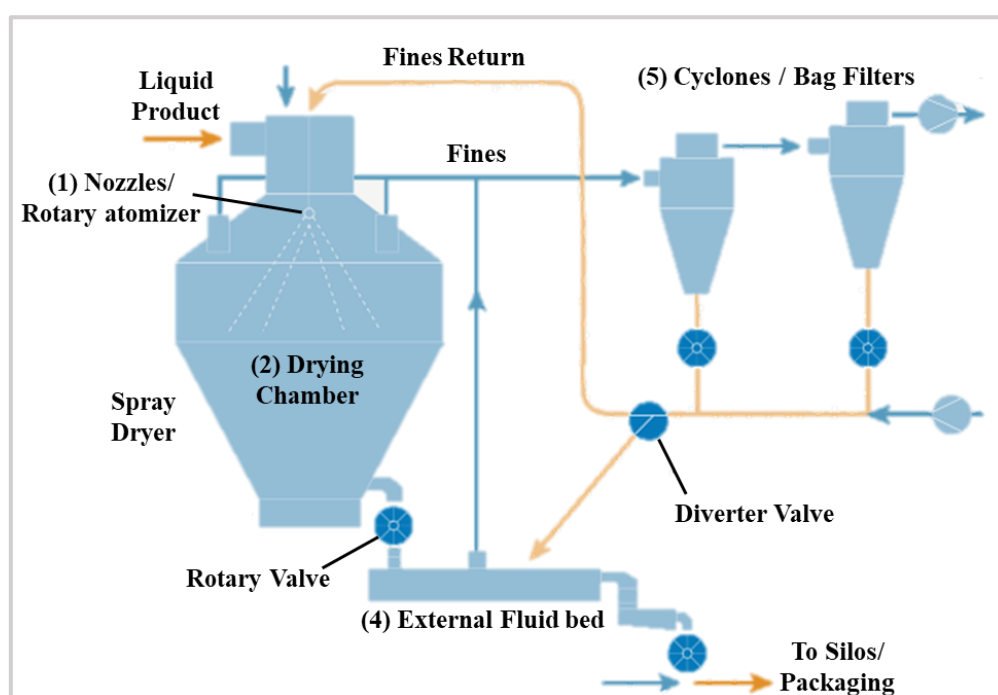


Figure 21. Spray drying process flow for milk powder production.

(Adapted from: <https://www.coperion.com/en/industries/food-pet-food/dairy-nutrition>).

The aim of atomization (1) is to form droplets fine enough to dry quickly, but not too fine as to escape with the outlet air following drying. Producing a very fine powder would also cause subsequent dissolution problems, as wetting is impaired by increased powder particles total surface. The rotary atomizer and the stationary atomizer are the most common atomizer designs. Both spraying mode cause the milk particles to form a spherical shape during drying, due to surface tension influence. The

droplet/particle size conditions properties such as bulk density, shape, size distribution of the powder particles, and the occluded air and moisture content (Caric 1993; Caric et al. 2009).

There is a variety of drying chamber designs (2). Usually, they consist of a cylindrical cone set at 40–60°, which allows the powder to exit the chamber using gravity. These chambers are also designed with flat bottoms, so that scraper or suction devices can be attached in order to remove the powder from the chamber. The temperature of the air in the chamber is 170–250°C, while the product temperature remains between wet bulb temperature (40–50°C) and outlet temperature (70–90°C); conversely, the droplet surface humidity decrease from saturation to final powder water content, whereas the air relative humidity increase from less than 0.1% to 12–20% depending on the dryer type. This temperature and humidity gradients cause rapid heat and water transfers and, therefore, rapid and gentle drying is achieved. Heat denaturation in the product during the sole drying stage is very limited, although it cannot be completely avoided. In order to minimize the degradation of sensitive products such as IFs, the drying process can be completed in several stages, with each stage having a different time–temperature profile. The first processing stage is completed in the drying chamber (2) and the second in fluid bed drying (3) devices. Dry air with a lower temperature than that of the drying chamber passes through the vibrating fluid bed, thus further increasing the removal of moisture.

It is occasionally necessary to include a third and final processing stage in an external fluid bed (4), which involves using a flow of dry and hot air with a temperature below 100°C, but most of the time makes it possible to stabilize the resulting powder by cooling it down the glass transition temperature. This final drying and cooling stage (4) is then conducted outside of the drying chamber (Montagne et al. 2009).

A more or less important amount of powder escapes the plant during the drying and cooling stages. This powder is separated from the air using cyclones (5). Cyclones present high efficiency and are easy to clean and maintain, but need additional mean to separate the finer particles. A final cleaning is therefore completed using bag filters, which consist of numerous bags or filters arranged so that each bag receives equal quantities of air (Vestergaard 2004; Westergaard 2002).

This multistage spray dryer (Figure 21) is currently usually used for the production of IFs, as it can provide improved product quality in terms of agglomeration, solubility, dispersibility, wettability, etc. (Pisecky 1990). Moreover, the formulation of IF may involve the mixing of over 40 different constituents before the evaporation and spray drying processing stages. These variations in product composition may exhibit different drying behaviors and stickiness-solubility patterns. The drying of such products may require adjustment of outlet gas temperature and outlet gas humidity (in a cooler and drier way). In other words, due to the diversity and complexity of IFs, the operational parameters for one IF cannot be applied systematically to another formula (Schuck, 2002), and thus optimisation and adjustment should be conducted for each IF formulation.

1.4.1.7. IF quality control

Spray dried IF powders are often packed in big-bags pending the outcome of quality control. Firstly, a microbiological examination is essential and needs to be performed to guarantee the microbiological safety of the product, especially for the detection of *Salmonella*, *Enterobacteria* and *Enterobacter sakazakii*. It aims to ensure that the final product was not recontaminated after the pasteurization step. This is all the more important because IFs are usually given to infants without further heat treatment. Moreover, chemical contaminants should also be controlled to ensure IFs quality such as organic pollutants (dioxins, polychlorinated biphenyls, melamine, pesticides...), heavy metals (aluminium, manganese, cadmium, lead) or endocrine disrupters (bisphenol A).

Secondly, the nutrient content of powdered IFs must be checked to ensure that it can fully meet the nutritional requirements of infants (e.g. composition of proteins, AAs, fat, mineral and vitamin content). According to the CAC guideline (Codex Alimentarius Commission 1981) or the applicable European regulation (EU 2016), infant formula should contain no less than 60 kcal and no more than 70 kcal of energy per 100 mL. The use of additives is allowed in IFs (thickeners, emulsifiers, acidity regulators, antioxidants, and packaging gases) but it is also strictly regulated (EU 1995; WHO & FAO 2007).

Finally, physical analyses, such as powder solubility, dispersibility, density, granulometry profile and bottle test, should also be performed to assure the physiochemical quality of the powder. Consequences of not controlling these parameters lead to quality impairments in IFs, such as free fat, flecking, Maillard reactions, lactose crystallization (*i.e.*, caking) and poor solubility (Schuck et al. 2007).

1.4.1.8. Packaging

Once powder quality is consistent, the powder can be then packed in small metal cans in an aseptic environment. The powder is usually packed in a nitrogen atmosphere to prevent oxidation of the fat component (Montagne et al. 2009).

1.4.1.9. Future trends in IF production

The techniques for production of powdered IFs have not change much in the last 20 years. The two main issues confronting manufacturers and researchers are product quality and cost.

The first issue can be summarized as follows: how to preserve as much as possible the different components during the production process and, at the same time, how to control powder characteristics to improve its stability during transport and storage and its convenience for customer use (such as instant solubility). Consideration must be given to thermal and dehydration practices used during manufacture of IF. These preservation techniques determine the final quality of both liquid (ready to feed) and powdered IFs; attributes such as emulsion stability in liquid IF and rehydration properties in the case of powder are essential to ensure homogeneity of nutrients in the finished product. The emergence of new protein ingredients with different dissolution properties, e.g., milk protein concentrate, pea, rice and legume proteins suggests the need for new handling and hydration technologies (Amagliani et al. 2016). A number studies have demonstrated the benefits of using high intensity ultrasound to aid dissolution of powders, with some success for milk protein concentrate (McCarthy et al. 2014). And other proteins such as pea (McCarthy et al. 2016) and soy (Jambrak et al. 2009).

Microwave heating is also an emerging technology with specific interest and potential for IF manufacturers. In essence the technology focuses on the delivery of focused microwave energy in a tubular heating system with specific permeability to microwaves (Laguerre et al. 2011). A new approach to IF manufacture has been developed with low temperature microfiltration (Crowley et al. 2015, 2016; McCarthy et al. 2017). Among the benefits of this new approach, include a more sustainable process, reduced thermal load on nutrients, a protein profile closer to human milk and potentially altered digestion properties.

The second issue is how to control the costs of the raw materials and the production costs for IFs. For instance, feeding the spray dryer with a concentrate at higher total solids contents represents a strong opportunity to reduce the overall energy cost of powder production. However, the extent of such increase in concentration is limited due to the simultaneous increase in product viscosity, the actual spraying systems being unable to spray high viscous products (Schuck et al., 2016). Schuck et al. (2015) tested an alternative technology including vacuum concentration and crystallization that was able to handle highly viscous concentrates to produce powders with no spray-dryer step required. This new processing design led to powder properties closed to those obtained with a conventional spray drying system and also conducting in lower energy consumption (10 to 30%).

Other improvements such as new packaging formats with tableting, twist-cap mechanisms which can store and release powder when opened, and the integration of E-commerce and virtual and augmented reality with packaging design can all provide alternative supply chain options for the IF area. Moreover, social media on nutritional information and convenience of IFs are continuing to shape and drive consumer demand.

1.4.2. Effect of processing on IF quality

1.4.2.1. Effect on protein digestibility

Heat treatments are known for affecting the digestibility of milk proteins due to several mechanisms. Dupont et al. (2010a) reported that heat processing during milk powder manufacture causes caseins to aggregate and thereby increases its resistance to *in vitro* digestion. Using proteomic techniques to compare protein modifications during different heat treatments, Wada & Lönnerdal (2014) showed that lactulosyllysine, a Maillard reaction product, is an indicator of digestibility; a high level corresponds to low protein digestibility as observed with in-can sterilized and UHT milk. This study also suggests that heat treatment decreases protein digestibility (Wada & Lönnerdal 2014). Sarwar et al. (1989) demonstrated that heat treatments (122°C for 8 min ; 132°C for 5 min) applied during the manufacture of ready-to-feed liquid IFs reduced the apparent and true digestibility of protein in rats (74% to 76%; 88% to 90%) compared with powder forms of IFs (79% to 83%; 93% to 97%). The true digestibility of lysine, methionine, and cysteine in liquid products was also 5% to 13% lower than in powder products.

Moreover, the structure of IFs may affect the rate and extent of protein digestion. The proteolysis rate of a milk emulsion containing native milk fat globules during *in vitro* gastric digestion is lower when compared to homogenized or homogenized/pasteurized IFs (Bourlieu et al. 2015). The adsorption of proteins to lipid droplet interfaces in an emulsified food or the protein-lipid interactions can change the

accessibility of cleavage sites to digestive enzymes and modify the local flexibility of substrates (Mackie & Macierzanka 2010). De Oliveira et al. (2016) used an *in vitro* dynamic system to study the gastrointestinal digestion of human milk either raw or pasteurized under infant digestive conditions. Gastric digestion was marked by a strong emulsion destabilization, due to acid and enzymatic-induced aggregation. The pasteurization of human milk reduced its gastric destabilization (the particle size profile remained quasi-steady during all the gastric phase), whereas an important destabilization was observed in raw human milk. As observed by confocal laser scanning imaging, the aggregated particles were based on clusters of milk fat globules around which protein aggregates built up (Bourlieu et al. 2015; Gallier et al. 2013).

1.4.2.2. Effect on protein functionality

PROTEIN SOLUBILITY

Reconstituted milk powder should ideally reflect the organoleptic, nutritional and colloidal properties of fresh milk. However, an issue that is often encountered upon dispersion of the powder in water is the presence of insoluble particles (Schuck et al. 2016; Singh & Ye 2010). Insolubility is not a simple concept as it may have several different origins. Schuck et al. (2016) used the term “flecking” in infant milk formula powder covering all insoluble material originating from protein interactions, fat coalescence or flocculation, or insoluble powder particles.

Insoluble particles with various sizes may form under several mechanisms at different stages of the manufacturing process. Insolubility originates from heat instability of the system before or during drying and depends on the composition, pretreatment (homogenisation, pasteurisation), characteristics of the concentrate (pH, solids content, viscosity), drying parameters (nozzle design, inlet and outlet temperatures) and finally, transport and storage conditions (Sadek et al. 2015; Schuck et al. 2016; Sharma et al. 2012). Lactose, native whey proteins and some inorganic monovalent salts are the only completely water soluble components in milk powder. Therefore, fat globules, casein micelles, divalent minerals, denatured whey protein and their combinations have the ability to form insoluble material (McKenna 2000).

Protein insolubility in standard cow milk-based IFs is initiated by the unfolding of whey proteins followed by aggregation with caseins (Baldwin 2010), and in fat-containing systems, this results in formation of protein-bridged fat globule clusters (Singh & Ye, 2010). The formation of insoluble material is hypothesized to originate from heat and shear applied during spray drying resulting in changes in fat-protein interactions. Also, emulsion quality before drying and fat crystallization as well as whey protein denaturation (Joyce et al. 2017) at different stages of drying may play a role. Lactose crystallization during humid storage has been reported to result in disruption of fat globules, higher amount of free fat and rougher particles in model IF powders made with different protein-to-fat ratios (McCarthy et al. 2013). This could also contribute to insoluble material formation during storage.

Toikkanen et al. (2018) studied the origin and mechanisms of the formation of insoluble particles (white flecks) in model IF powders. These authors found that white flecks consisted of 75 % fat and 22% protein (casein and whey protein). Increased fat-protein clustering in the powder was associated with white flecks formation. White flecks had at least two structure types originating either from the fusion of

spherical particles or from sharp-edged morphologies containing lactose crystals. The identification of the two different types of white flecks particles highlighted that both, the drying process and the storage conditions, need consideration to reduce white flecks formation. The stability of the fat globules could be achieved by addition of an emulsifier and by lowering the fat-to-protein ratio. Fat globule clustering should be avoided as well as humid storage. Humid storage promotes lactose crystallization in powders, which is known to cause remarkable changes in the powder structure, resulting in disruption of the fat globules and reconstituting behavior.

PROTEIN DENATURATION AND AGGREGATION

As previously mentioned, most IFs are cow milk protein-based and are enriched in whey proteins. The major whey proteins in cow milk are β -lactoglobulin and α -lactalbumin. β -lactoglobulin has two disulphide bonds and one free thiol group, which are deemed responsible for the irreversible thermal aggregation and gelling properties of this protein (Brodkorb et al. 2016). α -lactalbumin has a single polypeptide chain, containing four disulphide bonds, and has no free sulphydryl group; native molecules of α -lactalbumin consist of two domains, a large α -helical domain and a small β -sheet domain, which are connected by a calcium-binding loop (Permyakov & Berliner 2000).

The mechanisms of denaturation and aggregation of whey proteins have been extensively studied (Oldfield et al. 2005) and reviewed recently (Brodkorb et al. 2016; Wijayanti et al. 2014). The mechanism of denaturation and aggregation of β -lactoglobulin was first proposed with two distinct stages; the initial stage is the unfolding of the globular protein at temperatures >65 °C, while the second stage involves irreversible association of the unfolded molecules to form complexes, i.e. aggregates. Denaturation and aggregation have been shown to impact functionality of whey proteins during processing of different whey protein-containing systems (Mulcahy et al. 2016; Ryan et al. 2012).

Previous studies have investigated the influence of temperature and ionic strength on whey protein denaturation and aggregation during thermal processing and the enhancement of heat stability of model IF protein systems by increasing α -lactalbumin: β -lactoglobulin ratio (Crowley et al. 2016). Joyce et al. (2017) have studied the influence of pre-denaturation and aggregation of whey proteins on viscosity and processing properties of a model IF. These authors showed that a pre-heat treatment of whey protein helped to reduce susceptibility to protein aggregation when incorporated into a model IF system. Moreover, it was shown that the physical reactivity of whey protein particles strongly influenced protein-protein interactions and their ability to contribute to viscosity during thermal processing. Finally, they showed that aggregation was promoted by the addition of low levels of calcium ions prior to heat treatment. These findings are important in facilitating an enhanced understanding of protein-protein interactions and their influence on viscosity development and processing performance of formulated nutritional beverages such as IFs.

1.4.2.3. Effect on structure and thermal properties of IFs

Lopez et al. (2015) studied the effect of technological processes, mainly homogenization, on the structure and interface composition of processed lipid droplets found in milks and IFs. As mentioned earlier, the smaller size of lipid droplets found in IF compared to human milk (Figure 9) results from the homogenization step involved in the manufacture of IFs. The composition of the fat/water interface is found different between human milk fat globules and the processed lipid droplets in IFs. The CLSM images and patterns previously presented in Figure 9 show the differences in interface composition: processed lipid droplets in IFs are mainly covered by milk proteins while human milk fat globules are enveloped by polar lipids and membrane proteins organized in the milk fat globule membrane (Lopez and Ménard 2011). Moreover, the structural analysis of various commercialized IFs revealed the presence of aggregates of proteins or of complexes formed between lipid droplets and proteins (Figure 22). These protein aggregates and lipoprotein complexes may result from the thermo-induced denaturation of proteins occurring during the heat treatments performed for the microbial safety of IFs. Such lipoprotein complexes induced by the industrial process raise questions about the accessibility of FA and proteins by the digestive enzymes in the gastro-intestinal tract of newborns and then about the nutritional and health impacts.

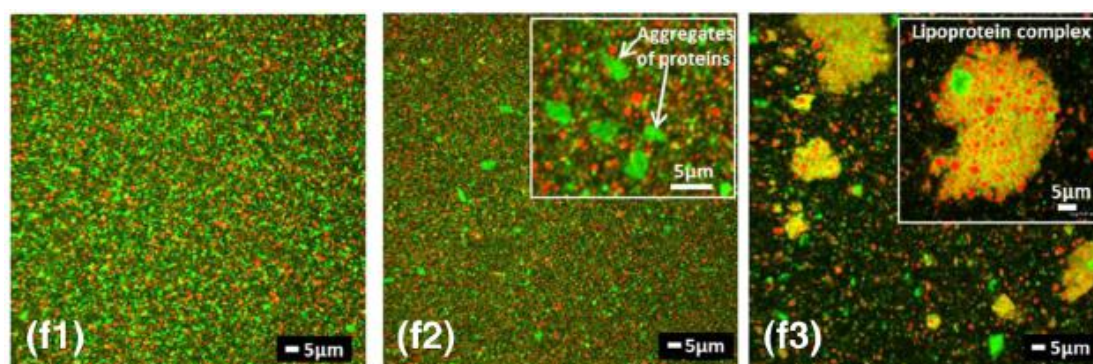


Figure 22. CLSM (confocal laser scattering microscopy) images showing the microstructure of three different commercialized infant formulas (f1, f2, f3) after hydration in a bottle. Total fat was labelled with Nile red fluorescent dye and proteins with Fast green FCF. (Lopez et al. 2015).

Pasteurization and spray drying may promote interactions between proteins and lactose due to heat treatment and mechanical stress (Birlouez-Aragon 2004; Joyce et al. 2017). The properties of IFs, for example colour, solubility, storage stability, and even allergenicity can be affected by the component interactions (Li et al. 2016). Previous studies mostly focused on the effects of processing on the nitrogen-distribution, mineral distribution and protein damages (Guo et al. 1998, 1999). Sun et al. (2018) investigated the effects of unit operations on the microstructure, thermal properties and structural characteristics of IFs. Results indicated that homogenization, pasteurization, and spray drying had the most pronounced impact on the last mentioned properties, due to the interactions between protein–protein, protein–lipid, and protein–carbohydrate. Thus, unit operations as well as the components used to prepare IFs have a great impact on the structure and the physicochemical properties of the IFs.

2. Objectives & Experimental strategy

As stated in the previous literature review, protein intake early in life is essential for the development of infants. Therefore, the nutritional value of proteins is a key point for IFs that are the only food of many infants during the first months of life. Then, nutritional value of proteins depends on AA composition and their digestibility that are both directly determined by the nature of proteins included in the food and by the processing applied. At the same time, the interest in alternative proteins to animal proteins is growing since the global human population is increasing: the demand for animal proteins is thus expected to double by 2050. This change is promoted through recent advances in research and development that led to improve the nutritional and functional properties of many plant protein sources. The aim of IFs is to mimic the composition and function of human milk and to provide formula-fed infants with the same growth and development as exclusively breastfed infants. Scientific and technological advances are pushing forwards new fields of innovations in infant nutrition such as formulas with new protein sources compared to those currently authorized. However, only few studies related the use of other protein sources than the conventional cow, goat, soy and hydrolysed rice proteins that could be suitable for 1st age IF.

In this context, the aim of the project was to develop 1st age IFs in which whey protein concentrate usually added to the skimmed cow milk and representing 50% of the total protein in IFs will be replaced by innovative plant proteins. These new protein sources are not yet allowed according to the applicable regulation, but the project aimed to investigate future possibilities in this field.

The main research question that raised was:

How do protein sources influence the functional properties and the digestibility of infant formulas?

Three different sub-questions were stated:

1. Which protein sources would be suitable to replace whey protein concentrate in IFs?
2. How do protein sources affect the manufacturing and the functional properties of IFs compared to a standard dairy IF?
3. How do protein sources affect the digestibility of IFs compared to a standard dairy IF?

To answer these questions, the experimental strategy presented in Figure 23 has been set up. First, alternative protein sources have been selected, mainly on nutritional aspects. Then, IFs have been developed at a pilot scale and their digestibility was evaluated using an *in vitro* static model. Finally, the most promising IFs have been produced at a semi-industrial scale and their digestibility was investigated using an *in vitro* dynamic model. This experimental design using a multi-scale approach should let us to conclude on the aptitude of new protein sources to partially substitute whey protein in 1st age IFs on a functional and a nutritional point of view

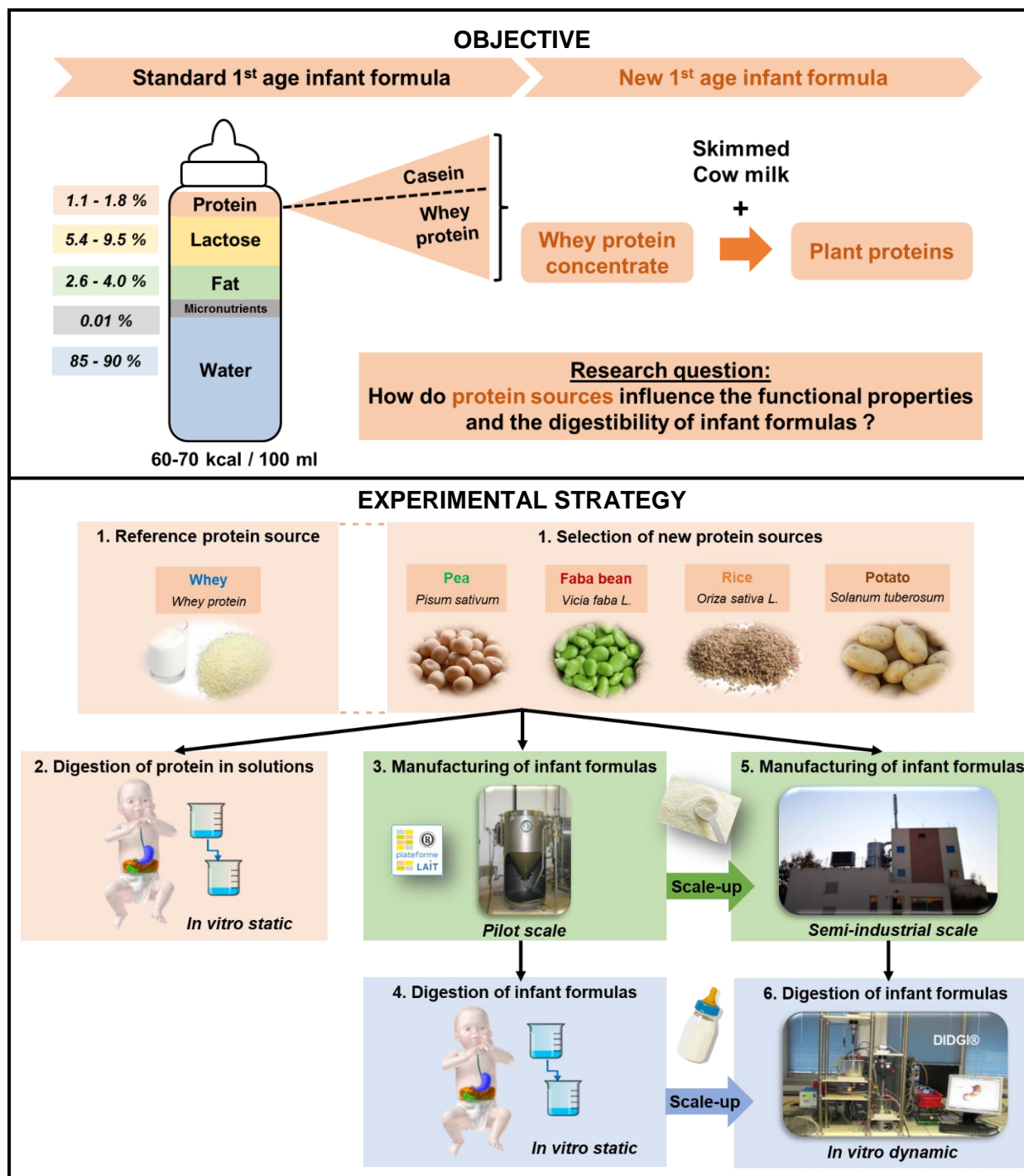


Figure 23. Objective & Experimental strategy of the present study.

3. Materials & Methods

This section has been drawn from the related publications and summarized hereafter in order to avoid repetitions and redundancies throughout the results section.

3.1. 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). N-p-tosyl-L-arginine methyl ester hydrochloride (TAME) was supplied by Sigma Aldrich (St Louis, MO, USA). The fluorescent dyes used for CLSM analysis were HCS LipidTOX™ Green Neutral Lipid Stain (H34475) obtained from Thermo Fisher Scientific (Illkirch, France), RD-DOPE Liss Rhod PE 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (810150C) obtained from Merck (Darmstadt, Germany), Fast Green FCF (Sigma F7258, Sigma-Aldrich, St Louis, USA), and Nile Red fluorescent probe (5H-Benzo, α -phenoxazine-5-one, 9-diethylamino; Sigma – Aldrich, St Louis, USA). The standards used for size exclusion chromatography were purchased from Phenomenex (Waters Inc., Milford, USA) (No. ALO-3042 for bovine thyroglobulin, IgA, IgG, ovalbumin and myoglobin) and from Sigma-Aldrich (cytochrome C (C2506) and human angiotensin II (A9525)). All other chemicals were of standard analytical grade.

3.2. Model Infant formula ingredients

Skimmed 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* L., 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. 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”. The nutritional composition of all ingredients is presented in Table 11. The composition of the model IFs was determined according to the nutritional requirements reported in the latest European regulation for 1st age infant formula (EU 2016). The model IFs composition is available in Table 12.

All IFs rehydrated in baby bottles were iso-proteinaceous at approximately 1.6 g of protein / 100 mL of IF, except for IFs digested within *in vitro* dynamic model where too much water has been erroneously

added and thus 1.3 g / 100 mL was obtained in these baby bottles instead of the targeted value of 1.6 g / 100 mL.

Table 11. Nutritional composition of the ingredients for infant formula development.

	Skim med Milk	Lactose	Maltodextrin	Lactarmor™ DM 90	Protarmor™ 80	Nutralys® XF	Vitesse™ 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	20* ¹	NA	3.8* ²	NA
Ashes (w/w% DM)	5.5*	0.2*	0.03*	0.4*	1.6*	5*	NA	6*	0.04*	NA

*technical data from suppliers.

N.A: not available data.

¹Carbohydrates (14% dietary fiber; 2% starch ; 2% sugars; 2% sucrose).

²Carbohydrates (3.5 % dietary fiber; 0.1% starch ; 0.2% sugars).

Table 12. Infant formulas ingredient composition (Reference IF; Pea IF; Faba bean IF; Rice IF; Potato IF). Data are expressed in kg / 100 kg of infant formula at 24 w/w% DM.

	Reference IF	Pea IF	Faba bean IF	Rice IF	Potato IF
Skimmed milk			3.72		
Maltodextrin			0.56		
Oil blend			5.23		
Lactose	6.76	13.30	13.30	13.30	13.30
Protarmor™ 80	0.62				
Lactarmor™ DM 90	7.73				
Nutralys® XF		2.09			
VITESSENCE™			2.33		
RicePro NG BIO				2.08	
Solanic® 200					1.78
Water	75.38	75.09	74.86	75.10	75.41

3.3. Infant formula processing

3.3.1. Pilot scale

Skimmed 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 at 45°C under stirring at 35 Hz for 1 h (Figure 24). The protein concentrates represented 50 w/w% of the total protein content of the formulas whereas the others 50 w/w% came from skimmed cow milk proteins. Neither vitamins nor minerals were added since this study was primarily focused on protein fraction and explain the expression of “model infant formula” used in the present work. The solution was then pasteurized at 80°C for 35 s, before concentration 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 $65 \pm 2 \text{ ml} \cdot \text{min}^{-1}$. The inlet and outlet air temperatures were set at $175 \pm 5^\circ\text{C}$ and $75 \pm 5^\circ\text{C}$, respectively. The evaporation capacity was approximately $3.25 \text{ kg} \cdot \text{h}^{-1}$. The resulting powders were finally stored in light- proof plastic bags at 20°C during maximum 4 weeks pending for characterizations. Each IF was manufactured only once.

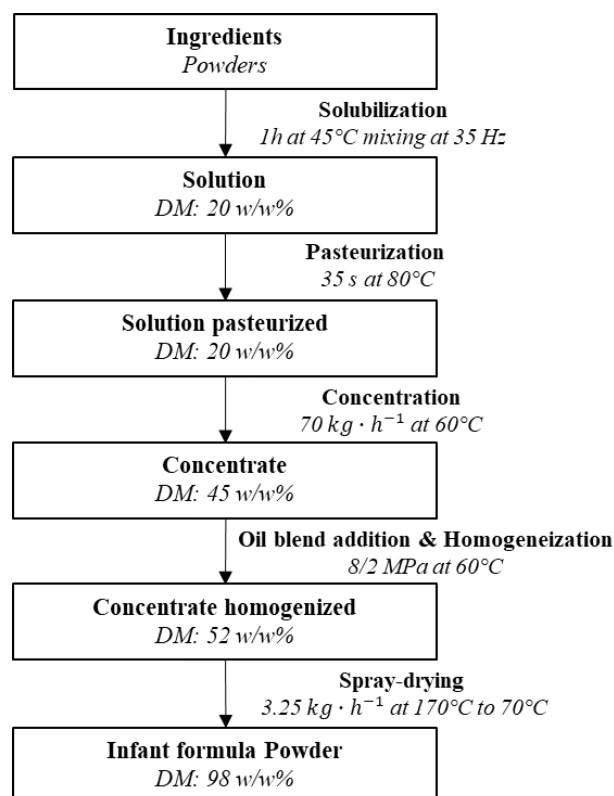


Figure 24. Pilot process flow diagram for model infant formula production.

3.3.2. Semi-industrial scale

The model IFs were manufactured at Bionov (Rennes, France) according to the technological diagram presented in Figure 25, which included two processing routes (1 and 2). Skim bovine milk powder, lactose, maltodextrin and the respective protein concentrates (whey protein as the reference, pea protein or faba bean protein as the plant protein sources) were solubilized in water at 20% w/w DM at 45°C under stirring at 35 Hz for 45 min. The protein concentrates represented 50 w/w% of the total protein content of the formula whereas the other 50 w/w% came from skimmed bovine milk. Neither additional vitamins nor minerals (apart from those provided by the ingredients) were added since this study was primarily focused on protein fraction. The solution was then pasteurized at $80 \pm 2^\circ\text{C}$ for 35s, before concentration to approximately 48% w/w% DM in a two-stage semi-industrial scale falling film vacuum evaporator (GEA Process Engineering, St Quentin-en-Yvelines, France) with an evaporation capacity of $280 \text{ kg} \cdot \text{h}^{-1}$ at $60 \pm 4^\circ\text{C}$. The concentrate was then cooled to 45°C before storage in a tank. The oil blend was added to the concentrate before homogenization at 60°C and either 8/2 MPa or 14/4 MPa for the processing routes 1 and 2, respectively. Finally, the solution was spray-dried from 53 w/w%

DM to 97 w/w% DM in a semi-industrial-scale Niro Atomizer (GEA-PE, Saint Quentin en Yvelines, France) spray dryer at Bionov (Rennes, France) (Bimbenet, Schuck, Brulé, Roignant & Méjean, 2002), which maximum theoretical evaporation capacity is approximately $90 \text{ kg} \cdot \text{h}^{-1}$. The dryer was equipped with a pressure nozzle (orifice diameter: 0.73 mm, with four core slots with a nominal slot width of 0.51 mm) providing a spray angle of 60° . The inlet air temperatures were set at either 165°C or 150°C for the processing routes 1 and 2, respectively. The outlet air temperatures were set at either 75°C or 65°C for the processing routes 1 and 2, respectively. The concentrate homogenized flow rates were $100 \pm 10 \text{ L} \cdot \text{h}^{-1}$ and the major air flow rate was $3200 \pm 100 \text{ kg} \cdot \text{h}^{-1}$. The resulting powders were finally stored in light proof plastic bags at 20°C during maximum 4 weeks pending for characterizations. Each IF was manufactured only once with two processing routes for each.

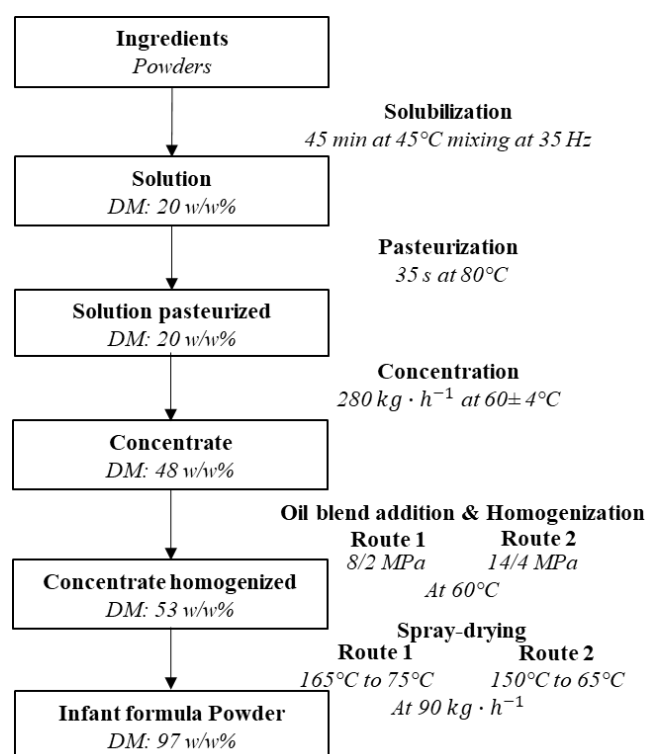


Figure 25. Semi-industrial process flow diagram for model infant formulas production including processing routes (1) or (2).

3.4. Physicochemical analysis

3.4.1. Dry matter, ash and protein content

Total dry matter (DM; in $\text{g} \cdot 100 \text{ g}^{-1}$) was determined gravimetrically after heating at $102 \pm 2^\circ\text{C}$ for 5 h, and ash content after incineration at $525 \pm 25^\circ\text{C}$ 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 and for the reference cow milk protein based IF (Mariotti et al. 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 calculate the protein content in the plant based ingredients. All measurements were carried out in duplicate.

3.4.2. 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 350g. The free fat content was determined gravimetrically after evaporation of the solvent. Total and free fat analyses were carried out in duplicate (AFNOR, 1990 ; Schuck et al. 2012).

3.4.3. Water activity and glass transition temperature

Water activity (a_w) was measured at $25 \pm 0.1^\circ\text{C}$ using the Novasina aw-meter (Novasina, Switzerland). The glass transition temperature (T_g) was determined on the powders after equilibration in a 20% relative humidity atmosphere using the SPSx-1 μ Sorption Test System (ProUmid GmbH & Co. KG, August-Nagel-Str., Germany). The T_g was then determined at this constant sorption point by using a modulated temperature differential scanning calorimetry method according to Schuck et al. (2012). T_g measurements were carried out in triplicate.

