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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 1st age 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. In comparison to RIF, PIF and FIF were difficult to disperse, thus conducting to remaining insoluble particles. Thus, the protein source greatly influences IFs properties, and process parameters need to be adapted for each formulation to meet IFs quality criteria.

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

1. Introduction

Infant formulas (IFs) can be defined as substitutes for human milk, which are mostly spray-dried to a powdered form. IFs are prepared to closely mimic the nutritional composition of the benchmark human milk, comprising of macronutrients (carbohydrates, fat and proteins) and micronutrients (minerals and vitamins), in order to provide the required nutrients for proper growth, body composition, neurodevelopment, appetite and hormonal regulation of the infants (Michaelsen & Greer, 2014). In the absence of breastfeeding, the nutritional requirements of infants must be satisfied by supplying IF products until they become accustom to complementary food (Agostoni et al., 2008; EU, 2016).

According to the applicable European regulation (EU, 2016), the sources of proteins allowed for 1st age IFs (0 to 6 months) are either bovine's milk protein, goat's milk protein, soy protein isolate or hydrolysed rice protein. Furthermore, the demand for animal proteins is expected to double by 2050, driven by population growth and by the emerging middle classes in developing countries (Egbert & Payne, 2009; FAO, 2006). Therefore, it seems essential to search for alternative protein sources that show nutritional and functional qualities close to that of breast milk or to the IFs currently on the market that include animal protein. In that respect, there is a growing interest in utilizing plant proteins as partial replacers of animal proteins in food (Ainis, Ersch, & Ipsen, 2018). Due to high nutritive quality, good techno-functional properties (Barac et al., 2012) and acceptable cost, legume proteins, for instance soy, pea, chickpea, faba bean or lupine proteins, represent a potential alternative to proteins of animal origin (Ainis et al., 2018; Alves & Tavares, 2019; Chihi, Mession, Sok, & Saurel, 2016). Especially, pea proteins (Pisum sativum) are becoming a viable alternative to soy protein products because of their high essential amino acid content (Boye, Zare, & Pletch, 2010) and relatively good digestibility (O'Kane, Vereijken, Gruppen, & Van Boekel, 2005). Furthermore, pea protein has fat- and water-binding capabilities, emulsification and gelation properties (Sandberg, 2011). Faba bean (Vicia faba L.) is another source of good quality proteins, particularly rich in lysine and threonine. The most recent research is promoting its use for novel food applications, as a potential soy substitute, and as a beneficial crop having important functions for vital and sustainable agroecosystems (Crépon et al., 2010). However, some functional properties of plant proteins such as the ability to stabilize emulsions are known to strongly depend on pH, ionic environment, presence of other ingredients, variation in pre-treatment processing of the proteins and thermal processing of emulsion-based foods (Day, 2013; Tang & Sun, 2011). Nevertheless, the interfacial properties of plant proteins are only partially known. In general, plant proteins form 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 (Gharsallaoui, Cases, Chambin, & Saurel, 2009; Wong et al., 2012).

Therefore, the question arises whether alternative plant proteins to soy or rice proteins could be conceivable in 1st age IFs.

Some 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, Valencia, & Garcia, 1988). Some others were focused on the capacity of probiotics encapsulation using plant proteins in follow-on IFs, as for example pea protein in Kent & Doherty (2014) study or different legume proteins (chickpea, faba bean, lentil and pea) in Khan, Korber, Low, & Nickerson (2013) study. Recently, a process for preparing a 1st age IF based on potato protein, naturally hypoallergenic, that is suitable for infants with cow's milk protein allergy, has been patented (WO2018 115340 A1). These relevant studies on the use of plant proteins in IFs need to be furthered with other protein sources that would be suitable to infant needs from birth, on a nutritional and functional point of view.

IFs are produced by spray drying a concentrated solution, which extends their shelf-life and aids handling (Blanchard, Zhu, & Schuck, 2013). The manufacture of powdered IFs usually includes the following unit operations: mixing, pasteurization, evaporation, homogenization and spray drying. 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, Wang, Wang, & Guo, 2018). The properties of IFs, for example colour, solubility and storage stability can be affected by the component interactions (Li, Zhu, Zhou, Peng, & Guo, 2016), as well as by the unit operations. Sun et al. (2018) demonstrated that homogenization, pasteurization and spray drying steps strongly influenced the microstructure, thermal properties and structural characteristics of IFs. IF powders and the emulsions resulting from their reconstitution in water should be stable in order to avoid quality problems such as fat release, flecking, Maillard reactions, lactose crystallization (i.e., caking) and poor solubility. A greater understanding of the interactions between composition, manufacturing conditions and product-process interactions is essential to solve the above stated problems.

