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Formulation, pilot-scale preparation, physicochemical characterization and digestibility of a lentil protein-based model infant formula powder

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Abstract

BACKGROUND: Infant formula is a human milk substitute for consumption during the first months of life. The protein component of such products is generally of dairy origin. Alternative sources of protein, such as those of plant origin, are of interest due to dairy allergies, intolerances, and ethical and environmental considerations. Lentils have high levels of protein (20–30%) with a good amino acid profile and functional properties. In this study, a model lentil protein-based formula (LF), in powder format, was produced and compared to two commercial plant-based infant formulae (i.e., soy; SF and rice; RF) in terms of physicochemical properties and digestibility.

Results: The macronutrient composition was similar between all the samples; however, RF and SF had larger volume-weighted mean particle diameters (D[4,3] of 121–134 μm) than LF (31.9 μm), which was confirmed using scanning electron and confocal laser microscopy. The larger particle sizes of the commercial powders were attributed to their agglomeration during the drying process. Regarding functional properties, the LF showed higher D[4,3] values (17.8 μm) after 18 h reconstitution in water, compared with the SF and RF (5.82 and 4.55 μm, respectively), which could be partially attributed to hydrophobic protein–protein interactions. Regarding viscosity at 95 °C and physical stability, LF was more stable than RF. The digestibility analysis showed LF to have similar values ($P < 0.05$) to the standard SF.

Conclusion: These results demonstrated that, from the nutritional and physicochemical perspectives, lentil proteins represent a good alternative to other sources of plant proteins (e.g., soy and rice) in infant nutritional products.

Keywords: lentil; protein; emulsion; powder; functionality; digestibility; infant formula

INTRODUCTION

Infant or first-age infant formula (0–6 months) is an industrially produced, human milk substitute, designed for infant consumption during the first months of life, through to the introduction of appropriate complementary feeding.[1,2] Such products are typically based on the milk from cows or goats, combined with other ingredients (e.g., lipids, carbohydrates and minerals), which have been proven suitable for infant feeding.[3] The most widely used protein ingredients in the formulation of infant nutritional products are those of dairy origin. However, infants can have intolerances or allergies to dairy sources, such as milk protein allergy and lactose intolerance.[4,5] Furthermore, there are parents who choose alternatives to animal-based infant formula due to environmental and animal-welfare considerations.[6] In this regard, there are alternatives to dairy-based formulations available commercially, such as those formulated using plant proteins (e.g., soy- and rice-based infant formulae).

The most common plant-based infant formulae available commercially are those prepared using soy proteins,[7,8] followed by

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those prepared using rice proteins.[9] This is due mainly to the ready commercial availability of soy and rice protein ingredients, which have been well characterized, for formulation of such products. It is also due to the general acceptance of such protein sources by consumers.[9–12] Indeed, as far as the authors are aware, these are the only plant-based infant formula available commercially. The use of soy and rice protein-based ingredients in the formulation of such products is not without its own challenges; for example, soy-based formulae have been reported in a number of previous studies to negatively influence the hormonal system of infants, due to the presence of isoflavones found naturally in soy.[13,14] With regards to rice, the protein content found in the seed (~10%) is low[10,15] compared to legumes for example; therefore, to obtain a purified rice protein ingredient it is necessary to apply intensive extraction, enrichment, and isolation – approaches that can negatively impact on the environment. Rice protein ingredients generally have low solubility and so often present challenges in expressing techno-functional properties (e.g., solubility and interfacial properties) of importance in the formulation of nutritional beverages.[11,16] In addition, rice has an ability to accumulate arsenic during the growth phase and rice-based products can sometimes have relatively high arsenic levels.[17] Le Roux *et al.*[18] reported that the inclusion of rice protein in infant formula results in technological and functional challenges, leading to lower production efficiency during the manufacturing process, due to the impaired solubility of the rice protein ingredient. For all these reasons, it is of scientific and commercial relevance to identify and develop alternative protein sources to dairy, soy or rice that can provide the desired nutritional and functional properties in infant formulations.

A review of the literature demonstrates that there are few scientific studies available that have investigated the substitution of dairy proteins with plant proteins in first-age infant formula, with a particular emphasis on physicochemical properties. Relevant recent work by Le Roux *et al.*[18,19] involved studying the effects of partial substitution of dairy proteins with faba bean, potato, pea and rice protein on the physicochemical properties and digestibility of first-age infant formula. In these two studies, the authors demonstrated the feasibility of producing plant protein-based infant formulations close to a bovine milk-based reference infant formula in terms of physicochemical and functional properties. However, some of the plant protein-based ingredients, such as rice and potato, showed low solubility and very high viscosity, respectively, which both negatively impacted production and the quality of the infant formula. Moreover, the type of protein tested in those studies affected protein digestibility, showing the pea protein-based infant formula to have higher *in vitro* digestibility than the reference formula, while the faba bean infant formula had similar digestibility to the reference formula.

The formulation of food products using plant proteins can present some challenges as these proteins generally have higher molecular weights and lower solubility than dairy proteins.[20,21] The incorporation of plant proteins in infant nutritional products requires that the protein-based ingredients have specific functional properties (e.g., solubility, emulsification, and heat stability) that influence processability (e.g., during homogenization, thermal treatment, and concentration) and key quality attributes of the products. In nutritional product formulation, the proteins from pulses can represent a good alternative to dairy proteins as they have been shown to contain amphiphilic proteins that form relatively thick interfacial layers around oil droplets, thereby enhancing emulsion formation and stability.[21,22] In this regard, lentil proteins were shown to have good ability to form and stabilize oil-in-water emulsion systems, owing to their surface hydrophobicity and/or formation of thick viscoelastic films around oil droplets,[18,21] which make lentil-based emulsions very stable to environmental and compositional stresses such as heat, pH, and added salts.[22,23]

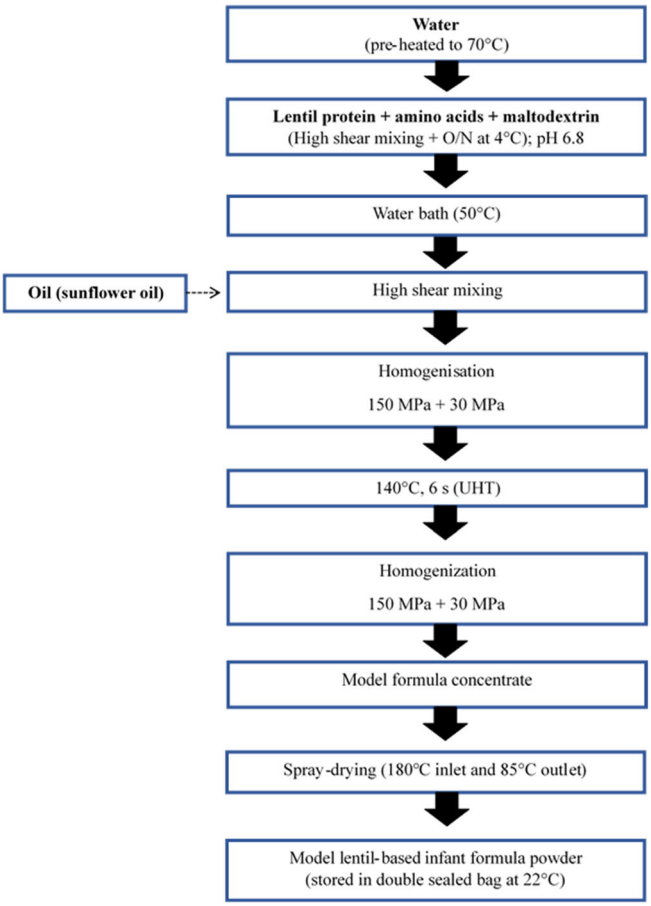

Figure 1. Process flow diagram for the pilot-scale process developed for production of the lentil-based model infant formula.