3.4.4. Particle size distribution

The powder size distribution was determined using a laser scattering granulometer (Mastersizer 2000, Malvern Instruments Ltd, Malvern, UK) with a 300-mm measurement cell (0.5-880 mm range) in dry channel. The refractive index of dried particles was 1.45, and 30 kPa air pressure was used. The median diameter $d(0.5)$, i.e. the particle diameter below which 50% of the material volume exists, was chosen to describe the particle size distribution.

The particle size distribution of the dispersed elements present in solutions during manufacturing and during digestion were determined using the same laser scattering granulometer in liquid channel. The particle size distribution was based on volume and expressed as sphere-equivalent diameter. The refractive index used was 1.45 for blends of vegetable oils in infant formulas. The refractive index of 1.33 was used for water. The samples taken from concentration and homogenization process steps were diluted 1:1 in water prior measurement. About 0.2 mL of sample was diluted in 100 mL of water directly in the measurement cell of the apparatus in order to reach 10% obscuration. The diameter mode (diameter of the most frequent population of particles in the volume distribution) and the $D[4.3]$ (mean volume diameter of the distribution) were chosen to describe particle size distribution in solutions.

The measurements were performed in triplicate for each sample (powder and solutions).

3.4.5. 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. Color measurements were carried out in triplicate.

3.4.6. 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- μ m mesh-size sieve. The solubility index is the v/v% of soluble particles (i.e., remaining in the supernatant after centrifugation at 160g 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). These experiments were carried out in duplicate.

3.4.7. 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 ($\pm 0.1^\circ\text{C}$). Apparent viscosity was measured on homogenized samples (52-53 w/w % DM) 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. The viscosity was determined using Newton law or Power law model depending on the behaviour of the fluids measured (Newtonian or rheofluidifiant). These experiments were carried out in triplicate.

3.4.8. Trypsin inhibitor activity

The trypsin inhibitor activity in each protein ingredients 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 protein ingredient in question. 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 $\mu\text{g/mL}$ and 50 μL of protein solution at 1.6 g/100 mL (or 50 μL water for the blank) were mixed in a 4-mL quartz cell with a 1-cm light path, before absorbance measurement at 247 nm for 10 min at 30-s intervals at 25°C. The activity was expressed in TAME units where one unit hydrolyzes 1 mmol TAME per minute at 25°C.

3.4.9. Confocal laser scanning microscopy (CLSM)

3.4.9.1. CLSM analysis of IFs during manufacturing

Confocal laser scanning microscopy (CLSM) observations were performed using an inverted microscope NIKON Eclipse-TE2000-C1si (NIKON, Champigny sur Marne, France). Samples collected during the process (before concentration, after concentration, after homogenization in presence of lipids) were stored at 50°C in a laboratory oven during the staining step and during the CLSM observations thanks to a temperature-regulated stage (Linkam Scientific Instruments Ltd, Tadworth Surrey, England). The powdered IFs obtained were rehydrated, stained and observed at ambient temperature. Fast Green FCF fluorescent probe was used for the labelling of proteins (Excitation = 632 nm). The lipid-soluble Nile Red fluorescent probe was used to label the lipids (Excitation laser = 543 nm). After labelling, the samples were stored for at least 30 min before microstructural analysis. A He-Ne laser operating at 543 nm wavelength excitation and 565 nm to 615 nm emission, and a diode operating at 633 nm with emission detected with a long pass filter > 650 nm were used. The observations were performed using

a x40 and a x100 oil immersion objectives. The two-dimensional images had a resolution of 512 x 512 pixels and the pixel scale values were converted into micrometers using a scaling factor. In the multiple labelled samples, different colors were used to locate the fluorescent probes. At least five to ten images were taken on each samples.

3.4.9.2. CLSM analysis of IFs during digestion

The microstructure of the IFs and of the digestas was observed using Confocal laser scanning microscopy (CLSM) was performed using a Nikon C1Si inverted microscope TE2000-E (Nikon, Champigny-sur-Marne, France) equipped with a x100 oil immersion objective, as previously described by (Bourlieu et al. 2015); three fluorescent dyes were added to the samples (Lipidtox™ for apolar lipids ; Rhodamine-PE for amphiphilic compounds and Fast Green FCF for proteins). At least five to ten images were taken on each samples.

3.5. Theoretical indicators for nutritional quality evaluation

The following theoretical indicators were used to evaluate the nutritional quality of the different protein sources analysed in the results chapter 1 (section 4.1.):

$$\text{EAA recovery ratio} = \frac{\text{EAA content of the protein}}{\text{EAA content of the reference}} \quad (4)$$

In this part of the study, the reference pattern corresponded to either the deficiency to cover 50% of total protein content in IF or a standard cow milk 1st age IF (EU 2016).

$$\text{PDCAAS-like score (\%)} = (\text{EAA recovery ratio (LEAA)} \times \text{Protein digestibility}) \times 100 \quad (5)$$

where LEAA is the most limiting EAA.

PDCAAS-like score is an adaptation of the PDCAAS score (protein digestibility amino acid score). Protein digestibility in this part of the study corresponded to protein digestibility obtained from literature data.

3.6. In vitro digestion

3.6.1. In vitro static digestion

Either protein sources in solution (i.e., whey protein concentrate (WPC), pea protein concentrate (PPC), faba bean protein concentrate (FPC), rice protein concentrate (RPC) and potato protein isolate (PPI)) or IFs produced at a pilot scale (i.e., reference IF, pea IF, faba bean IF, rice IF and potato IF) were subjected to *in vitro* digestion in static conditions. The meals were prepared by solubilizing powders (either protein ingredients or IFs) 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 the meals were 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) and has also been presented in Section 1.3.3.2.

Briefly, the gastric pH was initially set at 5.3 with 1 M HCl, with a 63:37 meal:gastric secretion ratio (v:v) 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 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. The protocol described above is summarized in Figure 26.

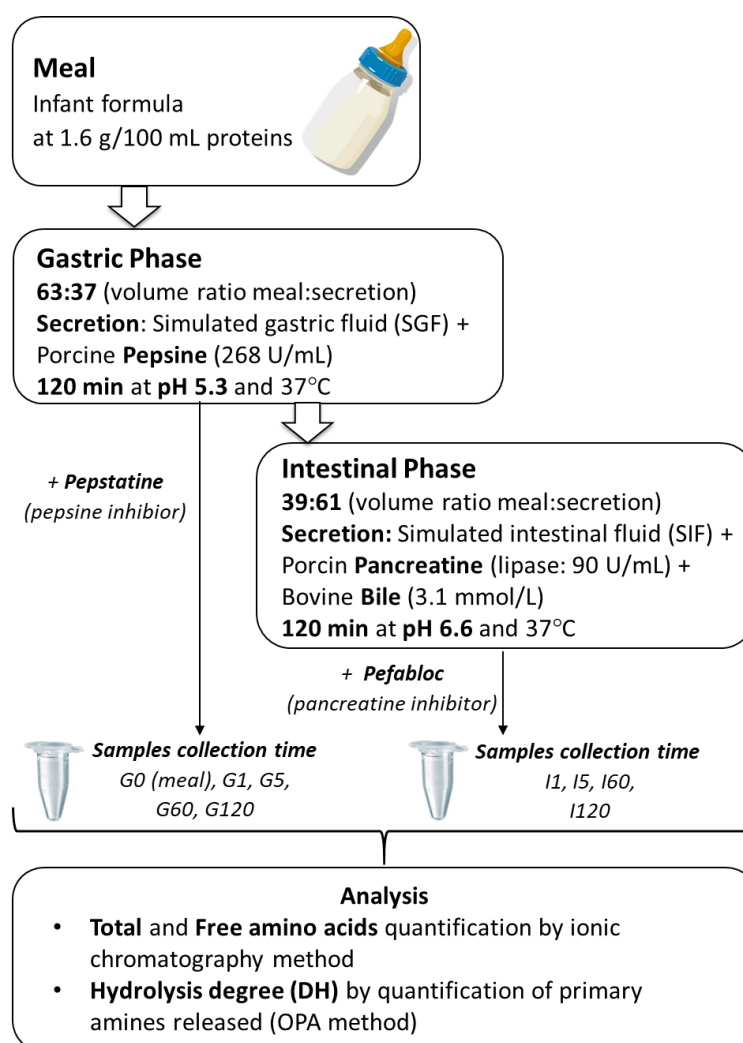


Figure 26. Illustration of *in vitro* static simulating infant digestion protocol used in the present study.

(Adapted from Ménard et al. 2018).

3.6.2. *In vitro* dynamic digestion

Gastrointestinal digestions of RIF (reference IF), PIF (pea IF) and FIF (faba bean IF) produced at a semi-industrial scale (through process route 1, Figure 25) were performed in an *in vitro* dynamic system (DIDGI®, INRA, Paris, France). Parameters for gastric and intestinal phases were chosen to mimic the digestive conditions of term newborn fed with human milk at the postnatal age of four weeks and have been adapted from de Oliveira et al. (2016) as described in Table 13. The main change was the half-time of gastric emptying ($t_{1/2}$) of 78 min for an IF (Bourlieu et al. 2014). The *in vitro* dynamic system was controlled by the STORM® software, which allows regulating and monitoring the digestive parameters. No gastric lipase was used since the objective of the present study was focused on protein digestion.

Table 13. Gastrointestinal conditions for *in vitro* dynamic digestions. (Adapted from de Oliveira et al. 2016).

Gastric conditions (37°C)		
SGF (stock solution adjusted at pH 6.5)	Na ⁺	94 mmol/L
	K ⁺	13.2 mmol/L
	Cl ⁻	122 mmol/L
Fasted state / initial conditions	SGF	2 mL
	pH	3.1
Infant formula ingested	Total volume	100 mL
	Flow rate	10 mL/min from 0 to 10 min
Gastric pH (acidification curve)	pH = -0.0155*t + infant formula pH with t : time after ingestion in min	
SGF + enzymes (pepsin)	Pepsin	268 U/mL
	Flow rate	1 mL/min from 0 to 10 min
		0.5 mL/min from 10 to 180 min
Gastric emptying (Elashoff, 1982 ; Ewer, 1994)	$t_{1/2}$	78 min
	β	1.2
Intestinal conditions (37°C)		
SIF (stock solution adjusted at pH 6.2)	Na ⁺	164 mmol/L
	K ⁺	10 mmol/L
	Ca ²⁺	3 mmol/L
Intestinal pH	6.2	
SIF + bile	Bile salts	3.1 mmol/L
	Flow rate	0.5 mL/min from 0 to 180 min
SIF + pancreatic	Pancreatic lipase	90 U/mL
	Flow rate	0.25 mL/min from 1 to 180 min
Intestinal emptying (Elashoff fitting)	$t_{1/2}$	200 min
	β	2.2

Digestion experiments were performed over three hours and in triplicate for each IF. Samples were collected from the IFs before digestion (time 0 min) and in both compartments at 30, 60, 90, 120 and 180 min after the beginning of the digestion. Digesta emptied from the intestinal compartment was collected on ice over the three hours of digestion. Structural analyses by confocal microscopy and laser light scattering were performed immediately after sampling. The samples taken for proteolysis analysis were immediately mixed with protease inhibitors, namely 10 μ L of 0.72 mM Pepstatin A per mL gastric digesta or 50 μ L of 0.1 M Pefabloc per mL intestinal digesta, and then frozen at -20 °C. More details on

in vitro dynamic digestion model is available in Ménard et al. (2014) and has also been presented in the bibliography review section 1.3.3.2. The protocol described above is summarized in Figure 27.

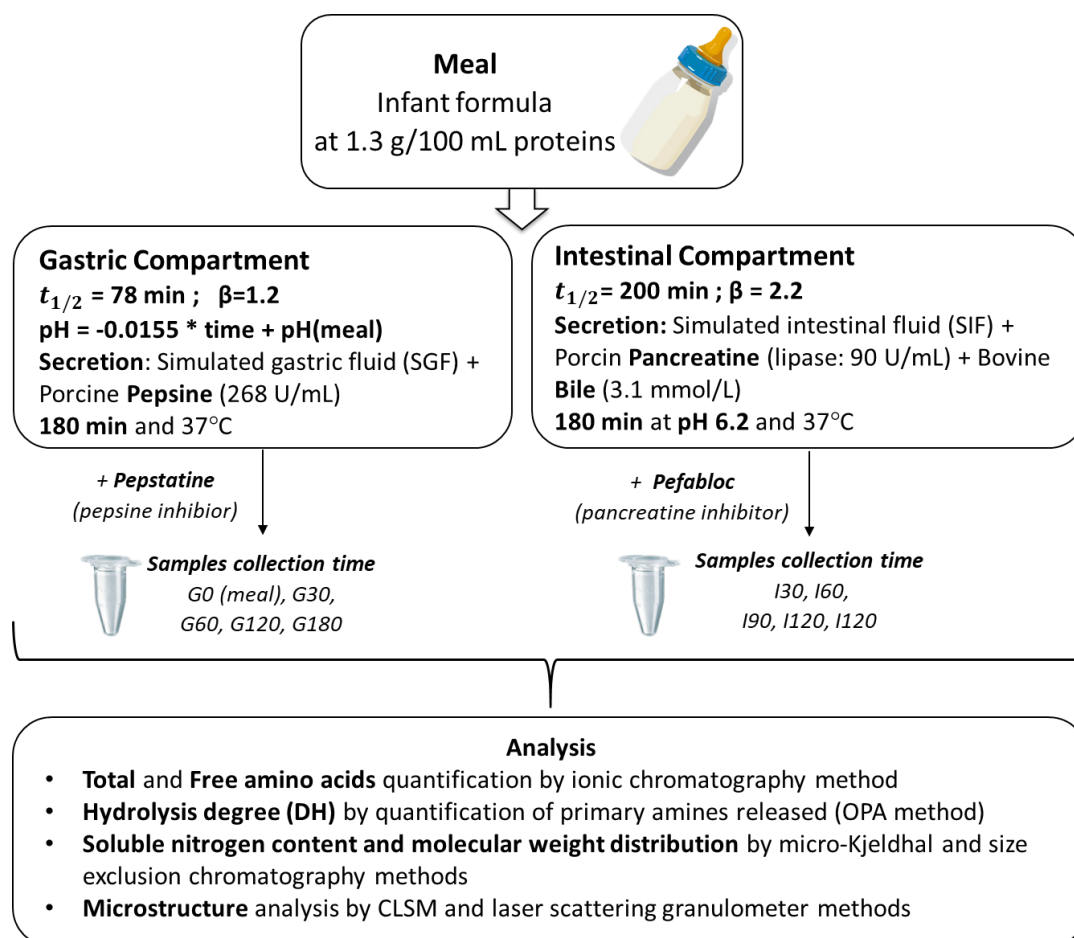


Figure 27. Illustration of *in vitro* dynamic simulating infant digestion protocol used in the present study.

(Adapted from Ménard et al. 2014).

3.7. Digested sample analysis

3.7.1. 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 10,000g and 4°C, using the o-phthaldialdehyde (OPA) method according to Darrouzet-Nardi, Ladd & Weintraub (2013). The absorbance was measured 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). All measurements were carried out in triplicate.

The DH was calculated as follows for *in vitro* static digestion:

$$\% \text{ DH} = 100 \times \left[\frac{\text{NH}_2(t) - \text{NH}_2(t_0)}{\text{NH}_2(\text{tot}) - \text{NH}_2(t_0)} \times F \right] \quad (6)$$

with $\text{NH}_{2(t)}$ the amount of primary amines after t min digestion expressed in mg NH_2 per L of digesta; $\text{NH}_{2(t0)}$ the amount of initial primary amines before digestion (meal + secretions) expressed in mg NH_2 per L of meal diluted with the gastric secretions; $\text{NH}_{2(\text{tot})}$ the maximum amount of primary amines (after total acid hydrolysis of the meal); F the dilution factor to express $\text{NH}_{2(\text{tot})}$ in mg NH_2 per L of digesta (F value depends on gastric or intestinal phase).

The DH was calculated as follows for *in vitro* dynamic digestion:

$$\% \text{ DH} = 100 \times \left[\frac{\text{NH}_{2(t)} - \text{NH}_{2(t0)}}{\text{NH}_{2(\text{tot})} - \text{NH}_{2(t0)}} \right] \quad (7)$$

with $\text{NH}_{2(t)}$ the concentration of primary amines after t min digestion; $\text{NH}_{2(t0)}$ the concentration of primary amines in the IF before digestion; $\text{NH}_{2(\text{tot})}$ the concentration of the total primary amines measured after total acid hydrolysis (HCl 6 N, 110°C, 24 h) of the IF. All values were expressed as g per 100 g IF.

3.7.2. Amino acid analysis

3.7.2.1. Total AA

The total AA contents were determined after acid hydrolysis of each protein ingredients and IFs, according to (Davies & Thomas 1973). Acid hydrolysis of IF powder (20 mg) was performed by adding 2 mL of 6 N HCl and heating at 110 °C for 24 h in vacuum sealed glass tubes. The sulfuric AA (cysteine and methionine) were measured as methionine sulphone and cysteic acid after performic acid oxidation. The determination of tryptophan was not possible using ionic chromatography, due to its degradation following acid hydrolysis. Total AA content of each IF was determined in duplicate.

3.7.2.2. Free AA (or AA bioaccessibility)

The free AA contents were determined after deproteinization of the samples according to the method of Mondino, Bongiovanni, Fumero, & Rossi (1972). To this end, sulfosalicylic acid was added to digesta (0.05 g/mL), followed by incubation for 1 h at 4 °C and then centrifugation at 5000g for 15 min at 4 °C. The supernatants were filtered through a 0.45 µm pore-size membrane (Sartorius, Palaiseau, France) and diluted five times with a 0.2 mol/L lithium citrate buffer (pH 2.2) before injection. Free AA content was determined once for each digestion experiment, i.e. in triplicate for each IF.

The AA analysis (both total AA and free AA) was carried out by a cation exchange chromatography on a Biochrom30 automatic AA Analyser (Biochrom Ltd., Cambridge, G.B.) 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 et al. (1958). The quantity of AA released during digestion was expressed as the percentage of free AA (expressed in g / 100 g IF) related to the total AA (g / 100 g IF).

3.7.3. Soluble nitrogen content and molecular weight distribution

IFs, intestinal digesta in the intestinal compartment at 3 h of digestion or emptied from the intestinal compartment over 3 h were analyzed for total N and soluble N (micro-Kjeldahl method) after the removal of insoluble particles by a 20 min centrifugation at 10,000g and 4°C. Molecular weight distributions of

the resulting soluble fractions were determined by size exclusion chromatography (SEC), using a Biosep-SEC-2000 Phenomenex column connected to a Waters e2695 separation module equipped with a Waters e2489 UV/Visible detector (Waters Inc., Milford, USA). Samples were eluted at 40°C under isocratic 0.8 mL/min flow of 50 mM phosphate buffer pH 7 containing 0.2 M NaCl. Detection was performed at 214 nm. The column was previously calibrated by injection of eight molecular weight markers: bovine thyroglobulin (670 kDa), IgA (300 kDa) and IgG (150 kDa) from human gamma globulin, ovalbumin (44 kDa), myoglobin (17 kDa), cytochrome C from horse heart (12 kDa) and human angiotensin II (1.05 kDa). The calibration curve (Log molecular weight vs retention time) allowed to determine the retention times defining the limits of each molecular weight range: > 10 kDa, 10-5 kDa, 5-2 kDa, 2-1 kDa and 1-0.2 kDa. The proportion of soluble proteins and peptides in a given molecular weight range was determined as the percentage of area under the curve between the respective limits (% Area SEC). Analysis was carried out in duplicate for each sample.

The soluble N fraction corresponds to the nitrogen contained in the proteins, peptides and free AA of the soluble fraction. For each molecular size range, and because free amino acids are supposed to be undetectable at 214 nm, the proportion of soluble N in this range (% N SEC) was calculated as follows:

$$\% \text{ N SEC (x kDa)} = \left[\frac{\text{Total soluble N (Digesta)} - \text{Soluble N (Free AA)}}{\text{Total N (IF)}} \right] \times \% \text{ Area SEC (x kDa)} \quad (8)$$

Total soluble N (Digesta) corresponded to the quantity of soluble N (mg) in the digesta at the end of digestion in the intestinal part (both intestinal compartment and intestinal emptied fraction). Total N (IF) corresponded to the total N in the IF (mg). Soluble N (Free AA) was calculated as the quantity of soluble N corresponding to free AAs (mg).

3.7.4. *In vitro* protein digestibility and PDCAAS-like score

***In vitro* apparent protein digestibility** was determined based on the soluble N lower than 10 kDa, i.e. as measured in the peptides by SEC and cumulated to the free AA N. It was determined in the intestinal compartment at 180 min and in the intestinal fraction emptied over 180 min. In both compartments, it was calculated as follows:

$$\text{In vitro Apparent Protein Digestibility (\%)} = \left[\frac{(\sum [\text{N SEC (<10 kDa)}] + \text{Soluble N (Free AA)})}{(\text{N}_{\text{IF}} + \text{N}_{\text{Secretions}}) \times \% \text{ substrate}} \right] \times 100 \quad (9)$$

where N_{SEC} and Soluble N (Free AA) (expressed as mg / kg digesta) was the soluble N content in the intestinal compartment or in the intestinal emptied fraction; %substrate was the percentage of the IF initially introduced in the digester that was present in the intestinal compartment or in the intestinal emptied fraction (g IF / 100 g digesta), estimated using the emptying equation and the known flows in the digester; N_{IF} (expressed as mg / kg IF) was the total N content of the meal introduced in the digester; $\text{N}_{\text{Secretions}}$ (expressed as mg / kg IF) was the total nitrogen content of the simulated bile secretion and pancreatin solution. Both digestibility values (intestinal compartment and emptied fraction) were averaged after weighting each value according to the substrate repartition in these two fractions.

PDCAAS-like score (protein digestibility-corrected amino acid score) was calculated by adapting the FAO/WHO (1991) in which the true fecal protein digestibility (normally determined in growing rats for PDCAAS calculation) was replaced by the *in vitro* apparent protein digestibility calculated as described above and considering the most limiting EAA score (LEAA). The equation was then as follows:

$$\text{PDCAAS-like score} = \text{Amino acid score (LEAA)} \times \text{In vitro Apparent Protein Digestibility} \quad (10)$$

$$\text{Amino acid score} = \frac{\text{AA content of the infant formula}}{\text{AA content of the reference pattern}} \quad (11)$$

As the present study focused on infants, the reference AA pattern used was that recommended for 0 to 6 months infants (FAO, 2013).

3.8. Statistical analysis

Statistical analyses were conducted with the use of R version 3.5.2 (The R Foundation 2014). The residues of the linear model with meal and digestion time (and their interaction) as factors were not normal for the kinetics of hydrolysis (DH) and the kinetics of EAA release, using the Kolmogorov-Smirnov test ("lillie.test" from the "nortest" package) (Fernandez 1992). Therefore, a non-parametric analysis for repeated measurements was conducted for these two variables, taking the type of meal and the digestion time (and their interaction) into account, using the "f1.lid.f1" function of the package "nparLD" (Noguchi et al. 2012). In the event of a significant treatment effect, the function "npar.t.test" or "nparcomp" of the R package "nparcomp" (Konietschke et al. 2012) was systematically used. In the event of a significant interaction effect, a linear mixed effect model with a random intercept on experiments to take into account the repeated measurements was performed and followed by the "diffsmeans" of the "lmerTest" package (Kuznetsova et al. 2017).

Regarding the nitrogen size distribution and the *in vitro* apparent digestibility, as well as the PDCAAS-like score, a one-way ANOVA ("anova.lme" function from the "nlme" package) was conducted with meal as 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 ($p < 0.05$). All results were expressed as mean \pm SD.

4. Results & Discussion

The main results of the present study are reported hereafter through publications submitted or in project for submission to peer-reviewed journals (except for the first result chapter). For each result chapter, the *Introduction* and *Materials & Methods* sections have been removed in order to avoid repetitions; the *Materials & Methods* sections have been gathered and moved to the previous section 3.

The first chapter focuses on the selection of the alternative protein sources mainly on a nutritional point of view. The second chapter handles the physicochemical properties and the *in vitro* static digestibility of model IFs manufactured at a pilot scale. The third chapter deals with the scale-up of the model IFs manufacturing and the influence of protein source on physicochemical and microstructure properties of IFs. Finally, the fourth chapter focuses on the nutritional quality of these later IFs, investigated in more physiological conditions using an *in vitro* dynamic model.

Chapter 1. Alternative proteins : which ones should be selected ?

IFs are the key nutritional source for infants who cannot be breastfed. The current protein sources allowed in the applicable European regulation are either cow milk protein, goat milk protein, soy protein isolate or hydrolysed rice proteins. For sustainability reasons, alternative to animal proteins in food have to be considered. Plant proteins offer interesting nutritional and functional benefits for the development of innovative IFs. Moreover, several research groups and industrials become more and more interested in new fields of innovations in infant nutrition.

Therefore, in this chapter, the aim will be to identify **which alternative protein sources could be nutritionally adapted to replace the whey protein concentrate usually added to skimmed cow milk and representing 50% of the total protein in 1st age IFs.**

Since the first objective of the project was to select new protein sources based on nutritional criteria regarding the applicable European regulation (EU 2016), the strategy involved first comparing several plant protein sources with the targeted requirements stated in the specification list (Table 14). Then, from literature data, several protein sources have been analyzed firstly on their nutritional properties, but also on their organoleptic characteristics as well as the presence of allergens and antinutritional factors. Finally, only protein sources that were available in purified form (concentrate or isolate) have been selected for this study. Secondly, in order to characterize the digestibility of these new protein sources alone, *in vitro* digestion in static conditions simulating infant digestion have been conducted. The results obtained were compared to that obtained for the reference whey protein.

4.1.1. Specifications for alternative protein sources selection

The different specifications used to select the alternative protein sources for partially replace whey protein in 1st age IF are listed in Table 14.

Table 14. Specifications for the choice of alternative protein sources to replace whey protein concentrate in 1st age infant formula.

Nutritional criteria	Organoleptic criteria	Availability
Essential amino acid profile should cover infant nutritional needs*.	Without organoleptic defects (flavor, color).	Should be commercially available in a purified form (concentrate or isolate).
Good digestibility (ideally > 70%).		
No allergens.		
No antinutritional factors (ANF).		
Should be innovative protein sources compared to the protein already allowed by the regulation (EU 2016) Φ.		

*up to 50% of the total protein content compared to the recommendations for a 1st age infant formula (EU 2016).
 Φ According to the applicable European regulation, the sources of proteins allowed for 1st age IFs are either cow milk protein, goat milk protein, soy protein isolate or hydrolysed rice protein (EU 2016).

4.1.2. Nutritional criteria: EAA profile and digestibility

As already mentioned, the aim of the study was to replace the whey protein concentrate fraction usually supplemented in standard cow milk 1st age IF, considering that this whey protein fraction represents 50% of the total protein content in IFs.

Table 15 shows the EAA profile for a standard 1st age cow milk based IF produced at a semi-industrial scale for the project (Table 15, column A). Knowing that 50% of the protein in the present IF comes from skimmed cow milk (Table 15, column B), the deficiency to cover the other 50% of protein (Table 15, column C) was calculated as followed: $C = A - (0.5 * B)$ (12)

Table 15. Essential amino acid profiles (mg / g of protein) of a standard 1st age (0-6 months) IF produced at a semi-industrial scale in this study, skimmed cow milk ingredient (used to prepare the IF) and the calculated deficiency to cover 50 % of the protein in the infant formula.

	A	B	C
	Standard 1 st age infant formula developed for the study	Skimmed cow milk	Deficiency
	mg / g of protein		
Lys	91	77	53
AAA	90	93	34
Leu	107	92	62
Ile	57	45	35
SAA	50	32	44
Val	62	53	36
His	24	23	12
Thr	72	40	52
Trp	18*	16*	10
References	Data are from intern AA analysis *EU (2016)	Data are from intern AA analysis *Derbry (2001)	Theoretical calculation

AAA: aromatic amino acids; SAA: sulfuric amino acids.

Then, the **EAA profile determined for the deficiency** to cover 50% of the protein to substitute in the IFs will be compared to the EAA profiles of different protein sources from literature data (Table 16).

Table 16. EAA profile (mg / g of protein) and protein digestibility value of different plant protein sources based on literature data compared to the deficiency to cover 50% of the total protein content in a standard 1st age IF. (WPC: whey protein concentrate).

Protein	Protein family	Ly s	AAA	Leu	Ile	SAA	Val	His	Thr	Trp	Protein Digestibility	Reference (AA profile)	Reference (protein digestibility)*
Deficiency	Animal	53	34	62	35	44	36	12	52	10		Determined from AA analysis (Table 15)	
Cow milk	Animal	77	93	92	45	32	53	23	40	16	95	Plouvien (supplier) ; AA analysis	TFD (Bos et al. 2003; Gaudichon et al. 2002)
WPC	Animal	78	54	93	49	37	48	17	54	16	98	Mathai et al. (2017)	TID (Mathai et al. 2017)
Oat	Cereal	29	83	67	34	34	46	19	25	11	86	Cervantes-Pahm et al. (2014)	TID (FAO, 1991)
Wheat	Cereal	11	61	50	20	14	23	14	18	7	91.5	Millward (1999)	TID (Bos et al. 2005)
Barley	Cereal	51	107	96	51	52	69	33	44	13	61.5	Cervantes-Pahm et al. (2014)	AID (Cervantes-Pahm et al. 2014)
Rye	Cereal	50	86	81	46	47	62	32	40	11	57.2	Cervantes-Pahm et al. (2014)	AID (Cervantes-Pahm et al. 2014)
Corn	Cereal	26	55	84	26	30	35	23	24	4	87	FAO (1991)	TFD (Hopkins 1981)
Rice	Cereal	56	85	75	39	34	56	29	38	14	94.8	Amagliani et al. (2017)	TFD (Amagliani et al. 2017)
Quinoa	P-cereal	52	77	69	39	31	47	23	42	35	91.6	Abugoch et al. (2008)	TID (Ruales & Nair 1992)
Rapeseed	Oilseeds	57	70	72	34	49	43	28	48	30	84	Bos et al. (2005)	TFD (Bos et al. 2007)
Sunflower	Oilseeds	45	88	80	55	46	63	30	45	17	90	FAO (1991)	TID (Gilani et al. 2011)
Faba bean	Legumes	60	72	66	37	20	41	26	37	13	86	Koivunen et al. (2016)	TID (Gilani et al. 2011)
Lentil	Legumes	74	80	75	35	37	48	55	49	11	85	Seidu et al. (2015)	TFD (Sarwar & Peace 1986)
Lupin	Legumes	51	108	81	46	30	37	26	41	10	90	Ciquel table	TFD (Mariotti et al. 2002)
Pea	Legumes	71	128	82	47	19	46	25	35	10	90.3	Overduin et al. (2015)	TID (Gausserès et al. 1997; Mariotti et al. 2001)
Soya bean	Legumes	61	82	74	44	27	46	25	40	13	91.5	FAO (1991)	TID (Bos et al., 2003 ; Gaudichon et al. 2002)
Potato	Tubers	67	110	97	39	32	44	18	66	10	94.8	He et al. (2013)	TFD (Jørgensen et al. 2008)
Chlorella	Microalgae	84	84	88	38	36	84	20	48	21	76.6	Becker (2007)	DC Becker (2007)
Spirulina	Microalgae	48	106	98	37	35	71	22	62	13	77.6	Becker (2007)	DC Becker (2007)
Chia	Protein crop	37	67	56	37	27	46	22	29	26	78.9	Olivos-Lugo et al. (2010)	IVD (Sandoval-Oliveros & Paredes-López 2013)
Hemp	Cannabaceae	42	84	66	40	16	50	28	46	8	94.9	Wang et al. (2008)	TID (House et al. 2010)

*TID: true ileal digestibility; TFD: true fecal digestibility; AID: apparent ileal digestibility; IVD: *in vitro* digestibility; DC: digestibility coefficient.

P-cereal: pseudo-cereal.

Secondly, the relevance of a given protein in covering the deficiency was estimated by calculating the **EAA recovery ratio** (Materials & Methods, section 3.4, Equation (4)) between the EAA profile (mg/g protein) of the different protein sources presented in Table 16 and the EAA profile (mg / g protein) of the deficiency. These ratios are presented in Table 17.

Table 17. EAA recovery ratio of the different plant protein sources compared to the deficiency to cover 50 % of the protein in a standard 1st age IF. (WPC: whey protein concentrate).

Protein	Protein family	Lys	AAA	Leu	Ile	SAA	Val	His	Thr	Trp
Cow milk	Animal	1.45	2.11	1.51	1.29	0.94	1.47	1.88	0.77	1.57
WPC	Animal	1.46	1.23	1.52	1.40	1.08	1.33	1.39	1.04	1.57
Oat	Cereal	0.54	1.88	1.09	0.98	0.99	1.27	1.57	0.48	1.06
Wheat	Cereal	0.21	1.39	0.82	0.57	0.41	0.64	1.14	0.35	0.69
Barley	Cereal	0.96	2.43	1.57	1.46	1.54	1.93	2.68	0.85	1.29
Rye	Cereal	0.93	1.95	1.33	1.32	1.39	1.71	2.58	0.77	1.07
Corn	Cereal	0.48	1.26	1.38	0.76	0.88	0.98	1.87	0.46	0.43
Rice	Cereal	1.05	1.93	1.23	1.12	1.00	1.55	2.37	0.73	1.38
Quinoa	P-cereal	0.98	1.75	1.13	1.12	0.91	1.30	1.88	0.81	3.44
Rapeseed	Oilseeds	1.07	1.59	1.18	0.97	1.44	1.19	2.29	0.92	2.95
Sunflower	Oilseeds	0.85	2.01	1.31	1.57	1.34	1.76	2.49	0.86	1.64
Faba bean	Legumes	1.13	1.64	1.08	1.06	0.59	1.14	2.13	0.71	1.28
Lentil	Legumes	1.39	1.82	1.23	1.00	1.09	1.33	4.50	0.94	1.08
Lupin	Legumes	0.96	2.45	1.33	1.32	0.88	1.03	2.13	0.79	0.98
Pea	Legumes	1.33	2.91	1.34	1.34	0.56	1.28	2.04	0.67	0.98
Soya bean	Legumes	1.14	1.87	1.22	1.27	0.79	1.27	2.01	0.76	1.31
Potato	Tubers	1.26	2.50	1.59	1.12	0.94	1.22	1.47	1.27	0.98
Chlorella	Microalgae	1.58	1.91	1.44	1.09	1.06	2.33	1.64	0.92	2.07
Spirulina	Microalgae	0.90	2.41	1.61	1.06	1.03	1.97	1.80	1.19	1.28
Chia	Protein crop	0.69	1.52	0.92	1.06	0.79	1.28	1.80	0.56	2.60
Hemp	Cannabaceae	0.79	1.91	1.08	1.14	0.46	1.39	2.29	0.88	0.79

In red: ratios <1.0; in black: ratios >1.0.

P-cereal: pseudo-cereal.

When **EAA recovery ratio** is below 1.0 means that the EAA is limiting regarding the objective of covering the deficiency. Table 17 highlights that all plant protein sources considered had one or more limiting EAA with respect to the objective. In particular proteins from cereal sources show very low scores for several EAA. Only the animal protein WPC presents a balanced EAA profile compared to the deficiency. However, rice, lentil, chlorella and spirulina showed only one limiting EAA.

Lastly, for each protein source, the **lowest EAA recovery ratio** was multiplied by the **protein digestibility** reported in literature for the different protein sources (Table 16) in order to calculate a **PDCAAS-like score** (Materials & Methods, section 3.4, Equation (5)). The resulting **PDCAAS-like scores** are presented in Figure 28. Plant proteins cover 19 to 87 % of the deficiency, consistently with the previously mentioned EAA limitations for the majority of the plant protein sources. It is the reason why these proteins have to be provided in higher amount in order to meet the nutritional recommendations, or need to be supplemented in some EAAs or with other protein sources (Young & Pellet, 1994). Moreover, infant nutritional requirements are higher than the one for older children or adults (Table 3, section 1.1.2.), that is why the theoretical **PDCAAS-like scores** calculated here are quite low. Moreover, it should be noted that the protein digestibility data reported from the literature and

used for the calculation have been determined with animal models in adults, and thus may be overestimated compared to infant conditions.