In a previous study (Le Roux et al., submitted 2019a), four plant proteins have been selected for the preparation of innovative 1st age IFs at a pilot scale. Selection was based on the following criteria: the proteins should contain an amino acid profile suited to infant needs (UE, 2016), have no known allergens or organoleptic defects, be commercially available and should be innovative alternative protein sources to animal or plant proteins already used in 1st age IFs (EU, 2016). The aim of this study was to investigate the capability of a partial substitution (50%) of bovine milk proteins by plant proteins in IFs, considering a standard cow milk protein IF as a reference (RIF). 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 to pave the way to future innovation in this field. Physicochemical properties of these new IFs have been evaluated and *in vitro* static digestion assays have been performed respecting most of the infant physiological conditions. It was concluded that rice

and potato proteins IFs showed limitations in terms of manufacturing (very high insolubility for rice IF and high viscosity for potato IF) as well as digestibility impairments (low proteolysis and low amino acid bioacessibility for both IFs, particularly for potato IF). On the contrary, pea and faba bean IFs (PIF and FIF) showed physicochemical properties and overall digestibility closer to the RIF.

In the present study, the semi-industrial scale-up of PIF, FIF and RIF preparation has been investigated in order to explore more representative processing conditions with regard to industrial realities and to confirm the first encouraging results previously obtained with the screening of the plant-based IFs produced at a pilot scale (Le Roux et al., submitted 2019a). The influence of the process parameters, namely homogenization pressure and spray drying temperatures, on the physicochemical properties and the microstructure of the IFs was investigated using two different processing routes. To the best of our knowledge, this is the first time that 1st age IFs (0 to 6 months infants) containing plant proteins other than soy, hydrolysed rice have been designed and their behaviour during processing investigated.

2. Materials and Methods

2.1. IF ingredients

Skim bovine milk powder (35.1 w/w% protein, 54.5 w/w% lactose) was purchased from SILL, Plouvien, France. Maltodextrin (GLUCIDEX® Maltodextrin Premium 19, 89.0 w/w% maltodextrin) was purchased from ROQUETTE, Lestrem, France. Lactose (96.0 w/w%), whey protein concentrate (Protarmor™80, 81.4 w/w% protein), and demineralized whey protein concentrate (Lactarmor™ DM 90, 12.0 w/w% protein, 81.6 w/w% lactose) were all purchased from ARMOR PROTEINES in Loudéac, Saint-Brice-en-Coglès and Pontmain, France, respectively. Pea protein concentrate (*Pisum sativum*, Nutralys® XF, 71.7 w/w% protein) was purchased from ROQUETTE FRERES, Vic-sur-Aisne, France. Faba bean protein concentrate (*Vicia faba*, VITESSENCE™ Pulse CT 3602, 60.9 w/w% protein) was purchased from INGREDION, Hamburg, Germany. An oil blend adapted to infant formulas (91.1 w/w% saturated fatty acids) was purchased from CARGILL REFINED OILS EUROPE, Izegem, Belgium. The composition of IFs was based on the nutritional requirements from the latest European regulation for 1st age infant formula (EU, 2016). For 100 ml of reconstituted IFs at 13.4 ± 0.6 % DM, the nutritional composition was the following: 69.6 ± 2.0 kcal, 1.5 ± 0.1 % protein, 3.2 % ± 0.2 fat and 8.3 % ± 0.4 carbohydrates. The IFs ingredient composition is available in supplementary data (Table 3).

2.2. IF processing

The IFs were manufactured at Bionov (Rennes, France) according to the technological diagram presented in Fig. 1, 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 skim bovine milk; therefore, all infant powders were iso-nitrogenous. Neither additional vitamins or 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°C for 35 s, 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 $kg \cdot h^{-1}$ at 60 ± 4 °C. The concentrate was then cooled to 45°C and stored in a tank. The oil blend was added to the concentrate and was homogenized 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 $kg \cdot h^{-1}$. The dryer was equipped with a pressure nozzle of 0.73 mm orifice diameter and 15 MPa for RIF and PIF, 0.63 mm orifice diameter and 17 MPa for FIF, both (both providing a spray angle of around 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 ± 10 $L \cdot h^{-1}$ and the major airflow rate was 3200 ± 100 $kg \cdot h^{-1}$. The resulting IF powders were finally stored in plastic bags at 20°C. Each IF, namely "formulation x processing route", was manufactured once.