The objectives of this study were to formulate, prepare at pilot scale, characterize physicochemically (i.e., powder physical and reconstitution properties), and evaluate the *in vitro* digestibilty of a model plant-based infant formula using a novel lentil protein isolate ingredient. The product will also be benchmarked against two commercially available plant-based infant formula products prepared using soy and rice protein.

MATERIALS AND METHODS

Materials

Lentil protein isolate (85.1% protein; w/w), obtained by isoelectric precipitation using a method described by Alonso-Miravalles *et al.*,[24] was provided by the Fraunhofer Institute (Munich, Germany). Maltodextrin, with a dextrose equivalent (DE) value of 17, was supplied by Tereos (Lille, France). Sunflower oil was obtained from a local retail outlet (Tesco, Welwyn Garden City, Hertfordshire, UK). Commercial first-age rice- and soy-based infant formulae from Italy and Ireland, respectively, were included in this study for benchmarking purposes. All the reagents used in this study were of analytical grade and supplied by Merck (St. Louis, Missouri, United States), unless otherwise stated.

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Preparation of model lentil-based infant formula powder

A pilot-scale process was developed to prepare the model lentil-based formula powder as described in Fig. 1. The formulation (~30% total solids; w/w) were prepared by reconstituting the lentil protein powder, amino acids and maltodextrin ingredients, in that order, in pre-heated (70 °C) de-ionized water using a high shear mixer (Silverson AXR, Silverson Machines, UK) operating at 6000 rpm for 2 or 4 h, at a 10 or 20 kg liquid batch size, respectively, until a homogeneous dispersion was obtained. Ingredient quantities were calculated to give a target model infant formula composition of 4.75, 8.22 and 17.0 g/100 mL of protein, oil and carbohydrate, respectively. The free amino acids L-cysteine, L-methionine and L-tryptophan were added at 30.3, 3.55 and 15.1 mg/100 mL, respectively, to ensure that the minimum regulatory requirements[25] were satisfied. After all dry ingredients were incorporated and dispersed, the dispersion was mixed at low speed with an overhead stirrer overnight at 5 °C to facilitate complete rehydration. Afterwards, the dispersion was adjusted to 50 °C using a water bath, followed by the addition of sunflower oil while using high shear mixing. The dispersion was homogenized using two-stage valve homogenization at first- and second-stage pressures of 150 and 30 bar, respectively, followed by high heat treatment at 140 °C for 6 s using a MicroThermics unit (MicroThermics Inc., North Carolina, USA). The heat-treated formulation was again homogenized downstream using an in-line two-stage valve homogenizer (Model NS2006H, Niro Soavi, Parma, Italy) with first and second stage pressures of 150 and 30 bar, respectively. The emulsion was spray-dried using a GEA-Niro Production Minor spray-dryer (Copenhagen, Denmark) equipped with a rotary disc atomizer (27 000 rpm). Inlet and outlet temperatures of the spray dryer were set at 180 and 85 °C, respectively. The powder was collected in a double-sealed sterilized bag and maintained at room temperature until further analysis. Two independent liquid batches (10 and 20 kg) of the model infant formula were prepared using this process.

Characterization of the powders

Chemical composition

The moisture, ash, fat, and protein content of the three products was determined according to the standard methods of the Association of Analytical Chemists.[26] Moisture was determined by oven drying at 103 °C for 5 h. The ash content was analyzed by dry ashing in a muffle furnace at 500 °C for 5 h. The fat content was determined using the Röse-Gottlieb method. The free fat content was measured after extraction with petroleum ether and was determined gravimetrically after evaporation using the method described by Schmidmeier *et al*.[27] The total nitrogen content was determined using the Kjeldahl method using nitrogen-to-protein conversion factors of 6.25 for soy and lentil protein and 5.95 for rice protein. Total carbohydrate content was calculated by difference (i.e., 100 – (sum of protein, fat, ash and moisture)).

Powder particle-size distribution

The particle-size distribution of the powders was determined using a Malvern Mastersizer 3000 laser diffraction instrument with an Aero S dry dispersion unit (Malvern Instruments, Worcestershire, UK) operating at a feed rate of 25%, using a hopper gap of 3.5 mm, and a pressure of 0.4 bar on the standard venturi disperser. The particle refractive index and density were set to 1.45 and 1.33 g cm^{-3}, respectively.

Scanning electron microscopy

The powders were mounted on aluminium stubs using double-sided adhesive carbon tape and sputter coated with a 5 nm layer of gold/palladium (Au:Pd = 80:20) in a Q150R ES (Quorum Technologies, Loughton, East Sussex, UK) coating system. Subsequently, the powders were imaged using a JSM-5510 scanning electron microscope (Jeol Ltd, Tokyo, Japan) coupled with a Everhart-Thornley secondary electron detector operated at an accelerating voltage of 5 kV. At least three images were taken per sample using a magnification of 500× at a working distance between 6 to 7 mm until a good-quality image was achieved.

Confocal laser scanning microscopy

Microstructural analysis of the powders was performed using a Leica TCS SP Confocal Laser Scanning Microscope (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany). Powder samples were placed onto a glass slide and labeled using a dye mixture of Fast Green FCF and Nile Red to visualize the protein and fat phases, respectively. The mixture consisted of Fast Green FCF dissolved in water (0.01 g/100 mL) and Nile Red (0.1 g/100 mL), mixed in a ratio that allowed diffusion of the dyes into the powder particles whilst restricting their hydration. More specifically, for the rice formula sample, Nile Red was dissolved in polyethylene glycol 400 to prevent otherwise rapid hydration and solubilization of the powder particles. Visualization of oil and protein in emulsions was carried out using an Ar laser (excitation 488 nm, emission 520–620 nm) and a He—Ne laser (excitation 633 nm, emission 650–730 nm) for oil (green) and protein (red), respectively. The observations were performed using 40× and 60× oil immersion objectives.