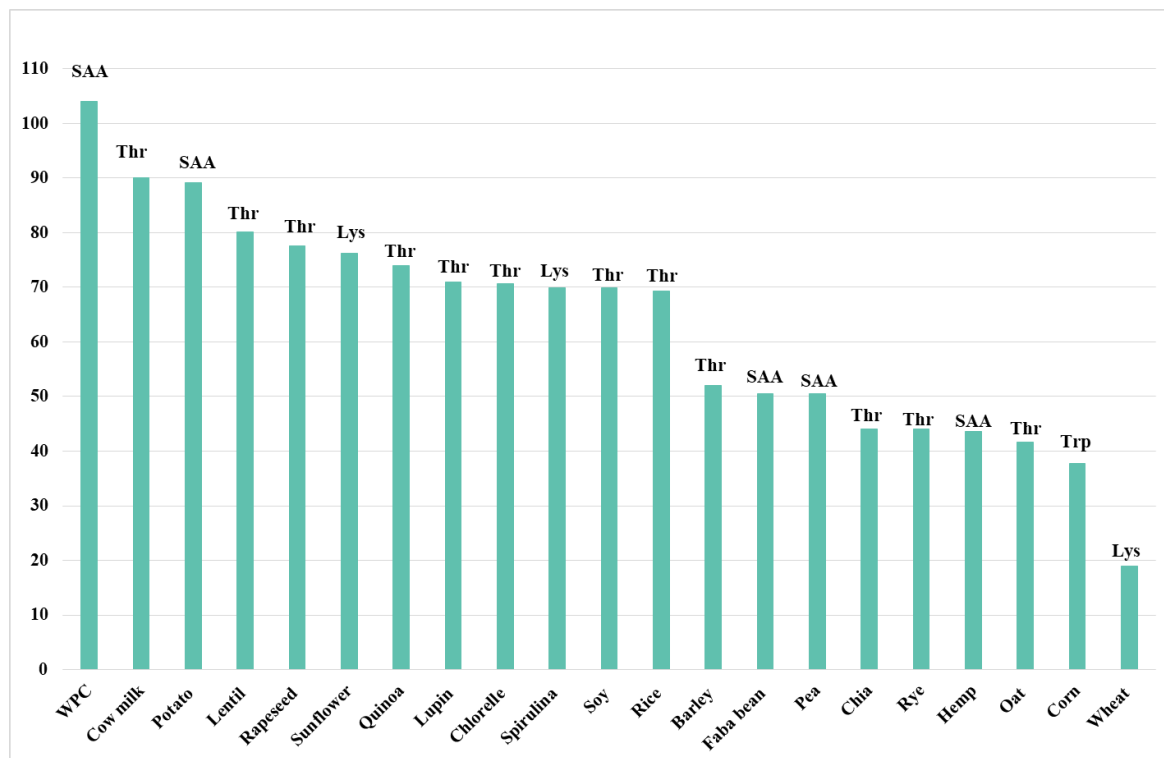


Figure 28. PDCAAS-like score (%) of the different protein sources compared to the reference pattern of the deficiency to cover 50 % of the protein in a 1st age IF (deficiency). Amino acid above bars corresponded to the limiting EAA. (WPC: whey protein concentrate; SAA: sulfuric AA).

In order to compare more precisely the ability of plant protein sources to cover the deficiency in IF, **EAA recovery ratios** were determined as previously, but considering this time, the EAA reference pattern of 1.6 g protein for 100 mL IF (compared to 1 g previously). It was chosen to provide 1.6 g protein for 100 mL IF to meet the nutritional requirements for 1st age infant formula (EU 2016) made up with cow milk proteins or soy proteins (Table 18). Thus, each plant protein source (presented in Table 16) was used to calculate the **EAA profile of a simulated IF** providing 1.6 g of protein/ 100 mL, with 50 % of the protein coming from skimmed cow milk and 50 % from each alternative protein. As previously, the **EAA recovery ratios** was calculated dividing the EAA profile of the plant based IFs compared to the EAA profile of the reference cow milk based IF (Table 19). The corresponding **PDCAAS-like scores** were calculated by multiplying the EAA recovery ratios with the protein digestibility (taking into account 50% of the plant protein digestibility and 50 % of the cow milk protein digestibility). These last results are presented in Figure 29.

Table 18. European regulation for protein requirements for 1st age infant formula based on cow milk or goat milk proteins and soy protein isolate.

European regulation for 1 st age infant formula (EU 2016)						
kcal / 100 mL			Cow milk or goat milk proteins		Soy protein isolate	
min	max		min	max	min	max
60	70	g / 100 kcal	1.8	2.5	2.3	2.8
		g / 100 mL	1.1	1.8	1.4	2.0

Table 19. EAA recovery ratio of the simulated plant based IFs compared to a standard 1st age IF, all containing 1.6 g of protein / 100 mL.

Infant formula	Protein family	Lys	AAA	Leu	Ile	SAA	Val	His	Thr	Trp
Cow milk IF	Animal	1.36	1.31	1.38	1.32	1.10	1.30	1.33	1.04	1.42
Oat IF	Cereal	0.93	1.56	1.19	1.11	1.05	1.27	1.41	0.72	1.19
Wheat IF	Cereal	0.77	1.37	1.06	0.91	0.74	0.98	1.23	0.64	1.02
Barley IF	Cereal	1.13	1.78	1.40	1.35	1.35	1.58	1.86	0.93	1.29
Rye IF	Cereal	1.11	1.59	1.29	1.28	1.27	1.48	1.82	0.89	1.20
Corn IF	Cereal	0.90	1.32	1.32	1.00	0.99	1.14	1.53	0.71	0.91
Rice IF	Cereal	1.17	1.58	1.25	1.18	1.06	1.41	1.73	0.87	1.33
Quinoa IF	P-cereal	1.13	1.51	1.20	1.18	1.01	1.29	1.53	0.91	2.27
Rapeseed IF	Oilseeds	1.18	1.45	1.23	1.11	1.30	1.24	1.70	0.98	2.04
Sunflower IF	Oilseeds	1.07	1.61	1.28	1.40	1.24	1.50	1.78	0.94	1.45
Faba bean IF	Legumes	1.20	1.47	1.18	1.15	0.83	1.21	1.63	0.86	1.29
Lentil IF	Legumes	1.33	1.54	1.25	1.12	1.10	1.30	2.60	0.99	1.20
Lupin IF	Legumes	1.13	1.79	1.29	1.28	0.99	1.16	1.63	0.90	1.16
Pea IF	Legumes	1.30	1.96	1.30	1.29	0.82	1.28	1.60	0.83	1.16
Soya bean IF	Legumes	1.21	1.56	1.24	1.25	0.94	1.27	1.59	0.89	1.30
Potato IF	Tubers	1.27	1.80	1.41	1.18	1.02	1.25	1.37	1.18	1.16
Chlorella IF	Microalgae	1.42	1.57	1.35	1.16	1.09	1.77	1.43	0.98	1.64
Spirulina IF	Microalgae	1.10	1.77	1.42	1.15	1.07	1.60	1.50	1.13	1.29
Chia IF	Protein crop	1.00	1.42	1.11	1.15	0.94	1.28	1.50	0.77	1.88
Hemp IF	Cannabaceae	1.05	1.57	1.18	1.19	0.76	1.33	1.70	0.96	1.07

In red: ratios <1.0; in black: ratios >1.0. P-cereal: pseudo-cereal.

Compared to the previous results (Table 17), when providing 1.6 g of protein / 100 mL of IF, the EAA recovery ratios suggest less limiting EAAs. However, only potato IF and spirulina IF show a whole balanced EAA profile compared to the reference cow milk based IF, even if all the EAA recovery ratios were relatively high (range between 0.64 to 0.99) compared to previously (0.21 to 0.98). As expected, the corresponding **PDCAAS-like scores** (Figure 29) are higher than previously (Figure 28), meaning that when proteins are provided in higher amount, the nutritional requirements targeted can almost be covered.

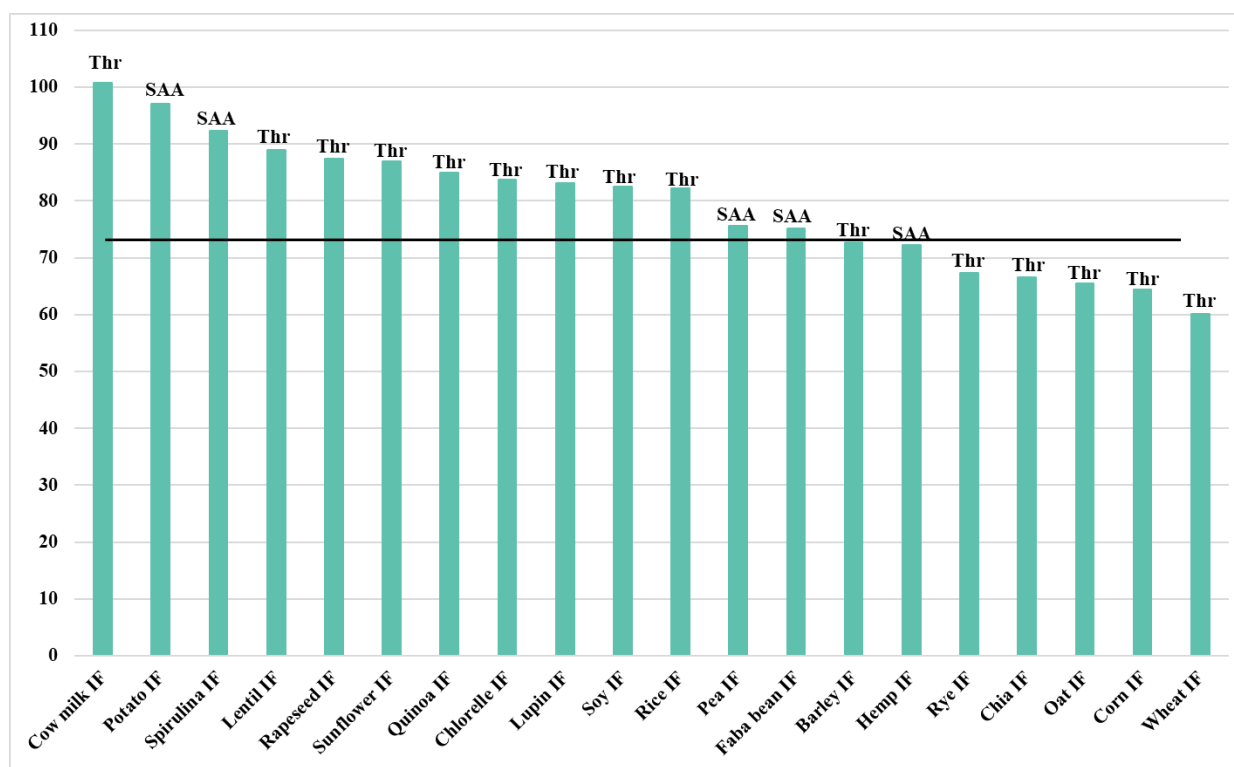


Figure 29. PDCAAS-like score (%) of the different plant protein based IFs compared to a reference pattern of a 1st age cow milk based IF with 1.6 g of protein/100mL. Amino acid above bars correspond to the limiting amino acid.

In overall, from the 19 plant protein sources studied, **the majority presented one or more limiting EAA compared to both the deficiency to cover 50 % of the protein in IF and the standard cow milk based IF (with 1.6 g of protein/ 100 mL)**. However, it should be kept in mind that all these theoretical calculations were carried out using literature data. Therefore, the EAA profiles of the different protein sources that would be promising for the study had to be experimentally checked prior selection to guarantee their nutritional balance compared to the infant nutritional requirements.

Finally, it was then decided that the **protein sources with a PDCAAS-like score lower than 70 % were excluded (i.e., rye, chia, oat, corn and wheat)**. The other protein sources were analyzed on the other nutritional and organoleptic criteria as mentioned in the specifications (Table 14).

4.1.3. Allergenicity, antinutritional factors and organoleptic criteria

Table 20 reminds the protein digestibility and PDCAAS-like score (based on a 1st age IF at 1.6 g protein/100 mL) of the 14 protein sources previously selected (PDCAAS-like score > 70 %). However, along with these protein sources, 12 are deemed to contain one or more anti-nutritional factors (ANF). Besides, soy bean as well as barley and lupin contain allergens. Hemp, as well as spirulina show organoleptic defaults (green color and grassy flavor for hemp ; blue color and iodine taste for spirulina).

Table 20. Protein digestibility, PDCAAS-like scores, organoleptic defects, allergens and antinutritional factors of different protein sources.

Protein (scientific name)	Protein digestibility ^a	PDCAAS- like score ^b (LEAA)	Organoleptic defaults	Allergen ¹	ANF ²	References
Barley (<i>Hordeum sativum</i>)	61,5	73 (Thr)	NO	YES	Phytic acid (11.9 g/kg product); Tannins content (5.5-12.3 g/kg product)	¹ food.gouv.uk ² (Belitz et al. 2009; Jansman 1993)
Rice (<i>Oriza sativa</i> L.)	94,8	82 (Thr)	NO	NO	Phytic acid (3-55 g/kg product)	² (Harland & Narula 1999; Ravindran et al. 1999)
Quinoa (<i>Chenopodium quinoa</i>)	91,6	85 (Thr)	NO	NO	Phytic acid (10 g/kg product); Saponin (3-21 g/kg product); Tannins (5 g/kg)	² (Ahamed et al. 1998; Belitz et al. 2009)
Rapeseed (<i>Brassica napus</i>)	84	88 (Thr)	NO	NO	Phytic acid (27 g/kg product); Glucosinolates (5-21 µmol/g oil free DM)	² (Ravindran et al. 1999)
Sunflower (<i>Helianthus</i>)	90	87 (Thr)	NO	NO	Phytic acid (27 g/kg product)	² (Ravindran et al. 1999)
Faba bean (<i>Vicia faba</i>)	86	75 (SAA)	NO	NO	Tannins content (0.1-24.1 g/kg product)	² (Crépon et al. 2010; Jansman & Longstaff 1993)
Lentil (<i>Lens culinaris</i>)	85	89 (Thr)	NO	NO	Phytic acid (7 g/kg product)	² (Porres et al. 2002) Porres et al.
Lupin (<i>Lupinus albus</i>)	90	83 (Thr)	Bright yellow ; very bitter	YES	Phytic acid (8 g/kg product); Tannins (0,1-3,7 g/kg product)	¹ food.gouv.uk ² (Pettersson & Fairbrother 1996)
Pea (<i>Pisum Sativum</i>)	90,3	75 (SAA)	Slightly bitter	NO	Trypsin inhibitor activity (2.1-11.9 mg/g protein); Tannins (0.6-10.5 g/kg product)	² (Alajaji & El-Adawy 2006; Jansman & Longstaff 1993)
Soya bean (<i>Glycine max</i>)	91,5	89 (SAA)	NO	YES	Trypsin inhibitor activity (20.3-122.6 mg/g protein); Phytic acid (10-41 g/kg product); Saponin (4-6 g/kg product)	¹ food.gouv.uk ² (Belitz et al. 2009; Chitra et al. 1995; Harland & Narula 1999; Sarwar et al. 2012a)
Potato (<i>Solanum tuberosum</i>)	94,8	97 (SAA)	Light brown	NO	Proteases inhibitor (100-240 g/kg product)	² (Waglay et al. 2014)
Chlorella (<i>Chlorella vulgaris</i>)	76,6	84 (Thr)	NO	NO	NA	
Spirulina (<i>Spirulina platensis</i>)	77,6	92 (SAA)	Blue ; Iodine flavor	NO	NA	
Hemp (<i>Cannabis sativa</i>)	94,9	72 (SAA)	Green ; Strong grassy flavor	NO	Phytic acid (61-74 g/kg DM); Tannins (1.4-2.1 g/kg DM); Trypsin inhibitor (10.8-28.4 U/mg); Saponins (58-81 mg/kg DM)	² (Russo 2013)

^aprotein digestibility from literature data (Table 16).

^bPDCAAS-like scores based on a standard 1st age IF with 1.6 % of protein containing 50 % cow milk proteins and 50% coming from the different protein sources analyzed (Figure 29).

LEAA: limiting essential amino acid. ANF: antinutritional factor. NA: not available data.

Finally, from the protein sources nutritionally relevant to cover the EAA deficiency due to the replacement of 50 % of the total protein content in IF, it was decided to exclude protein sources that contain allergens (soya beans, barley and lupin), and those that show too much organoleptic defects (spirulina and hemp). However, almost all protein sources contain ANF.

ANF are chemical compounds synthesized in natural food and / or feedstuffs by the normal metabolism of species and by different mechanisms (for example inactivation of some nutrients, diminution of the digestive process or metabolic utilization of food/feed) which exerts effect contrary to optimum nutrition (Fekadu Gemede 2014; Kumar 1992). ANF may occur endogenously or may be formed during heat/alkaline processing of proteins. Examples of major naturally occurring **ANF include trypsin inhibitors and hemagglutinins in legumes, tannins in legumes and cereals, phytates in cereals and oilseeds, glucosinolates in mustard and canola protein products, gossypol in cottonseed protein products, and uricogenic nucleic acid bases in yeast protein products** (Arendt & Zannini 2013; Sarwar Gilani et al. 2005).

Different methods have been studied in order to inactivate or inhibit most of these ANF from the plant products. For instance, trypsin inhibitors in soya beans (about 17–27 mg/g sample or 35–123 mg/g protein) can be readily reduced by heat processing (Gatel 1994). Optimal heat processing can inactivate up to 80% of the trypsin inhibitor activity in commercial soya protein products used for human consumption such as protein concentrates and isolates, soya-based IFs and soya-based beverages (Liener 1994). Dehulling, soaking, boiling, blanching, autoclaving, roasting, steaming, extrusion, germination, fermentation, gamma irradiation, genetic manipulation and other processing methods have been relevant to reduce ANF contents in plant based products (Kalpanadevi & Mohan 2013; Le Gall et al. 2005; Rizzello et al. 2016; Soetan & Oyewole 2007; Xu & Chang 2008).

In overall, as the majority of ANF in plant protein sources can be inhibited or inactivated by mechanical, chemical or heat treatments as previously mentioned, it was decided to not take this criterion into account for the choice of the alternative protein sources (otherwise, all plant proteins studied should be excluded). Thus, **chlorella, faba bean, rapeseed, quinoa, potato, lentil, sunflower, pea and rice** were considered possible alternative protein sources to replace 50 % of the protein in a 1st age IF.

Finally, only five protein sources were commercially available in purified form and thus selected for this study:

- **Pea protein concentrate (PPC)**
- **Faba bean protein concentrate (FPC)**
- **Potato protein isolate (PPI)**
- **Rice protein concentrate (RPC)**

and **Whey protein concentrate (WPC)** for the reference standard cow milk 1st age IF.

4.1.4. Nutritional quality of the selected proteins in solution

In vitro static digestions simulating infant conditions were carried out with the previously selected protein sources in order to experimentally characterize their digestibility. These protein sources were dissolved in water at the protein concentration targeted in this study, i.e. 1.6 %. The total EAA content of the different protein sources were analysed. Then, the digestibility of the protein sources were investigated by measuring protein hydrolysis degree (DH) during digestion as well as EAA release (bioaccessibility) at the end of the digestion.

4.1.4.1. Total essential amino acids close to the targeted values

Table 21 presents the EAA profile for the different protein sources compared to the deficiency to cover 50 % of the total protein in IF previously presented (Table 15). In comparison to the theoretically determined deficiency, only WPC seemed to be entirely balanced. Then, PPC and FPC showed limitation for SAA and threonine, RPC for lysine and threonine and PPI for SAA only. However, PPI showed a relatively balanced EAA profile with a high content in almost all the EAA and comparable to the reference WPC. FPC showed the lowest amount of EAA almost for all EAA except lysine. These results were expected since it was previously demonstrated, based on literature data, that plant proteins alone do not meet the infant nutritional requirements. Hereafter the EAA profile of the whole IFs will be analysed and compared to the recommendations.

Table 21. Composition in total essential amino acids (EAA) of the five selected protein sources: WPC: whey protein concentrate; PPC: pea protein concentrate; FPC: faba bean protein concentrate; RPC: rice protein concentrate; PPI: potato protein isolate compared to the Deficiency to cover 50 % of the protein in IF. Data are means \pm SD (n=2). Values with a different superscript letter for each essential amino acid (EAA) are significantly different ($p < 0.05$).

	Deficiency*	WPC	PPC	FPC	RPC	PPI
Protein (g / 100 mL solution)			1.6 \pm 0.02			
Dry matter (g / 100 mL solution)			2.1 \pm 0.2			
		Total EAA mg / g protein				
Lys	53	115.9 \pm 4.4 ^a	76.1 \pm 2.1 ^c	60.3 \pm 1.0 ^d	32.9 \pm 1.0 ^e	87.5 \pm 0.7 ^b
AAA	34	83.8 \pm 2.5 ^d	93.9 \pm 3.0 ^c	70.2 \pm 2.8 ^e	110.6 \pm 1.7 ^b	135.1 \pm 4.3 ^a
Leu	62	141.0 \pm 3.8 ^a	84.6 \pm 2.5 ^c	69.5 \pm 1.8 ^d	85.3 \pm 1.6 ^c	116.4 \pm 2.7 ^b
Ile	35	66.0 \pm 1.7 ^a	48.4 \pm 0.8 ^c	38.9 \pm 0.7 ^d	45.2 \pm 0.4 ^c	60.8 \pm 1.2 ^b
SAA	44	59.9 \pm 2.5 ^a	14.1 \pm 3.1 ^c	21.4 \pm 1.8 ^b	54.7 \pm 2.1 ^a	26.4 \pm 1.5 ^b
Val	36	61.2 \pm 1.8 ^a	51.9 \pm 1.6 ^b	41.5 \pm 0.5 ^c	63.4 \pm 0.8 ^a	63.7 \pm 1.8 ^a
His	12	22.9 \pm 2.9 ^a	24.6 \pm 1.3 ^a	22.4 \pm 0.8 ^a	21.3 \pm 1.8 ^a	23.3 \pm 0.1 ^a
Thr	52	59.5 \pm 2.9 ^b	37.4 \pm 3.5 ^c	31.7 \pm 0.8 ^c	37.6 \pm 1.9 ^c	75.5 \pm 1.6 ^a

*deficiency theoretically determined (Table 15).

4.1.4.2. Pea and whey proteins in solution result in the highest proteolysis degree after *in vitro* static digestion

The kinetics of proteolysis was determined from the quantification of the free primary amines detected in the soluble fraction of the digested protein. It is expressed as a percentage/degree of hydrolysis (DH), defined as the proportion of cleaved peptide bonds in a protein (Adler-Nissen 1976). During the gastric phase of static *in vitro* digestion (the time 0 min on Figure 30 corresponded to the end of the gastric digestion), proteolysis was very limited (DH < 3%). It was expected since a reduced pepsin:protein ratio coupled with a high pH were used in order to mimic infant's stomach conditions (pH 5.3 vs pH 2 for pepsin optimal activity) (Agunod et al. 1969; Davidson & Lönnerdal 1987; Henderson et al. 2001; Johnson 2014).

As soon as the intestinal enzymes were added, proteolysis drastically increased for all protein solutions, except RPC (Figure 30). Then, proteolysis slightly increased reaching almost a plateau at 60 min with 22.2 ± 1.4 %, 14.0 ± 3.1 % and 16.0 ± 1.3 % for FPC, RPC and PPI, respectively, whereas proteolysis continued to increase until the end of the intestinal phase (120 min) for WPC and PPC. At the end of the intestinal digestion (120 min), DH ranged from 13.2 ± 2.7 % to 42.5 ± 1.2 % for PPC and PPI, respectively. During the entire intestinal phase, PPC showed a DH similar to the WPC and significantly higher than FPC, RPC and PPI. Proteolysis did not significantly differ ($p > 0.05$) between FPC, RPC and PPI at the end of the intestinal digestion. However, PPI showed significantly higher DH than RPC and similar to FPC during the first 5 minutes of intestinal digestion, but after that its DH value remained constant until the end of the digestion.

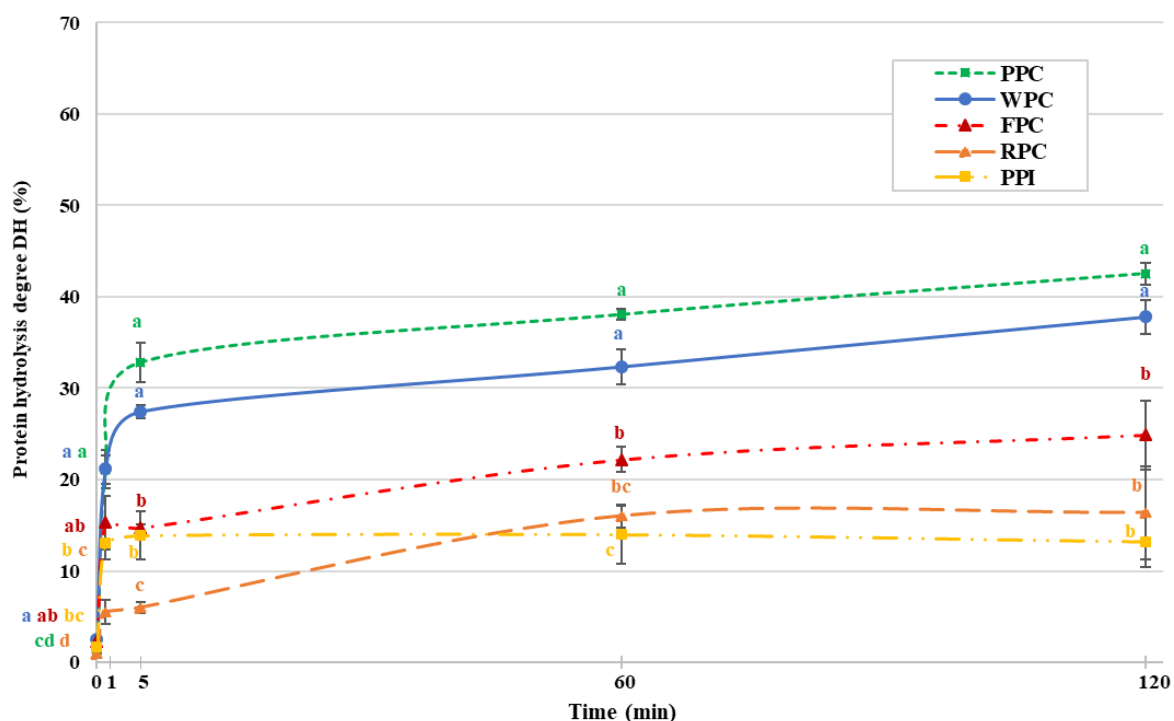


Figure 30. Degree of protein hydrolysis (DH) during the gastro-intestinal phase of *in vitro* static digestion of the five selected protein sources in solutions (WPC: whey protein concentrate; PPC: pea protein concentrate; FPC: faba bean protein concentrate; RPC: rice protein concentrate; PPI: potato protein isolate). Data are means \pm SD ($n=3$). Values with a different superscript letter for a given digestion time are significantly different ($p < 0.05$).

4.1.4.3. *In vitro* essential amino acid bioaccessibility is not really a discriminating criterion

The overall EAA bioaccessibility after *in vitro* static digestion (Table 22) was the highest for WPC, except for lysine and leucine. PPC was very close to the reference WPC, as well as FPC and RPC. However, PPI released extremely low amount of EAA, 5 to 20 times less than the other protein sources. It can be assumed that this protein source contain components that impaired its digestion. This hypothesis has been investigated in the next chapter 4.2.

Table 22. Bioaccessibility of essential amino acids determined at the end of the gastro-intestinal *in vitro* static digestion of the five selected protein sources in solution. WPC: whey protein concentrate; PPC: pea protein concentrate; FPC: faba bean protein concentrate; RPC: rice protein concentrate; PPI: potato protein isolate (% w/w of total EAA). Data are means \pm SD (n=3). Values with a different superscript letters for a given EAA and between the five IFs indicate a significant difference ($p < 0.05$).

	WPC	PPC	FPC	RPC	PPI
	% w/w of total EAA				
Lys	65.0 \pm 2.6 ^b	47.5 \pm 1.5 ^c	53.0 \pm 3.1 ^c	73.6 \pm 2.1 ^a	6.8 \pm 1.1 ^d
Tyr	77.5 \pm 8.7 ^a	45.7 \pm 1.0 ^{bc}	54.6 \pm 1.0 ^b	42.3 \pm 0.9 ^c	2.1 \pm 0.7 ^d
Phe	73.6 \pm 6.2 ^a	43.5 \pm 1.7 ^b	48.4 \pm 2.6 ^b	46.7 \pm 4.1 ^b	5.0 \pm 0.4 ^c
Leu	34.9 \pm 3.7 ^b	44.4 \pm 4.0 ^a	35.8 \pm 1.7 ^b	35.8 \pm 4.5 ^{ab}	7.6 \pm 0.3 ^c
Ile	28.8 \pm 6.7 ^a	28.9 \pm 2.3 ^a	18.2 \pm 0.7 ^b	28.1 \pm 2.8 ^a	4.5 \pm 0.2 ^c
Met	30.9 \pm 4.3 ^a	30.5 \pm 3.5 ^a	35.4 \pm 1.9 ^a	21.0 \pm 2.0 ^b	5.2 \pm 0.3 ^c
Val	25.5 \pm 0.9 ^a	25.5 \pm 2.2 ^a	18.1 \pm 0.5 ^b	25.6 \pm 2.9 ^a	3.9 \pm 0.3 ^c
His	28.5 \pm 3.8 ^a	19.9 \pm 1.1 ^b	11.1 \pm 2.3 ^c	30.2 \pm 2.6 ^a	5.4 \pm 0.4 ^d
Thr	13.2 \pm 3.9 ^{ab}	15.4 \pm 0.8 ^a	8.9 \pm 0.1 ^b	15.7 \pm 1.4 ^a	2.5 \pm 0.5 ^c

4.1.5. Conclusion

Protein sources from different origins have been investigated in this chapter: legumes, algae, oil seeds, cereals... Some of these protein sources were almost nutritionally balanced compared to the requirements for replacing 50 % of the total protein content in a 1st age IF containing 1.6% protein. However, protein from cereals sources showed very limited EAA recovery ratios and PDCAAS-like scores, thus were not adapted to meet the infant nutritional needs except for rice and barley. Then, the protein sources that did not contain allergens nor organoleptic defaults were considered good candidates to partially replace whey protein in IF. Finally, only five protein sources were commercially available in purified form and selected to this study: WPC (whey) as the reference protein, PPC (pea), FPC (faba bean), RPC (rice) and PPI (potato).

In order to characterize the nutritional quality of these plant protein sources, *in vitro* static digestions within simulating infant conditions were conducted. Although these five selected proteins showed EAA profiles not fully balanced compared to the requirements (deficiency), it should be noted that the EAA profiles of the plant proteins are not the EAA profiles of the full IFs that will be investigated in the next chapter. Then, protein hydrolysis was shown very low during gastric phase for all proteins, but higher proteolysis was obtained during intestinal phase, especially for WPC and PPC. Finally, the overall EAA bioaccessibility was a bit higher for WPC, but PPC, FPC and RPC showed very close results compared

to the reference WPC. However, PPI showed very poor EAA bioaccessibility assuming that a component in this protein might impair its digestion.

HIGHLIGHTS

The main findings of this chapter was that, although not fully adapted to cover infant nutritional requirements because of limitations in one or more EAA, several protein sources resulted in EAA profiles close to the requirements when provided in proper amount. When excluded the protein sources that showed allergens and organoleptic defects, the following protein were considered possible alternative to replace whey protein concentrate in 1st age IF: chlorella, faba bean, rapeseed, quinoa, potato, lentil, sunflower, pea and rice. Finally, out of these proteins, only four plant proteins were commercially available in purified form and selected for the project: **pea (PPC), faba bean (FPC), potato (PPI) and rice proteins (RPC); whey protein (WPC)** was selected as the reference for a standard dairy 1st age IF.

In the second part of this chapter, the digestibility of the previously selected protein sources was investigated using an *in vitro* static digestion model simulating infant conditions. It was concluded that **PPC was very close to the reference WPC in terms of protein hydrolysis and EAA release. Secondly, FPC and RPC also showed results relatively close to the reference. However, PPI showed lower protein hydrolysis and very few EAA release compared to the reference.**

In the next chapter, the five selected protein sources will be used to prepare IFs using spray drying process at a pilot scale. The functional properties of the resulting IFs powders will be characterized, as well as their digestibility during *in vitro* static digestion simulating infant conditions. Finally, the presence of ANF, namely trypsin inhibitor, will be evaluated in order to find a possible explanation of the poor digestibility of PPI previously highlighted.

Chapter 2 - *In vitro* digestion reveals how plant proteins modulate model IF digestibility

Infant formulas (IFs) are the key nutritional source for infants who cannot be breastfed. IFs are prepared to closely mimic the nutritional composition of human milk and is often based on cow milk proteins supplemented with whey protein concentrate (which represents 50 % of the total protein in 1st age IF). The other protein sources allowed in the applicable European regulation for 1st age IF are goat milk, soy, and hydrolysed rice proteins. However, other plant proteins offer interesting nutritional and functional benefits, and thus could be good candidates to replace whey protein concentrate in IFs.

In this chapter, the aim was to develop at a pilot scale model 1st age IFs using the previously selected plant protein sources, i.e. pea, faba bean, rice, potato to replace whey protein concentrate in a standard dairy 1st age IF. These innovative plant-based IFs have been analysed for their physicochemical properties after manufacturing. To assess their nutritional quality, an *in vitro* static gastrointestinal model simulating infant digestion was used. *In vitro* experiments have been preferred because of the ethical constraints relating to infant, or even animal studies. A static *in vitro* model was chosen since it is simpler and easier to use than dynamic models, while remaining convenient as screening technique to compare products. The IFs digestibility was assessed through proteins hydrolysis and EAA bioaccessibility was determined at the end of the digestion.

Results in this chapter will be presented through the following submitted publication (from which the *Introduction* was removed and *Materials & Methods* was moved to the section 3 of the present manuscript):

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

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HIGHLIGHTS

- Plant protein-based infant formulas (IFs) can be produced close to a dairy reference
- The protein source has an impact on the *in vitro* digestibility of IFs
- Pea and faba bean IFs showed *in vitro* digestibility close to a dairy reference
- Rice and potato IFs showed lower *in vitro* digestibility compared to a dairy reference

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

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 “dairy 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 dairy 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.

4.2.1. Results and discussion

The aim of this study was to assess the possibility of substituting whey protein concentrate usually added to the skimmed cow milk and representing 50% of the total proteins in model 1st age IFs with alternative plant protein sources. These proteins were selected on the basis of different criteria. First, the infant requirements for EAA had to be covered. Then, the alternative protein sources should not contain known allergens or organoleptic defects. Finally, they should be innovative protein sources not yet used according to the European regulation (EU 2016) and commercially available in a purified form. 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 dairy “reference IF”. The five 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.

4.2.1.1. 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 reference values of 26% fat whole milk powder (WMP) (as described in Schuck 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 23).

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 protein and fat contents were equal to 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 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 flavour (Nasirpour et al. 2006). The free fat content should normally remain below 5% for a 26% fat WMP (Vignolles et al. 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 et al. (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 and 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 (T_g) and the water activity (a_w) (Schuck et al., 2007). The mean water activity (a_w) reported for all IFs was 0.15 ± 0.03 , i.e., slightly lower than the optimal value of 0.2 as defined by Efstathiou, Feuadent, 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 (T_g) values of all the powders were not significantly different, with $49.4 \pm 2.1^\circ\text{C}$ as T_g mean inflexion value at 0.2 water activity, regardless of the protein source. For a regular WMP, T_g is usually in the range of $42 \pm 2^\circ\text{C}$ at 0.2 water activity (Schuck et al., 2012), i.e., slightly lower than the T_g values measured 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 (Schuck et al. 2007).

The dispersibility of the powders ranged between $85.4 \pm 0.6\%$ and $96.4 \pm 0.7\%$ for the potato and rice IFs, respectively. 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 obtained 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 and 6.8, and around 6.8 in the rehydrated IF powder, closer to neutral than acidic conditions, thus it can easily explain the solubility limitations observed for rice protein both during process and in the final product. 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 $89.5 \pm 2.2\%$ (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 the composition and the structure of powders but also on the environmental conditions experienced during process. Thus, the powder dissolution could be even enhanced through process parameters improvement such as speed and temperature of mixing (Jeantet et al. 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 (Schuck et al., 2005). For an optimal spray, the viscosity of the concentrate being dried for an IF should be around 60 mPa.s (Vestergaard, 2004) and should not exceed 200 mPa.s to allow subsequent spray drying. This means that the viscosity of potato IF was far too high for an optimal drying (more than 20 times than the recommendations). Therefore parameters should be adjusted to improve powder quality for this IF.