2.3. Physicochemical analysis

2.3.1. Ash and protein content

Ash content was determined after incineration at 525 ± 25°C in a muffle furnace, according to Schuck, Dolivet and Jeantet (2012).

Total nitrogen content was determined according to IDF (2001a) using the Kjeldhal method, and a conversion nitrogen factor of 6.38 for the reference bovine milk protein based IF (Mariotti, Tomé, & Mirand, 2008). For the IFs composed of 50% bovine milk proteins and 50% plant proteins, the conversion factor used was the average of the one of bovine milk proteins (6.38) and 5.4 for pea and faba bean proteins (Mariotti et al., 2008). All measurements were carried out in duplicate.

2.3.2. Dry matter

Dry matter (DM; in $g \cdot 100\ g^{-1}$) was gravimetrically determined by drying 1 g sample mixed with sand in a forced air oven at 102 ± 2°C for 5 h. Measurements were carried out in duplicate.

149 2.3.3. Fat and free fat content

150 The total fat content was measured by Gerber's acid-butyrometric method after dissolution of proteins by the addition of
151 sulfuric acid and of amyl alcohol to facilitate the separation of milk fat by centrifugation at 350g. The free fat content was
152 obtained after extraction with petroleum ether and was determined gravimetrically after evaporation of the solvent. Total
153 and free fat analyses were carried out in duplicate (AFNOR, 1990 ; Schuck et al. 2012).

154 2.3.4. Water activity and glass transition temperature

155 Water activity (a_w) was measured at 25°C ± 0.1°C using the Novasina aw-meter (Novasina, Switzerland).
156 The glass transition temperature (Tg) was determined on the powders after equilibration in a 20% relative humidity
157 atmosphere using the SPSx-1μ Sorption Test System (ProUmid GmbH & Co. KG, August-Nagel-Str., Germany). Tg was
158 determined at this constant sorption point by using a modulated temperature differential scanning calorimetry method
159 according to Schuck et al. (2012). Water activity and Tg measurements were carried out in triplicate.

160 2.3.5. Particle size distribution

161 The powder size distribution was determined using a laser scattering granulometer (Mastersizer, Malvern Instruments Ltd,
162 Malvern, UK) with a 300-mm measurement cell (0.5-880 mm range). The refractive index of dried particles was 1.45, and
163 30 kPa air pressure was used. The median diameter d(0.5) was chosen to describe the particle size distribution of infant
164 powders.
165 The particle size distribution of the dispersed elements present in solutions during process was determined using the same
166 laser scattering granulometer in liquid channel. The particle size distribution was based on volume and expressed as
167 sphere-equivalent diameter. The diameter Mode (the population of the particles the most frequent in the volume
168 distribution) as well as and the D[4.3] (the mean volume diameter) were calculated. The refractive index used was 1.45 for
169 blends of vegetable oils in infant formulas. The refractive index of 1.33 was used for water. The samples taken from
170 concentration and homogenization steps were half-diluted in water prior measurement. About 0.2 mL sample was diluted
171 in 100 mL water directly in the measurement cell of the apparatus in order to reach 10% obscuration. The experiments
172 were performed in triplicate for each sample.

173 2.3.6. Color

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

2.3.7. Rehydration properties

Dispersibility and solubility were determined according to Schuck et al. (2012). The dispersibility index is defined as 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-mm sieve. The solubility index (SI) is defined as the v/v% of soluble particles (i.e., remaining in the supernatant after centrifugation of 160*g* for 5 min) after 13 g powder were added to 100 g water and two droplets of defoaming agent (octan-1-ol) at 40°C and mixed in a blender for 90 s. These experiments were carried out in duplicate.

2.3.8. Viscosity

Apparent viscosity was measured using a controlled-stress rheometer (Rheometer, TA DHR2 Hybrid Instruments, Crawley, UK), equipped with a coaxial cylinder geometry and a solvent trap. Temperature was controlled by a Peltier apparatus (±0.1°C). Apparent viscosity was measured in triplicate on homogenized samples (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).

2.4. Confocal laser scanning microscopy

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 20°C. Fast Green FCF fluorescent probe (Sigma F7258, Sigma-Aldrich, St Louis, USA) was used for the labelling of proteins (Excitation = 632 nm). The lipid-soluble Nile Red fluorescent probe (5H-Benzo, α-phenoxazine-5-one, 9-diethylamino; Sigma – Aldrich, St Louis, USA) was used to label the lipids (Excitation laser = 543 nm). After labelling, the samples were kept for at least 30 min before microstructural analysis. A He-Ne laser operating at 543 nm wavelength excitation and emission detected between 565 nm and 615 nm, 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.