Properties of reconstituted infant formula powders

To study the properties of reconstituted model lentil-based and commercial soy- and rice-based formulae, the powders were reconstituted in pre-heated (70 °C) ultrapure water or 0.2% sodium dodecyl sulfate (SDS). All samples were reconstituted to the same target protein concentration (1.90% protein; w/w), the pH was adjusted to 6.8 with 1 Mol L^{-1} NaOH or HCl, for 2 h at 22 °C, followed by 18 h at 5 °C, after which samples were adjusted to 22 °C, and the pH re-adjusted to 6.8 if necessary, and the following analyses were performed.

Heat treatment and viscosity

The changes in viscosity during heat treatment of the formulae reconstituted in water or SDS, were determined using an AR-G2 controlled-stress rheometer, equipped with a starch pasting cell geometry (TA Instruments Ltd, Waters LLC, Leatherhead, Survey, UK); the internal diameter of the cell was 36.0 mm, the diameter of the rotor was 32.4 mm, and the gap between the two elements at the base of the geometry was 0.55 mm. All measurements of viscosity were performed at a fixed shear rate of 15 rad s^{-1}. To simulate industrial high temperature-short time thermal processing, the samples (28 g) were conditioned and held at 15 °C during 2 and 5 min, respectively, and the temperature increased to 95 °C (10 °C min^{-1}) and held at 95 °C during 30 s, after which the temperature was decreased to 15 °C (10 °C min^{-1}) and maintained at this temperature for 5 min. Data for viscosity was continually recorded throughout all heating, holding, and cooling stages of the thermal treatment.

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Particle size distribution

The particle size distribution (PSD) of the different formulations was measured after 2 and 18 h of reconstitution in water or 0.2% SDS and after high temperature short time (HTST) treatment at 95 °C for 30 s. The PSD of the emulsions was measured using static laser light diffraction (Mastersizer 3000, Malvern Instruments Ltd, Malvern, UK) with water as a dispersant. The refractive index was set at 1.47 and the absorption and dispersant refractive indices used were 0.001 and 1.33, respectively. The emulsion systems were equilibrated at 22 °C and introduced into the dispersing unit using ultrapure water as dispersant until a laser obscuration of 12% was achieved. Data for the relevant PSD parameters was extracted and analyzed in accordance with the Mie theory.

Physical stability

Separation rates of the different samples after 18 h of reconstitution in water or SDS, and after HTST treatment at 95 °C for 30 s, were measured using an accelerated stability analyzer (LUMiSizer®; LUM GmbH, Berlin, Germany). Samples were subjected to centrifugal force (145×*g*, 8 h, 22 °C), while near-infrared light illuminated the entire sample cell to measure the intensity of transmitted light as a function of time and position over the entire sample length. Data were extracted and analyzed in accordance with the method of O'Sullivan *et al.*[28]

In vitro protein digestibility

Infant gastrointestinal digestion was simulated by modifying a previously developed static human adult-stage *in vitro* protein digestibility (IVPD) method[29,30] to mimic the different pH conditions occurring during infant digestion.[31] The plant-based infant formulas were initially weighed in triplicate to contain 25 ± 0.1 mg protein on a dry matter basis, followed by dilution in ultrapure water (10 mL) and adjustment of the pH to 5.25 using 0.5 Mol L^{-1} HCl. Free alanine amino acid (Merck, Darmstadt, DE) samples, blank samples, as well as bovine serum albumin (BSA) (Sigma-Aldrich, Copenhagen, DK) samples serving as a reference protein source, were also included in the same amounts as the protein samples in all experiments. Prior to enzymatic digestion, aliquots of untreated samples (200 µL) were withdrawn and diluted in sodium borate buffer (800 µL, 0.05 mol L^{-1}, pH 10.0) to prevent inherent enzymatic activity. Pepsin digestion (1 h, 37 °C) was initiated by adding pepsin (Merk, Copenhagen, DK) solution (1 mL, 0.5 mg mL^{-1} pepsin freshly prepared in 0.05 M acetate buffer, pH 4.5) to all samples, corresponding to an enzyme to substrate (E:S) ratio of 2.0% (w/w). The final pH of sample solutions after addition of pepsin was in the range pH 5.0–5.3. Sample aliquots after pepsin digestion (200 µL) were withdrawn and diluted in sodium borate buffer (800 µL, 0.05 Mol L^{-1}, pH 10.0) to stop enzymatic hydrolysis. Pancreatic (Sigma-Aldrich) digestion (1 h, 37 °C) was initiated by adding sodium bicarbonate buffer (2.35 mL, 0.6 Mol L^{-1}), HCl (1.8 mL, 6 Mol L^{-1}), and sodium cholate solution (0.05 mL, 100 mg mL^{-1} sodium cholate hydrate freshly prepared in 0.6 Mol L^{-1} sodium bicarbonate buffer) to the sample solution. Then pancreatin solution (2.5 mL, 1 mg mL^{-1} pancreatin freshly prepared in 1 mM HCl) was added to all samples, corresponding to an E:S ratio of 10.4% (w/w). The final pH of sample solutions after addition of pancreatin was in the range pH 6.3–6.6. Sample aliquots after pancreatin digestion (200 µL) were withdrawn and diluted in sodium borate buffer (800 µL, 0.05 M, pH 10.0) to stop enzymatic hydrolysis.

The IVPD (%) of samples was quantified using a previously described trinitrobenzenesulfonic acid (TNBS)-based assay.[29] The IVPD (%) results were expressed relative to an alanine standard solution and the alanine samples that represented 100% protein digestibility at each stage of digestion. The starting level of hydrolysis in untreated samples was subsequently subtracted to obtain corrected values for pepsin digestibility (1 h), pancreatin digestibility after pepsin hydrolysis (1 h), and sequential pepsin-pancreatin digestibility (1 + 1 h). A correction for the value of blank samples containing only buffer and enzymes was included to account for enzymatic self-digestion during the IVPD procedure.

Statistical data analysis

Two independent pilot scale liquid batches (10–20 kg) of the model lentil-based formula were prepared and all compositional and physicochemical analyses were conducted in triplicate. The data generated were subject to one-way analysis of variance (ANOVA) using R i386 version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria) and for the *in vitro* digestibility the program GraphPad Prism (version 8.3.0; GraphPad Software Inc., San Diego, CA, USA) was used. A Tukey's paired comparison test was used to determine statistically significant differences ($P < 0.05$) between mean values for different samples.