Table 23. Biochemical and physical composition of the five infant formulas (IFs). Data are means \pm SD (n=2 or 3). 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 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
α_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 ($^{\circ}\text{C}$)	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 index (SI, %)	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 index (DI, %)	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 ± 0.4^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

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 dairy reference IF 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.

4.2.1.2. 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 24). 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 (corresponding to 1.6 g/100 mL of IF in the present study), and were all in agreement with the European regulation (EU 2016) with significantly higher EAA content compared to the standard protein from the regulation. 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 1999), it would be therefore necessary to determine further its content in the innovative IFs designed in this study in order to confirm their agreement with the European regulation requirements (EU, 2016).

Table 24. Nutritional composition of the five infant formulas (IFs) compared to the European regulation. Data are means \pm SD (n=2). Values with a different superscript letter for each essential amino acid (EAA) are significantly different ($p < 0.05$).

	European regulation ²	Reference IF	Pea IF	Faba bean IF	Rice IF	Potato IF
Energy (kcal / 100 mL)	60-70			66.5 \pm 1.7		
Protein (g / 100 kcal)	1.8-2.8 ¹			2.4 \pm 0.2		
Fat (g / 100 kcal)	4.4-6.0			4.4 \pm 1.2		
Carbohydrates (g / 100 kcal)	9-14			12.8 \pm 0.9		
mg essential amino acid / 100 kcal						
Lys	113 ^e	227.6 \pm 0.2 ^a	188.4 \pm 6.7 ^{bc}	173.0 \pm 0.5 ^c	156.4 \pm 3.1 ^d	198.0 \pm 3.8 ^b
Tyr	76 ^d	88.5 \pm 3.1 ^{bc}	91.7 \pm 3.4 ^b	86.6 \pm 1.2 ^c	108.5 \pm 4.1 ^a	116.2 \pm 7.2 ^a
Phe	83 ^d	102.0 \pm 1.0 ^c	121.3 \pm 3.6 ^b	108.3 \pm 1.4 ^c	126.1 \pm 0.2 ^b	136.9 \pm 1.9 ^a
Leu	166 ^f	270.4 \pm 0.2 ^a	219.4 \pm 2.6 ^d	205.2 \pm 0.2 ^e	232.4 \pm 2.6 ^c	254.8 \pm 1.4 ^b
Ile	90 ^e	150.9 \pm 2.4 ^a	124.9 \pm 7.5 ^{cd}	114.5 \pm 1.0 ^d	124.6 \pm 3.8 ^c	131.8 \pm 1.2 ^b
Met ³	23 ^d	67.4 \pm 1.4 ^b	60.2 \pm 4.8 ^b	48.1 \pm 3.8 ^c	77.0 \pm 2.6 ^{ab}	81.8 \pm 2.2 ^a
Val	88 ^d	149.2 \pm 0.7 ^b	139.5 \pm 2.6 ^{bc}	127.8 \pm 1.2 ^c	158.6 \pm 0.7 ^a	144.1 \pm 2.2 ^b
His	40 ^c	56.5 \pm 0.7 ^b	59.9 \pm 6.7 ^{ab}	60.4 \pm 2.6 ^a	62.1 \pm 4.6 ^a	58.0 \pm 0.5 ^{ab}
Thr	77 ^c	144.4 \pm 0.2 ^a	94.8 \pm 4.1 ^b	103.2 \pm 1.7 ^b	107.3 \pm 0.7 ^b	148.9 \pm 2.6 ^a

¹1.8 g corresponded to the minimum value of protein content when using cow's or goat's milk proteins and 2.8 g is the maximum value 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 (see Table 18).

²The 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)

³Cysteine AA was not presented in the EAA results as inconsistent data were obtained for this AA suggesting analysis issue.

4.2.1.3. 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 (ANF) 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 et al. 2012).

The activity of porcine trypsin (the same enzyme used in the digestion protocol) did not significantly differ when measured in the presence of whey proteins (used to formulate the dairy reference IF), pea, faba bean and rice protein concentrates, with comparable value to the control (105.1 ± 5.0 U/mg). However, it was significantly lower (24.1 ± 2.8 U/mg) when measured in the presence of potato protein (Table 25). This result suggests that the potato protein used in the present study may contain porcine trypsin inhibitors.

However, porcine trypsin was used in the present test (as in the digestion protocol), 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 et al. (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. 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 et al. 2018). In any case, if low digestibility is reported in the presence of potato protein in this study, it could be explained by the results reported in Table 25, but it will not mean that the same results would be observed in the presence of human enzymes.

Table 25. Porcine trypsin activity (U/mg) measured in the presence of each protein source in solution and the substrate only (control). Data are means \pm SD. Values with a different superscript letter are significantly different ($p < 0.05$).

	Control	Reference ¹	Pea	Faba bean	Rice	Potato
Trypsin activity (U/mg)	105.1 ± 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

¹The reference protein corresponded to whey protein in the dairy reference IF of the present study

4.2.1.4. Pea and faba bean IFs are equivalent to the dairy 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 IFs 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 (Adler-Nissen 1976). During the gastric

phase of *in vitro* digestion, the proteolysis was very limited (DH < 2% measured at the end of the gastric phase and corresponding to the time 0 min on Figure 31). 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 et al. 1969; Davidson & Lönnerdal 1987; Henderson et al. 2001; Johnson 2014).

Then, as soon as the intestinal enzymes were added, proteolysis drastically increased for all formulas, except for that of potato (Figure 31). 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 dairy reference IF. At the end of the intestinal digestion, DH ranged from $28.8 \pm 3.3\%$ to $51.4 \pm 3.2\%$ for potato and pea IFs, respectively. During the entire intestinal phase, the pea IF showed a DH higher than or similar to the dairy 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 ($p < 0.05$).

He et al. (2013) studied potato protein in solution (exactly the same potato protein isolate as the one used in the present study) in comparison to different reference proteins (whey, soy and pea). These authors used an *in vitro* static digestion 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) and, as mentioned, the gastric pH is higher in the infant model compared to the adult one (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 which is closer to DH value of whey protein obtained in He et al. (2013). Moreover, protein in solutions were digested in He et al. (2013) compared to IFs based on cow milk and plant proteins in the present study. This could mainly explained the different results observed between the two studies.

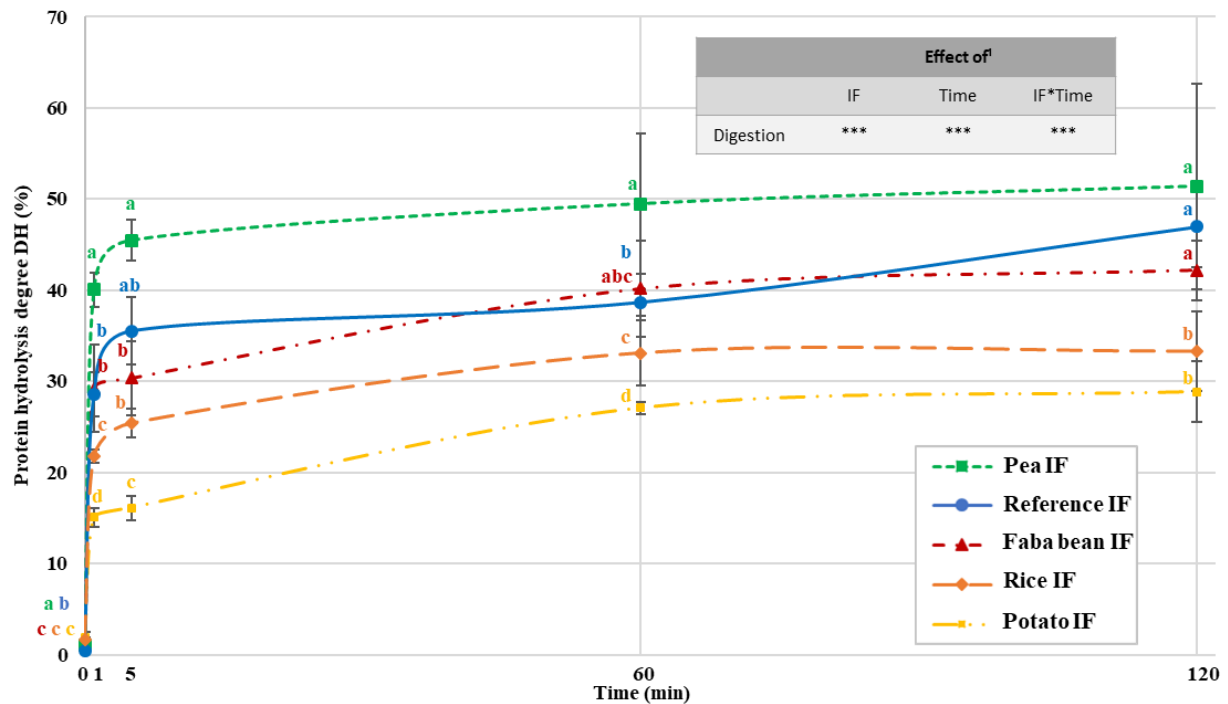


Figure 31. Degree of protein hydrolysis (DH) during the intestinal phase of *in vitro* static digestion of the five infant formulas (IFs). Data are means \pm SD (n=3). Values with a different superscript letter for a given digestion time and between the five infant formulas are significantly different ($p < 0.05$). ¹Table represents effect of IF, Time and interaction between IF and Time for DH (%) during intestinal digestion of the five IFs (Statistical significance: $p < 0.001$ (***) ; $p < 0.01$ (**); $p < 0.05$ (*); $p < 0.1$ (NS))

4.2.1.5. Pea and faba bean IFs are equivalent to the cow dairy 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 (Figure 32). 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 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 (Bourlieu 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 et al. 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 -Lactoglobuline digestibility as the protein unfold due to heat treatment above 65°C and thus became more sensitive to proteolysis (Mandalari et al. 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 (Figure 32).

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 24), 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 25).

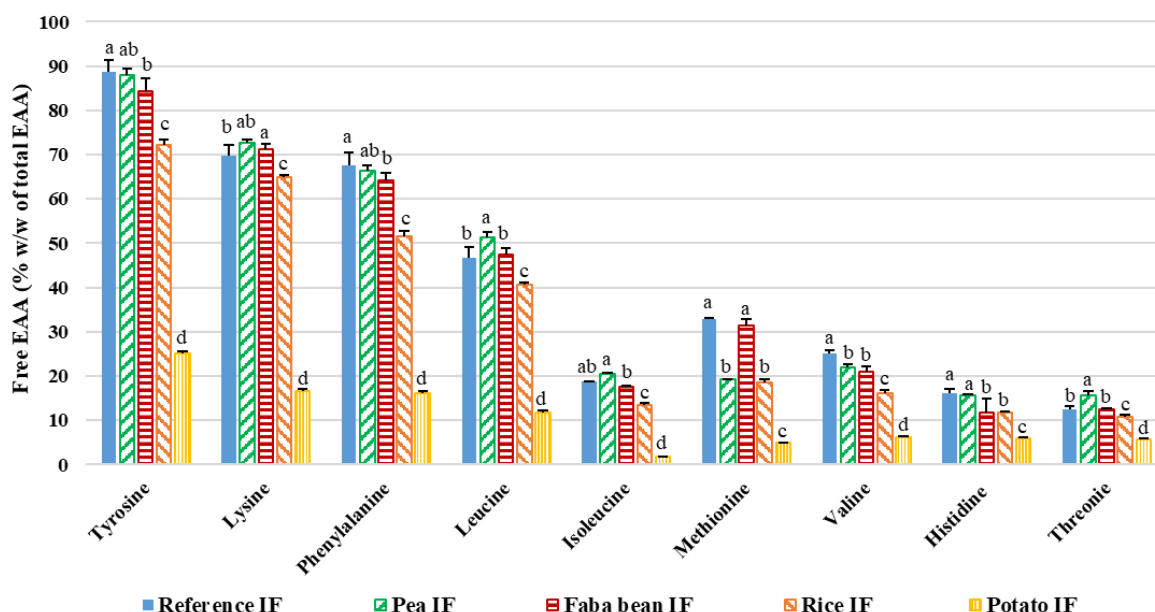


Figure 32. Bioaccessibility of essential amino acids (EAA) determined at the end (120 min) of the gastro-intestinal *in vitro* static digestion for the five IFs (% w/w of total EAA). Data are means \pm SD (n=3). Values with a different superscript letters for a given EAA and between the five IFs indicate a significant difference ($p < 0.05$). Cysteine AA was not presented in these results as inconsistent data were obtained for this AA suggesting analysis issue.

4.2.2. Conclusion

This is the first time that model 1st age 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. However, the rice protein IF showed solubility limits that negatively impacted IF production and powder quality. 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.

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. It was mainly highlighted that the type of protein sources tested in the present study had a great 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 dairy reference IF; 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 and functional 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 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 further with the reference, pea and faba bean IFs. This would let 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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HIGHLIGHTS

In this chapter, it was highlighted that protein sources had a great impact both on the manufacturing at a pilot scale and on the digestibility of IFs using an *in vitro* static model simulating infant digestion. In fact, rice IF showed limits in terms of solubility that created matter losses during process as well as a very high amount of free fat content that would affect rice IF powder quality. Potato IF also showed a high viscosity prior to drying that would not be appropriate to an optimal spray drying. Despite the fact that all the five IFs showed balanced nutritional recommendations compared to the European regulation for 1st age IF, some differences were noticed in terms of digestibility. Pea and faba bean IFs showed similar proteolysis and EAA bioaccessibility than the reference dairy IF. However, rice IF and even more so potato IF showed significantly lower digestibility than the three other IFs. This latter result was partly explained by the poor solubility of rice protein and the presence of trypsin inhibitor in potato protein.

In the next chapter, a scale-up of the manufacturing will be handled by producing IFs at a semi-industrial scale. Taking into account the results achieved with the pilot scale study and summarized just above, **it was decided to exclude rice and potato proteins for these experiments at a semi-industrial scale**, in order to focus more deeply on pea and faba bean proteins that showed promising results in terms of manufacturing and digestibility. Then, pea and faba bean IFs will be compared to the milk-reference IF in terms of structure and functional properties after manufacturing.

Chapter 3 - Scale-up of IFs manufacturing

In this chapter, pea and faba bean IFs as well as the milk-reference IF will be manufactured closer to industrial realities using semi-industrial installations. The functional properties and the stability of the emulsion will be assessed through physicochemical and microstructure analysis. The aim will be to investigate the protein source influence on the process conditions and the IFs functional properties during manufacturing as well as in the final product.

The results of this chapter will be presented through the following submitted publication (from which the *Introduction* was removed and the *Materials & Methods* was moved to the section 3 of the present manuscript):

Plant proteins partially replacing dairy proteins greatly influence infant formula functionalities

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HIGHLIGHTS

- Plant protein dispersibility is the limiting factor all along IFs manufacturing
- Similar emulsion stability and free fat were found for plant and milk-reference IFs
- Homogenization partly improved dispersion of plant protein aggregates
- Protein source had a great impact on IF manufacturing and functionalities

KEYWORDS: infant formula; plant proteins; emulsion; homogenization; spray drying

ABSTRACT

Infant formulas (IFs) can be defined as substitutes for human milk, which are mostly based on cow milk proteins. For sustainability reasons, alternative to animal proteins in food have to be considered. Plant proteins offer interesting nutritional and functional benefits for the development of innovative IFs. However, the behaviour of these proteins during processing and storage must ensure the physical stability and ability to reconstitution of IF powders, and that needs to be tested. This work aimed to study how a partial substitution of dairy proteins by plant proteins may influence the functional properties of IFs. Three IFs were developed at a semi-industrial scale using two different processing routes. The IFs composition was identical, except that 50% of the proteins were whey proteins in the “reference IF” (RIF), and pea or faba bean proteins in the “plant IFs” (PIF and FIF, respectively). After reconstitution, the three IFs result in similarly stable emulsions with equivalent free fat release. Nevertheless, in comparison to RIF, PIF and FIF were difficult to disperse, thus conducting to remaining insoluble particles in solutions. The protein source greatly influences IFs properties, and thus process parameters need to be adapted for each formulation in order to meet IFs quality criteria.

4.3.1. Results & Discussion

In an innovation purpose, this study aimed to assess the possibility of substituting a fraction (50%) of cow milk proteins in IFs with alternative plant protein sources previously demonstrated to be relevant from a functional and a nutritional point of view (Le Roux et al. 2020a). Thus, pea and faba bean proteins were tested in the present study to design “plant IFs” at a semi-industrial scale and testing two different processing routes, in comparison to a reference IF including only dairy proteins. The three IFs, namely PIF, FIF and RIF were characterized for their physicochemical properties, their microstructure using confocal microscopy, as well as the stability of emulsion after reconstitution.

4.3.1.1. Physicochemical properties of IFs

The six IFs, namely PIF, FIF and RIF produced according to the processing routes 1 and 2 (Figure 25), were equivalent in terms of dry matter (DM), ash, protein and fat contents with mean values of respectively 96.7 ± 0.6 w/w% DM, 1.6 ± 0.2 w/w% ash content, 10.9 ± 0.6 w/w% proteins and 23.3 ± 0.2 w/w% fat.

As previously mentioned, IFs generally contain a relatively large amount of unsaturated, and consequently oxidisable fatty acids. Hence, it is essential to control lipid stability and encapsulation during storage to ensure their nutritional value and flavour (Nasirpour et al. 2006). The fat stability is generally considered as satisfactory when the free fat content remains below 5% in a whole milk powder (WMP) (Vignolles et al. 2007). In the present study, free fat content was equal to 2.2 ± 0.0 w/w% of DM at T0 (measurement after process), regardless of the IFs and the process parameter sets. McCarthy et al. (2013) found similar results with a free fat level of 2.0 ± 0.2 % in dairy based IF powder (with a protein: fat ratio of 0.43, *i.e.* a fat content a bit higher compared to our IFs with a protein: fat ratio of 0.47). After four months storage at 20°C (T4), free fat content increased for all IFs, with a rise between 22% and 122% for RIF1 and RIF2, respectively (Table 26). Although all IFs contained less than 5% free fat, it is noticeable that such amount of free fat was already initially significant and increased over time,

especially for RIF 2, which value was very close to 5% after 4 months storage at 20°C (4.3 ± 0.6 w/w%). The high value of free fat measured in RIF 2 at T4 could be partly explained by the smaller particle size of this powder ($d(0.5)$ diameter of 105.81 ± 0.59 μm) compared to the other IFs ($d(0.5)$ ranging between 111.8 ± 0.1 μm and 141.2 ± 1.6 μm), resulting in a higher surface exchange area, leading to less fat retained in the particles, and consequently, more free fat released (Buma 1971).

Table 26. Physicochemical properties of the three powdered IFs (RIF: reference infant formula ; PIF: pea infant formula ; FIF: faba bean infant formula) and tested for the two processing routes: (1) 8/2 MPa homogenization and 165°C to 75°C drying temperatures; (2) 14/4 MPa homogenization and 150°C to 65°C drying temperatures. T0: measurement immediately after process; T4: measurement after 4 months storage at 20°C. Data are expressed as mean \pm SD. For a given characteristic and between the six IFs, values with a different superscript letter are significantly different ($p < 0.05$).

	Process parameters 1			Process parameters 2		
	RIF 1	PIF 1	FIF 1	RIF 2	PIF 2	FIF 2
Free fat T0 (w/w% total fat)	1.9 ± 0.3^a	2.6 ± 0.6^a	2.1 ± 0.1^a	1.9 ± 0.3^a	2.6 ± 0.1^a	2.2 ± 0.2^a
Free fat T4 (w/w% total fat)	2.4 ± 0.2^b	3.2 ± 0.3^{abc}	2.9 ± 0.7^{abc}	4.3 ± 0.6^c	3.4 ± 1.2^{ab}	2.8 ± 0.3^{bc}
$d(0.5)$ (μm)	133.7 ± 0.6^b	129.6 ± 1.2^c	141.2 ± 1.6^a	105.8 ± 0.6^f	123.2 ± 0.2^d	111.8 ± 1.0^e
a_w	0.14 ± 0.02^d	0.18 ± 0.02^c	0.18 ± 0.01^c	0.21 ± 0.01^{ab}	0.19 ± 0.01^{bc}	0.22 ± 0.01^a
Tg ($^{\circ}\text{C}$)	66.7 ± 0.3^a	56.9 ± 5.5^b	60.5 ± 1.8^{ab}	55.9 ± 1.4^b	55.9 ± 4.7^b	55.9 ± 0.8^b
Solubility index (SI; %)	100.0 ± 0.1^a	97.5 ± 0.5^b	97.0 ± 0.5^c	100.0 ± 0.1^a	97.5 ± 0.5^b	97.0 ± 0.5^c
Dispersibility index (DI; %)	99.7 ± 0.9^a	99.5 ± 0.6^{ab}	98.7 ± 0.4^{ab}	97.9 ± 1.5^b	99.0 ± 0.9^{ab}	98.6 ± 0.5^{ab}
Viscosity (Pa.s)	0.03 ± 0.01^e	0.80 ± 0.04^b	0.06 ± 0.02^{de}	0.24 ± 0.02^c	1.55 ± 0.01^a	0.09 ± 0.01^d
Color parameters						
L	69.6 ± 1.0^a	69.6 ± 1.0^a	69.6 ± 1.0^a	70.5 ± 0.8^a	69.5 ± 1.0^a	70.9 ± 0.9^a
a	-4.4 ± 0.1^f	-1.9 ± 0.1^a	-4.0 ± 1.4^d	-3.7 ± 0.1^c	-2.2 ± 0.1^b	-4.2 ± 1.2^e
b	12.4 ± 1.9^{ab}	14.7 ± 1.6^{ab}	16.5 ± 0.8^a	10.7 ± 2.1^b	15.9 ± 1.3^a	16.5 ± 1.9^a

In the present study, the emulsions corresponding to the different IFs were moderately stabilized regardless of the protein source, as indicated by the free fat content measured, especially after 4 months storage. This suggests that the processing parameters should be optimized to decrease free fat level, processing route 1 appearing preferable than route 2. The emulsion stability could be also improved by adding emulsifiers or producing bigger powder particles.

Spray-drying behaviour and storage ability of milk powders depend very much on both glass transition temperature (Tg) and water activity (a_w) (Schuck et al., 2007). The mean a_w was 0.19 ± 0.03 , i.e. close to the optimal value of 0.2 as defined by Efsthathiou et al. (2002) (Table 26). Therefore, the long-term quality of the IFs should be guaranteed, these powders being free from phenomena such as lipid oxidation, caking or browning that are likely to occur when a_w is not at its optimal value. The Tg mean value at 0.2 a_w was $58.7 \pm 4.3^{\circ}\text{C}$ for all the powders, and was significantly higher for RIF1 and FIF1 compared to the four other IFs with $66.7 \pm 0.3^{\circ}\text{C}$ and $60.5 \pm 1.8^{\circ}\text{C}$, respectively. Tham et al. (2017) found comparable Tg values for IFs compared to the present study and showed a good storage stability at 25°C. McCarthy et al. (2013) reported a Tg value of $55.5 \pm 1.1^{\circ}\text{C}$ for a dairy protein based IF powder ($a_w = 0.23$), also in accordance with our results.

Dispersibility is defined as the capacity of wet aggregates to uniformly disperse when in contact with water. A powder is considered dispersible when the dispersibility index (DI) is higher than 85% (Schuck et al. 2012). The DI of the IF powders prepared in this study ranged between $99.7 \pm 0.9\%$ and $97.9 \pm 1.5\%$ for the RIF1 and RIF2, respectively (Table 26). On the other hand, the solubility index (SI) represents the loss of granular structure when the powder is solubilized in water. A powder is considered soluble when the SI is above $89.5 \pm 2.2\%$ (Schuck et al. 2012). In this study, the RIFs presented a SI value of 100%, while PIFs and FIFs showed SI values of 97.5% and 97%, respectively (Table 26). Hence, all the IF powders prepared in this study can be considered as dispersible and soluble according to these methods. Even more so as the rehydration ability can be enhanced by increasing temperature or stirring speed during the reconstitution step (Jeantet et al. 2010). However, it was visually noticeable that PIFs and FIFs encountered dispersion impairments when dissolved in water. In fact, insoluble particles (or so called “flecking”) were observed during manufacturing as well as in the rehydrated powders. This behavior has also been noticed previously by Schuck et al. (2016) as well as by Singh & Ye (2010).

The color of the three IF powders were quite similar with the same brightness (L) value. However, PIF seemed to reach out towards grey color (lower a value), FIF towards yellow color (higher b value) and RIF was quite in a middle of the two other IF powders with more beige color. These color parameters were quite in accordance with WMP color parameters (71.9 ± 0.2 , 6.0 ± 0.1 and 17.4 ± 0.4 , respectively for the parameters L, a, b).

Lastly, after homogenization, the viscosity of some of the IFs studied was significantly higher than usually recommended for an effective spray-drying. The highest value was measured for PIF2 with 1.55 Pa.s (Table 26). For an optimal spray, the viscosity of a concentrate IF should be around 60 mPa.s (Vestergaard 2004), and should not exceed 200 mPa.s to allow subsequent spray drying. Moreover, the viscosity of a concentrate influences the quality of the powder (bulk density, solubility, etc.) by varying the size of the spray droplets (Schuck et al. 2005). Despite this, the high viscosity measured in this study seemed to not have affected the drying characteristics, neither the physicochemical properties of the final products (Table 26). Nevertheless, it is obvious that the high viscosity measured for PIFs did not correspond to optimal conditions for spray drying, and thus process optimization would be required. Moreover, it was noticeable that the viscosity significantly increased for all the IFs between processing routes 1 and 2, which correspond to homogenization pressures of 10 MPa and 18 MPa, respectively. This observation was consistent with the viscosity increase when pressure increases reported by Pouliot et al. (1990) for a study on IFs. These authors suggested that high homogenization pressure results in more casein spreading on fat globules, which finally increases their ability for interactions, up to gelation. However, it is likely that the poor solubilisation obtained for PIF at the powder rehydration stage was further completed by the different processing steps, including homogenization, thus leading to additional solubilisation of plant proteins. This latter could explain by itself the higher viscosity reported for PIF.

To sum up, it seems possible to produce IFs at semi-industrial scale in which dairy proteins are partially replaced by pea or faba bean proteins with regard to the key physicochemical criteria usually considered. However, some improvements should be done, notably to enhance the dispersibility and/or

solubility of plant proteins, as well as a reduction of the free fat level. The viscosity of the concentrate to be dried should be lessened too. In this way, the processing route 1 seemed to provide better physicochemical properties than processing route 2, in particular regarding the free fat release and the viscosity value prior drying.

4.3.1.2. Effect of unit process operations on the microstructure of IFs

The microstructure of the plant protein based IFs (PIF and FIF) and the reference IF (RIF) during process and after powder rehydration was investigated by confocal laser scanning microscopy (CLSM). This highlighted differences in size distribution, composition and architecture of lipid droplets and proteins between the three IFs and during process (Figure 33 and Table 27).

Table 27. Mode diameter and D[4.3] of PIF and FIF samples during process and in the rehydrated powder in baby bottles. 1 and 2 correspond to processing routes 1 and 2. Data for D[4.3] are expressed as mean \pm SD.

		D[4.3] (μm)	Mode 1 (μm)	Mode 2 (μm)	Mode 3 (μm)
RIF	Solubilization	0.8 ± 0.1	0.3	2.1	
	Concentration	4.6 ± 1.9	0.4	2.8	
	Homogenization 1 (10 MPa)	0.8 ± 0.1	0.5	2.8	
	Homogenization 2 (18 MPa)	0.9 ± 0.1	0.6	2.4	
	Baby bottle 1	0.4 ± 0.1	0.5		
	Baby bottle 2	0.4 ± 0.1	0.4		
PIF	Solubilization	48.3 ± 0.1	58.9		
	Concentration	45.4 ± 0.3	58.9		
	Homogenization 1 (10 MPa)	34.0 ± 0.2	14.5	66.9	
	Homogenization 2 (18 MPa)	31.6 ± 1.4	58.9		
	Baby bottle 1	18.9 ± 0.4	0.8	56.4	
	Baby bottle 2	15.3 ± 2.0	0.9	39.7	
FIF	Solubilization	20.9 ± 0.1	11.2	46.6	
	Concentration	18.9 ± 0.1	11.2	51.8	
	Homogenization 1 (10 MPa)	9.9 ± 1.7	0.7	2.4	12.7
	Homogenization 2 (18 MPa)	7.6 ± 0.3	0.7	6.7	
	Baby bottle 1	6.2 ± 0.4	0.6	8.9	
	Baby bottle 2	5.6 ± 0.1	0.6	10.0	

Firstly, after solubilisation of the different ingredients except oil blend, the modes of the particle size were 0.3 and 2.1 μm for RIF, whereas bigger particles were observed in plant-based formulas, with mode values of 58.9 μm for PIF, 11.2 and 46.6 μm for FIF. The bigger particles found in PIF and FIF suggests an incomplete solubilisation of plant proteins, as previously mentioned, but protein aggregates created during the technological processes might be also involved. Indeed, heating of globular proteins above their denaturation temperature (Amagliani & Schmitt 2017; Guo et al. 1998, 1999) leads to their unfolding, exposure of hydrophobic patches and irreversible aggregation by forming hydrophobic interactions, hydrogen bonds and/or disulfide bonds. Protein aggregation may be to a greater or lesser extent, depending on the protein nature and the physicochemical conditions (pH vs isoelectric point, nature and concentration of salts, etc.), but in any case it influences the solubility of the proteins (Benjamin et al. 2014; Corredig & Dalgleish 1995; Malaki Nik et al. 2009). Barac et al. (2015) measured the solubility of pea protein isolate at pH 7.0 (i.e. close to pH 6.6-6.8 applied in the present study) which was between $59.7 \pm 2.3 \%$ and $57.9 \pm 0.1 \%$, depending on if the isolate was non-heated

or heated at 90°C for 3 min. Similarly, Karaca et al. (2011) measured a solubility of 61.4 ± 0.8 % for pea protein isolate at pH 7.0 and at room temperature. At the end, such low solubility values were consistent with the large protein aggregates observed for the PIF in the present study. In the same conditions (pH 7.0, room temperature), Karaca et al. (2011) measured a higher solubility for faba bean protein isolate (89.7 ± 0.2 %), which was not observed in our case where big particle size and aggregate particles were observed in FIF, even if they were smaller compared to PIF (Figure 32). In overall, different protein structures and size were observed for the three IFs from the beginning of the process, indicating that plant protein based IFs should require specific processing for better solubilisation.

After the concentration step, the size distribution seemed to be quite similar in all IFs, but lactose crystals appeared (Figure 32). Bigger particles were still observed in the concentrated PIF and FIF solutions compared to RIF. In addition, circular particles could be observed in concentrated FIF that could be fibers. As expected, small lipid droplets (in red) appeared after addition of the oil blend and homogenization. The pressure applied upon homogenization was adjusted to obtain small size droplets (i.e. mainly <1 μm) in order to ensure the physical stability of the emulsion during long storage of the powder and after rehydration in a baby bottle (Vignolles et al., 2007). Using the homogenization process parameters 1 (i.e., 10 MPa), a majority of lipid droplets around 1 μm were observed in RIF (modes of 0.6 and 2.7 μm). In the homogenized PIF, a mix of small fat droplets and protein aggregates were observed in CLSM images, with mode values of 14.5 and 66.9 μm . Homogenized FIF also showed small fat droplets, as well as big protein particles but these last seemed to be lower than before homogenization (modes between 0.7 and 12.7 μm compared to 11.2 and 51.8 μm before homogenization). These results suggest that the protein aggregates present in PIF and FIF were dispersed to a higher level thanks to homogenization process. Only slight differences were noticed between homogenization 1 and 2 in terms of particle size. However, heterogeneous distribution of fat and proteins were observed in PIF and FIF after homogenization 2 and could be due to protein aggregation induced by heat treatments and mechanical treatments such as homogenization (Guo et al. 1998, 1999; Joyce et al. 2017). Lactose crystals with the characteristic of “Tomahawk shape” were observed from the concentration step and still after the homogenization step, with no change in average size and appearance. This was due to the very high concentration of lactose in the concentrate (dry matter around 50 % and lactose concentration around 30-33 %, that is close to lactose solubility limit). Conversely, lactose crystals disappeared after dilution of the samples. It should be reminded also that samples taken from concentration and homogenization steps were half-diluted in water prior granulometry measurement. Thus, lactose crystals were dissolved and could not be observed on particle size distribution graphs (Figure 33).

Finally, the rehydration of IF powders in baby bottles obtained after either processing routes 1 or 2 showed homogeneous and almost unimodal distribution of the fat droplets and the proteins in RIF with modes of 0.5 ± 0.1 μm (i.e. <1 μm). In PIF and FIF baby bottles, a bimodal particle size distribution was still observed with on the one hand, the proteins and the fat droplets <1 μm , and on the other hand, the protein aggregates (modes of 0.8-0.9 and 39-56 for PIF ; 0.6 and 9-10 for FIF). It could be noticed that the particle size decreased more than 3 times from the beginning to the end of the process, meaning that the process had probably an impact on the plant protein structure and re-dispersion of the aggregate

particles. These observations were in accordance with previous studies (Guo et al. 1998; Sun et al. 2018) in which it was highlighted that the homogenization and the thermal process steps had a key role on the microstructure of the infant milk formulas. In the present study, although particle size in the two plant-based IFs seemed to have decreased thanks to the process, it is clear that such an effect could be observed because plant proteins were initially not completely dispersed. Therefore, it is not possible to conclude which of the protein effect and the process effect has the major impact on the microstructure of PIF and FIF. In any case, additional analysis as well as replication of the manufacturing should be conducted further in order to clearly elucidate why the particle size of the plant protein-based IFs is so high and how it can be possible to improve it. Especially, homogenization prior concentration step should be tested for improvement of the plant protein solubilisation.

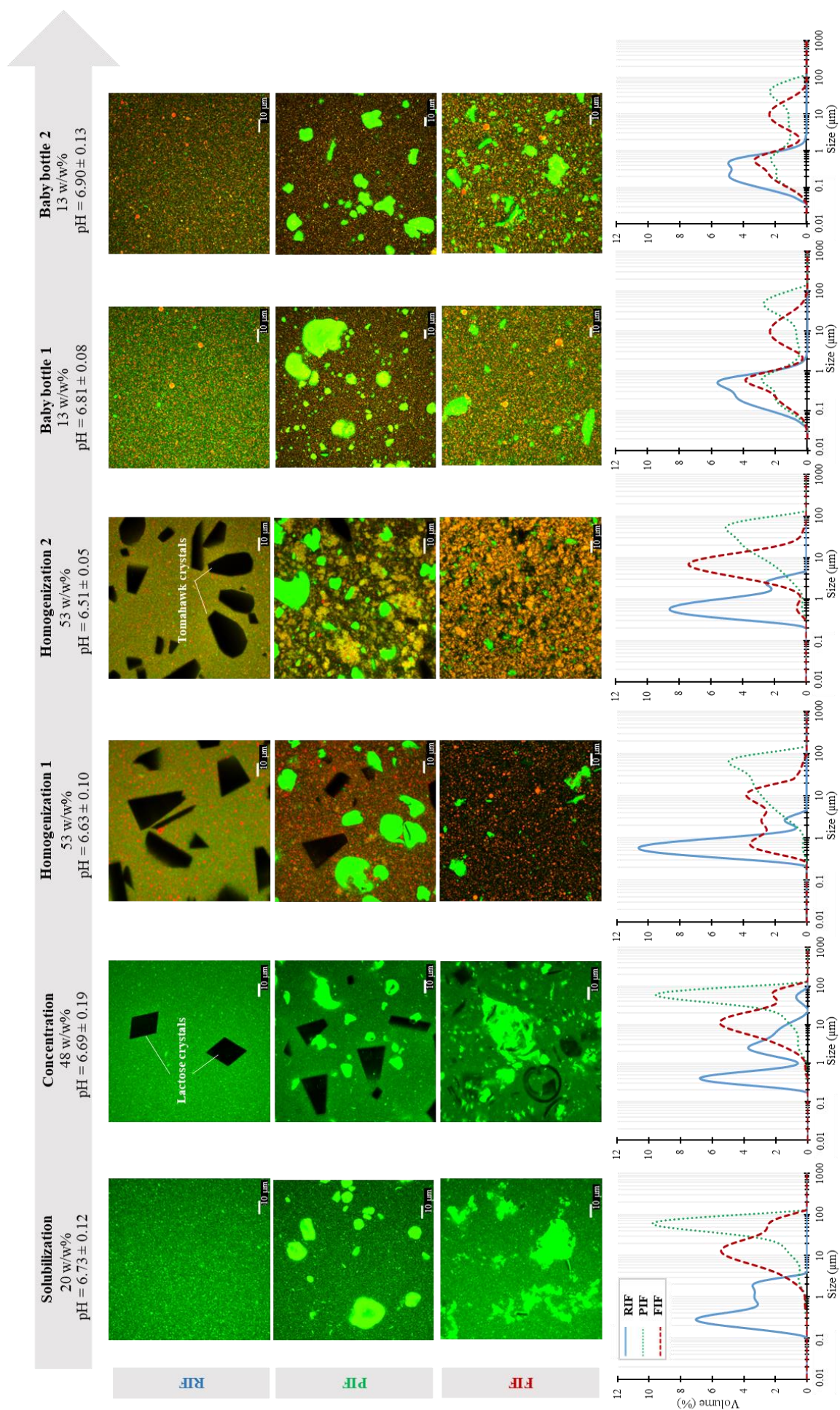


Figure 33. Spatial organization of lipids (in green) and proteins (in red) as evidenced by confocal laser scanning microscopy (CLSM) in the three IFs (RIF: reference infant formula; PIF: pea infant formula; FIF: faba bean infant formula) after the different process steps, from solubilisation of the ingredients to the rehydration of the IF powders in baby bottle. Homogenization 1: 8/2 MPa, homogenization 2: 14/4 MPa. Baby bottle 1 and 2 were obtained after the rehydration of the IFs obtained with the process parameters 1 and 2 (homogenization and drying described in Section 3.3). Size distributions of processed IFs were determined by laser light scattering.