2.5. Statistical analysis

Statistical analyses were conducted with the use of R version 3.5.2 (The R Foundation, 2014).

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

3. Results & Discussion

In an innovation purpose, this study aimed to assess the possibility of substituting a fraction (50%) of bovine's 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. 2019a, submitted). 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.

3.1. Physicochemical properties of IFs

The six IFs, namely PIF, FIF and RIF produced according to the processing routes 1 and 2 (Fig.1), were equivalent in terms of dry matter (DM), ash, protein and fat contents with mean values of respectively 96.9 ± 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.

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, Scher, & Desobry, 2006). The fat stability is generally considered as satisfactory when the free fat content remains below 5% in whole milk powder (Vignolles, Jeantet, Lopez, & Schuck, 2007). In the present study, free fat content was equal to 2.2 ± 0.3 w/w% of DM at T0, regardless of the IF and the process parameter sets. McCarthy et al. (2013) found similar results with a free fat level of 2.0 ± 0.2 % in dairy protein-based IF powder (with a protein: fat ratio of 0.43, i.e. a fat content a bit higher compared to IFs here tested 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 1). 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 (4.3 ± 0.6 w/w%) was very close to 5% after 4 months storage at 20°C. 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 105.8 ± 0.6 µm) compared to the other IFs (d(0.5) diameters ranging

237 between 111.8 ± 1.0 µm and 141.2 ± 1.6 µm), resulting in a higher surface exchange area leading to less fat retained in the
238 particles and, consequently, more free fat released (Buma, 1971).
239 Besides, pea protein isolate has been defined as a good emulsifier for preparing oil in water emulsions (Franco, Partal,
240 Ruiz-Marquez, Conde, & Gallegos, 2000; Lu, Quillien, & Popineau, 2000). However, its emulsifying capacity, as well as
241 solubility, has been reported to be reduced when pH is close to its isoelectric point, i.e. 5.2-6.1 (Karaca, Low, &
242 Nickerson, 2011). Moreover, the heat treatment of pea protein resulted in emulsions in which inter-droplet hydrophobic
243 interactions are favoured, which can increase the droplet flocculation and thus destabilize the emulsion (McClements,
244 2004; Peng et al., 2016). In the present study, the emulsions corresponding to the different IFs were moderately stabilized
245 regardless of the protein source, as indicated by the free fat content measured, especially after 4 months storage. This
246 suggests that the processing parameters should be optimized to decrease free fat level, processing route 1 appearing
247 preferable than route 2. The emulsion stability could be also improved by using emulsifiers or producing bigger powder
248 particles.
249 Spray-drying behaviour and storage ability of milk powder depend very much on both glass transition temperature (Tg)
250 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
251 defined by Efstathiou, Feuardent, Méjean & Schuck (2002) (Table 1). Therefore, the long-term quality of the IFs should be
252 guaranteed, these powders being free from phenomena such as lipid oxidation, caking or browning that are likely to occur
253 when a_w is not at its optimal value. The Tg mean value at 0.2 a_w was 58.7 ± 4.3°C for all the powders, and was
254 significantly higher for RIF1 and FIF1 compared to the four other IFs with 66.7 ± 0.3°C and 60.5 ± 1.8°C, respectively.
255 Tham, Yeoh, & Zhou (2017) found comparable Tg values for IFs compared to the present study and showed a good
256 storage stability at 25°C. McCarthy et al. (2013) reported a Tg value of 55.5 +/- 1.01 °C for a dairy protein based IF
257 powder (a_w= 0.23), also in accordance with our results.
258 Dispersibility is defined as the capacity of wet aggregates to uniformly disperse when in contact with water. A powder is
259 considered dispersible if the dispersibility index (DI) is higher than 85% (Schuck et al., 2012). The DI of the infant
260 formula powders prepared in this study ranged between 99.7 ± 0.9% and 97.9 ± 1.5% for the RIF1 and RIF2, respectively
261 (Table 1). On the other hand, the solubility index (SI) represents the loss of granular structure when the powder is
262 solubilized in water. A powder is considered soluble when the SI is above 89.5 ± 2.2% (Schuck et al., 2012). In this study,
263 the RIFs presented a SI value of 100%, while PIFs and FIFs showed SI values of 97.5% and 97%, respectively (Table 1).
264 Hence, all the powders prepared in this study can be regarded as dispersible and soluble according to these methods. Even
265 more so as the rehydration ability can be enhanced by increasing temperature or stirring speed during the reconstitution
266 step (Jeantet, Schuck, Six, Andre, & Delaplace, 2010). However, it was visually noticeable that PIF and FIF encountered

dispersion impairments when powder was dissolved in water, with insoluble particles produced during manufacturing of the plant-based IF powders and that create flecking when rehydrated. This behavior has also been noticed by (P. Schuck et al., 2016; 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 are in accordance with the one found for a whole milk powder parameters (71.9 ± 0.2, 6.0 ± 0.1 and 17.4 ± 0.4, respectively for the parameters L, a, b).