RESULTS AND DISCUSSION

Macro-chemical composition of infant formula powders

The macro-chemical composition of the plant-based infant formula powders is shown in Table 1. The protein content of the three plant-based infant formula powders ranged from 12.9 to 15.2%, with the lentil-based formula (LF) having intermediate protein content between that of the soy-based (SF, 12.9%) and rice-based formulae (RF, 15.2%). The fat content of the LF (21.2%) was significantly lower ($P < 0.05$) than those of the RF (23.9%) and SF (25.6%) products. The slight differences in macro-chemical composition between the formulae are likely due to differences in protein quality between the principal protein ingredients used, and differences in macronutrient formulation targets between the products. The free-fat content was significantly higher ($P < 0.05$) for the SF (4.66% of total powder) than either the LF

Table 1. Macro-chemical composition (%; w/w) of lentil-based formula (LF), soy-based formula (SF) and rice-based formulae (RF)

	Moisture	Ash	Protein	Fat	Free fat	Carbohydrates
LF	3.22 ± 0.40^{b}	0.98 ± 0.02^{a}	14.3 ± 0.19^{b}	21.2 ± 0.05^{a}	2.53 ± 0.16^{b}	57.7 ± 1.88^{b}
SF	1.46 ± 0.04^{a}	3.11 ± 0.03^{b}	12.9 ± 0.02^{a}	25.6 ± 0.03^{b}	4.66 ± 0.83^{c}	52.3 ± 1.03^{a}
RF	1.69 ± 0.08^{a}	3.24 ± 0.02^{c}	15.2 ± 0.14^{c}	23.9 ± 0.07^{b}	0.88 ± 0.02^{a}	55.1 ± 0.37^{ab}

$^{(a\text{-}d)}$ Samples not sharing a common letter in each column differed significantly ($P < 0.05$).

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(2.53%) or RF (0.88%) products. For example, it is known that high levels of free fat in spray-dried food powders are related to properties of the feed such as the presence of surfactants (e.g., lecithin), quality of the emulsion formed during homogenization and the spray-drying conditions used, as these can all potentially influence the migration of fat components to the atomized droplet surfaces during drying.[32,33] In addition, high levels of free fat can negatively influence bulk-handling properties (e.g., flowability) of powders due to increased cohesiveness, mediated by the fat present at powder particle surfaces.

In addition, rehydration properties of the powders can be impaired by high levels of free fat.[34,35] The ash content of the three products ranged from 0.98 to 3.24%, with the LF having a significantly ($P < 0.05$) lower ash content (0.98%), due to the fact that no mineral fortification was applied in the preparation of the LF (i.e., measured ash content represented innate minerals only). The macro-chemical composition of the three plant-based products was generally in line with previously published scientific reports for first-age infant nutritional products, making the model LF potentially suitable for the development of next-generation pulse-based infant formulae.[3,18,36]

Particle size distribution, morphology and confocal microscopy of powders

The particle size distribution (PSD) parameters of the three infant formula powders are shown in Fig. 2. The volume-weighted mean particle diameter (D[4,3]) of the powders ranged from 31.9 to 134 μm, with the LF having significantly smaller ($P < 0.05$) D[4,3] values (31.9 μm) than the RF (121 μm) or SF (134 μm); a similar trend was evident for Dv(50) (i.e., particle size below which 50% of the sample volume is found), with values of 27.6, 107, and 118 μm for LF, RF, and SF, respectively. The range of values measured is in agreement with literature data for spray dried food powders (from 10 to 250 μm).[37,38] The larger particle sizes for the RF and SF products is aligned with expected data for commercial products, as these would be expected to be agglomerated,[39] unlike the LF, which was dried using a single-stage pilot-scale dryer with no fines return or forced, secondary agglomeration capability.

Scanning electron micrographs of the powder particles are presented in Fig. 3. In agreement with the data for powder particle size, the LF generally had smaller, more uniformly shaped primary powder particles, interspersed with a smaller proportion of fine powder particles. In contrast, the other two powders (i.e., SF and RF) had much larger, more irregularly shaped powder particles, with clear evidence of agglomeration having taken place during drying. The morphology of the SF and RF powders displayed fine powder particles incorporated into the agglomerated structure for both, with surface pools of coalesced fat evident in morphological analysis of the SF powder.[40]

Analysis of microstructure of the powders using confocal microscopy (Fig. 4) confirmed the differences in size distribution reported earlier in this study, and the architecture of protein and lipids in the powder particles. The LF powder displayed an even distribution of fat and protein on the primary powder particles, with few pools of fat. Similar to the LF, the RF displayed well-defined, small fat globules on the surface of powder particles, indicative of the retention of good emulsion quality during spray drying, which is also associated with the low free fat content reported earlier. In contrast with both the LF and RF powders, the SF powder showed evidence of large protein aggregates embedded within the powder microstructure. These aggregates may have been mediated by covalent bonding (i.e., disulfide bonds) between protein molecules.[12] Such protein aggregation in infant nutritional products has been shown previously to contribute inferior emulsification properties of proteins and result in coalescence of fat and high free fat content, as reported earlier for the SF powder.[41,42]

Reconstitution properties of infant formula powders

Heat treatment and viscosity

The initial apparent viscosity values for the LF, SF and RF, before heating (i.e., at 15 °C) were not significantly ($P > 0.05$) different, with values ranging from 17.5 to 18.0 mPa·s (Table 2). The initial viscosity values of the LF and SF were 17.6 and 17.5 mPa·s, respectively. On increasing the temperature to 95 °C, all samples showed a decrease in viscosity, while the SF formula at 15.3 min showed a slight increase in viscosity (Fig. 5). The final viscosity

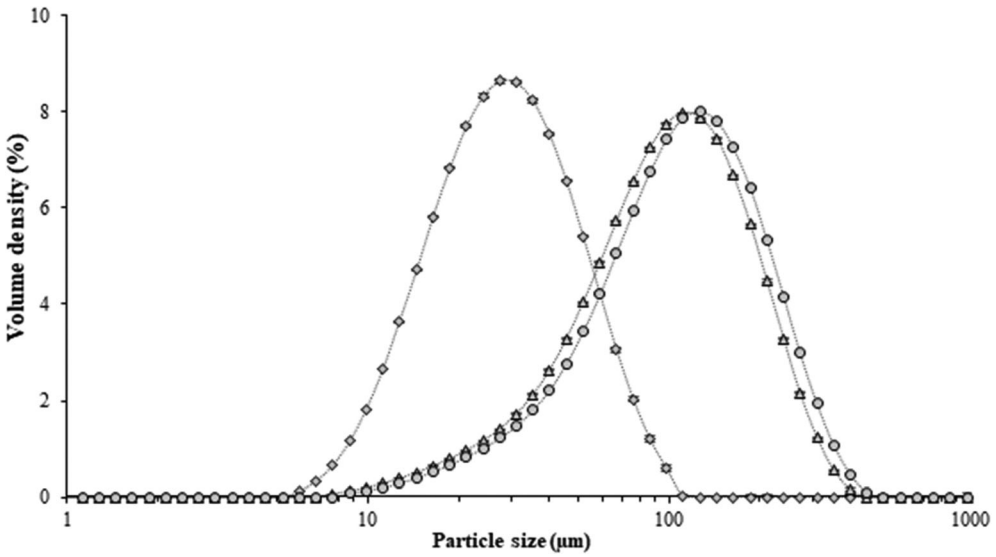

Figure 2. Particle size distribution of lentil-based formula (LF;◇), soy-based formula (SF;○) and rice-based formula (RF;△) powders.