4.3.1.3. Critical concentration of protein to stabilize emulsion in IFs

The oil-water interface has to be stabilized by surface-active molecules which can form a coat surrounding fat droplets of less than 1 μm in diameter. It ensures a good emulsion stability and a subsequent protection of fat droplets during drying and storage (Dalgleish 1997; Turchiuli et al. 2005). Proteins are surface-active elements that play an important role in oil-water interfaces during the homogenization step, and then in air-liquid interfaces during drying. For instance, adsorbed proteins in homogenized milk result in steric repulsions, which allow emulsion stability (Vignolles et al., 2007). In fact, instability during emulsion formation occurs if there is insufficient surfactant to cover the entire oil-water interface created by the homogenizer. Adsorbed protein spread out to cover the maximum area, but if there are gaps in the interfacial layer, fat droplets may coalesce, decreasing the total surface area, until it is totally covered by the available surfactant (Fang & Dalgleish 1993). The concentration of proteins in the milk-reference IF after homogenization had been determined in order to verify whether it was sufficient to stabilize the emulsion. The critical protein concentration, namely the minimum protein concentration needed for encapsulating the fat content was estimated as follows.

First, the fat droplet number (kg^{-1}) was determined according to:

$$\text{Number of fat droplets} = \frac{\text{Total volume of fat droplets}}{\text{Fat droplet volume}} \quad (13)$$

in which the total volume of fat droplet was calculated according to:

$$\text{Total volume of fat droplets} = \frac{\text{Total concentration of fat droplets}}{\text{Density of fat blend}} = \frac{0.12}{900} = 1.3 \cdot 10^{-4} \text{ m}^3 \text{ kg}^{-1} \quad (14)$$

given that the fat content in the IFs after homogenization was 12 w/w % and the density of the fat blend was $900 \text{ kg} \cdot \text{m}^{-3}$. The mean fat droplet diameter d chosen was 1 μm (close to $D[4.3]$ values obtained after homogenization of RIF, Table 28) the fat droplet volume (m^3) was calculated as:

$$\text{Fat droplet volume} = \pi \frac{d^3}{6} = 5.2 \cdot 10^{-19} \text{ m}^3 \quad (15)$$

Equations 12, 13 and 14 came with a number of fat droplets equal to $2.5 \cdot 10^{14} \text{ kg}^{-1}$.

Then, the area of the fat droplet interface ($\text{m}^2 \cdot \text{kg}^{-1}$) was obtained from the number of fat droplets and the fat droplet surface, given by:

$$\text{Fat droplet surface} = \pi d^2 = 3.1 \cdot 10^{-12} \text{ m}^2 \quad (16)$$

$$\text{Area of the fat droplet interface} = \text{Number of fat droplets} \cdot \text{Fat droplet surface} = 800 \text{ m}^2 \cdot \text{kg}^{-1} \quad (17)$$

Last, the minimum protein concentration (w/w %) was determined on the basis of area of the fat droplet interface and the droplet coverage by the proteins. Cow milk proteins are well-known to widely spread on oil-water interface in emulsions (Courthaudon et al. 1991; Hunt & Dalgleish 1994), and authors generally consider that the amount of milk proteins absorbed at the surface of fat droplets is around 1.5 to $3.0 \cdot 10^{-6} \text{ kg of proteins} \cdot \text{m}^{-2}$ surface after homogenization (McCarthy et al. 2012; Pelan et al. 1997; Ye et al. 2002):

Minimum protein concentration = Protein adsorbed at the fat droplet interface ·

$$\text{Area of the fat droplet interface} = 1.2 \text{ to } 2.4 \cdot 10^{-3} \text{ kg of proteins} \cdot \text{kg}^{-1} \text{ of homogenized concentrate} \quad (18)$$

In conclusion, **1.2 to 2.4 g of proteins per kg of emulsion** were necessary to cover the fat droplets in the IFs. Then, the protein concentration in the IFs after homogenization ($30 \text{ g} \cdot \text{kg}^{-1}$) was ten times higher than the critical concentration calculated. This probably explained why the stability of the emulsion did

not significantly differ (structure and free fat release) regardless of the protein source, as the protein content was in excess in all IFs and thus enough to stabilize all the fat droplets. In other words, even in the plant-based IFs, soluble proteins (from dairy and plant origin) might be in sufficient concentration to stabilize the emulsion, despite the insoluble fractions noticed.

4.3.2. Conclusion

This study handled the feasibility of producing, at a semi-industrial scale, plant protein-based IFs close to a milk-reference IF in terms of physicochemical and functional properties. It was seen that pea and faba bean proteins were hardly dispersed all along the manufacturing of the plant-based IFs, resulting in bigger particles, as well as flecking in the reconstituted powder compared to the milk reference IF.

It seemed that particle size in the two plant-based IFs have decreased thanks to the process, but it is clear that plant proteins were still in part aggregated, and contributed to high particle size values observed for PIF and FIF. Therefore, additional analysis as well as replication of the manufacturing should be conducted further in order to clearly elucidate why the particle size of the plant protein based IFs is so high and how it can be possible to decrease it. Especially, homogenization prior concentration step should be tested for increasing the plant protein solubilisation.

Moreover, the calculation of the theoretical quantity of proteins required to cover the oil-water interface let to think that dairy proteins would have been in sufficient concentration to stabilize the emulsion, including in the plant based IFs. This likely explains the similar results between the three IFs in terms of emulsion stability with equivalent free fat release regarding the conditions applied in the present study.

In addition, high viscosity was reported in the concentrate to be dried for some of the IFs, in particular for PIF using processing route 2. Despite this high viscosity seemed to not have affected the drying characteristics (Table 26, physicochemical properties of PIF 1 and PIF 2), the viscosity measured for PIF does not correspond to optimal conditions for spray drying and process optimization would be required. Moreover, few differences were noticed between processing routes 1 and 2, *i.e.* slightly lower free fat content (after 4 months storage) and higher dispersibility for RIF1 compared to RIF2; as well as lower viscosity for RIF1 and PIF1 compared to processing route 2. Thus, the choice would fell for processing route 1 if a decision should be taken.

In overall, it was seen that protein source had a great impact on IFs properties. This means that process parameters should be adapted for each formulation in order to provide satisfactory IFs quality. Nevertheless, it should be remembered that the results of this exploratory study need validations. And beyond this, this study will further be extended through process optimization and industrial development as well as *in vivo* studies for nutritional assessment.

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HIGHLIGHTS

In overall, it was seen that protein source had a great impact on IFs functional properties. The main issue that was highlighted in this part of the study was the poor solubility of the plant proteins in IFs. In fact, the difficult dispersion of pea and faba bean proteins during manufacturing of IFs and in the final product caused aggregated particles and flecking that impaired IF quality during storage and in the reconstituted IFs. However, it was revealed that particle size in the two plant-based IFs decreased thanks to the process treatments. Therefore, additional experiments as well as replication of the manufacturing of IFs are needed in order to improve and optimize the quality of these new IFs.

In the next (and final results chapter), the nutritional quality of the IFs manufactured at a semi-industrial scale (the one obtained after processing route 1) will be investigated through an *in vitro* dynamic infant digestion. A dynamic digestion model has been used to characterize the plant based IFs, and to compare them to the milk-reference IF in a more physiological approach than previously investigated with static digestions (see chapter 2 results).

Chapter 4 - *In vitro* dynamic digestion of pea and faba bean IFs compared to the reference IF

In the previous chapter, the consequences of whey protein substitution by pea and faba bean proteins on the manufacturing and functional properties of 1st age IFs were investigated. Despite process should still be improved to make the plant-based IFs closer to the milk-reference IF, the manufacturing of pea and faba bean IFs appears conceivable. Then, the question arises of the nutritional quality of such plant-based IFs. To address this issue, the innovative pea and faba bean IFs, produced at a semi-industrial scale, have been digested using an *in vitro* dynamic system simulating infant digestion that would let to identify more accurately the influence of protein source on IFs digestibility in comparison to the static model previously investigated. Digestibility has been assessed by proteolysis measurements, EAA bioaccessibility, apparent protein digestibility as well as microstructure analysis.

The results of this chapter will be presented through the following publication in preparation (from which the *Introduction* was removed and the *Materials & Methods* was moved to the section 3 of the present manuscript):

Are faba bean and pea proteins potential whey protein substitute in infant formulas? An *in vitro* dynamic digestion approach.

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HIGHLIGHTS

- Microstructure differences between the 3 IFs were mainly observed in gastric phase.
- DH was limited in the gastric phase for all 3 IFs (< 10 %).
- DH during the intestinal phase was higher for FIF compared to RIF and PIF.
- 80 % of proteins were cleaved into peptides < 10 kDa at the end of digestion.
- Apparent *in vitro* digestibility was similar for RIF and FIF, but lower for PIF.

KEYWORDS: infant formula; plant protein; *in vitro* digestion; microstructure; proteolysis; apparent protein digestibility

ABSTRACT

Infant formulas (IFs) are used as substitutes for human milk, mostly based on cow milk proteins. For sustainability reasons, alternative to animal proteins in food are considered, and plant proteins offer interesting nutritional and functional benefits for the development of innovative IFs. This study aimed to assess how a partial substitution (50%) of dairy proteins by faba bean and pea proteins influenced the digestibility of IFs under simulated dynamic *in vitro* digestion, set up to mimic infant digestion. Pea and faba bean-based IFs (PIF and FIF, respectively) have led to a faster aggregation than the reference milk-based IF (RIF) in the gastric compartment but that did not affect the digesta microstructure at the end of digestion. Proteolysis extent, estimated by the hydrolysis degree, was the highest for FIF (73%), and the lowest for RIF (50%), but finally, apparent *in vitro* protein digestibility and PDCAAS-like scores were similar for RIF and FIF (90% digestibility; 75% PDCAAS), but lower for PIF (75%; 67%). Therefore, this study confirms that faba bean proteins could be a good candidate for partial substitution of whey proteins in IFs from a nutritional point of view, provided that these *in vitro* results are confirmed *in vivo*.

4.4.1. Results & discussion

This study aimed to explore the possibility of substituting the whey proteins usually added to skimmed cow milk to formulate 1st age IF in which they represent 50% of the total protein content. Especially, pea and faba bean proteins were considered worthy of previous investigation because of their EAA profile compatible with the nutritional requirements of the infants, and also since their behaviour was shown to be consistent with processing constraints (Le Roux et al. 2020a). Therefore, one milk-reference IF (RIF) and two innovative plant-based IFs (PIF and FIF for pea and faba bean IFs, respectively) have been produced at a semi-industrial scale (Le Roux et al. 2020b) and submitted to an *in vitro* dynamic model of digestion, in order to compare some relevant indicators of their protein nutritional quality.

4.4.1.1. Composition and essential amino acid (EAA) content of IFs

In order to assess the effect of the protein source, the three IFs were designed equivalent in terms of calorie, protein, fat, and carbohydrates contents (Table 28). The EAA contents were not significantly different between the two plant-based IFs, except for methionine, phenylalanine and lysine. The contents were lower in FIF compared to PIF, but the difference was of less than 7%. However, contents in EAAs were significantly lower in the two plant-based IFs compared to the reference RIF, except for tyrosine, phenylalanine and histidine. The slightly higher content of non-essential AAs in pea and faba bean proteins compared to milk proteins (Supplementary material 1) cannot explain the lower total content of EAAs in plant-based IFs that are here observed. Therefore, this suggests a higher proportion of non-protein nitrogen in the plant protein isolates compared to the milk protein powders, which would lead to overestimation of the protein content of pea and faba bean isolates.

Table 28. Nutritional composition of the three reconstituted IFs (RIF, PIF and FIF). Essential amino acid composition is expressed in mg amino acid / 100 mL IF. Data are means \pm SD (n=2). Different superscript letters indicate statistically significant difference (p<0.05).

Energy (kcal / 100 ml)	60.1 \pm 2.1		
Protein (g / 100 ml)	1.3 \pm 0.03		
Fat (g / 100 ml)	2.8 \pm 0.1		
Carbohydrates (g / 100 ml)	7.3 \pm 0.3		
EAA (mg/100 mL IF)	RIF	PIF	FIF
Lys	123.9 \pm 0.4 ^a	111.5 \pm 3.4 ^b	103.4 \pm 0.8 ^c
Tyr	54.8 \pm 1.9 ^b	61.1 \pm 3.5 ^a	58.8 \pm 1.2 ^{ab}
Phe	54.5 \pm 0.8 ^b	68.4 \pm 3.9 ^a	60.9 \pm 0.9 ^b
Leu	145.2 \pm 0.9 ^a	124.5 \pm 7.8 ^b	118.0 \pm 0.1 ^b
Ile	77.4 \pm 4.4 ^a	65.4 \pm 2.5 ^b	60.1 \pm 0.2 ^b
Met	41.2 \pm 5.3 ^a	37.5 \pm 0.7 ^a	28.3 \pm 0.7 ^b
Cys	27.5 \pm 0.2 ^a	16.3 \pm 0.3 ^b	16.3 \pm 0.1 ^b
Val	83.9 \pm 4.9 ^a	76.2 \pm 3.6 ^{ab}	71.5 \pm 0.8 ^b
His	32.1 \pm 1.5 ^b	36.7 \pm 0.5 ^a	36.9 \pm 1.3 ^a
Thr	98.2 \pm 1.6 ^a	76.1 \pm 3.0 ^b	71.8 \pm 1.1 ^{bc}

Supplementary material 1. Amino acid composition in the reconstituted IFs (RIF, PIF and FIF) is expressed in mg amino acid / 100 mL IF. EAAs and non-EAAs are expressed in % compared to the total AAs. Data are means \pm SD (n=2).

AA (mg/100 mL IF)	RIF	PIF	FIF
Lys	123.9 \pm 0.4 ^a	111.5 \pm 3.4 ^b	103.4 \pm 0.8 ^c
Tyr	54.8 \pm 1.9 ^b	61.1 \pm 3.5 ^a	58.8 \pm 1.2 ^{ab}
Phe	54.5 \pm 0.8 ^b	68.4 \pm 3.9 ^a	60.9 \pm 0.9 ^b
Leu	145.2 \pm 0.9 ^a	124.5 \pm 7.8 ^b	118.0 \pm 0.1 ^b
Ile	77.4 \pm 4.4 ^a	65.4 \pm 2.5 ^b	60.1 \pm 0.2 ^b
Met	41.2 \pm 5.3 ^a	37.5 \pm 0.7 ^a	28.3 \pm 0.7 ^b
Cys	27.5 \pm 0.2 ^a	16.3 \pm 0.3 ^b	16.3 \pm 0.1 ^b
Val	83.9 \pm 4.9 ^a	76.2 \pm 3.6 ^{ab}	71.5 \pm 0.8 ^b
His	32.1 \pm 1.5 ^b	36.7 \pm 0.5 ^a	36.9 \pm 1.3 ^a
Thr	98.2 \pm 1.6 ^a	76.1 \pm 3.0 ^b	71.8 \pm 1.1 ^{bc}
Arg	40.3 \pm 0.8	78.9 \pm 3.2	95.6 \pm 1.5
Asx ¹	135.1 \pm 0.9	138.8 \pm 5.8	133.3 \pm 0.3
Glx ²	286.6 \pm 1.9	279.4 \pm 23.5	265.8 \pm 9.2
Ser	74.1 \pm 0.4	76.1 \pm 6.1	71.6 \pm 3.0
Pro	104.1 \pm 2.0	96.4 \pm 6.6	94.7 \pm 3.0
Gly	26.0 \pm 0.1	39.9 \pm 1.4	41.9 \pm 0.6
Ala	59.4 \pm 0.2	54.6 \pm 2.4	52.6 \pm 0.7
Amino nitrogen	25.1 \pm 0.1	25.2 \pm 0.2	26.9 \pm 0.2
EAA/AAT (%)	47.9 \pm 0.4	44.5 \pm 1.2	43.2 \pm 0.4
non-EAA/AAT (%)	52.1 \pm 0.4	55.5 \pm 0.6	56.8 \pm 0.4

¹ Asx: aspartic acid + asparagine

² Glx: glutamic acid + glutamine

4.4.1.2. Structural changes in gastric and intestinal compartments during digestion of IFs

Gastric compartment

Before digestion (0 min, Figure 34A), homogeneous and small particles of proteins and fat droplets were observed for RIF with an unimodal distribution, whereas bimodal distribution was observed for PIF and FIF. In these latter IFs, small fat droplets and protein particles coexisted with larger protein particles, especially in PIF. The modal diameters before digestion (0 min) were 0.6 μm for RIF, 0.8 and 10.0 μm for FIF, and 0.8 and 56.4 μm for PIF (Table 29). After 60 min of digestion, a majority of large aggregated particles of proteins and lipids were observed in the gastric compartment for PIF (31.7 μm modal diameter) and FIF (12.6 μm), whereas no large variations were seen for RIF (0.6 μm). After 120 min of digestion, only large aggregated particles were observed for all IFs, with proteins and lipids co-located (modes of 17.8 μm for RIF, 44.8 μm for PIF, 14.2 μm for FIF), except FIF in which a small proportion of much smaller particles (0.1-0.2 μm) were noticed (Figure 34A).

The bigger particles found in PIF and FIF before digestion (0 min) suggest an incomplete solubilization of plant proteins, but protein aggregation during the technological processes might also play a role. Indeed, heating of globular proteins above their denaturation temperature leads to their unfolding, exposure of hydrophobic patches and irreversible aggregation by forming hydrophobic interactions, hydrogen bonds and/or disulfide bonds (Amagliani & Schmitt 2017; Guo et al. 1998, 1999). Protein aggregation may depend, to a greater or lesser extent, on the protein nature and the physicochemical conditions (pH vs isoelectric point, nature and concentration of salts, etc.), but in any case it influences the protein solubility (Benjamin et al. 2014; Corredig & Dalgleish 1995; Malaki Nik et al. 2009). After 60 min of digestion, the smallest particles initially present in the gastric compartment for PIF and FIF almost totally disappeared and were replaced by even larger aggregated particles, likely due to acidification (Bourlieu et al. 2015; Raikos 2010). After 120 min of digestion, the microstructure of gastric digesta for PIF and FIF slightly differed compared to that at 60 min of digestion. While for RIF, a strong aggregation occurred resulting in only large particles (mode of 17.8 μm), whereas the initial small particles completely disappeared. These observations were in accordance with Nguyen et al. (2018) who compared the static *in vitro* gastrointestinal digestion of dairy and soy protein based IFs. These authors found the same size range values for the dairy IF compared to RIF in our study (0.02 to 3.0 μm at 0 min increasing up to 20 μm at the end of the gastric phase), and the results for soy IF were comparable to the one obtained for PIF in our study (bimodal distribution with a small population of 0.6–1.0 μm and a large population of 1–80 μm in the undigested IF with slight changes in size and aggregation observed at the end of the gastric digestion).

From this, it is clear that the aggregation was delayed for RIF compared to PIF and FIF, most likely due to the protein source specificity. At 120 min of digestion, pH 4.9 was reached in the gastric compartment, close to the isoelectric point of the major cow milk caseins, *i.e.* β -casein and α s-casein (pI 4.2-5.1), and consequently induced casein aggregation in RIF. Since the net charge of the main whey proteins, *i.e.* β -lactoglobulin and α -lactalbumin (pI 5.1-5.2) also decreased in these pH conditions, aggregation and/or interaction between whey proteins and uncharged lipids might also be involved (Adamson et al. 1988; Boirie et al. 1997; Sondheimer et al. 1985). However, some of the major pea proteins have much higher isoelectric point, such as legumin (Uniprot: P15838) with a theoretical pI of 6.11 (calculated from the

amino acid sequence of the protein using the Compute pI/MW tool of ExPASy, see *Table 5 in the Bibliography review section*). Similarly, the legumin (Uniprot: P05190) of faba bean protein has a theoretical pI of 5.78. Thus, the presence in PIF and FIF of proteins which isoelectric points higher than that of whey proteins might explain the earlier aggregation in these IFs during the gastric phase, namely at 60 min of digestion, when pH reached was 5.9. Then, casein aggregation likely occurred in addition during the end of the gastric phase, similarly in all three IFs.

Intestinal compartment

At 60 min of digestion, the largest aggregates observed for PIF and FIF at the same time in the gastric compartment had largely disappeared in the intestinal compartment (Figure 34B). As observed by Ménard et al. (2014), when arriving into the intestine, proteins are instantaneously hydrolyzed which can be explained by a higher solubilisation of the proteins due to the neutralisation of the pH and the large excess of digestive proteases (Ménard et al. 2014). This very likely reflects the intestinal hydrolysis, mostly responsible for the amphiphilic compounds observed on the CLSM images (Figure 34B), and corresponding to various products of digestion (peptides, fatty acids, etc.). Consequently, the modes at 60 min of intestinal digestion were comparable between the three IFs (modes for RIF: 0.2, 1.1, 5.6 μm ; PIF: 0.3, 1.2, 15.8 μm ; FIF: 0.6, 11.2 μm ; Table 29). Even more, the particle size distributions were nearly the same for RIF and PIF (Figure 34B). After 120 min of digestion, the particle size distributions in the intestinal compartment were rather similar among the three IFs with a slight increase compared to at 60 min (modes of RIF: 0.2, 0.6, 15.8 μm ; PIF: 0.2, 0.8, 17.8 μm ; FIF: 0.6, 1.3, 15.8 μm ; Table 29), especially for RIF and PIF. These observations were in accordance with the previously mentioned study of Nguyen et al. (2018) who described a dissolution of the particles in the intestinal phase compared to the gastric phase both for the dairy and the soy based IFs (particle size < 6 μm for the dairy IF, slightly lower than RIF and < 20 μm for soy based IF, comparable to PIF and FIF values).

To sum up, structural differences were mainly observed in the gastric compartment with different aggregation rates and protein particle size between the plant-protein based IFs and the milk-reference based IF. These physical differences during the gastric phase may affect *in vivo* the rate of gastric emptying (Armand et al. 1996; Cavell B. 1981; Henderson et al. 2001) and/or may result in non-homogeneous emptying from the stomach to the intestine. This, in turn, could have an effect on the absorption of nutrients in the upper part of the intestine and global nutrient metabolism in infants. Unfortunately, these phenomena could not be considered in the *in vitro* model used in the present study as the same rate of gastric emptying was applied for RIF, PIF and FIF due to a lack of *in vivo* data on this point.

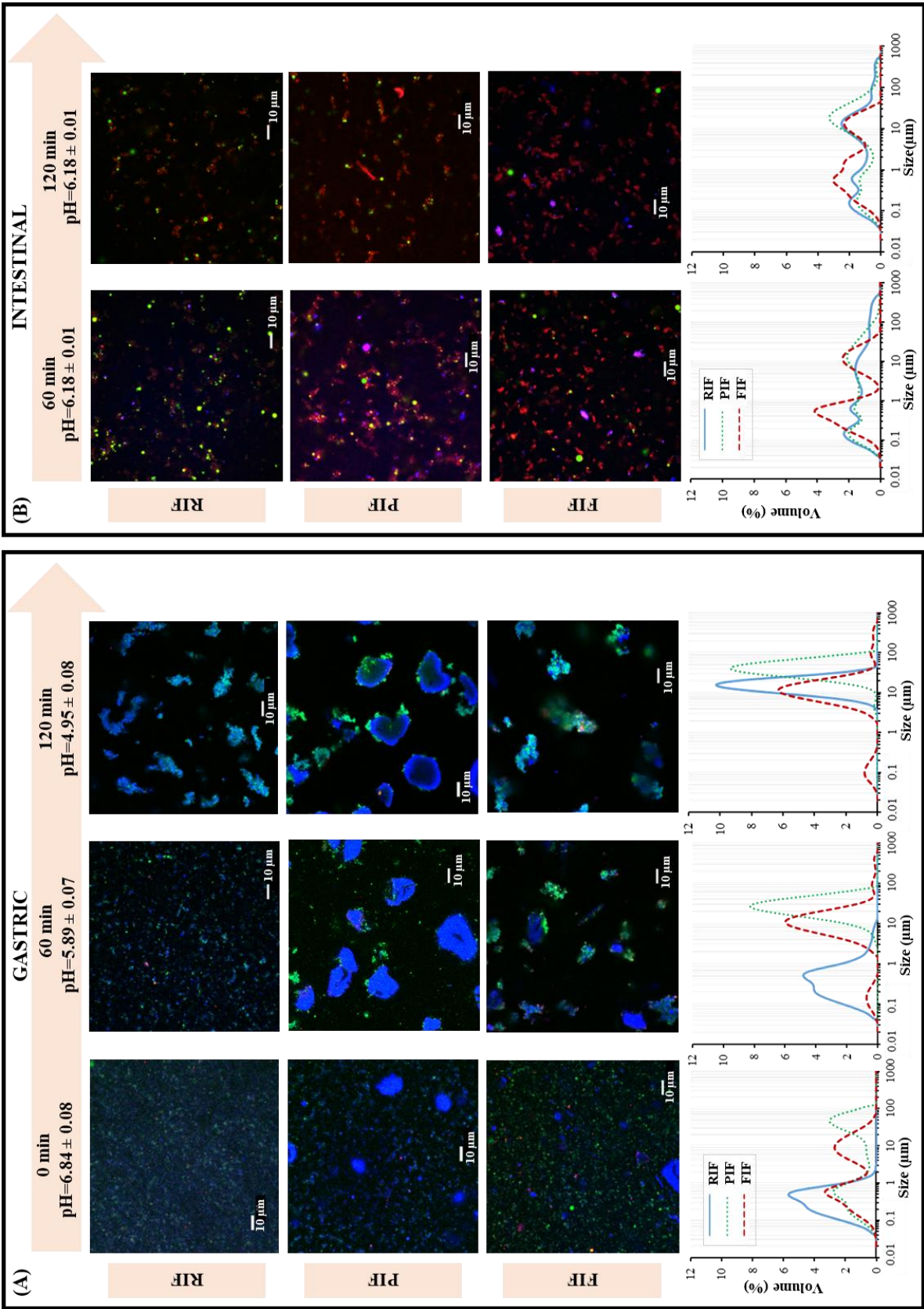


Figure 34. Confocal laser scanning microscopy images of RIF (reference IF), PIF (pea IF) and FIF (faba bean IF) in gastric (A) and intestinal (B) compartments over *in vitro* dynamic digestion and their corresponding particle size distribution profiles, as determined by laser light scattering. Proteins are colored in blue (FastGreen), apolar lipids in green (LipidTox) and amphiphilic compounds in red (Rhodamine-PE).

Table 29. Mode diameters (μm) of RIF, PIF and FIF samples in gastric and intestinal compartments over *in vitro* dynamic digestion.

Infant formula	Compartment and time	Mode 1 (μm)	Mode 2 (μm)	Mode 3 (μm)
RIF	G0	0.6		
	G60	0.6		
	G120	17.8		
	I60	0.2	1.1	5.6
	I120	0.2	0.6	15.8
PIF	G0	0.8	56.4	
	G60	31.7		
	G120	44.8		
	I60	0.3	1.2	15.8
	I120	0.2	0.8	17.8
FIF	G0	0.8	10.0	
	G60	0.2	12.6	
	G120	0.1	14.2	
	I60	0.6	11.2	
	I120	0.6	1.3	15.8

4.4.1.3. Kinetics of proteolysis

The kinetics of proteolysis were determined from the changes in the degree of hydrolysis (DH), defined as the proportion of cleaved peptide bonds (Adler-Nissen 1976). In the gastric compartment, the IF effect was not significant ($p > 0.05$) but there were significant effects of time ($p < 0.001$) and of IF x time ($p < 0.01$). The DH significantly increased from 30 to 180 min, but to a limited extent, as indicated by the DH reached after 180 min of digestion: $4.2 \pm 1.0 \%$, $8.2 \pm 2.0 \%$ and $10.7 \pm 0.7 \%$ for RIF, PIF and FIF, respectively (Figure 35A). These low values of gastric proteolysis can be explained by the reduced pepsin to protein ratio used, coupled with a relatively high gastric pH (pH between 4 and 6, not optimal for pepsin activity, closer to 2), both chosen to mimic infant's stomach conditions. Nevertheless, these DH values were higher than those measured ($< 2\%$) at the end of the gastric phase when *in vitro* digestion was performed in static conditions (Le Roux et al. 2020a), and above all, the three IFs significantly differed with a higher DH value for FIF than PIF, both higher than RIF one. These results highlight differences between the three IFs during the dynamic *in vitro* gastric digestion that were not perceptible in static conditions, and indicate a higher sensitivity to gastric conditions for pea and faba bean proteins compared to whey proteins.

As soon as the chyme entered the intestinal bowl, proteolysis drastically increased for all IFs, to reach $35.9 \pm 2.5 \%$, $44.3 \pm 2.3 \%$ and $49.7 \pm 4.4 \%$ DH after 30 min of digestion for RIF, PIF and FIF, respectively (Figure 34B) after which, DH remained constant in this compartment up to 90 min of digestion ($p > 0.05$). Then, DH slightly increased in the intestinal bowl to finally reach $49.7 \pm 4.2 \%$, $66.3 \pm 4.0 \%$ and $72.8 \pm 5.4 \%$ at 180 min of digestion for RIF, PIF and FIF, respectively. Besides the expected significant effect of time ($p < 0.001$), IF also significantly impacts on DH in the intestinal compartment ($p < 0.001$). Actually, the DH measured in the intestinal compartment significantly differed for the three IFs during the entire digestion (except at 90 min digestion), with DH values systematically higher for FIF than for PIF and RIF, explaining that the IF x time effect was not significant in the intestinal compartment ($p > 0.05$). These differences could be explained by the respective sensitivity of each protein to the

intestinal enzymes, linked to the specificity of these enzymes. The porcine pancreatic enzymes used in the present study contained trypsin, which specifically cleaves carboxyl bonds after basic AAs (lysine and arginine), chymotrypsin, which cleaves carboxyl bonds after aromatic AAs (phenylalanine, tyrosine and tryptophan), and finally carboxypeptidases A and B, which cleave carboxyl bonds after aromatic AAs and basic AAs, respectively. FIF and PIF seemed to contain more aromatic AAs and arginine, whereas cow's milk protein contained more lysine (Supplementary material 1). Thus, the pancreatic enzymes used in the present study would more likely hydrolyse FIF and PIF than RIF. In any event, as with the gastric step of the digestion, the intestinal step exhibited significant differences between the three IFs when performed in dynamic conditions, while no significant differences were measured in static conditions previously (Le Roux et al. 2020a). Moreover, the final DH were higher in dynamic conditions (around 50% up to 73%) than in static conditions (around 42% up to 52%) (Le Roux et al. 2020a).

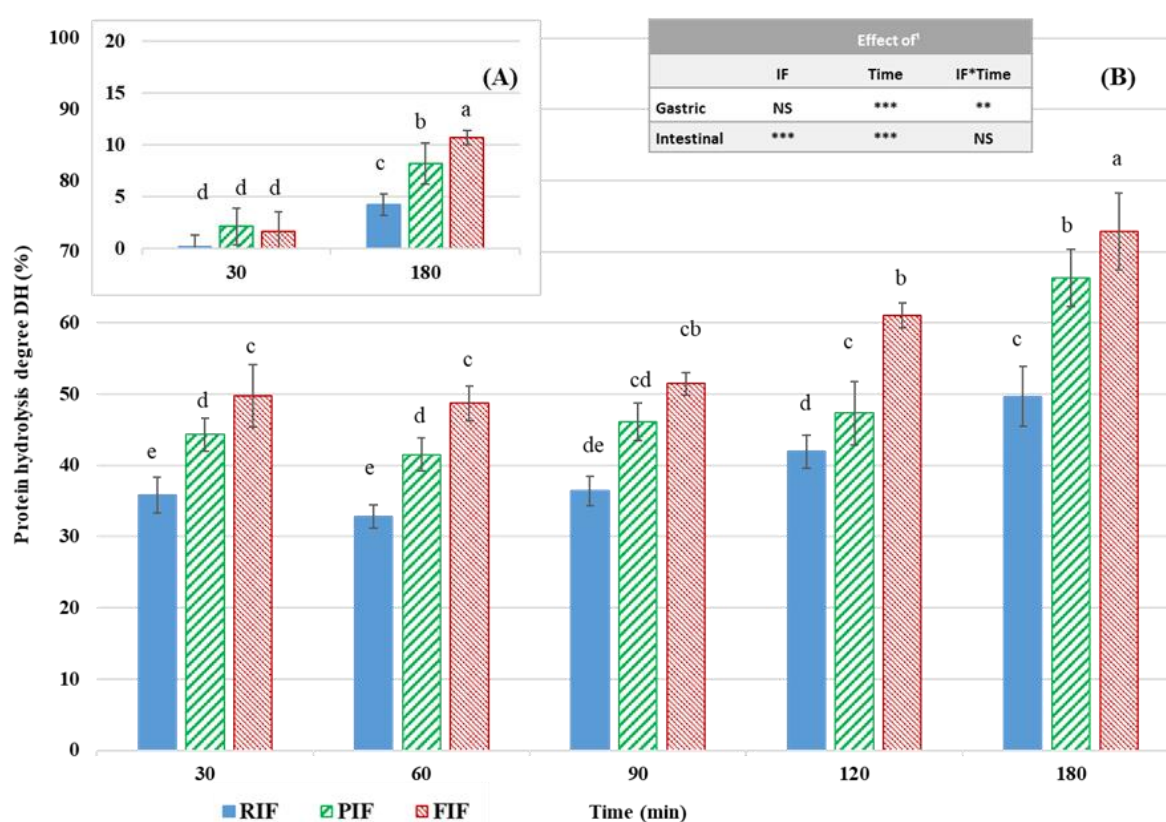


Figure 35. Degree of protein hydrolysis (DH) in gastric (A) and intestinal (B) compartments over in vitro dynamic digestion of RIF (reference IF), PIF (pea IF) and FIF (faba bean IF). Data are means \pm SD ($n=3$). Different superscript letters indicate significant differences ($p < 0.05$). The effects of IF, time and IF \times time in the gastric and intestinal compartments are presented in the insert with statistical significance: $p < 0.001$ (***) ; $p < 0.01$ (**) ; $p < 0.05$ (*) ; $p < 0.1$ (NS).

The difference observed between the static and the dynamic models can be explained by one-time vs continuous enzyme addition that might limit the enzymatic activity decrease, as a result of autolysis; and one whole concentration (in the beaker) vs the progressive emptying of the products of the digestion that might create inhibition of the digestive enzymes by some reaction products (Egger et al., 2019; Gauthier et al., 1982). In any case, the dynamic model is closer to physiological conditions despite the same gastric emptying was applied for the three IFs while structural differences would suggest different

emptying rate *in vivo*, as previously mentioned (Figure 34A, B). And overall, the present study suggests a higher proteolysis for the two plant-based IFs, compared to the reference IF, with the highest value obtained for FIF. Nevertheless, *in vivo* experiments are needed in order to confirm these *in vitro* observations.