Lastly, after dispersion in water and homogenization, the viscosity of some of the IFs studied here was significantly higher than usually recommended for an effective spray-drying. The highest value was measured for PIF2 at 1.55 Pa.s (Table 1). 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, Méjean, Dolivet, Beaucher, & Famelart, 2005). Despite this, the high viscosities measured in this study seemed to not have affected the drying characteristics, neither the physicochemical properties of the final products (Table 1). Nevertheless, it is obvious that the viscosities measured for PIF did not correspond to optimal conditions for spray drying and that 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 is thus consistent with the viscosity increase when pressure increases as reported by Pouliot, Britten, & Latreille (1990) for a study on IFs. These authors suggested that high pressures homogenization result 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.

3.2. 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 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 between the process steps (Fig. 2 and Table 2).

After solubilisation of the different ingredients except oil blend, the modes of the particle size were about 0.3 and 2.1 µm for RIF, whereas bigger particles could be observed in plant-base IFs, 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 suggest an incomplete solubilisation of plant proteins, 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, Hendricks, & Kindstedt, 1998, 1999) leads to their unfolding, exposure of hydrophobic patches and irreversible aggregation by forming hydrophobic interactions, hydrogen bonds and/or disulphide bonds. Protein aggregation may be to a greater or lesser extent, depending on the protein nature and the physicochemical conditions (pH versus isoelectric point, nature and concentration of salts, etc.), but in any case it influences the solubility of the proteins (Benjamin, Silcock, Beauchamp, Buettner, & Everett, 2014; Corredig & Dalgleish, 1995; Malaki, Tosh, Woodrow, Poysa, & Corredig, 2009). The solubility of pea protein isolate at pH 7.0 (i.e. close to pH 6.6-6.8 applied in the present study) was measured as 59.77 ± 2.34 % or 57.94 ± 0.21 % depending on the isolate was non-heated or heated at 90°C for 3 min (Barac, Pesic, Stanojevic, Kostic, & Bivolarevic, 2015). Karaca, Low, & Nickerson (2011) obtained a similar result (61.42 ± 0.77 %) when determining the solubility of pea protein isolate at pH 7.0 and at room temperature. At the end, such low solubility values are 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 (89.65 ± 0.24 %), 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 (Fig. 2). The solubility of whey protein isolate (87.67 ± 0.02 %) reported by Pelegrine & Gasparetto (2005) was pretty close to our observations for RIF where particle size was much smaller (Fig. 2). In overall, different protein structures 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 (Fig. 2). 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 might be fibbers. As expected, small lipid droplets (in red, Fig. 2) 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 baby bottle (Vignolles et al., 2007). Using the homogenization process parameters 1, a majority of lipid droplets close to 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 much smaller protein particles than before homogenization (modes between 0.7 and 12.7 µm compared to 11.2 and 51.8 µm after concentration). These results suggest that the protein aggregates present in PIF and FIF have been 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 might be due to protein aggregation induced by heat treatments and mechanical treatments such as homogenization (Guo et al., 1998, 1999; Joyce, Brodkorb, Kelly, & O'Mahony, 2017; Peng et al., 2016). Lactose crystals with the characteristic "Tomahawk shape" were observed from the concentration step, and still after the homogenization step, with no change in average size and appearance. Conversely, lactose crystals disappeared after dilution of the samples. It should be reminded that samples taken from concentration and homogenization steps were half-diluted in water prior measurement. Thus, lactose crystals were dissolved and not observed on particle size distribution graphs (Fig. 2).

Finally, the rehydration in baby bottles of the IF powders obtained after either processing routes 1 or 2 showed a homogeneous and unimodal distribution of the fat droplets and the proteins in RIF with modes of 0.5 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 decrease it. Especially, homogenization prior concentration step should be tested for improvement of the plant protein solubilisation.