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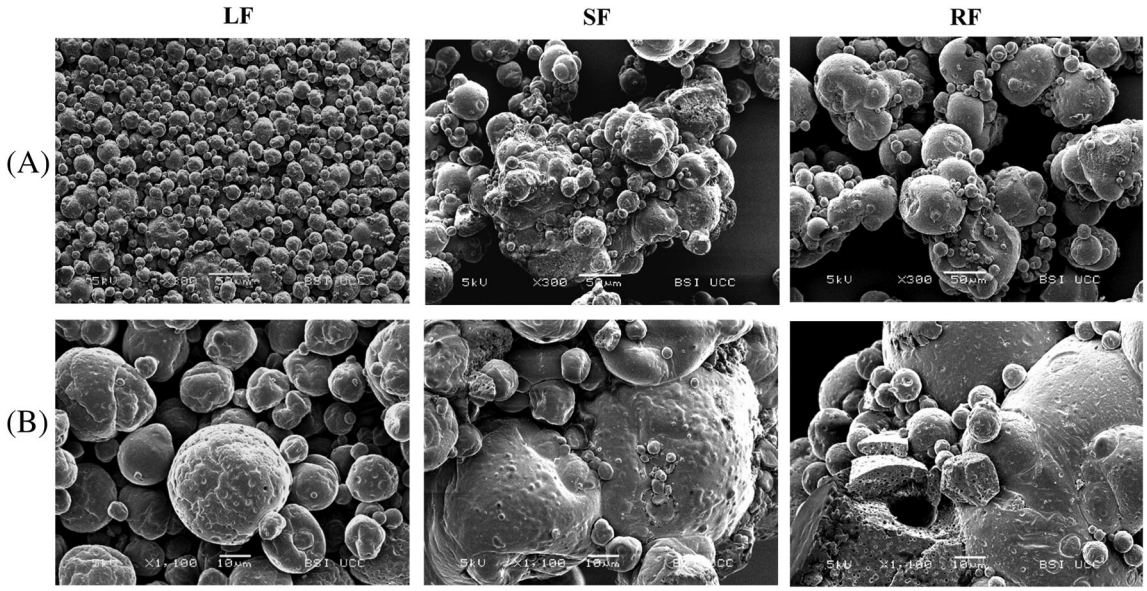

Figure 3. Scanning electron micrographs of lentil-based formula (LF), soy-based formula (SF) and rice-based formula (RF) powders. Magnification of 300× (a) and 1100× (b) with 50 and 10 μm scale bar, respectively.

values were slightly higher than the initial viscosity values, with the greatest increase observed for the SF formula, increasing from 17.5 to 21.1 mPa·s. The SF formula reconstituted in sodium dodecyl sulfate (SDS) displayed lesser extent of change in viscosity during heat treatment.

The measured changes in viscosity may be associated with changes in the physical properties of the proteins (i.e., unfolding of polypeptide chains, disruption of hydrophobic interactions and aggregation by covalent and non-covalent bonding), generally conferring increased viscosity.[43] Previous work demonstrated that a first-age infant formula (1.40 g/100 mL dairy protein; 12% total solids) had an initial viscosity value of 19.5 mPa·s,[36] which was similar to that reported in the present study for all the plant-based model infant formulae (1.90 g/100 mL; 12.5–14.7% total solids). Drapala *et al.*[43] analyzed the viscosity during heat treatment of a whey protein-based model infant formula (1.55%

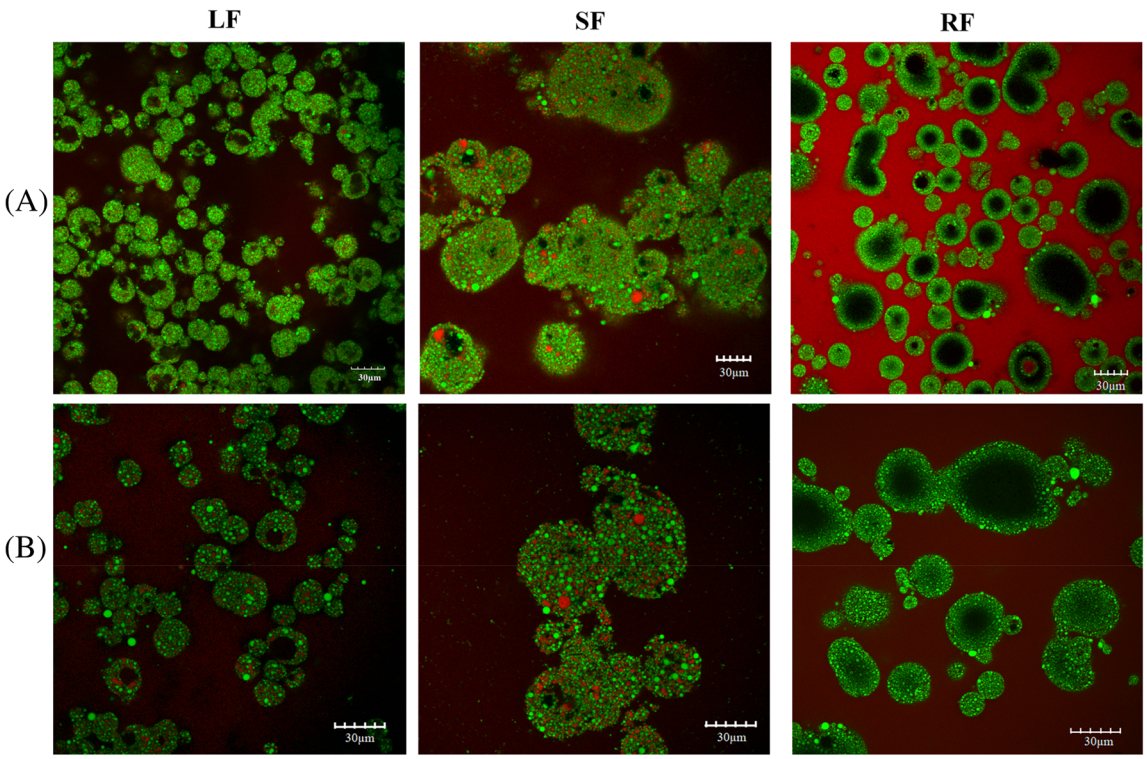

Figure 4. Confocal laser scanning micrographs of lentil-based formula (LF), soy-based formula (SF) and rice-based formula (RF) powders at 40x (a) and 60× (b) magnification. Scale bar is 30 μm. Fat = green; red = protein.