4.4.1.4. Essential amino acid bioaccessibility

During the course of the digestion in the intestinal compartment, the bioaccessibility of EAAs progressively and significantly increased with mostly effects of IF and time separately ($p < 0.001$ or $p < 0.05$), whereas the IF x time effect was not significant ($p > 0.05$) (Table 30). This was the case for all EAAs, except for isoleucine where no significant difference was noticed between the three IFs, and for threonine where a significant interaction between IF x time was noticed. In overall, RIF showed significantly higher bioaccessibility for tyrosine, phenylalanine, methionine and cysteine compared to the two plant-based IFs. PIF was similar or slightly higher than RIF for lysine, leucine, histidine and was significantly higher for threonine than RIF and FIF. However, FIF showed the lowest values among the three IFs for lysine, leucine, cysteine and histidine. The three IFs were equivalent for isoleucine and valine. Although comparisons between the three IFs are difficult because EAA bioaccessibility varied from one EAA to another, it suggests a higher quantity of free EAA for RIF compared to both plant-based IFs. This might be seen in contradiction with the higher DH measured for FIF and the lowest for RIF (Figure 35), but it should be kept in mind that the degree of hydrolysis is an indicator of all peptide bond cleavages, regardless it results in free AAs or peptides release, and regardless essential or non-essential AAs are concerned. Lastly, both EAA bioaccessibility and DH should be considered jointly to understand digestion at best.

In the previous study already mentioned (Le Roux et al. 2020a), the differences observed in terms of EAA bioaccessibility between RIF, PIF and FIF after 120 min of intestinal digestion in static conditions, were low and of the same magnitude as those observed in dynamic conditions. However, much higher values of EAA bioaccessibility were obtained in static conditions (from 12 to 88 % for threonine and tyrosine, respectively) compared to the present study (from 7 to 54 % for threonine and tyrosine, respectively). This difference might result from the continuous emptying while digestion occurs in dynamic conditions, whereas all the digestion products remain in the beaker in static conditions. Thus, more concentrated products and more free AAs are expected in static than in dynamic conditions.

Table 30. Bioaccessibility of essential amino acids (EAA) in the intestinal compartment (I) during *in vitro* dynamic digestion for RIF (reference IF), PIF (pea IF) and FIF (faba bean IF); bioaccessibility is expressed as % w/w of total amino acid. Data are means \pm SD (n=3).

EAA	IF	I30	I60	I90	I120	I180	Effect of ¹		
							IF	Time	IF * Time
Lys	RIF ^a	41.6 \pm 1.3	46.2 \pm 1.2	48.4 \pm 3.9	55.0 \pm 2.4	65.3 \pm 3.4			
	PIF ^{ab}	38.1 \pm 4.7	41.7 \pm 2.1	46.6 \pm 1.3	49.4 \pm 1.0	56.6 \pm 3.4	***	***	NS
	FIF ^b	33.6 \pm 1.5	36.0 \pm 2.1	42.3 \pm 2.2	45.3 \pm 2.1	54.0 \pm 0.7			
Tyr	RIF ^a	53.4 \pm 1.1	55.0 \pm 6.2	56.6 \pm 7.5	54.3 \pm 1.8	76.4 \pm 3.3			
	PIF ^b	42.5 \pm 4.8	44.6 \pm 1.6	50.0 \pm 0.7	52.1 \pm 0.6	58.2 \pm 3.5	***	***	NS
	FIF ^c	40.7 \pm 0.6	43.4 \pm 1.2	49.8 \pm 1.9	51.5 \pm 1.1	61.7 \pm 0.7			
Phe	RIF ^a	35.9 \pm 3.0	42.4 \pm 4.5	42.3 \pm 3.5	48.1 \pm 5.7	57.6 \pm 3.8			
	PIF ^b	24.8 \pm 4.4	29.8 \pm 2.9	34.9 \pm 1.4	35.9 \pm 1.1	42.9 \pm 4.5	***	***	NS
	FIF ^c	23.2 \pm 1.1	27.8 \pm 2.5	33.1 \pm 0.7	36.0 \pm 1.4	43.9 \pm 1.9			
Leu	RIF ^a	19.2 \pm 0.2	23.6 \pm 0.8	24.7 \pm 3.1	29.4 \pm 1.8	38.7 \pm 2.2			
	PIF ^{ab}	18.9 \pm 3.0	22.0 \pm 1.8	25.8 \pm 1.5	28.4 \pm 1.3	37.8 \pm 2.9	***	***	NS
	FIF ^b	13.4 \pm 0.8	16.5 \pm 1.4	21.0 \pm 1.6	24.1 \pm 1.6	33.2 \pm 0.8			
Ile	RIF ^a	6.2 \pm 0.3	7.5 \pm 0.4	7.9 \pm 1.4	10.0 \pm 0.9	14.8 \pm 0.5			
	PIF ^a	6.4 \pm 1.2	7.1 \pm 0.6	8.8 \pm 1.0	10.3 \pm 0.8	18.1 \pm 1.3	NS	***	NS
	FIF ^a	5.7 \pm 0.1	6.3 \pm 0.4	8.2 \pm 0.6	9.7 \pm 0.7	13.5 \pm 0.5			
Met	RIF ^a	16.9 \pm 0.7	18.0 \pm 1.9	19.1 \pm 2.0	22.9 \pm 1.4	26.5 \pm 1.7			
	PIF ^b	12.6 \pm 1.2	12.8 \pm 1.6	15.5 \pm 1.0	16.6 \pm 0.3	18.9 \pm 0.3	***	***	NS
	FIF ^c	11.5 \pm 1.1	12.3 \pm 0.7	14.7 \pm 0.6	17.4 \pm 1.4	21.1 \pm 1.7			
Cys	RIF ^a	22.3 \pm 1.8	26.2 \pm 5.2	27.7 \pm 5.3	29.3 \pm 0.9	18.1 \pm 0.9			
	PIF ^b	9.6 \pm 5.2	14.5 \pm 3.5	17.3 \pm 4.3	15.9 \pm 4.4	14.6 \pm 2.4	***	**	NS
	FIF ^c	4.7 \pm 2.0	10.3 \pm 4.8	9.4 \pm 2.4	16.5 \pm 5.6	12.2 \pm 2.5			
Val	RIF ^a	8.2 \pm 0.2	10.4 \pm 0.9	10.4 \pm 1.6	13.3 \pm 1.5	18.5 \pm 0.1			
	PIF ^a	8.5 \pm 1.2	9.9 \pm 0.3	11.4 \pm 0.8	12.9 \pm 0.6	19.4 \pm 1.9	*	***	NS
	FIF ^a	7.4 \pm 0.2	8.0 \pm 0.4	10.2 \pm 0.5	11.5 \pm 0.4	16.5 \pm 0.3			
His	RIF ^a	9.6 \pm 0.3	7.6 \pm 0.3	7.7 \pm 0.7	9.6 \pm 1.0	16.2 \pm 1.2			
	PIF ^{ab}	10.2 \pm 3.5	7.3 \pm 0.5	8.6 \pm 0.8	9.5 \pm 0.7	13.5 \pm 2.3	*	***	NS
	FIF ^b	7.2 \pm 0.6	6.2 \pm 0.6	7.0 \pm 0.6	8.2 \pm 0.1	10.4 \pm 0.6			
Thr	RIF ^b	6.8 \pm 0.1 ^b	6.7 \pm 0.3 ^a	6.4 \pm 1.0 ^b	7.5 \pm 0.5 ^b	11.0 \pm 1.1 ^b			
	PIF ^a	8.6 \pm 0.6 ^a	7.5 \pm 0.4 ^a	8.1 \pm 0.6 ^a	9.1 \pm 0.9 ^a	14.1 \pm 1.3 ^a	***	***	*
	FIF ^{ab}	7.9 \pm 0.4 ^b	6.6 \pm 0.3 ^a	7.4 \pm 0.2 ^b	8.3 \pm 0.2 ^{ab}	10.8 \pm 0.3 ^b			

¹ Statistics were conducted on data from I30 to I180. Statistical significance: p<0.001 (***); p<0.01 (**); p<0.05 (*). In case of a significant meal effect or of a significant interaction effect (IF*Time), multiple comparisons of means were conducted and differences between meals are indicated by a different superscript letter (p<0.05), either in the IF column or at a given time point. Differences over time are not represented

4.4.1.5. Size distribution of soluble nitrogen fraction and *in vitro* apparent protein digestibility of IFs

Size distribution of soluble nitrogen fraction

Thanks to centrifugation, size exclusion chromatography and micro-Kjeldhal methods, the nitrogen fraction of *in vitro* digestas was categorised into seven classes according to solubility and molecular size (Figure 36). Around 15% of the total nitrogen was in the insoluble fraction, whereas around 80% corresponded to soluble peptides smaller than 10 kDa and free AAs. Nitrogen fraction smaller than 10

kDa was considered as potentially absorbable form by the intestinal epithelium (Cave 1988; Huang et al. 2000; Moughan 1999). The majority of the nitrogen was in the 5-2 kDa class. There was no difference between the three IFs, except for FIF, which showed significantly more nitrogen in the soluble class higher than 10 kDa (0.5 point more) and less nitrogen in the free AA fraction (2 to 3 points less), in accordance with the slightly lower EAAs release observed after digestion of FIF (Table 30). It should be noted that about 95% of the soluble nitrogen released from the three IFs was digested into either small peptides (< 10 kDa), that could potentially be reduced by the brush border enzymes into absorbable di or tri-peptides, or free AAs that are directly absorbable by the intestinal epithelium (Cave 1988; Moughan et al. 1999; Huang et al. 2000).

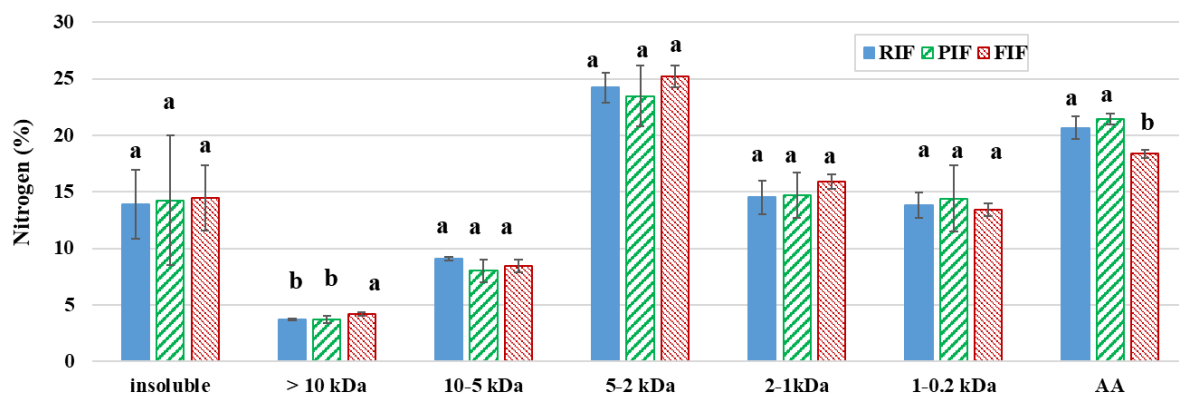


Figure 36. Nitrogen molecular size distribution (%) of RIF (reference IF), PIF (pea IF) and FIF (faba bean IF) determined at the end of *in vitro* dynamic digestion (in intestinal compartment and intestinal emptied fraction mixed). Data are means \pm SD (n=3). Values with a different superscript letter are significantly different ($p < 0.05$).

In vitro apparent protein digestibility

When *in vitro* digestion models are used to simulate the complex *in vivo* conditions of digestion, it is recognized that the apparent digestibility of protein can be estimated by calculating the percentage of the nitrogen present in the fraction of soluble peptides smaller than 10 kDa, as suggested by the previously mentioned authors, namely Cave (1988), Moughan et al. (1999) and Huang et al. (2000). This last author used a dialysis tubing that retains molecular compounds of 12 kDa (Huang et al. 2000), close to the 10 kDa chosen in our study.

In the present study, the apparent protein digestibility calculated was 74.9 ± 6.7 , 89.2 ± 3.9 and 91.1 ± 3.1 for PIF, RIF and FIF, respectively (Table 31). The limiting EAAs are the aromatic AAs for RIF and isoleucine for PIF and FIF, with resulting PDCAAS-like scores of 67.5 ± 6.0 , 75.4 ± 2.5 and 76.1 ± 3.3 for PIF, FIF and RIF, respectively. PIF showed significantly lower *in vitro* digestibility compared to RIF and FIF and a lower PDCAAS-like score compared to RIF. The lower value obtained for PIF results mainly in a lower quantity of nitrogen recovered in the intestinal compartment and in the emptied fraction after 180 min of digestion compared to the nitrogen introduced into the digester. This may be associated with the large aggregates observed in the gastric compartment for PIF (Figure 34A) which also adsorbed on the wall of the digester (visual observation), resulting in a smaller quantity transferred into the intestinal bowl. In contrast, FIF was not significantly different from RIF for both *in vitro* digestibility and PDCAAS-like score.

Rudloff & Lonnerdal (1992) studied the *in vitro* digestion of different IFs and determined a mean protein digestibility of 78.5 ± 4.2 % for powder IFs (cow milk-based), a bit lower than our results for RIF and FIF, but closer to PIF values. Similarly, Nguyen et al. (2015) reported *in vitro* digestibility of 81.5 ± 0.04 % and 76.4 ± 0.04 % for a standard cow milk-based IF and a soy-based IF, respectively. Ulloa et al. (1988) tested chickpea protein concentrate as a potential milk substitute for follow-on IFs and measured an apparent protein digestibility (determined *in vivo* in growing rats) of 88.0 % compared to 93.0 % for a milk-based IF. Therefore, all these results are in agreement with the present results obtained for RIF and FIF, but somewhat higher than that for PIF, thereby confirming the high nutritional quality of faba bean proteins, and to some lower extent of pea proteins, with the aim of partially substituting whey proteins with plant proteins in IFs. Moreover, this means also that the method proposed in the present study to estimate protein digestibility using *in vitro* digestion coupled with size exclusion chromatography (with a threshold of 10 kDa) seems to be relevant since the results are in accordance with the literature data including *in vitro* and *in vivo* results.

Table 31. Limiting essential amino acid (LEAA), amino acid score (AAS), apparent *in vitro* digestibility (nitrogen fraction < 10 kDa) and PDCAAS-like score for RIF (reference IF), PIF (pea IF) and FIF (faba bean IF). Data are means \pm SD (n=3). Different superscript letters indicate significant differences ($p < 0.05$).

Infant Formulas	LEAA	AAS	Apparent <i>in vitro</i> digestibility (%)	PDCAAS-like (%)
RIF	AAA*	0.85	89.2 ± 3.9^a	76.1 ± 3.3^a
PIF	Ile	0.90	74.9 ± 6.7^b	67.5 ± 6.0^b
FIF	Ile	0.83	91.1 ± 3.1^a	75.4 ± 2.5^{ab}

*AAA corresponds to aromatic amino acids (methionine + cysteine)

4.4.2. Conclusion

This study aimed to investigate the digestion of two plant-based IFs (PIF and FIF) compared to a dairy reference IF (RIF) using an *in vitro* dynamic model simulating infant digestion. In terms of microstructure, changes were observed only during gastric digestion with a faster aggregation for PIF and FIF, compared to RIF, likely due to specific sensitivity to pH drop.

Very limited proteolysis was noticed during gastric phase, whereas all three IFs were extensively hydrolysed as soon as the chyme were submitted to the pancreatic proteases. Surprisingly, FIF showed higher DH value than RIF and PIF in the intestinal compartment after 3 hours of digestion whereas no significant difference was observed between these three IFs in static conditions in a previous study (Le Roux et al. 2020a).

Finally, FIF and RIF showed similar *in vitro* apparent protein digestibility and PDCAAS-like score, higher than PIF. Thus, faba bean protein could be a good candidate to partially replace whey protein in IFs without altering the nutritional quality, and ensuring balanced nutrients intake compared to the infant needs. However, pea protein showed a lower protein digestibility than the reference under *in vitro* dynamic digestion and might likely need better dispersion to improve its nutritional properties. Nevertheless, the digestion model used in the present study, as well as the calculation chosen present

some limits and need to be completed with *in vivo* studies in order to come even closer to infant physiological conditions and to confirm these promising results.

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HIGHLIGHTS

This last chapter dealt with the *in vitro* digestion of pea (PIF) and faba bean (FIF) IFs compared to the milk reference IFs (RIF) using an *in vitro* dynamic model simulating infant digestion. Despite microstructure differences highlighted during the gastric digestion, the end of the intestinal digestion showed similar structure between the three IFs. Surprisingly, hydrolysis degree (DH) was higher for FIF than for RIF and PIF, a difference that was not observed in static conditions (section 4.2.2.4.). Finally, FIF and RIF showed similar *in vitro* apparent protein digestibility, both higher than PIF due to solubility impairments of PIF during digestion that might induced matter losses and therefore lower its digestibility. Nevertheless, these experiments need to be completed with *in vivo* studies in order to measure the nutritional quality of pea and faba bean IFs in even more realistic conditions (closer to infant physiological conditions) and to confirm these promising results.

5. General discussion & Perspectives

5.1. General discussion

This PhD project aimed at consider possibilities in the field of innovation in IFs in which whey protein concentrate would be replaced by plant protein sources. Namely, this project investigated the influence of protein source on the functional properties and the digestibility of innovative IFs compared to a dairy protein IF (reference).

Firstly, four plant protein sources (i.e., pea, faba bean, rice and potato) were selected mainly in order to comply with the nutritional requirements of infant. In addition, they should also be free of any organoleptic defects or antinutritional factors (ANF) and be commercially available in purified form. The four plant-based IFs as well as the dairy reference IF were manufactured at a pilot scale and digested using an *in vitro* static model simulating infant digestion. After these first screening experiments, it was decided to exclude rice and potato proteins as they both showed functional and nutritional deficiencies in comparison to the reference IF. However, pea and faba bean IFs showed results closer to the reference IF.

The next step consisted in scaling-up the manufacturing of pea, faba bean and the reference IFs. It was mainly concluded that pea and faba bean IFs were difficult to disperse and led to insoluble particles in solutions all along the IF manufacturing, as well as in the final reconstituted IF. Despite their poor solubility, emulsions with similar stability and equivalent physicochemical properties were obtained with the two plant protein IFs and the dairy reference IF.

Last, the digestibility of the three different IFs was studied in a frame to come closer to physiological conditions using an *in vitro* dynamic model and to compare some relevant indicators of protein nutritional quality between pea, faba bean and the dairy proteins used to formulate IFs (PIF, FIF and RIF, respectively). Overall, a faster aggregation was observed for the two plant-based IFs compared to RIF in the gastric compartment, whereas the same microstructure was obtained at the end of the digestion. A higher final proteolysis (DH) was found for FIF (72.8%) and PIF (66.3%) compared to the RIF (49.7%). Finally, FIF and the reference RIF showed similar apparent protein digestibility (89.2 and 91.1%) and PDCAAS-like score (76.1 and 75.4%), both higher than PIF (74.9 and 67.5%, for protein digestibility and PDCAAS, respectively). Thus, faba bean protein was considered as a good candidate to partially replace whey protein in IFs. However, pea protein in IF showed a lower protein digestibility than the reference IF under *in vitro* dynamic digestion mainly due to the lack of solubility of pea protein. Therefore PIF might likely need better dispersion to improve its nutritional properties.

In any case, these promising results need to be completed both with process optimisations and with *in vivo* studies in order to come even closer to industrial realities and physiological conditions, as well as to confirm the aptitude of such plant proteins to partially replace dairy protein in 1st age IFs.

As mentioned, the originality of our findings consisted mainly in bringing information about future possibilities in the field of innovative IFs using a multi-scale process and digestion approaches. Indeed, studies in infant nutrition have focused mainly on the protein sources currently allowed by the European regulation for 1st age IF, *i.e.* cow milk, goat milk, soy or hydrolysed rice proteins (Bourlieu et al. 2015; Bouzerzour et al. 2012; Chatterton et al. 2004; Hodgkinson et al. 2018, 2019; Lonnerdal 2014; Maathuis et al. 2017; Nguyen et al. 2015; Reche et al. 2010; Sakai et al. 2000; Wada & Lönnerdal 2015a). Other authors worked on the use of plant proteins in follow-on IFs (Kent & Doherty 2014; Khan et al. 2013; Malunga et al. 2014; Ulloa et al. 1988) or on 1st age IF based on potato protein [WO2018 115340 (A1)]. However and to the best of our knowledge, no information concerning the digestive and techno-functional behaviour of new protein sources in 1st age IFs was available to date. Our findings bring some insights that may be useful for future considerations on new protein sources used to formulate 1st age IFs.

In order to combine results and to offer more refined and consolidated conclusions about IF manufacturing, the key parameters and the main results obtained at pilot and semi-industrial scales are summarized in Table 32. The comparison between the digestion of proteins in solution and the proteins in IFs using *in vitro* static digestion model is summarized in Table 33. Lastly, the *in vitro* static and dynamic approaches to study IF digestion are summarized in Table 34.

5.1.1. Scale-up effect during IFs manufacturing of IFs

As observed in Table 32, the manufacturing of the five IFs at pilot scale highlighted two key issues: on the one hand, significant matter losses were obtained with the rice IF (2 to 3 points below the other IFs for protein and fat contents) due to poor solubility during manufacturing. On the other hand, a very high viscosity was obtained for the potato IF (5 Pa.s), much higher than the recommended value for an optimal spraying ability prior to drying (0.2 Pa.s maximum). Therefore, it was decided to exclude the rice and potato IFs that showed several impairments in terms of manufacturing and physicochemical properties whereas the pea and faba bean IFs behaved more closely to the reference IF.

In comparison, the manufacturing at semi-industrial scale for the reference, pea and faba bean IFs (RIF, PIF and FIF, respectively) led to higher fat content (23.3 vs 21.1 % fat in IFs at semi-industrial vs pilot scales, whereas 21% fat content was expected from the formulation). This slight difference might come from the lack of accuracy of the method used to quantify fat content in the samples. In fact, this method is usually used to analyse dairy fat-based products more than vegetal fat-based products, thus made it difficult to accurately determine fat content in the vegetal oil fat based IFs. Then and as expected, almost **all physicochemical parameters were improved after semi-industrial manufacturing of IFs**, with lower free fat released, larger powder particle size, higher solubility and dispersibility indexes as well as water activity closer to 0.2 and higher Tg values (Table 32). This improvement can be explained by the implementation of controlled operating parameters on a well-designed equipment that let to reach optimal parameters, closer to industrial conditions. Only the viscosity of PIF was found higher after semi-industrial manufacturing. Regarding the color parameters, it seems that the IF powders produced at semi-industrial scale have a lower L value (brightness) than the one at pilot scale. The L value is expected to decrease as browning occurs (Subhashree et al. 2017). The browning index (BI) was

calculated (details of calculation in Annexes section 6.2) and was effectively higher for the IF powders produced at a semi-industrial scale. This means that the process parameters and/or equipments used at this scale caused a bit more damages on the powders, and in this case, Maillard reaction did probably occur. This observation could be confirmed by analyzing some of the Maillard reaction products such as furosine.

All together, these findings highlighted a **scale-up effect on the manufacturing of the IFs between pilot and semi-industrial scales**. Although some physicochemical properties still need improvements after semi-industrial manufacturing, notably to enhance the poor dispersion of pea and faba bean proteins, it seems that the IFs produced at semi-industrial scale with these latter finally matched with the reference IF in terms of physicochemical and functional properties, and could thus provide satisfactory IFs quality. As mentioned, **these experiments still need to be further extended through process optimization and industrial development in order to confirm and improve these first promising findings**.

Table 32. Pilot vs semi-industrial scale manufacturing of IFs (RIF: reference IF; PIF: pea IF; FIF: faba bean IF). SI: solubility index; DI: dispersibility index.

Manufacturing scale	Pilot	Semi-industrial ¹
Infant formula	Reference, pea, faba bean, rice, potato IFs.	RIF 1, PIF 1, FIF 1.
Process parameters		
<i>Pasteurization</i>	Pasteurization: 35s 80°C.	
<i>Concentration</i>	Concentration: 70 kg/h 60°C.	Concentration: 280 kg/h 60°C.
<i>Homogenization</i>	Homogenization: 8/2 MPa 60°C.	
<i>Drying</i>	Drying: 170°C – 70°C and 3.25 kg/h.	Drying: 165-75°C and 90 kg/h.
Composition	Similar composition for all IFs.	
<i>Protein</i>	Except for rice IF with lower protein and fat contents due to high insolubility during manufacturing resulting in matter losses. 20.1 ± 0.1 % fat (mean value without rice IF).	
<i>DM</i>		
<i>Ash</i>		
<i>Fat</i>		
Physicochemical properties		
<i>Free fat</i>	Free fat > 5% for all IFs and > 20 % for rice IF.	Free fat release < 2.6 % for all IFs but increase after 4 months storage for PIF (still < 5%).
<i>Viscosity² d(0.5)</i>	Higher viscosity for potato IF (5 Pa.s>0.2). Low particle size (35 µm < 150 µm).	High viscosity for PIF (0.8 Pa.s > 0.2 Pa.s). High/standard particle size (106-142 µm ~150 µm).
<i>SI</i>	SI 97.0 ± 2.7 % > 90%.	SI 98.2 ± 1.6 % > 90%.
<i>DI</i>	DI 89.8 ± 4.1 % > 85%.	DI 99.3 ± 0.5 % > 85%.
<i>a_w</i>	<i>a_w</i> 0.15 ± 0.03 < 0.2.	<i>a_w</i> 0.19 ± 0.03 = 0.2.
<i>Tg (at a_w 0.2)</i>	Tg 49.4 ± 2.1°C.	Tg 58.7 ± 4.3°C.
Color		
<i>L (brightness 0-100)</i>	Bright for all IFs (73-76); darker for potato IF (66).	Darker for all IFs (69-70).
<i>a (-60 green to +60 red)</i>	a 0.8 (potato); -2.0 (rice); -3.8 (faba bean); -2.3 (pea); -3.1 (reference).	a -4.0 (FIF); -2.0 (PIF); -4.4 (RIF).
<i>b (-60 blue to +60 yellow)</i>	b 11.0 (potato); 9.5 (rice); 15.9 (faba bean); 13.3 (pea); 9.8 (reference).	b 16.5 (FIF); 14.7 (PIF); 12.4 (RIF).
<i>BI (Browning Index)³</i>	BI 18.6 (potato); 11.5 (rice); 19.9 (faba bean); 17.2 (pea); 10.4 (reference).	BI 21.9 (FIF); 21.0 (PIF); 14.2 (RIF).

Structure		
<i>CLSM</i>	N.A.	Bigger particles in PIF and FIF compared to RIF, but seems to be reduced thanks to the process.
<i>Particle size distribution</i>		Similar emulsion stability.
<i>Emulsion stability</i>		
Main conclusions	<p>Manufacturing issues noticed for rice IF and potato IF with high insolubility and viscosity, respectively → Rice and potato proteins sources excluded.</p> <p>Fat content differences (analysis method not adapted to vegetal fat based products). Higher free fat at pilot vs semi-industrial scale. Smaller powder particle size at pilot scale. Higher/better Tg and a_w values at semi-industrial scale. Better solubility and dispersibility after semi-industrial scale. Higher viscosity for PIF after semi-industrial scale. Powder darker and higher BI at semi-industrial scale.</p> <p>Scale-up effect: improvement of almost all physicochemical properties after semi-industrial manufacturing of RIF, PIF and FIF compared to the same IFs produced at pilot scale.</p>	Same physicochemical properties, except higher viscosity for PIF and bigger particle size/poor dispersion of PIF and FIF during manufacturing.

¹results corresponded to samples collected during processing routes 1 (Figure 25).

²viscosity measured in the concentrate after homogenization and prior to drying.

³browning index was calculated for each IF powders (see Annexe 6.2, Table 37).

5.1.2. Proteins in solution vs IFs showed a great matrix and process effects when subjected to *in vitro* static digestion

As presented in Table 33, one or more EAAs were limiting in the plant protein sources compared to the infant requirements (EU 2016), whereas the reference protein (whey protein concentrate: WPC) showed a balanced **EAA profile**. However, when the protein sources were used to formulate IFs at a pilot scale, all EAA profiles seemed to be balanced compared to the infant needs. Moreover, it seems that WPC and potato protein isolate (PPI) contained more EAAs compared to pea protein concentrate (PPC), faba bean protein concentrate (FPC) and rice protein concentrate (RPC) for which more Non-EAAs were found.

In terms of **proteolysis (DH)**, the proteolysis was very limited during gastric phase with values lower than 3 % regardless of the meal (protein in solutions or IFs). However, DH increased in the intestinal digestion for all meals, but higher final DH values were obtained for IFs compared to the protein alone in solutions. The reference and pea showed higher DH values than rice and potato, both in protein solutions as well as in IFs. On the other hand, faba bean IF showed similar DH value than the reference and pea IFs whereas it was found lower after digestion in protein solution form.

The **EAA bioaccessibility** showed higher EAA release after IFs digestion compared to the protein in solutions. In any case, the reference showed higher EAA content than faba bean, rice and potato IFs. Pea showed similar results than the reference after IFs digestion and faba bean showed an intermediate behaviour, close to pea but lower than the reference. Moreover, potato showed very limited EAA bioaccessibility, at least much lower than the other protein sources in solution or in IFs.

Altogether, these findings highlight a great matrix and manufacturing effects since **DH and EAA bioaccessibility were found higher after the digestion of IFs than after the one of proteins alone in solution**. This could mainly be explained by the presence of other ingredients added to formulate IFs that were not present in the solution solely containing the different protein sources. In particular, as half

of the proteins came from good quality dairy proteins, the overall nutritional quality of all IFs was thus improved. Moreover, the addition of fat was found in droplets form in IFs (after homogenization), coming with a majority of the proteins adsorbed at the interface of these fat droplets in order to stabilize the emulsion, and therefore the proteins might be more accessible for the enzymes and prone to higher proteolysis (Bourlieu et al. 2015; Nguyen et al. 2018). However, it was **not possible to isolate the matrix effect with regard to the potential process effect on the protein digestibility**, since the formulation and the manufacturing of IFs were successively realised and no analysis were conducted on the IFs prior to the process in order to make this distinction possible.

Table 33. *In vitro* static digestion of protein in solutions compared to IFs in terms of composition and digestibility indicators. WPC: whey protein concentrate; PPC: pea protein concentrate; RPC: rice protein concentrate; FPC: faba bean protein concentrate; PPI: potato protein isolate. NEAA: non-essential amino acids.

Digestion model	<i>In vitro</i> static digestion	
Meal	WPC, PPC, FPC, RPC, PPI Proteins in solution	Reference IF, pea IF, faba bean IF, rice IF, potato IF Infant formulas (produced at pilot scale).
Composition	Compared to the European regulation: deficiencies in SAA and Thr for PPC and FPC solutions; Thr and Lys for RPC solution; SAA for PPI.	Compared to the European regulation: all IFs presented balanced EAAs compared to the requirements.
AA profile	WPC and PPI contained more EAAs (52 and 51%, respectively) whereas PPC, FPC and RPC contained more NEAAs (57, 60 and 56 %, respectively).	Potato IF contained half EAAs and NEAAs whereas the reference, pea, faba bean and rice IFs contained slightly more NEAAs (51, 54, 55 and 54 %, respectively).
Proteolysis (DH) <i>Gastric (end)</i>	Very low DH (< 2-3%).	
<i>Intestinal (end)</i>	WPC: 37.8 ± 1.8 % PPC: 42.5 ± 1.2 % FPC: 24.9 ± 3.8 % RPC: 16.4 ± 5.1 % PPI: 13.2 ± 2.7 % WPC = PPC > FPC = RPC = PPI.	Reference IF: 47.0 ± 4.4 % Pea IF: 51.4 ± 3.2 % Faba bean IF: 42.2 ± 3.3 % Rice IF: 33.3 ± 4.3 % Potato IF: 28.8 ± 3.3 % Reference IF=Pea IF=Faba IF>Rice IF=Potato IF.
EAA bioaccessibility <i>At the end of the intestinal phase</i>	Free EAA/ Total EAA (%) range: WPC: 13.2-77.5 % PPC: 15.4-47.5 % FPC: 8.9-54.6 % RPC: 15.7-73.6 % PPI: 2.5-6.8 % WPC>PPC=FPC=RPC>PPI.	Free EAA/ Total EAA (%) range: Reference IF: 12.4-88.7 % Pea IF:15.7-88.1 % Faba bean IF:11.8-84.3 % Rice IF:10.8-72.1 % Potato IF:1.8-25.2 % Reference IF=Pea IF>Faba IF>Rice IF>Potato IF.
Main conclusions	PPC and the WPC similar in terms of DH; lower for FPC, RPC and PPI. Higher EAA bioaccessibility for WPC, then PPC; lower for FPC and RPC and even lower for PPI.	Reference IF, pea IF and faba bean IFs similar DH > rice and potato IFs. Close EAA bioaccessibility between the reference, pea and faba bean IFs. Rice: intermediate results. Potato: very limited (because of trypsin inhibitor activity mainly).
Higher EAA than NEAAs in IFs than in protein solutions. Similar DH during gastric phase. Higher DH value at the end of intestinal digestion for IFs than proteins in solution. Higher EAA bioaccessibility at the end of intestinal digestion for IFs than proteins in solution.		
Matrix combined to process effects: improvements of almost all protein nutritional quality indicators after <i>in vitro</i> static digestion of IFs compared to the proteins in solution.		

5.1.3. Static vs dynamic *in vitro* digestion of IF

An *in vitro* static model elaborated by the INFOGEST network (Brodkorb et al. 2019; Minekus et al. 2014) for adult physiological conditions and adapted to infant digestion by Ménard et al. (2018) has been widely used to study digestion. It should be considered essentially as screening techniques to identify key parameters that may affect the hydrolysis of food macronutrients. Although static models are simple and easy to use, they are not very physiologically relevant as it is difficult to recreate the complexity of the digestive tract in beakers. In order to get closer to the physiological reality, *in vitro* dynamic digestion models have been developed and adapted to infant conditions and further validated *in vivo* (Ménard et al. 2014). These models are much more sophisticated than the static ones, *i.e.* include pH regulation, dynamic flows of food and controlled concentration of digestive enzymes in the different compartments.

Table 34 shows comparisons between *in vitro* static and dynamic digestions of pea, faba bean and the reference IFs (produced at pilot or semi-industrial scales). As expected because of their similar composition, all three IFs produced at a pilot scale or at a semi-industrial scale showed balanced EAA profile compared to the infant requirements (EU, 2016). Slightly higher EAAs were found for the reference IF compared to pea and faba bean IFs, for which more non-EAAs were found (Table 35).

As previously described, very limited proteolysis was observed at the end of the gastric digestion in static conditions (<2%). However, gastric proteolysis was found higher in dynamic conditions reaching DH values between 5 to 11 %. Similarly, the final DH in intestinal compartment were higher in dynamic conditions (49.7, 66.3 and 72.8% for RIF, PIF and FIF, respectively) than in static ones (47.0, 51.4 and 42.2% for the reference, pea and faba bean IFs, respectively). In any case, the gastric and the intestinal steps showed significant differences between the three IFs when performed in dynamic conditions, while no significant differences could be highlighted in static conditions. This difference observed between the static and the dynamic models may be explained by single vs continuous enzyme addition: the latter mode might limit the enzymatic activity decrease observed in the first mode, as a result of autolysis. Moreover, single batch concentration (in the beaker) might create inhibition of the digestive enzymes by some reaction products compared to progressive emptying of the products of the digestion (Egger et al. 2019). This phenomenon was also confirmed by Gauthier et al. (1982) who suggested that the increased frequency of buffer replacement and increased emptying rate may reduce the inhibition of enzyme action by digestion products. This confirmed that within dynamic conditions, the enzyme inhibition should be lower than in static conditions, and thus explained the higher DH values obtained after *in vitro* dynamic digestions.

Moreover, much higher values of EAA bioaccessibility were obtained in static (from 12 to 88 % for threonine and tyrosine, respectively) compared to dynamic conditions (from 7 to 54 % for threonine and tyrosine, respectively), regardless of the IF. This difference might result from the continuous emptying of the digestive content occurring in dynamic conditions, whereas all the digestion products remained in the beaker in static conditions. Thus, more concentrated products and more free AAs content were expected in static than in dynamic conditions.

In any case, the dynamic model was closer to physiological conditions despite the same gastric emptying was applied for the three IFs, while structural differences would suggest different emptying rates *in vivo*, as previously mentioned. The dynamic digestion results suggested a higher proteolysis for the two plant-based IFs compared to the reference RIF, the higher value being obtained for FIF. Finally, similar *in vitro* apparent protein digestibility was found for RIF and FIF, both significantly higher than PIF.

However, it should be mentioned that the *in vitro* digestion protocols used in the present study did not mimic the enzymes of the brush border intestinal membrane that would further hydrolysed nutrients before their absorption in the bloodstream (Woodley 1994). Therefore, *in vivo* experiments are needed in order to confirm and complete these first findings.