3.3. 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 RIF after homogenization had thus 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:

$$\textbf{Number of fat droplets} = \frac{\textbf{Total volume of fat droplets}}{\textbf{Fat droplet volume}} \quad (1)$$

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

$$\textbf{Total volume of fat droplets} = \frac{\textbf{Total concentration of fat droplets}}{\textbf{Density of fat blend}} = \frac{0.12}{900} = \mathbf{1.3\ 10^{-4}\ m^3 \cdot kg^{-1}} \quad (2)$$

given that the fat content in the IFs after homogenization was 12 w/w % and the density of the fat blend was $\mathbf{900\ kg \cdot m^{-3}}$. The mean fat droplet diameter **d** chosen was **1 µm** (close to D[4.3] values obtained after homogenization for RIF, Table 2). The fat droplet volume (m^3) was calculated as:

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

Equations 1, 2 and 3 came with a number of fat droplets equal to $\mathbf{2.5\ 10^{14}\ kg^{-1}}$.

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

$$\textbf{Fat droplet surface} = \pi d^2 = \mathbf{3.1\ 10^{-12}\ m^2} \quad (4)$$

$$\textbf{Area of the fat droplet interface} = \textbf{Number of fat droplets} \cdot \textbf{Fat droplet surface} = \mathbf{800\ m^2 \cdot kg^{-1}} \quad (5)$$

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. Bovine milk proteins are well-known to widely spread on oil-water interface in emulsions (Courthaudon, Dickinson, Matsumura, & Williams, 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 $3.0 \cdot 10^{-6}$ kg of proteins ·

m^{-2} surface after homogenization (McCarthy et al., 2012; Pelan, Watts, Campbell, & Lips, 1997; Ye, Singh, Taylor, & Anema, 2002):

$$\textbf{Minimum protein concentration} = \textbf{Protein adsorbed at the fat droplet interface} \cdot \textbf{Area of the fat droplet interface} = \textbf{1.2 to 2.4 } 10^{-3} \textbf{ kg of proteins} \cdot \textbf{kg}^{-1} \textbf{ of homogenized concentrate} \quad (6)$$

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 $g.kg^{-1}$) 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 might be in sufficient concentration to stabilize the emulsion, despite insoluble fraction was noticed.

4. Conclusion

This study handled the feasibility of producing, at a semi-industrial scale, plant protein-based IFs close to a reference dairy 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 lipid-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 for the concentrate to be dried for some of the IFs, in particular PIF using processing route 2. Despite this high viscosity seemed to not have affected the drying characteristics (Table 1, 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, no major differences were noticed between processing routes 1 and 2 except slightly lower free fat (after 4 months storage) and higher dispersibility for RIF1 compared to RIF2; and 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. That means process parameters should be adapted for each formulation in order to provide satisfactory IFs quality. Nevertheless, we have to remember that the results of this exploratory study needs validation. 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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589 ## Captions

590 Fig. 1. Semi-industrial process flow diagram for infant formulas (IFs) production including processing routes (1) or (2) (DM: dry matter;
591 w/w: weight/weight).

592 Table 1. Physicochemical properties of the three powdered IFs (RIF: reference infant formula ; PIF: pea infant formula ; FIF: faba bean
593 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
594 MPa homogenization and 150°C to 65°C drying temperatures. T0: measurement immediately after process; T4: measurement after 4
595 months storage at 20°C. Data are expressed as mean ± SD. For a given characteristic, values with a different superscript letter are
596 significantly different ($p < 0.05$).

597 Fig. 2. Spatial organization of lipids (in red) and proteins (in green) as evidenced by confocal laser scanning microscopy (CLSM) in the
598 three IFs (RIF: reference infant formula; PIF: pea infant formula; FIF: faba bean infant formula) after the different process steps, from
599 solubilisation of the ingredients to the rehydration of the IF powders in baby bottle. Homogenization 1: 8/2 MPa, homogenization 2:
600 14/4 MPa. Baby bottle 1 and 2 were obtained after the rehydration of the IFs obtained with the process parameters 1 and 2
601 (homogenization and drying described in Section 2.2). Size distributions of processed IFs were determined by laser light scattering; The
602 diameter Mode is the population of the particles the most frequent in the volume distribution and the D[4.3] is the mean volume
603 diameter, these results are summarized in the Table 2.

604 Table 2. Mode diameter and D[4.3] of RIF, PIF and FIF samples during process and in the rehydrated powder in baby bottles. Data for
605 D[4.3] are expressed as mean ± SD. (RIF: reference infant formula; PIF: pea infant formula; FIF: faba bean infant formula).