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Table 2. Apparent viscosity (mPa·s) of lentil-based formula (LF), soy-based formula (SF) and rice-based formula (RF) solutions (1.9% protein; w/w) reconstituted in water or sodium dodecyl-sulfate (SDS) at various stages of heat treatment

	Initial	Peak	End of cooling	Final
Lentil-based formula				
LF	17.6 ± 1.45^{a}	n.d.	17.6 ± 1.49^{ab}	18.1 ± 1.82^{b}
LF + SDS	17.5 ± 1.08^{a}	n.d.	18.2 ± 0.36^{bc}	18.7 ± 0.53^{bc}
Soy-based formula				
SF	17.5 ± 0.07^{a}	15.1 ± 1.03	21.4 ± 0.52^{c}	21.1 ± 0.32^{c}
SF + SDS	17.3 ± 0.37^{a}	n.d.	17.9 ± 0.09^{bc}	18.7 ± 0.15^{bc}
Rice-based formula				
RF	18.0 ± 0.38^{a}	n.d.	18.1 ± 0.16^{bc}	18.6 ± 0.12^{bc}
RF + SDS	15.2 ± 0.31^{a}	n.d.	14.8 ± 0.22^{a}	15.1 ± 0.14^{a}

$^{(a-c)}$ Values within a column, for same treatment, not sharing a common superscript differed significantly ($P < 0.05$). n.d. = Not detected.

protein, w/w; 12% total solids) and reported values for initial viscosity of ~14.0 mPa·s, similar to the initial viscosity values (17.5 to 18 mPa·s) of the plant-based infant formulae analyzed in this study (LF, SF, and RF). The model infant formula studied by Drapala *et al.*[43] was reported to be very unstable upon heat treatment, with an increase in viscosity, which is in line with the study reported by Crowley *et al.*.[44] In the present study, the viscosity of the formulae, especially the LF, was very stable upon heat treatment at 95 °C and this can be attributed to the formation of thick interfacial layers and steric repulsion.[23] Le Roux *et al.*[18] analyzed the viscosity of different plant-based model infant formulae, including those prepared with rice, fava bean, pea and potato protein blended with dairy protein, reporting viscosity values ranging from 10 to 550 mPa·s depending on the protein source; all formulae had viscosity values between 10–50 mPa·s, except the one formulated with potato protein, which developed extremely high viscosity values (5.4 Pa·s) during processing.

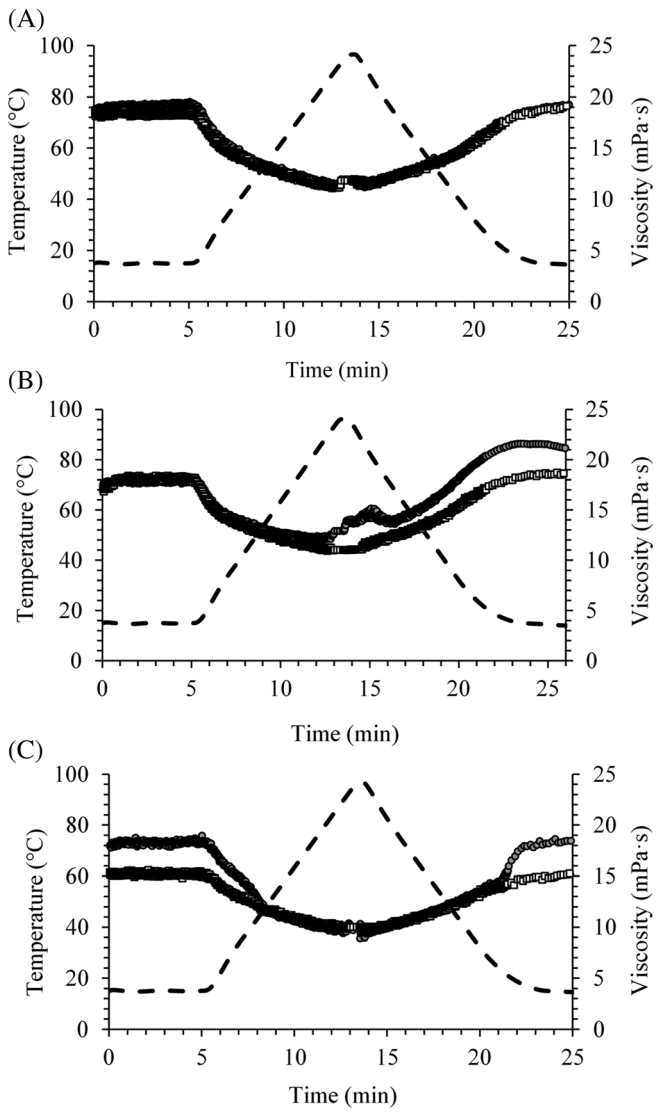

Figure 5. Temperature (dashed line) and viscosity (symbols) throughout the high temperature-short time thermal treatment for a lentil-based formula, b soy-based formula and c rice-based formula reconstituted in sodium-dodecyl-sulfate (SDS) (—□—) or water (—○—) during the heating ramp.

Particle size distribution of infant formula solutions

The particle size of the different plant-based infant formulae was measured at 2 and 18 h post reconstitution, before and heat treatment at 95 °C, in water or SDS (Table 3 and Fig. 6). After 2 h of reconstitution, the RF showed the smallest particle size with a volume-weighted mean particle diameter (D[4,3]) value of 4.28 μm followed by the SF (10 μm) and the LF (21.3 μm). Reconstitution of the formulae in SDS reduced considerably the particle size for SF and LF, with measured values of 2.92 and 5.72 μm, respectively, after 2 h, with a slight reduction in particle size also observed for the RF (2.90 μm). After 18 h of reconstitution in water the D[4,3] values of LF and SF decreased to 17.8 and 5.82 μm, respectively, while the fat globule size of the RF remained stable (4.55 μm). The samples reconstituted in SDS showed lower particle sizes after 18 h in comparison to when reconstituted in water. These results indicate that the effect of SDS as dissociating agent had an impact in the decrease of particle size, suggesting the presence of hydrophobic interactions in the SF and LF samples. After heat treatment at 95 °C, the particle-size distribution of the LF and RF did not change. However, the SF showed larger values for D[4,3] after heating, this being correlated to the increase in viscosity of the formulae. The particle sizes obtained for the plant-based infant formula are in agreement with the particle size values (D[4,3]) reported by Le Roux *et al.*[18] for pea and fava bean (18.9 and 6.2 μm), in comparison with a whey model infant formula, the latter having a value of 0.4 μm. In the same way, Nguyen *et al.*[7] reported a value of D(90) of 9.36 μm for a model infant formula with native soy protein. Similarly, Prakash, Ma and Bhandari (2014)[45] reported values ~5 μm for soy-based formula. The larger particle size parameter data reported for plant-based infant

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Table 3. Particle size distribution of lentil-based formula (LF), soy-based formula (SF) and rice-based formula (RF) after reconstitution in water or sodium-dodecyl-sulfate (SDS) and after heat treatment (95 °C × 30 s)