Table 34. Pea, faba bean and the reference dairy IFs showed different digestibility indicators after *in vitro* static vs dynamic digestions. NEAA: non-essential amino acids.

Digestion model	<i>In vitro</i> static	<i>In vitro</i> dynamic
Meal	Reference IF, pea IF, faba bean IF produced at pilot scale .	RIF, PIF, FIF produced at semi-industrial scale (route 1) .
Digestion conditions		
<i>Enzymes</i>	Static secretion (one-time).	Dynamic secretion flow.
<i>pH</i>	Static pH for each compartment.	Dynamic variation of pH.
<i>Emptying</i>	No gastric or intestinal emptying.	Gastric and intestinal emptying.
Composition		
<i>AA</i>	All EAAs of IFs were balanced compared to European requirements.	
	The reference IF, pea IF and faba bean IF contained slightly more NEAAs (51, 54 and 55 %, respectively).	RIF contained 48% EAAs and 52% NEAAs; PIF and FIF contained slightly more NEAAs (56 and 57 %, respectively).
Proteolysis (DH)		
<i>Gastric (end)</i>	DH (< 2%).	DH (4-11%).
<i>Intestinal (end)</i>	Reference IF: 47.0 ± 4.4 % Pea IF: 51.4 ± 3.2 % Faba bean IF: 42.2 ± 3.3 % Reference IF = Pea IF = Faba IF.	RIF: 49.7 ± 4.2 % PIF: 66.3 ± 4.0 % FIF: 72.8 ± 5.4 % FIF > PIF > RIF.
EAA bioaccessibility		
<i>At the end of the intestinal phase</i>	Free EAA/ Total EAA (%) range: Reference IF: 12.4 - 88.7 % Pea IF: 15.7 - 88.1 % Faba bean IF: 11.8 - 84.3 % Reference IF = Pea IF > Faba IF.	Free EAA/ Total EAA (%) range: RIF: 11.0 - 76.4 % PIF: 13.5 - 58.2 % FIF: 10.8 - 61.7 % RIF = PIF = FIF.
<i>In vitro</i> Apparent Protein digestibility	N.A.	RIF = FIF > PIF.
Structure		
<i>CLSM</i> <i>Particle size distribution</i>	N.A.	Larger particles in PIF and FIF; Faster aggregation rate in gastric phase for PIF and FIF than for RIF; Comparable final structure in the intestinal compartment.
Main conclusions	Very few differences between the 3 IFs after static digestion in terms of DH and EAA bioaccessibility.	Differences in DH values with FIF>PIF>RIF; Faster aggregation rate for PIF and FIF compared to RIF, but similar final microstructure in the intestinal compartment; No large differences in EAA bioaccessibility; Similar apparent protein digestibility between RIF and FIF, both higher than for PIF (insoluble particles).

Differences in DH value between the three IFs in dynamic conditions but not in static.
 Higher DH value in dynamic digestion than in static.
 Higher EAA bioaccessibility after static digestion than dynamic.
 Slight differences and same magnitude in EAA bioaccessibility in both digestion models.

Scale-up or digestion model effect: differences in terms of protein hydrolysis and EAA bioaccessibility due to difference in digestion conditions between static and dynamic models.

5.1.4. Limitations

- **Subject limitations:** few studies existed on the use of alternative protein sources in IFs, that was whereby the originality of the study, but at the same time it was difficult to compare our results with literature data. Moreover, very different process diagrams are used by research groups to develop IFs, as well as digestion models (Table 10) with specific conditions implemented to assess digestibility and bioaccessibility, making it difficult to compare results with each others.
- **Analytical limitations:** some of the methods used in the present study were routinely used for highly soluble dairy products. However, it was sometimes uncertain that these methods were suitable for animal and plant protein mixes, potentially introducing a bias and making it necessary to optimize the protocol. This was for example the case for the fat content determination, or for static digestion during which a stirring method was adapted in order to avoid flecking of the plant proteins during digestion.
- **Experimental replications:** data obtained from the IF manufacturing at pilot and semi-industrial scales are generally means of measurements obtained from one single trial. Due to high cost and effort to conduct these kind of experiments, it was difficult to perform several independent trials. However, in order to make our statistical analysis more significant, some replications of these trials should be done to confirm the results obtained in the present manuscript.

5.1.5. General conclusion

This is the first time that 1st age IFs, containing plant proteins other than soy and hydrolysed rice, have been designed and their behaviour during digestion investigated. This work was handled through a multi-scale approach from manufacturing to digestion of IFs in order to give a large overview of the aptitude of plant proteins to partially substitute whey protein in IFs.

Overall, it was concluded that protein source greatly influenced the functional properties and the digestibility of 1st age IFs. More precisely, rice and potato proteins were not fully adapted to partially substitute whey protein due to high insolubility for rice protein and high viscosity for potato protein. This resulted in not optimal spray-drying and thus caused powder quality impairments during storage (lipid oxidation, caking or Maillard reaction) that would also affect the nutritional quality of the IFs after rehydration. In addition, these two plant proteins showed low digestibility after *in vitro* static digestion, mainly due to solubility limits for rice IF and antinutritional factors in potato IF. Therefore, these two proteins were not kept for the further experiments.

On the contrary, pea and faba bean proteins were closer to the dairy reference IF, both in terms of functional properties after manufacturing, and of protein nutritional quality after digestion. Despite insoluble particles remained in solution during manufacturing and in the reconstituted powder (baby bottle) for these two plant protein IFs, they showed similar emulsion stability and physicochemical properties to the dairy reference IF. However, process optimization and additional experiments are still required in order to confirm these first results and to provide IFs with fully matching properties. Finally, it was concluded after *in vitro* dynamic digestions that faba bean protein used in IF showed the highest proteolysis and closest apparent digestibility to the dairy reference IF, thus making it the best candidate to partially replace whey protein in IFs. Pea protein showed a lower protein digestibility than the reference and might likely need better dispersion to improve its nutritional properties. Nevertheless, these first exploratory results must be completed by *in vivo* studies in order to come even closer to infant physiological conditions as well as confirming these promising results.

5.2. Perspectives

5.2.1. Short-term perspectives

5.2.1.1. Improvement of pea and faba bean IFs solubilisation during manufacturing

As mentioned earlier, one of the key prospect of the project will be to optimize the manufacturing of pea and faba bean IFs (PIF and FIF), in particular in improving their dispersion and thus decrease the remaining particle size in solution all along the process (previously described in part 4.3.2.2 of the manuscript). In this view, additional experiments were already conducted by adding homogenization step of 20 MPa prior to the concentration step compared to the initial process diagram used to develop IFs at a semi-industrial scale. The results, presented in the manuscript Annexes section 6.1 (Figure 36 and Table 37) can be compared to the results previously presented in manuscript section 4.3.2.2 (Figure 32 and Table 28).

As described in Figure 37 and Table 36 (Annex 6.1), the particle size was reduced by half for PIF when introducing an additional homogenization step prior to concentration, and up to the rehydrated powder stage (baby bottle). The particle size was also reduced by half for FIF, but similar particle size distribution was found in FIF baby bottle than without the additional homogenization step (Figure 32, Table 28).

Overall, these results highlighted that a homogenization step applied prior to concentration of PIF and FIF made it possible to reduce the particle size during process. However, particle size in the newly produced PIF and FIF were still larger than in the reference dairy IF. Moreover, the plant based IF powders still need longer solubilisation to obtain a properly disperse solution in the baby bottle. Thus, further experiments are needed to improve even more plant based IFs dispersibility and also to make replications and to confirm the results of these first trials.

5.2.1.2. Other short-term perspectives

(a) Dissociation of formulation and process effects on IF digestibility

As described in discussion part 5.1.2, it was not possible to dissociate the matrix effect (formulation) from the process effect when comparing the protein solutions and the IFs digestibility (*in vitro* static

model). In this way, it could be interesting to separately investigate the influence of the formulation and the thermal or mechanical treatment on the digestibility of the IFs. This could for example provide information on how the process impacts (positively or negatively) on the overall digestibility of IFs.

(b) Better characterized the ingredients used to formulate IFs

Better characterize the raw materials prior to the experiments, in particular concerning the fibre content in plant proteins or other complex sugar content, would have been interesting in order to be able to better explain the physicochemical reactions during the manufacturing and during the digestion of the IFs.

(c) Focus on the other IF components on the functional properties and the digestibility of IFs

As this work was only focused on the proteins, it could be interesting to analyze the other components of IFs, *i.e.* mainly lipids, lactose (and the other form of sugar) and micronutrients that might also have an influence on the functional properties and the protein digestibility of IFs.

(c) Conformity to the European regulation & further innovation aspects

In short-term perspectives, it should also be important to formulate the IFs in fully accordance with the European regulation (EU 2016) which should be applicable from February 2020. For instance, the addition of vitamins, mineral and DHA are currently mandatory but missing in the present formulated IFs, as the project was mainly focused on proteins.

Moreover, the addition of other components such as probiotics, prebiotics or other bioactive molecules should also be considered in order to come closer to the gold standard, human milk. As mentioned in the literature review, recent findings focused nowadays on IF innovation to resemble to human milk, particularly concerning the fat structure. In fact, lipids structure in IF has remained very different from human milk: human milk shows dispersed milk fat globules with an average size of 4 μm surrounded by a membrane so called milk fat globule membrane, whereas the fat droplets found in IFs are mostly from plant origin with an average size of 1 μm and stabilized by a neoformed membrane made of proteins or other tensioactive molecules. Such different structures may lead to differences in the kinetics of digestion and nutrients assimilation (Armand et al. 1996; Bourlieu et al. 2015; Bourlieu & Michalski 2015). That is why, several research groups worked on the incorporation of milk fat globule membrane extracts from bovine milk which is closer to human milk fat structure (Gallier et al. 2015; Le Huërou-Luron et al. 2018; Zou et al. 2016), or with the use of a mix of bovine and vegetal fat (Bourlieu et al. 2015; Delplanque et al. 2013; Lemaire et al. 2018).

(d) Financial aspects

Another aspect that was not yet mentioned is the food cost assessment of these new plant IFs compared to the standard dairy IF. Table 35 shows the food cost of the different IFs prepared in the present study on the basis of the cost of each ingredients. It can be seen that all IFs, except potato IF, show similar or even lower prices than the reference dairy formula, particularly interesting financially for pea and faba bean IFs.

Table 35. Estimation of the food cost (€/kg) of the five IFs studied (without vitamins and minerals).

	Reference IF	Pea IF	Faba bean IF	Rice IF	Potato IF
Price for the liquid formula at 24 % DM (€/kg)	0.48	0.43	0.41	0.47	0.62

5.2.2. Long-term perspectives

5.2.2.1. Industrial development

In long-term perspectives, these new IFs should be produced at an industrial scale in order to highlight the real production specifications and constraints of the use of these new protein sources in 1st age IFs.

5.2.2.2. *In vivo* studies and other digestion models

Human clinical trials remain the best approach to study food digestion (Deglaire et al. 2009). However, obtaining prior ethical approval for performing such experiments on humans is difficult, if not almost impossible when concerning healthy specific populations like infants. For those reasons, animal models have been widely proposed and used in research. Among the different animal models available, the pig is considered as the best to mimic the upper part (stomach and small intestine) of the human gastrointestinal tract (Deglaire & Moughan 2012; Rowan et al. 1994).

In this way, another important perspective of this work will be to conduct *in vivo* studies with piglet model (Moughan & Leenaars 1992) in order to highlight some physiological specificities that could not be demonstrated with *in vitro* experiments. For instance, the impact of the larger particle size found in the plant-based IFs on the gastric emptying could be conducted *in vivo*. Moreover, true digestibility value could be determined using *in vivo* model and could be compared with the apparent digestibility found *in* in the present study. *In vivo* studies could also be relevant to investigate the allergenicity of the new protein sources as well as the influence of these new protein sources on the infant microbiota. Another interesting think to explore could be the real physiological effect of the presence of insoluble particles (which might be fibers) and antinutritional factors in plant based IFs. For instance, it was mentioned by Wang & Zijlstra (2018) that the presence of fibers could modulate *in vivo* the retro-control of the digestion in response to the viscosity and volume increase generated by the fibers.

However, *in vivo* studies using animal model are still ethically and financially complex to perform. Therefore, other relevant digestion models could be considered such as Caco-2 cells that can be used to investigate intestinal absorption in the intestinal epithelium (Andersson et al. 2011; Lipkie et al. 2014; Vors et al. 2012). Human intestinal organoids have also been proposed to study specificities of the human gastrointestinal tract (Workman et al. 2017) and could be interesting alternatives to the other digestion models discussed in the present study. It could also be interesting to study digestion using other relevant *in vitro* dynamic models such as the infant digestive apparatus developed by Passannanti et al. (2017) or the new dynamic *in vitro* human stomach made up with silicone (Wang et al. 2019).

5.2.2.3. Regulatory approval

A last important perspective of this project will be to bring evidence of safety of these new protein ingredients regarding infant health as these plant proteins are not yet allowed in the European regulation for IFs. Currently, the applicable European regulation for 1st age IFs (EU 2016) indicates in the Article 3 that “*1st age IFs should be formulated with protein sources defined in Annexe I point 2 of European directive 2016/127 EC (i.e., cow milk, goat milk, soy and hydrolysed rice protein sources) and with other ingredients if their adequacy for infants have been demonstrated from accepted scientific data*”. This means that if the conformity of the new protein sources used in the present work is demonstrated, their utilisation in IFs and their marketing could be considered.

6. Annexes

6.1. Improvement of pea and faba bean IFs solubilisation during manufacturing at semi-industrial scale

Table 36. Mode diameter and D[4.3] of pea infant formula (PIF) and faba bean infant formula (FIF) samples during process and in the rehydrated powder. Data for D[4.3] are expressed as mean \pm SD.

		D[4.3] (μm)	Mode 1 (μm)	Mode 2 (μm)
PIF	Solubilization	57.6 \pm 0.3	5.2	76.0
	Homogenization 20 MPa	31.1 \pm 0.2	8.6	45.6
	Concentration	24.1 \pm 0.1	7.6	35.3
	Homogenization 10 MPa	15.6 \pm 0.2	1.1	8.7
	Baby bottle	9.2 \pm 0.1	0.6	22.4
FIF	Solubilization	20.9 \pm 0.1	16.4	58.9
	Homogenization 20 MPa	9.0 \pm 0.1	3.55	12.7
	Concentration	11.2 \pm 0.2	14.5	
	Homogenization 10 MPa	5.5 \pm 0.6	1.1	5.2
	Baby bottle	5.4 \pm 0.1	0.6	10.0

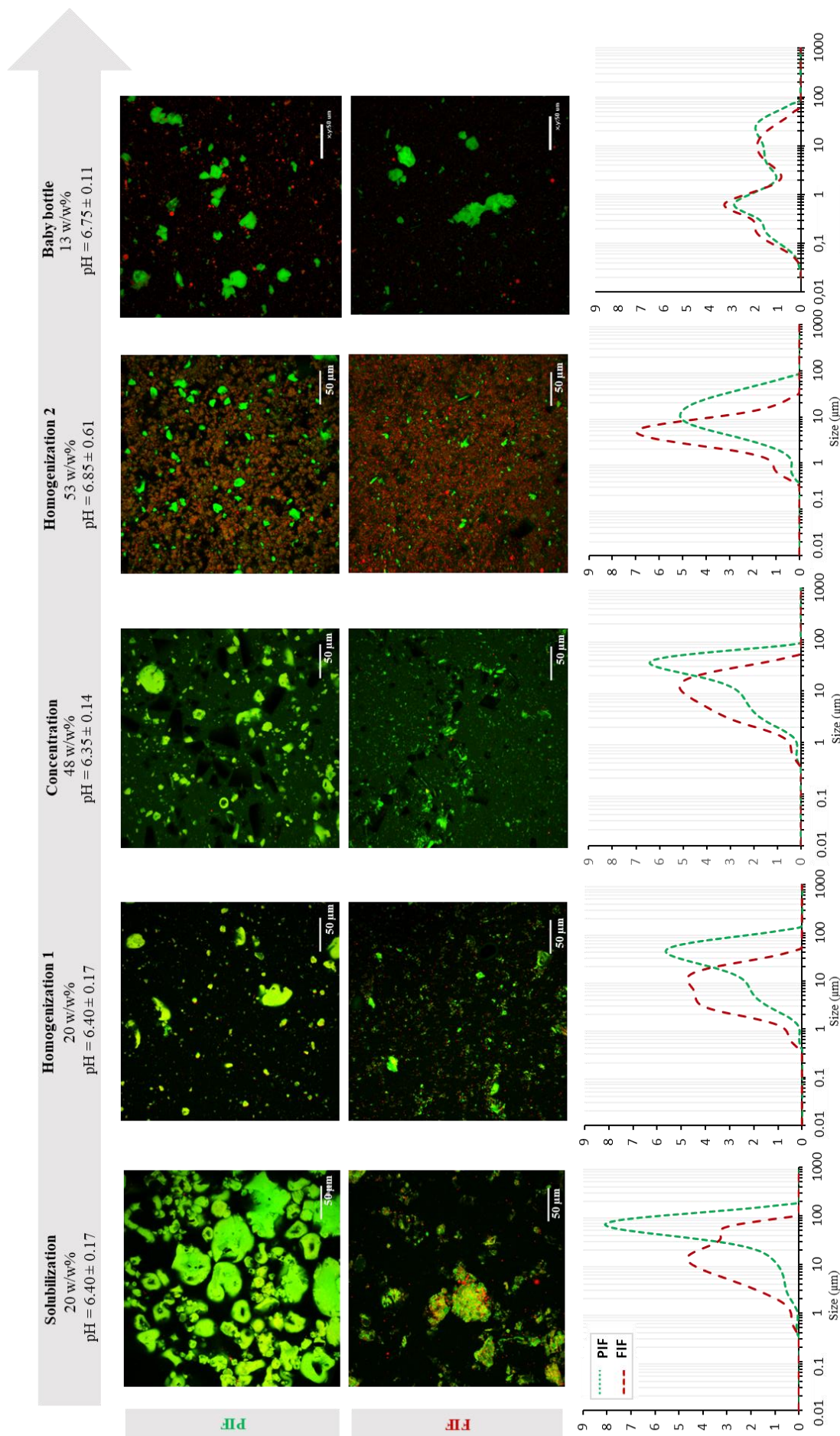


Figure 37. Spatial organization of lipids (in red) and proteins (in green) as evidenced by confocal laser scanning microscopy (CLSM) in two plant-based IFs (PIF: pea infant formula; FIF: faba bean infant formula) with an homogenization (1) step prior concentration. Homogenization 1: 15/5 MPa, homogenization 2: 8/2 MPa. Baby bottle was obtained after the rehydration of the IFs powder (160 and 65 °C for inlet and outlet drying temperature). Size distributions of processed IFs were determined by laser light scattering.

6.2. Browning index & images of the IF powders

Table 37. Browning index (BI) of the IFs powders based on L, a, b color parameters.

	Reference IF	Pea IF	Faba bean IF	Rice IF	Potato IF	RIF 1	PIF 1	FIF 1	RIF 2	PIF 2	FIF 2
Process scale	Pilot scale					Semi-industrial scale (processing route 1)			Semi-industrial scale (processing route 2)		
L	75.9	73.1	73.2	73.4	66.4	69.6	69.5	69.6	70.5	69.5	70.9
a	-3.1	-2.3	-3.8	-2.0	0.8	-4.4	-2.0	-4.0	-3.7	-2.2	-4.2
b	9.8	13.3	15.9	9.5	11.0	12.4	14.7	16.5	10.7	15.9	16.5
x	0.33	0.34	0.34	0.33	0.34	0.33	0.35	0.35	0.33	0.35	0.35
BI	10.4	17.2	19.9	11.5	18.6	14.2	21.0	21.9	12.1	23.0	21.3

where $x = \frac{a+1.75 L}{5.645 L+a-3.012 b}$ and $BI = \frac{100 (x-0.31)}{0.17}$ (equations from Subhashree et al. (2017)).



Figure 38. Images of the five IF powders produced at pilot scale (reference IF, potato IF, rice IF, pea IF and faba bean IF).

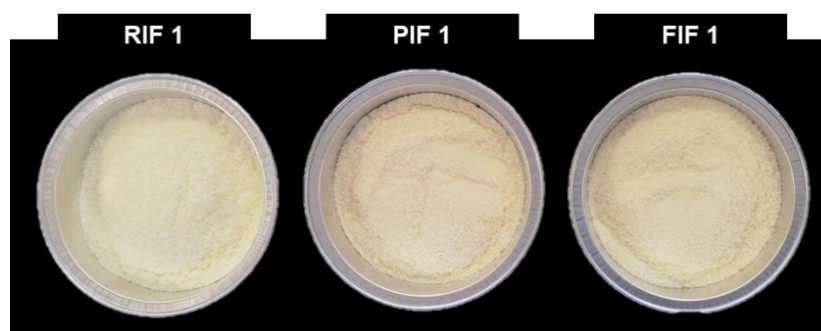


Figure 39. Images of the three IF powders produced at semi-industrial scale after processing route 1 (RIF (reference IF), PIF (pea IF) and FIF (faba bean IF)).

6.3. Substantial summary of the thesis in French

Titre : De la fabrication de formules infantiles innovantes en partie composées de protéines végétales à leur digestion *in vitro*: une approche multi-échelle

6.3.1. Contexte scientifique et socio-économique

Les formules infantiles sont destinées à l'alimentation des nourrissons pendant leurs 4 à 6 premiers mois de vie lorsque l'allaitement n'est pas suffisant ou non désiré. Elles doivent répondre à elles seules aux besoins nutritionnels des nourrissons jusqu'à l'introduction d'une alimentation complémentaire. Même si les recommandations nutritionnelles préconisent un allaitement exclusif pendant les 6 premiers mois de vie de l'enfant, les réalités s'en éloignent avec une grande disparité dans le monde. Ainsi au niveau mondial, seuls 38% des nourrissons âgés de 0 à 6 mois sont exclusivement allaités (WHO & UNICEF, 2017). C'est pourquoi la demande pour les formules infantiles est toujours en forte croissance avec des volumes de vente estimés à 2,7 million de tonnes en 2017 et 3 millions de tonnes attendues en 2020. La croissance du marché des formules infantiles est expliquée pour partie par des facteurs sociologiques et culturels. En effet, la durée des congés maternités n'est pas toujours en adéquation avec les recommandations nutritionnelles de l'allaitement : la reprise du travail pour les femmes, qui rend difficile la poursuite de l'allaitement, est souvent prématurée. D'autre part, le nombre de personnes issues de la classe moyenne, disposant des moyens de s'offrir des formules infantiles, est un critère expliquant la croissance à l'échelle mondiale. Mais un autre facteur est aussi bien évidemment déterminant : l'augmentation chaque année du nombre de naissances dans le Monde.

Historiquement, la première formule infantile a été inventée en 1867. Elle était composée de farine de blé, de lait de vache, de farine de malt et de carbonate de potassium. Il a fallu attendre plus d'un siècle pour voir apparaître le premier décret européen qui régleme la composition ainsi que la fabrication des formules infantiles. Ensuite, entre 1999 et 2018 se sont succédées plusieurs directives et réglementations européennes déterminant encore plus spécifiquement la composition des formules infantiles (aminogramme obligatoire, concentration en macro- et micro-nutriments, présence obligatoire de DHA etc.). La majorité des formules infantiles développées aujourd'hui sont fabriquées à partir de protéines de lait de vache auxquelles sont ajoutées des protéines de lactosérum afin de se rapprocher du ratio caséines/protéines de lactosérum retrouvées dans le lait maternel, mais surtout pour couvrir les besoins en acides aminés essentiels (AAE) du nourrisson. Or, des formules infantiles à base de protéines autres que les protéines du lait de vache existent. Notamment, des formules infantiles à base de protéines de soja, de riz hydrolysé ou encore de lait de chèvre sont autorisées aujourd'hui dans la réglementation, pour certaines depuis plusieurs années. Ainsi, le marché des formules infantiles a connu récemment de nombreuses évolutions et est aujourd'hui strictement réglementé. L'utilisation de protéines autres que les protéines du lait de vache a été autorisée relativement tôt dans l'histoire et est aujourd'hui en plein essor.

L'intérêt grandissant pour les protéines alternatives aux protéines animales est dû notamment à l'augmentation de la population mondiale qui devrait atteindre près de 10 milliards d'habitants d'ici 2050. Au rythme de consommation actuel, la demande en protéines animales pourrait alors entraîner de fortes

problématiques environnementales telles que l'augmentation d'émission de gaz à effet de serre que l'on sait en partie causée par l'agriculture. Au-delà de cet objectif de durabilité de notre système alimentaire, l'intérêt grandissant pour les protéines alternatives répond également à des préoccupations éthiques et de santé. Parmi les sources de protéines alternatives, on peut citer les micro-algues (50-70 % de protéines ; spiruline, chlorelle...), les légumineuses (20-40 % de protéines ; soja, pois, féverole, lupin, lentilles...), les oléagineux (13-35 % de protéines ; soja, tournesol, colza, graines de coton...), les céréales (7-15 % de protéines ; blé, riz, avoine, orge, seigle, maïs...) ou encore les tubercules (1-3 % de protéines ; pommes de terre, manioc...). Ces différentes sources de protéines végétales pourraient constituer d'intéressants substituts aux protéines animales, présentant à la fois des propriétés nutritionnelles et fonctionnelles intéressantes. En revanche, il existe des freins à leur utilisation, comme la limitation en un ou plusieurs acides aminés essentiels (AAE), la présence de facteurs antinutritionnels qui diminuent la digestibilité de ces protéines, ou encore la présence d'allergènes et certains défauts organoleptiques. Ainsi, pour faciliter l'utilisation de ces protéines végétales en alimentation humaine, des solutions devront être trouvées pour pallier à ces inconvénients.

L'apport en protéines tôt dans la vie est essentiel pour assurer le bon développement du nourrisson. Il affecte en effet sa croissance, son développement cérébral, son appétit ou encore sa régulation hormonale et ses défenses immunitaires. Une protéine est dite nutritionnellement équilibrée lorsqu'elle couvre les besoins métaboliques en azote et en AAE. Ceci dépend donc à la fois du profil en AAE de la protéine, mais aussi de sa digestibilité. En effet, la digestibilité d'une protéine détermine la quantité de nutriments absorbée et ensuite délivrée à l'organisme pour assurer ses différentes fonctions métaboliques. Le système digestif du nourrisson présente une certaine immaturité en comparaison de celui de l'adulte. Le pH gastrique est plus élevé (pH compris entre 3.2 et 6.5, comparé à un pH inférieur à 3 chez l'adulte), et la protéolyse est limitée au niveau gastrique du fait d'une moindre sécrétion de la pepsine. De plus, l'activité de la lipase au niveau intestinal est également limitée à la naissance. Ces spécificités digestives doivent être prises en compte pour garantir la pertinence des modèles *in vitro* utilisés pour étudier la digestion chez le nouveau-né.

Les formules infantiles sont le plus souvent commercialisées sous forme liquide (prête à l'emploi) ou sous forme de poudre à reconstituer. Les formules liquides sont plus faciles à utiliser et engendrent moins d'erreurs de dosage. En revanche, les formules infantiles en poudre sont plus avantageuses du point de vue de la conservation, du transport et du stockage. Au cours de ce projet, seules les formules infantiles en poudre ont été étudiées. Elles sont obtenues par un procédé de fabrication dit par « voie humide » qui consiste tout d'abord à mélanger les ingrédients secs avec de l'eau pour reconstituer une solution. Ensuite, une étape de pasteurisation est nécessaire pour garantir la sécurité sanitaire du produit. Une homogénéisation est réalisée après avoir ajouté la matière grasse ; elle consiste à appliquer une pression au produit permettant de créer une émulsion qui stabilise la matière grasse à la fois au cours de la fabrication mais également dans le produit fini. Une étape de concentration est ensuite effectuée et est indispensable dans ce type de schéma technologique pour éliminer un maximum d'eau avant l'étape de séchage, beaucoup plus énergivore. Enfin, l'étape de séchage en tour d'atomisation consiste à pulvériser le concentré liquide sous forme de très fines gouttelettes dans une enceinte dans laquelle circule un air chaud et sec, qui transforme en quelques secondes la gouttelette

liquide en grain de poudre. Les poudres obtenues sont finalement contrôlées sur différents critères de qualité (contrôles sanitaires, équilibre nutritionnel, solubilité, densité, profile granulométrique, test biberon etc.) avant d'être conditionnées.

Ce projet de thèse CIFRE s'intègre à une collaboration entre l'INRA Agrocampus Ouest UMR Sciences et Technologie du Lait et de l'œuf (Rennes, France) et l'entreprise SILL (Société Industrielle Laitière du Léon, Plouvien, France). Cette entreprise est spécialisée dans les produits laitiers, les jus de fruits, les soupes et les plats préparés. SILL a récemment investi dans le secteur du baby food et souhaite aujourd'hui élargir sa gamme de produits, notamment en se lançant dans la production de formules infantiles. Dans ce contexte, la SILL souhaite développer des formules infantiles innovantes au regard des enjeux environnementaux actuels, tout en respectant bien entendu les recommandations nutritionnelles. Poussée également par un manque d'approvisionnement en lactosérum qui est un des ingrédients clé pour la fabrication des formules infantiles, l'entreprise a souhaité axer l'innovation sur l'évaluation du potentiel de diverses protéines végétales comme substitut partiel à des protéines animales pour la fabrication de formules infantiles.

6.3.2. Objectif & Stratégie

La question posée dans ce travail de thèse était de savoir : **quelle serait l'influence du changement de source de protéines sur les propriétés fonctionnelles et la digestibilité des formules infantiles ?** Pour cela, la part des protéines de lactosérum habituellement ajoutées aux protéines de lait de vache pour fabriquer les formules infantiles (et représentant 50% des protéines totales), ont été remplacées par des sources de protéines alternatives, dans le but d'obtenir des formules infantiles innovantes (Figure 40). Ces nouvelles sources de protéines n'étant pas encore autorisées dans la réglementation, le but du projet était d'explorer de manière prospective de futures possibilités dans le domaine des formules infantiles.

Pour répondre à cet objectif, plusieurs étapes ont été prévues : tout d'abord la sélection des protéines alternatives, principalement selon des critères nutritionnels. Ensuite, le développement de ces nouvelles formules infantiles a été réalisé d'abord à échelle pilote, afin de cribler un grand nombre de produits, puis dans un deuxième temps à échelle semi-industrielle, afin de se rapprocher davantage des réalités de l'industrie. Enfin, la digestibilité de ces nouvelles formules infantiles a été étudiée en utilisant des modèles de digestion *in vitro* mimant les conditions physiologiques du nourrisson, en utilisant d'abord un modèle statique, puis un modèle dynamique. Ces éléments sont résumés dans la Figure 40 ci-dessous.

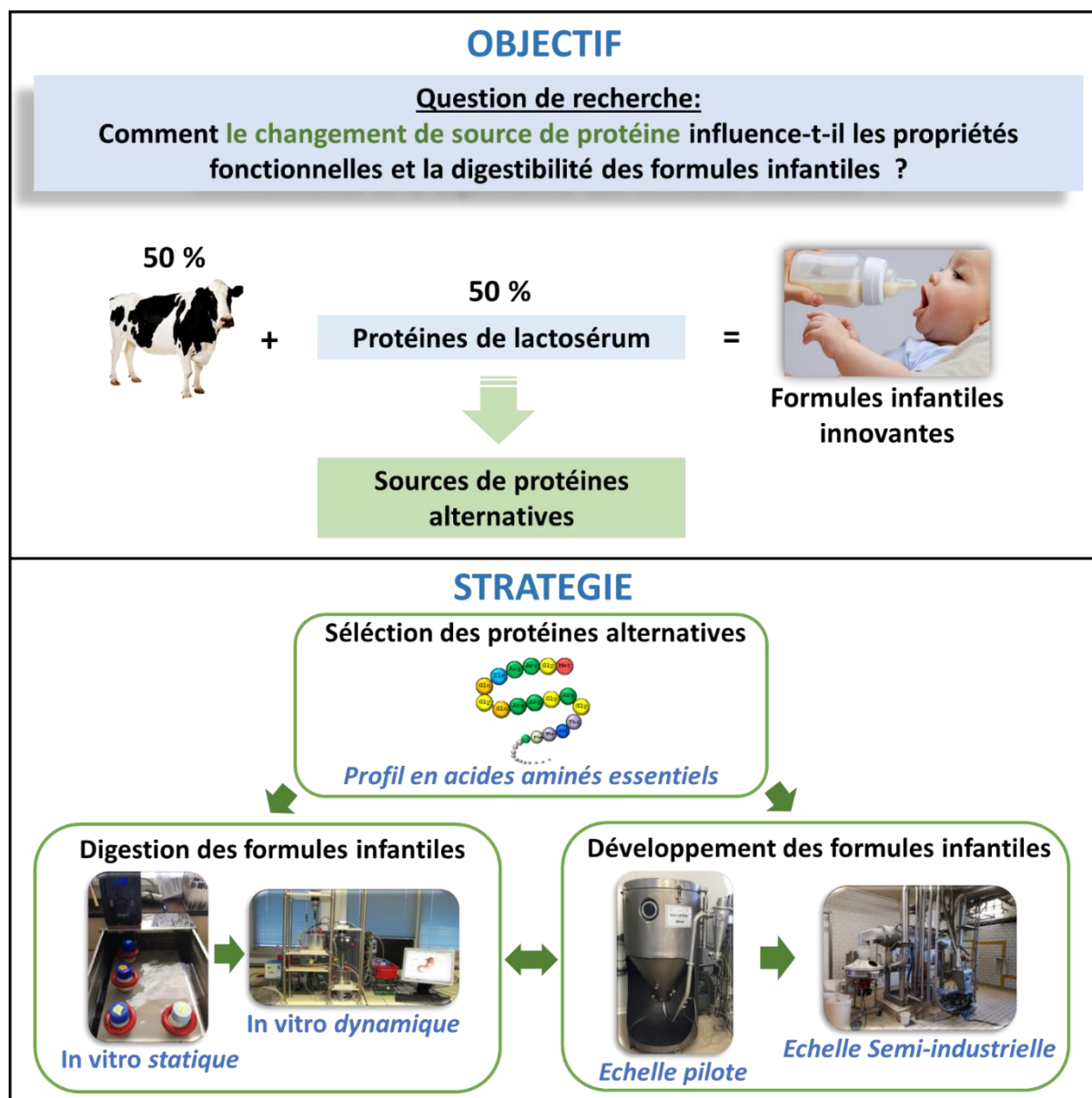


Figure 40. Objectif & Stratégie expérimentale de l'étude.

6.3.3. Résultats

6.3.3.1. Sélection des protéines alternatives

Le but de cette première partie du projet était de sélectionner les sources de protéines alternatives nutritionnellement adaptées au remplacement partiel des protéines de lactosérum dans les formules infantiles.

Pour cela, plusieurs sources de protéines, principalement d'origine végétale, ont été comparées à une formule infantile laitière classique, considérée comme la référence dans cette étude et respectant la réglementation européenne 2016/127 actuellement en vigueur. En partant de données de la littérature, la composition en acides aminés essentiels (AAE) de différentes sources de protéines a donc été comparée à celle d'une formule infantile classique. Un indicateur de la qualité nutritionnelle des protéines appelé PDCAAS (*protein digestibility corrected amino acid score*, exprimé en %) a également été calculé afin de pouvoir comparer ces protéines entre elles. Le PDCAAS se calcule en faisant le ratio entre la concentration de l'AAE limitant dans la protéine d'intérêt et la concentration de ce même AAE dans une protéine de référence (qui couvre entièrement les besoins nutritionnels et qui diffère selon la catégorie d'âge à laquelle on s'intéresse : nourrisson, jeune enfant ou adulte). Ce ratio doit ensuite être multiplié par la digestibilité fécale vraie de la protéine (qui doit normalement être déterminée *in vivo* chez un modèle de rat en croissance). Dans ce projet, nous avons calculé un PDCAAS légèrement modifié, en prenant en compte des compositions en AAE de protéines issues de la littérature, ainsi que des valeurs de digestibilité obtenues *in vivo* ou *in vitro*. En plus de ces critères nutritionnels, les nouvelles sources de protéines devaient dans l'idéal être exemptes d'allergènes, de défauts organoleptiques, de facteurs antinutritionnels et être disponibles commercialement sous forme purifiée. Pour finir, les protéines respectant le mieux ces derniers critères ont été digérées en utilisant un modèle de digestion *in vitro* statique afin d'évaluer leur digestibilité par mesure de protéolyse et de bioaccessibilité des AAE. La présence de facteurs antinutritionnels a également été testée par mesure d'activité de la trypsine.