606 Supplementary data. Table 3. Infant formulas ingredient composition for a liquid formula at 24 % DM. (RIF: reference infant formula;
607 PIF: pea infant formula; FIF: faba bean infant formula).

Fig. 1

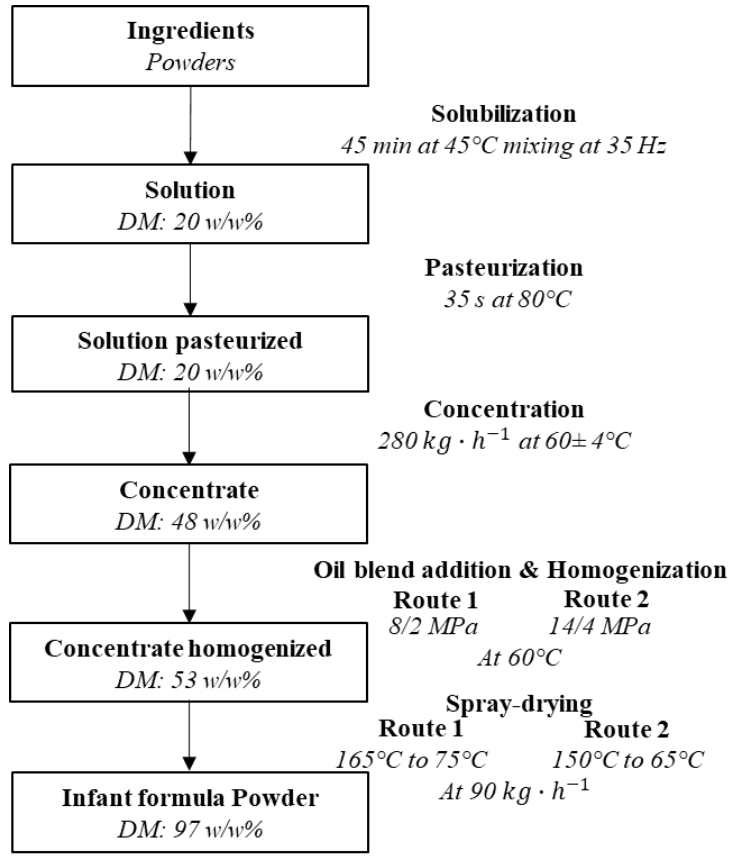
Ingredients
Powders
Solubilization
45 min at 45°C mixing at 35 Hz
Solution
DM: 20 w/w%
Pasteurization
35 s at 80°C
Solution pasteurized
DM: 20 w/w%
Concentration
280 kg · h−1 at 60± 4°C
Concentrate
DM: 48 w/w%
Oil blend addition & Homogenization
Route 1
8/2 MPa
Route 2
14/4 MPa
At 60°C
Concentrate homogenized
DM: 53 w/w%
Spray-drying
Route 1
165°C to 75°C
Route 2
150°C to 65°C
At 90 kg · h−1
Infant formula Powder
DM: 97 w/w%