	Water (2 h)			Water (18 h)			Water (after heating)		
	LF	SF	RF	LF	SF	RF	LF	SF	RF
Dv(10)	6.52 ± 0.98^{c}	3.28 ± 0.16^{b}	0.73 ± 0.04^{a}	4.18 ± 0.94^{b}	0.31 ± 0.04^{a}	0.83 ± 0.02^{a}	5.35 ± 1.20^{b}	3.23 ± 0.05^{a}	0.86 ± 0.13^{a}
Dv(50)	19.8 ± 1.18^{c}	7.33 ± 0.39^{b}	3.05 ± 0.06^{a}	16.0 ± 0.93^{b}	4.07 ± 0.17^{a}	3.22 ± 0.11^{a}	16.0 ± 0.93^{c}	6.36 ± 0.20^{b}	3.23 ± 0.18^{a}
Dv(90)	37.8 ± 1.42^{c}	19.9 ± 2.64^{b}	9.36 ± 0.41^{a}	34.4 ± 1.86^{b}	11.4 ± 0.63^{a}	9.68 ± 0.59^{a}	34.7 ± 1.23^{c}	13.8 ± 0.93^{b}	8.57 ± 0.48^{a}
D[4,3]	21.3 ± 0.84^{c}	10.0 ± 0.92^{b}	4.28 ± 0.20^{a}	17.8 ± 0.80^{b}	5.82 ± 0.35^{a}	4.55 ± 0.30^{a}	18.1 ± 0.72^{c}	8.46 ± 0.67^{b}	4.33 ± 0.45^{a}
D[3,2]	6.50 ± 0.31^{a}	6.00 ± 1.02^{a}	1.72 ± 0.08^{a}	5.34 ± 1.19^{b}	1.00 ± 0.15^{a}	1.89 ± 0.04^{a}	6.30 ± 2.05^{a}	5.70 ± 0.15^{a}	1.93 ± 0.21^{a}
	SDS (2 h)			**SDS (18 h)**			**SDS (after heating)**		
	LF	**SF**	**RF**	**LF**	**SF**	**RF**	**LF**	**SF**	**RF**
Dv(10)	0.50 ± 0.14^{a}	0.16 ± 0.03^{a}	0.66 ± 0.02^{a}	0.52 ± 0.31^{a}	0.24 ± 0.03^{a}	0.67 ± 0.03^{a}	0.44 ± 0.11^{a}	0.25 ± 0.01^{a}	0.63 ± 0.04^{a}
Dv(50)	4.53 ± 0.73^{b}	1.62 ± 0.11^{a}	2.55 ± 0.10^{a}	6.34 ± 2.08^{b}	1.54 ± 0.10^{a}	2.58 ± 0.07^{ab}	3.66 ± 0.83^{a}	1.70 ± 0.14^{a}	2.57 ± 0.09^{a}
Dv(90)	12.4 ± 1.76^{b}	4.90 ± 0.11^{a}	5.52 ± 0.14^{a}	11.4 ± 1.60^{a}	13.7 ± 1.43^{a}	6.00 ± 0.23^{a}	9.38 ± 1.47^{a}	14.9 ± 1.70^{a}	6.09 ± 0.18^{a}
D[4,3]	5.72 ± 0.90^{b}	2.92 ± 0.02^{a}	2.90 ± 0.09^{a}	5.64 ± 0.91^{a}	4.28 ± 0.52^{a}	3.13 ± 0.19^{a}	4.38 ± 0.85^{a}	4.96 ± 1.00^{a}	3.25 ± 0.15^{a}
D[3,2]	1.50 ± 0.42^{a}	0.43 ± 0.07^{a}	1.52 ± 0.04^{a}	1.40 ± 0.65^{a}	0.63 ± 0.07^{a}	1.56 ± 0.04^{a}	1.28 ± 0.36^{a}	0.67 ± 0.04^{a}	1.52 ± 0.08^{a}

D[4,3], volume-weighted mean particle diameter.
D[3,2], surface-weighted mean particle diameter.
Dv(10), particle size below which 10% of sample volume is found.
Dv(50), particle size below which 50% of sample volume is found.
Dv(90), particle size below which 90% of sample volume is found.
$^{(a-c)}$ Values within a column, for individual treatments, not sharing a common superscript differed significantly ($P < 0.05$).

formulae may be attributed to components other than protein in the plant-based protein ingredients such as starch granules and fiber.[46,47]

Physical stability

Differences in the extent of phase separation between the plant-based infant formulae were observed upon centrifugation (Fig. 7). Similar profiles were observed before and after heat treatment for all the samples, suggesting that minimal changes occurred during heat treatment at 95 °C. The RF showed considerably higher transmission values over time, in comparison with the LF and RF formulae. The LF and SF had almost identical integral transmission profiles, suggesting that both formulae have similar physical stability. Reconstitution in SDS decreased the transmission values of the samples. The low stability of RF formula can be attributed to the low solubility of rice proteins, especially the glutelin fraction which is extremely insoluble due to inter- and intra-molecular hydrophobic, hydrogen, and disulfide bonding.[16] In addition, the RF (as declared on the product packaging) is formulated using a rice protein hydrolysate ingredient and several studies have reported that the hydrolysis of proteins can negatively affect emulsion stability. This can be explained by different reasons such as: the poorer ability of smaller peptides to interact at the oil / water interface decreasing the viscoelasticity of the interfacial film,[48] the increase in hydrophilic groups (i.e., increased number of polar groups) which bind more weakly, or not at all, to the oil–water interface[49] and hydrolyzed proteins have a tendency to saturate the continuous phase rather than adhere to the water–oil interface.[50] The study of Le Roux *et al.*[18] supported the results of this study, where they reported low solubility for rice-

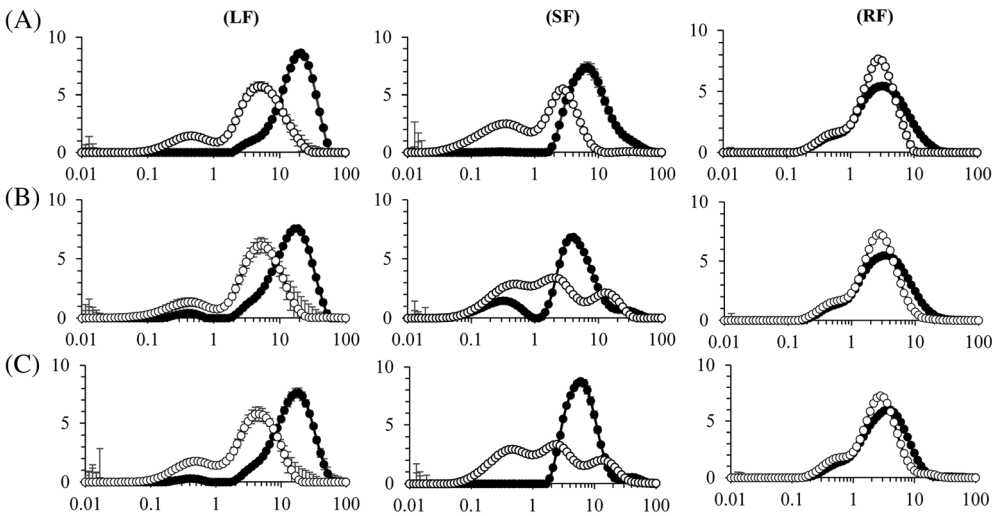

Figure 6. Particle size distribution for lentil- (LF), soy- (SF) and rice- based (RF) formula after a 2 h and b 18 h of reconstitution and c heat treatment at 95 °C with (—○—) or without (—●—) sodium dodecyl sulfate.