Sur les 19 sources de protéines initialement recensées, 14 ont été estimées de bonne qualité nutritionnelle par rapport aux besoins du nourrisson, avec un score au PDCAAS supérieur à 70% (Figure 41). Après avoir écarté les sources de protéines présentant un risque d'allergie, parmi la liste officielle à déclaration obligatoire, et celles présentant des défauts de couleurs ou d'odeurs, les sources de protéine commercialement disponibles ont finalement été sélectionnées, à savoir : les **protéines de pois, féverole, riz et pomme de terre**. Dans la suite du travail, ces différentes sources protéiques ont été systématiquement comparées aux protéines de lactosérum, retenue en tant que protéine de référence pour une formule infantile laitière classique. Les résultats de digestibilité ont montré une protéolyse très proche entre la protéine de pois et la référence, presque deux fois supérieure à celle de la féverole, du riz et en particulier de la pomme de terre. La libération des AAE en fin de digestion a montré des profils relativement proches entre la référence et les protéines végétales, excepté pour la protéine de pomme de terre, significativement inférieure à toutes les autres protéines. Enfin, la présence de facteurs antinutritionnels (anti-trypsiques), souvent retrouvés dans les protéines végétales, n'a été mise en évidence que dans la protéine de pomme de terre.

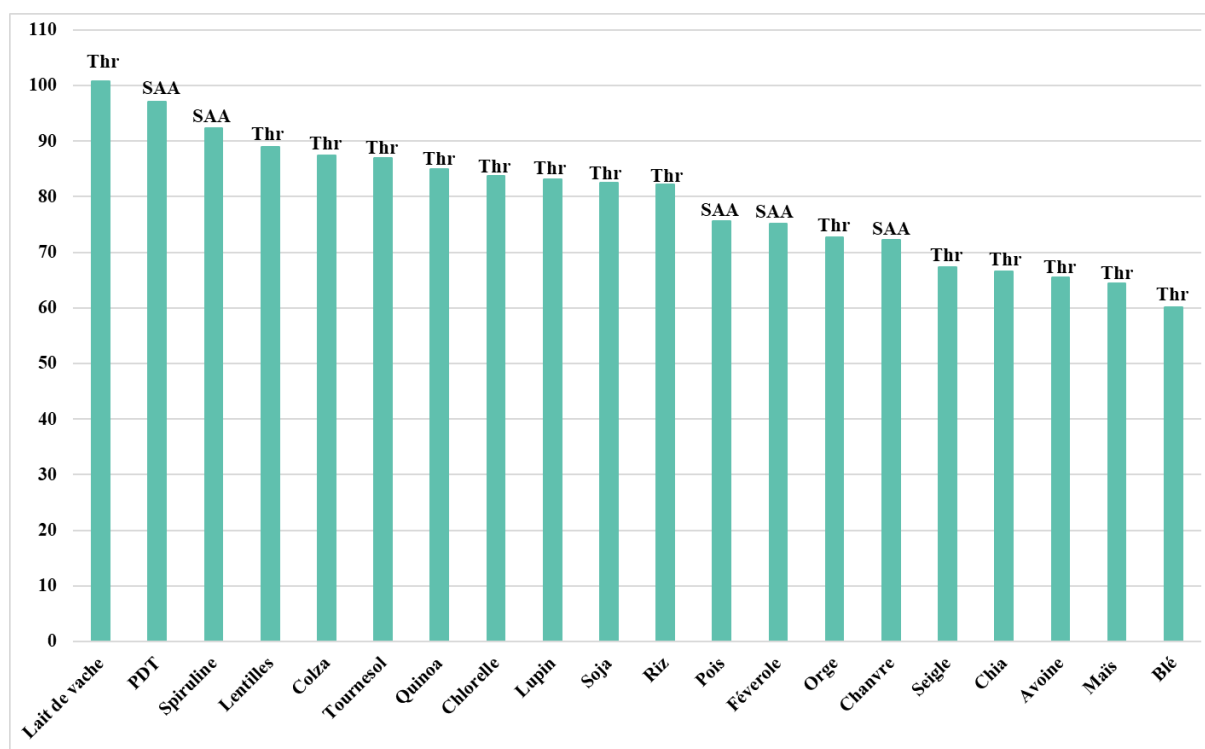


Figure 41. Score au PDCAAS (%) calculé pour différentes sources de protéines par rapport à la protéine de référence (formule infantile 1^{er} âge classique à 1,6 g protéine / 100 ml). Les acides aminés en haut de chaque barre correspondent à l'acide aminé limitant. SAA : acides aminés soufrés ; PDT : pomme de terre.

Dans cette première partie, il a été montré que toutes les sources de protéines ne sont pas nutritionnellement adaptées aux besoins du nourrisson. En effet, beaucoup de protéines, notamment d'origine végétale, présentent des déficiences en un ou plusieurs AAE ce qui diminue leur qualité nutritionnelle. D'autre part, la présence d'allergènes ou de défauts organoleptiques est également un frein à leur utilisation dans l'alimentation humaine et en particulier pour celle du nourrisson. Enfin, certaines protéines peuvent présenter des facteurs antinutritionnels qui ont un impact sur la digestibilité de la protéine.

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Pour conclure, parmi les 19 sources de protéines étudiées de manière théorique, seules 4 protéines végétales ont été sélectionnées pour leur capacité à répondre au mieux au cahier des charges: **le pois, la féverole, le riz et la pomme de terre**. Leur digestibilité a été évaluée et comparée à celle de la protéine de lactosérum : des valeurs similaires ont été obtenues avec la féverole, le riz et le pois. Les meilleurs résultats ayant été obtenus avec ce dernier. En revanche, une moindre digestibilité a été mesurée pour la protéine de pomme de terre, potentiellement due à la présence de facteurs antinutritionnels.

6.3.3.2. Développement des formules infantiles à échelles pilote et semi-industrielle

Les nouvelles sources de protéines ayant été sélectionnées, l'objectif à suivre était de vérifier s'il était possible de fabriquer des formules infantiles à partir de ces sources protéiques alternatives, et d'évaluer l'impact du changement de source de protéine sur les étapes de fabrication et sur les propriétés fonctionnelles des formules infantiles.

Pour cela, cinq formules infantiles (les quatre formules « végétalisées » et la formule infantile laitière de référence) ont été fabriquées à échelle pilote dans un premier temps, pour évaluer la faisabilité de la fabrication. Dans un deuxième temps, sur un choix plus restreint de formules, la fabrication a été réalisée à échelle semi-industrielle afin de se rapprocher davantage de la réalité industrielle. Le même schéma technologique a été suivi pour la fabrication à ces deux échelles, à savoir : le mélange en solution des ingrédients secs, sa pasteurisation et sa concentration, l'ajout de matière grasse (mélange d'huiles) et l'homogénéisation du mélange ainsi formé, puis son séchage par atomisation. De plus, deux voies technologiques différentes ont été testées à l'échelle semi-industrielle, à savoir : basse pression d'homogénéisation et haute température de séchage vs haute pression d'homogénéisation et basse température de séchage. Des analyses physico-chimiques et de microscopie confocale ont été réalisées au cours de la fabrication et sur le produit fini, afin de caractériser les propriétés fonctionnelles de ces formules infantiles innovantes.

Le développement à échelle pilote a permis de mettre en évidence certains points critiques, notamment des limites de viscosité lors de la fabrication de la formule infantile à base de protéines de pomme de terre, mais également des limites de solubilité pour la formule infantile à base de protéines de riz (Figure 42). A l'inverse, les formules infantiles à base de protéines de pois et de féverole ont montré à la fois des comportements au cours de la fabrication et des propriétés fonctionnelles relativement proches de la formule infantile de référence. Suite à ces premiers résultats, il a été décidé d'écarter les protéines de pomme de terre et de riz pour la suite du projet, en raison de leurs limites en termes de viscosité et de solubilité (Figure 42), mais également pour des raisons nutritionnelles (facteurs antinutritionnels probables dans la protéine de pomme de terre notamment), comme indiqué précédemment.



Figure 42. Illustrations des limites de solubilité pour la formule infantile à base de protéines de pomme de terre (PDT) après l'étape de concentration ; Limites de solubilité pour la formule infantile à base de protéines de riz comparée à la formule infantile à base de protéines de féverole au cours de leur fabrication (M : mélange ; P : pasteurisation ; C : concentration ; H : homogénéisation).

La fabrication à échelle semi-industrielle a donc été réalisée uniquement pour les formules infantiles de pois et de féverole en comparaison à la formule infantile laitière de référence. Le suivi de la fabrication de ces trois formules en microscopie confocale a permis de mettre en évidence des agrégats de protéines insolubles présentes tout au long de la fabrication dans les formules infantiles « végétalisées » (Figure 43). Les analyses physico-chimiques ont montré finalement peu de différences entre les trois formules infantiles, hormis une plus forte viscosité pour la formule infantile à base de protéines de pois. De plus, des résultats sensiblement meilleurs sont été obtenus avec les paramètres de la voie technologique basse pression d'homogénéisation et haute température de séchage, notamment en termes de viscosité du concentré avant séchage (moins visqueux) et de matière grasse libre (moins de matière grasse libre retrouvée dans la poudre). Des calculs théoriques ont également permis de montrer que la quantité de protéines laitières présentes dans les trois formules infantiles était suffisante pour stabiliser l'émulsion, malgré la présence d'agrégats protéiques en amas peu solubles dans les formules « végétalisées » (Figure 43).

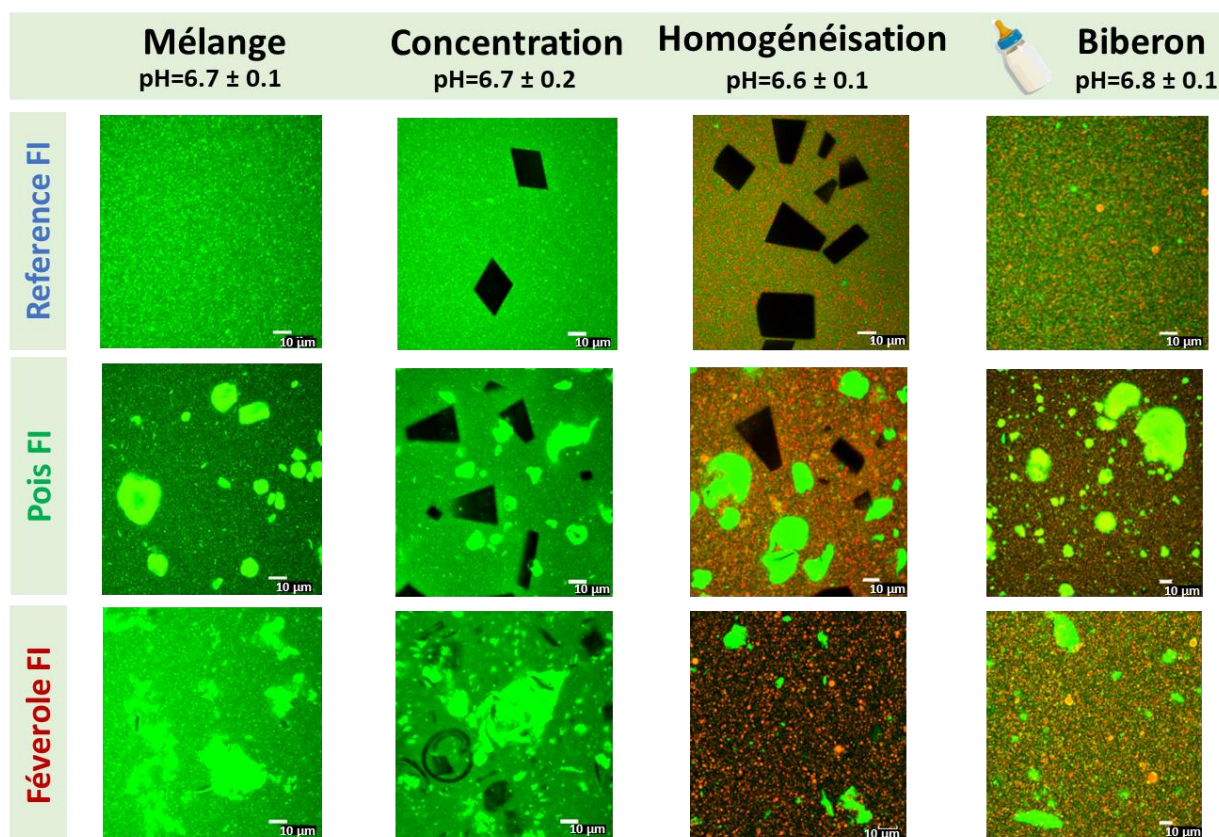


Figure 43. Organisation spatiale des lipides (rouge) et des protéines (vert) en microscopie confocale dans les trois formules infantiles (FI) au cours des différentes étapes de fabrication à échelle semi-industrielle (obtenues pour la voie technologique basse pression d'homogénéisation et haute température de séchage) : du mélange des ingrédients jusqu'à la reconstitution des poudres en biberons.

BILAN

La source de protéine semble avoir une réelle influence sur la fabrication et les propriétés fonctionnelles des formules infantiles. Il a été montré dans un premier temps, lors de la fabrication à échelle pilote, que les formules infantiles à base de protéines de pois et de féverole se rapprochaient davantage de la formule infantile laitière de référence en termes de comportement au cours de la fabrication et de propriétés physicochimiques. A l'inverse, les formules infantiles à base de protéines de pommes de terre et de riz qui ont montré des limites de viscosité et de solubilité et ont donc été écartées pour la suite du projet. Globalement à échelle semi-industrielle, la présence d'agrégats protéiques et d'insolubles dans les formules infantiles de pois et de féverole doit être améliorée pour garantir une fabrication optimale et des caractéristiques fonctionnelles encore plus proches de la formule laitière de référence.

6.3.3.3. Digestibilité *in vitro* des formules infantiles

Le but de ce troisième volet du projet était d'évaluer l'impact du changement de source de protéines sur la digestibilité des formules infantiles.

Pour cela des digestions *in vitro* simulant les conditions physiologiques du nourrisson ont été réalisées. Dans un premier temps, les cinq formules infantiles développées à échelle pilote (pois, féverole, riz, pomme de terre et référence laitière) ont été soumises à un modèle de digestion en conditions statiques, relativement simplifié et facile à mettre en œuvre. Puis, les trois formules développées à échelle semi-industrielle ont été évaluées en utilisant un modèle de digestion en conditions dynamiques, plus sophistiqué et qui se rapproche davantage des réalités physiologiques. Au cours de ces digestions, des échantillons ont été collectés, sur lesquels des mesures de protéolyse et de bioaccessibilité des acides aminés ont été réalisées. Pour les digestas obtenus avec le modèle dynamique, des mesures de répartition de taille des peptides, des bilans de matière et un suivi en microscopie confocale ont également été effectués.

L'étude de la composition nutritionnelle des cinq formules infantiles a montré qu'elles étaient toutes équilibrées nutritionnellement par rapport aux besoins du nourrisson. Suite aux digestions *in vitro* statiques, il a été montré que les formules infantiles à base de protéines de pois et de féverole étaient relativement proches de la formule de référence en terme de protéolyse et de libération d'AAE. A l'inverse, la formule infantile à base de protéines de riz, et encore plus celle à base de protéines de pomme de terre, ont montré une moindre digestibilité par rapport aux trois autres formules. Suite aux digestions *in vitro* dynamiques, le suivi de la microstructure a permis de mettre en évidence la présence d'agrégats protéiques dans les formules infantiles à base de protéines de pois et de féverole (comme au cours de la fabrication), mais aussi des différences notables dans le compartiment gastrique entre ces deux formules par rapport à la formule de référence (Figure 44). En effet, l'agrégation des protéines était retardée pour la formule de référence par rapport aux deux formules « végétalisées », probablement en raison des différences de nature et de sensibilité de chaque type de protéines vis-à-

vis de l'acidification. Dans le compartiment intestinal, les différences de structure étaient moins marquées et finalement, les trois formules infantiles semblaient assez proches en fin de digestion. Par ailleurs, la protéolyse était légèrement plus faible pour la formule infantile à base de protéine de féverole, alors que peu de différences étaient décelables en termes de libération des AAE et de répartition de tailles des peptides en fin de digestion entre les trois formules infantiles. Cependant, les calculs de digestibilité *in vitro* ont abouti à des valeurs significativement plus faibles pour la formule infantile à base de protéines de pois par rapport aux deux autres formules. Cette différence pourrait s'expliquer en partie par des problèmes de solubilité de la protéine de pois, qui aurait entraîné à minima une sous-estimation des bilans de matière.

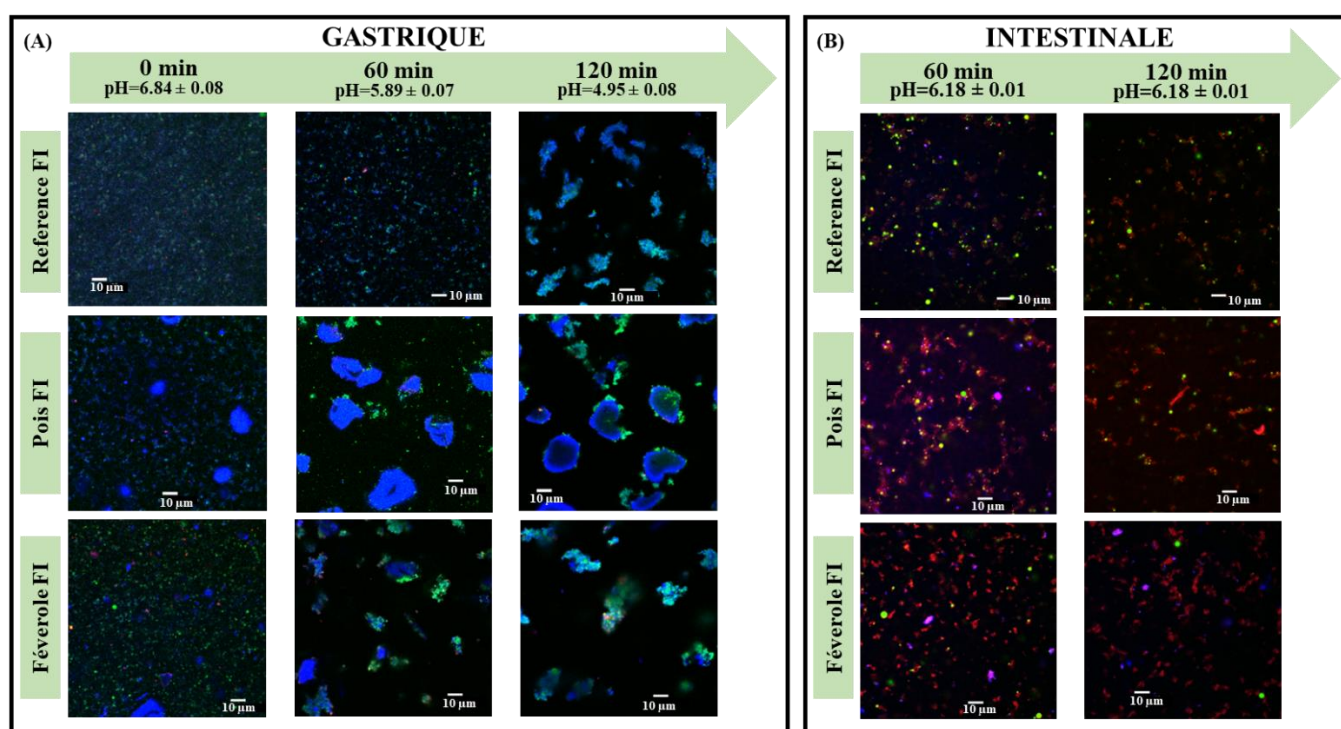


Figure 44. Organisation spatiale des lipides (vert), des protéines (bleu) et des composés amphiphiles (rouge) en microscopie confocale pour les trois formules infantiles (FI) dans le compartiment gastrique (A) et intestinal (B) au cours de la digestion *in vitro* dynamique.

BILAN

Ces expériences de digestions *in vitro* ont mis en évidence que le changement de source de protéines impacte effectivement la digestibilité des formules infantiles. Ainsi, les digestions *in vitro* statiques indiquent une moindre digestibilité des formules infantiles à base de protéines de riz et encore davantage pour celle à base de protéines de pomme de terre, par rapport à la formule de référence et aux formules à base de protéines de pois et de féverole. Les digestions *in vitro* dynamiques des formules infantiles de pois, féverole et de référence ont quant à elles montré des différences de structure dans le compartiment gastrique ainsi que des différences de protéolyse, mais qui ne semblaient finalement pas impacter les résultats de bioaccessibilité des AAE mesurées en fin de digestion. Cependant, une moindre digestibilité a été calculée pour la formule infantile à base de protéines de pois par rapport à celle à base de féverole et la référence, montrant ici de nouveau l'effet négatif de la moindre solubilité de la protéine de pois sur sa digestibilité *in vitro*.

6.3.4. Conclusion générale

C'est à notre connaissance la première fois que des formules infantiles contenant des protéines végétales autres que des protéines de soja ou de riz hydrolysé ont été développées, et que leur comportement au cours de la digestion a été étudié. Ce travail a été mené selon une approche multi-échelle en partant de la fabrication jusqu'à la digestion de ces formules infantiles innovantes, permettant ainsi d'apporter une vision globale de l'aptitude des protéines végétales candidates à remplacer partiellement les protéines de lactosérum dans les formules infantiles.

Ce travail a tout d'abord montré qu'il est possible de formuler des produits nutritionnellement adaptés aux besoins du nourrisson, sur le plan de la composition en AAE du moins, en substituant une partie des protéines de lactosérum par certaines protéines végétales. Cependant, certains critères physico-chimiques comme la solubilité des protéines et la viscosité qu'elles engendrent, doivent être pris en compte car ils conditionnent la faisabilité industrielle et les propriétés des formules infantiles. Ainsi, les protéines de riz et de pomme de terre se sont finalement avérées moins adaptées à la fabrication de formules infantiles, tout du moins pour ce qui concerne les concentrés protéiques utilisés dans ce projet. Ces limites technologiques des protéines de riz et de pomme de terre expliquant sans doute également leur moindre digestibilité. A l'inverse, les formules infantiles à base de protéines de pois et de féverole présentent des propriétés physicochimiques et nutritionnelles proches de la référence laitière. Ces résultats prometteurs éclairent les possibilités d'innovation en nutrition infantile. Ils devront toutefois être confirmés à échelle industrielle et complétés par des études *in vivo*.

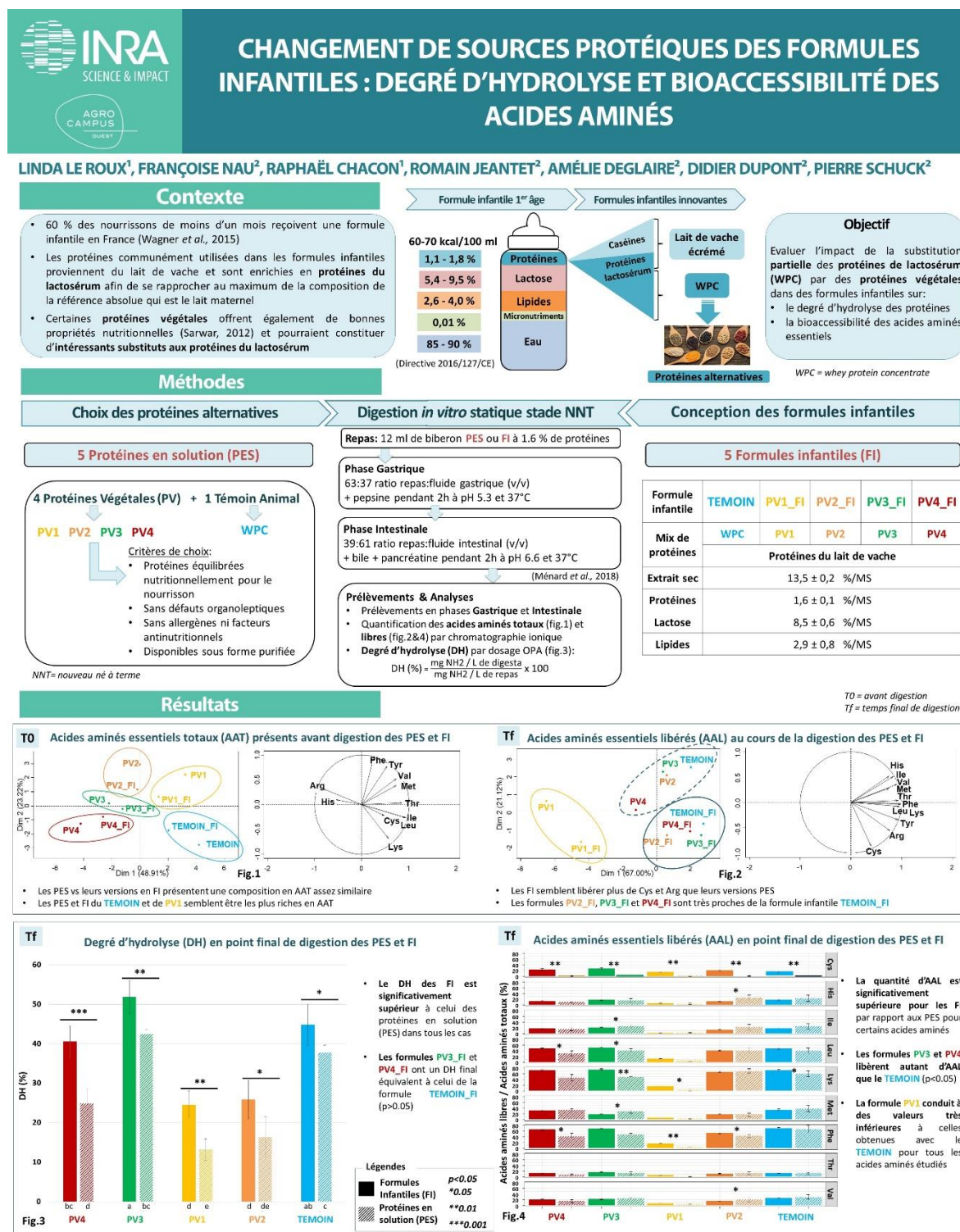
6.3.5. Perspectives

A court terme, la fabrication des formules infantiles « végétalisées » devra être optimisée, notamment pour améliorer leur solubilité au cours de la fabrication et dans le produit fini. Pour cela, des prétraitements mécaniques sur les protéines végétales en solution, comme par exemple une homogénéisation, pourraient être une piste pour diminuer la taille des agrégats protéiques et donc améliorer leur dispersion. L'hydrolyse des protéines pourraient également être une solution pour améliorer leur solubilité. L'aspect financier devra aussi être étudié et pris en compte pour le choix de l'utilisation de ces nouvelles protéines dans une perspective de commercialisation de ces produits.

A plus long terme, des essais industriels seront à mettre en place afin de confirmer la faisabilité de développement de ces nouvelles formules infantiles à échelle réelle. De plus, des études *in vivo* seront indispensables pour étudier le réel impact nutritionnel du changement de source protéique dans ces formules infantiles, mais aussi pour étudier l'éventuel effet allergène ou encore l'impact sur le microbiote de ces nouvelles sources de protéines. Pour finir, il faudra aussi obtenir l'autorisation réglementaire de conformité pour l'utilisation de ces nouvelles protéines dans les formules infantiles, notamment par la réalisation d'études scientifiques qui devront démontrer l'innocuité de ces protéines dans l'alimentation du nourrisson.

6.4. Poster communications

Journées Francophones de Nutrition (JFN) – 28 au 30 novembre 2018 – Nice (France)



STLOpen Days – 19 au 21 mars 2019 – Rennes (France)



Linda Le Roux



CIFRE PhD student

2016-2019



UMR INRA - Agrocampus Ouest
Science and Technology of Milk and Eggs

Teams

Bioactivity and Nutrition
Spray-Drying - Concentrated Matrices –
Functionalities

Key words

Infant formulas
Infant nutrition
In vitro digestion
Protein substitution
Spray drying

Funding



Infant formulas & New protein sources

Socio-economic context

Infant formulas are the key nutritional source for infants who cannot be breastfed:
60 % of the newborns aged of 1 month are fed with an infant formula in France



France is the number 1 in infant nutrition market in Europe
→ 945 million € turnover in 2013

Infant formulas are usually cow milk proteins based with a majority of **Whey Proteins**

When there is a **lack of whey production**, is it possible to **substitute cow milk whey proteins by alternative protein sources, innovatives, environnement-friendly** and respectful of the infant health ?



Research question

How do **protein sources** influence the **digestibility** and the **functionality** of infant formulas ?

Expected results

Identification of new protein sources

Amino acid balance, no antinutritional factors, no allergens and commercially available

Development of new infant formulas

Pilot and semi-industrial scales

Digestion of infant formulas

Static and dynamic in vitro models

Nutritional properties

Bioaccessibility of amino acids

Techno-functional properties

Stability and solubility of powders

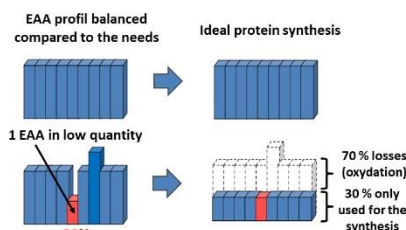
Infant formulas designed and optimised at a semi-industrial scale and nutritionally balanced

Research perspectives

- Industrial development of innovative infant formulas
- Evidence of conformity to the European regulation 2016/127 for marketing
- Expand the knowledge to the protein sources not yet exploited

Scientific context

The nutritional quality of proteins depends on the content of the **Essential Amino Acids (EAA)** and their **Digestibility**



Protein denaturation during process (pasteurization, concentration, spray drying) generates...

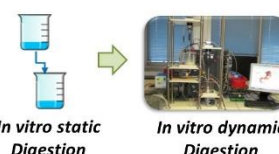
...**structural and functional** modifications of protein which can result in...

...**nutritional losses and/or technical issues**



Pilot Spray dryer

Semi-industrial Spray dryer



In vitro static Digestion

In vitro dynamic Digestion

7. Thesis outputs

Publications

Le Roux, L., Méjean, S., Lopez, C., Chacon, R., Dupont, D., Deglaire, A., Nau, F., Jeantet, R. Plant proteins partially replacing dairy proteins greatly influence infant formula functionalities. Accepted on 27th November 2019. LWT - Food Science and Technology Journal.

Le Roux, L., Chacon, R., Dupont, D., Jeantet, R., Deglaire, A., Nau, F. *In vitro* digestion reveals how plant proteins modulate infant formula digestibility. Accepted on 15th December 2019. Food Research International.

Le Roux, L., Ménard, O., Chacon, R., Dupont, D., Jeantet, R., Deglaire, A., Nau, F. Are faba bean and pea proteins potential whey protein substitute in infant formulas? An *in vitro* dynamic digestion approach. Submitted to Foods Journal on 16th January 2020 (foods-709871).

Communications

Oral communication

Le Roux, L., Chacon, R., Ménard, O., Dupont, D., Deglaire, A., Jeantet, R., Nau, F. Alimentation et digestion chez le nourrisson. 7^e Journée de l'animation transversale « Glandes mammaires, lait ». 15 November 2018. Rennes. France.

Le Roux, L., Chacon, R., Ménard, O., Dupont, D., Deglaire, A., Jeantet, R., Nau, F. *In vitro* digestion evidence of how plant proteins modulate infant formula digestibility. International Conference on Food Digestion (6th Edition). 2-4 April 2019. Grenada. Spain.

Le Roux, L., Chacon, R., Dupont, D., Deglaire, A., Nau, F., Jeantet, R. Influence of protein source on the functionality and the digestibility of infant formulas. International Congress on Engineering and Food (13th Edition). 23-26 September 2019. Melbourne. Australia.

Poster communication

Le Roux, L., Chacon, R., Dupont, D., Deglaire, A., Jeantet, R., Nau, F., Schuck, P. Changement de sources protéiques des formules infantiles: degré d'hydrolyse et bioaccessibilité des acides aminés. Journées Francophones de Nutrition. 28-30 November 2018. Nice. France.

Le Roux, L., Chacon, R., Ménard, O., Dupont, D., Deglaire, A., Jeantet, R., Nau, F. Infant formula & New protein sources. STLOpen Days. 19-21 March 2019. Rennes. France.

Formations

Année 2017

11/01/2017. Introduction à l'éthique de la recherche. Rennes.

21/01/2017. Initiation à Zotéro - Logiciel de gestion des références bibliographiques. Rennes.

15-16/02/2017. Mister 1 & 2 – Recherche bibliographique. Rennes.

20-21/02/2017. Concentration-Séchage-Perfectionnement. Plouvien.

16/03/2017. Table ronde « anciens doctorants ». Rennes.

04-06/04/2017. Participation et membre du comité d'organisation du congrès *International Conference on Food Digestion* (5th Edition). Rennes.

07/04/2017. Participation au Doctorama – Exercice de vulgarisation scientifique du sujet de thèse des doctorants. Rennes.

12/10/2017. Participation et membre du comité d'organisation du Festival des Sciences. Melesse.

13-15/12/2017. Journées Francophones de Nutrition. Nantes.

Année 2018

19/03/2018. La gestion des données sous R. Rennes.

03-06/04/2018. Doctoriales Bretagne Loire 2018. Angers.

12/04/2017. Membre du comité d'organisation du Doctorama – Exercice de vulgarisation scientifique du sujet de thèse des doctorants. Rennes.

02-04/05/2018. Planification expérimentale. Nantes.

Octobre 2018. Statistiques de base avec le logiciel R. Le Rheu.

Année 2019

04-05/07/2019. Journées scientifiques de l'école doctorale EGAAL. Rennes.

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Titre : De la fabrication à la digestion *in vitro* de formules infantiles innovantes en partie composées de protéines végétales : une approche multi-échelle

Mots-clés : digestion, formule infantile, homogénéisation, protéines végétales, protéolyse, séchage par atomisation

Résumé : Les formules infantiles (FI) sont la seule source nutritionnelle pour les nourrissons lorsque l'allaitement est impossible. Les FI en poudre sont obtenues par séchage par atomisation et sont composées de protéines de lait de vache, majoritairement des protéines du lactosérum. A l'avenir, les protéines végétales pourraient constituer une alternative nutritionnelle, fonctionnelle et durable à l'utilisation de protéines animales. L'objectif de ce travail était d'étudier l'influence de la substitution partielle des protéines laitières par des protéines végétales sur les propriétés fonctionnelles et la digestibilité de FI innovantes. A cet effet, des protéines de pois, féverole, riz et pomme de terre ont remplacé la moitié des protéines dans des FI végétalisées et ont été comparées à une FI de référence composée de protéines du lactosérum.

La digestibilité de ces FI, fabriquées à échelle pilote puis semi-industrielle, a été étudiée en digestions *in vitro* statique puis dynamique simulant les conditions physiologiques du nourrisson. La source de protéine influence les propriétés des FI, qui étaient cependant toutes nutritionnellement adaptées aux besoins du nourrisson. Les limites de solubilité et de viscosité observées pour les FI à base de riz et de pomme de terre affectent leur fabrication et leur digestibilité. A l'inverse, les FI à base de pois et de féverole présentent des propriétés physicochimiques et nutritionnelles très proches de la référence. Ces résultats prometteurs éclairent les possibilités d'innovation en nutrition infantile. Ils devront toutefois être confirmés à échelle industrielle et complétés par des études *in vivo*.

Title: From manufacturing to *in vitro* digestion of innovative infant formulas partly made of plant proteins: a multi-scale approach

Keywords: digestion, infant formula, homogenization, plant proteins, proteolysis, spray drying

Abstract: Infant formulas (IF) are the only source of nutrition for infants when breastfeeding is not possible. Usually, IF are spray-dried to a powder form and include cow milk proteins as well as whey protein concentrate. In the future, plant proteins could be an interesting alternative to the use of animal proteins for nutritional, functional and sustainable concerns. The objective of this work was to study the influence of the partial substitution of whey protein concentrate by plant proteins on the functional properties and the digestibility of IF. For this purpose, proteins from pea, faba bean, rice and potato replaced half of the proteins in plant-based IF and were compared to a reference IF based on whey proteins. The digestibility of these IF, manufactured at pilot scale then at semi-industrial

scale, was assessed through *in vitro* digestion simulating infant physiological conditions using static and dynamic models. Although all IF showed a balanced nutritional profile compared to the infant needs, it was demonstrated that the protein source had a great impact on IF properties. Indeed, solubility and viscosity limits observed for rice and potato IF affected their manufacturing as well as their digestibility. On the contrary, pea and faba bean IF showed physicochemical properties and protein nutritional quality very close to the reference. These promising results highlight future innovation possibilities in infant nutrition field. However, they need to be validated at the real industrial scale and extended through *in vivo* studies.