Fig. 2

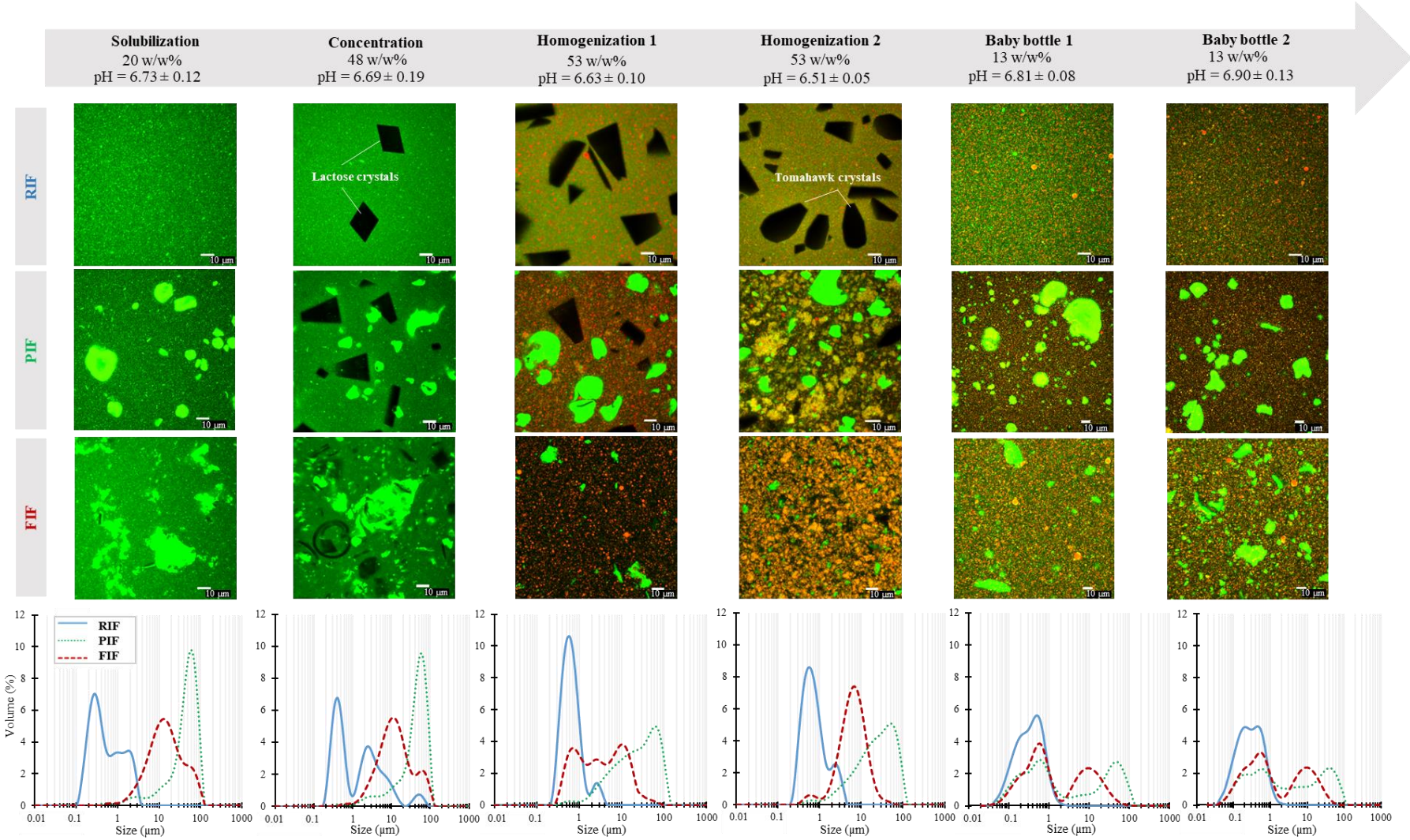
Solubilization
20 w/w%
pH = 6.73 ± 0.12
Concentration
48 w/w%
pH = 6.69 ± 0.19
Homogenization 1
53 w/w%
pH = 6.63 ± 0.10
Homogenization 2
53 w/w%
pH = 6.51 ± 0.05
Baby bottle 1
13 w/w%
pH = 6.81 ± 0.08
Baby bottle 2
13 w/w%
pH = 6.90 ± 0.13
RIF
PIF
FIF
Lactose crystals
Tomahawk crystals
10 µm
RIF
PIF
FIF
Volume (%)
Size (µm)
0.01
0.1
1
10
100
1000
0
2
4
6
8
10
12

Table. 1

	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 (°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}

Table 2.

		D[4.3] (µm)	Mode 1 (µm)	Mode 2 (µm)	Mode 3 (µm)
RIF	Solubilization	0.8 ± 0.4	0.3	2.1	
	Concentration	4.6 ± 1.9	0.4	2.8	
	Homogenization 1	0.8 ± 0.1	0.5	2.8	
	Homogenization 2	0.9 ± 0.3	0.6	2.4	
	Baby bottle 1	0.4 ± 0.1	0.5		
	Baby bottle 2	0.4 ± 0.4	0.4		
PIF	Solubilization	48.3 ± 0.1	58.9		
	Concentration	45.4 ± 0.3	58.9		
	Homogenization 1	34.0 ± 0.2	14.5	66.9	
	Homogenization 2	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	9.9 ± 1.7	0.7	2.4	12.7
	Homogenization 2	7.6 ± 0.3	0.7	6.7	
	Baby bottle 1	6.2 ± 0.4	0.6	8.9	
	Baby bottle 2	5.6 ± 0.2	0.6	10.0	

Supplementary data. Table 3.

kg ingredient / 100 kg infant formula	RIF	PIF	FIF
Skim milk		3.72	
GLUCIDEX®		0.56	
Oil blend		5.23	
Lactose	6.76	13.30	13.30
Protarmor™80	0.62		
Lactarmor™ DM 90	7.73		
Nutralys® XF		1.97	
VITESSENCE™			2.33
Water	75.38	75.22	74.86