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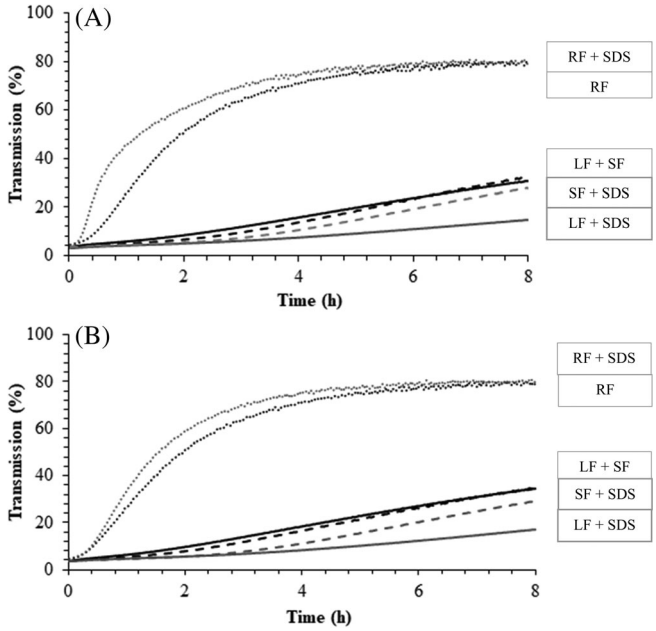

Figure 7. Separation profiles expressed as integral transmission as a function of time for lentil- (line line), soy- (dashed line) and rice-based (dotted line) formulae reconstituted in water (black) or in sodium-dodecyl-sulfate (grey) before a and after b heat treatment at 95 °C.

based infant formula in comparison with other plant-based protein sources (i.e., potato, pea, and faba bean).

In vitro protein digestibility

The *in vitro* protein digestibility (IVPD) of the plant-based infant formulas at each stage of digestion is shown in Table 4. The pepsin digestibility results showed similar values for all of the plant-based infant formulas, and these values were also comparable to the reference protein source bovine serum albumin (BSA), which is usually a highly digestible protein source under adult-stage IVPD conditions.[29] However, the pepsin digestibility values were markedly lower than those typically obtained during adult-stage digestion, which may be due to the higher pH used during pepsin digestion in the infant model system (pH 5.0–5.3 vs. pH 1–2), leading to a significantly lower activity of pepsin enzyme.

The sequential pepsin and pancreatin digestibility results showed significantly higher values for RF compared to all the other protein sources ($P < 0.05$). This is probably due to the fact that the RF (as declared on the product packaging), is formulated using a rice protein hydrolysate ingredient and appears to be more susceptible to pancreatin digestion than the other protein sources. The SF, which can be considered as the plant-based infant formula standard, had similar protein digestibility values to the LF during these stages of digestion. Furthermore, these two infant formulas showed no significant differences in the protein digestibility values to BSA. Again, except for RF, the pepsin-pancreatin sequential pepsin and pancreatin digestibility values were relatively low compared to those typically obtained during adult-stage digestion,[29] and also compared to those reported for other legume-based infant formulas in previous studies.[19,51] As previously mentioned, this deviation is likely due to a very low degree of pre-digestion by pepsin, thereby not priming the proteins for additional hydrolysis by pancreatin. This discrepancy may also be explained by the lower pH used during pancreatin digestion in this infant method compared to in the adult-stage digestion method (pH 6.3–6.6 vs pH 7–8),[30] which may be less optimal, especially for the activity of trypsin enzyme in pancreatin.

Infant formulas usually contain protein levels that are both higher than those found in human milk and greater than the levels shown to be adequate in clinical trials.[52,53] This is a precautionary approach to ensure a sufficient supply of amino acids, even from a protein source that may have a lower digestibility. The protein digestion in infants should ideally lead to generation of free amino acids, dipeptides, and tripeptides that can be transported into the intestinal enterocyte.[52] Therefore, in theory, the optimal IVPD value for an infant formula should be ≥33%, which corresponds to an average peptide chain length following digestion of three amino acids or less. In this respect, the results for LF and the other infant formulas obtained in this study are significantly lower than the optimal IVPD value. Besides the suboptimal conditions for proteolysis, this may be due to the presence of anti-nutritional compounds in the lentil protein isolate used for the preparation of LF. In a recent study, this lentil protein isolate obtained by isoelectric precipitation was shown to have a higher trypsin inhibitor activity and also a higher content of total galacto-oligosaccharides (GOS) than found in a similar lentil protein isolate prepared by ultrafiltration.[54] However, as antinutritional compounds were not tracked during the preparation of LF in this study, it remains uncertain whether these compounds contribute significantly to the relatively low IVPD values.

Table 4. *In vitro* protein digestibility (IVPD) of plant-based infant formulas according to the stage of digestion[a]

	Pepsin	Pepsin + Pancreatin
	1 h	1 + 1 h
LF	0.4 ± 0.4[a]	6.9 ± 0.3[b]
SF	0.3 ± 0.1[a]	7.4 ± 0.1[b]
RF	0.3 ± 0.5[a]	22.6 ± 1.0[a]
BSA	0.4 ± 0.1[a]	7.9 ± 0.3[b]

[a] Pepsin digestibility (1 h), and sequential pepsin and pancreatin digestibility (1 + 1 h).

CONCLUSION

Lentil proteins have been studied for their applicability in a model first-age infant nutritional product. A colloidally stable model lentil-based infant formula was produced successfully following the traditional steps involved in infant formula manufacture (i.e., high-shear mixing, homogenization, ultra-high temperature treatment, and spray drying). The lentil-based formula showed similar macronutrient composition to commercial plant-based infant formulae (i.e., soy and rice) with comparable reconstitution properties to such formulae and greater stability to downstream heat treatment. The model LF had similar digestibility and physical stability values to a commercial soy-based formula. This study has demonstrated for the first time that lentil proteins can be applied for the development of first-age infant nutritional products with excellent thermal and colloidal stability, broadening the range of options for use of plant proteins in such specialized nutritional applications.

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