



Impact of α -lactalbumin: β -lactoglobulin ratio on the heat stability of model infant milk formula protein systems



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ARTICLE INFO

Article history:

Received 6 March 2015

Received in revised form 17 July 2015

Accepted 18 July 2015

Available online 20 July 2015

Keywords:

Infant milk formula

Whey protein

Humanisation

Heat stability

Processing

ABSTRACT

Model infant milk formula systems (5.5% protein) were formulated to contain α -lactalbumin: β -lactoglobulin ratios of 0.1, 0.5, 1.3, 2.1 or 4.6 and assessed for heat stability and heat-induced changes. 'Humanising' the model formulas by increasing α -lactalbumin: β -lactoglobulin enhanced heat stability at 140 °C in the pH range 6.6–6.9. The model formulas were analysed after lab-scale high-temperature short-time heating at pH 6.8. Gel electrophoresis indicated that increased heat stability in high α -lactalbumin: β -lactoglobulin samples was due to decreased covalent interactions between proteins. In low α -lactalbumin: β -lactoglobulin formulas, protein–protein interactions caused marked increases in protein particle size and viscosity of the heated systems; conversely, covalent interactions between proteins were minimal in high α -lactalbumin: β -lactoglobulin formulas. Reduced protein–protein interactions with increasing α -lactalbumin: β -lactoglobulin has important implications for subsequent processing; for example, lower viscosity post-heating may affect bulk density in spray-dried products or physical stability in ready-to-feed products.

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1. Introduction

One of the key compositional differences between human and bovine milks is protein, in terms of both quantity and quality. For several decades, first-age infant milk formula products (IMFs) have been formulated to be whey protein-dominant (i.e., containing a whey protein:casein ratio of ~60:40 in the final product). Demineralised whey or whey protein concentrate/isolate, when added to skim milk in the correct proportions, can be used to attain the desired whey protein:casein ratio (De Wit, 1998; Fomon, 2001). This protein base, to which other ingredients (e.g., lipids, carbohydrates, minerals and vitamins) are added, is standardised to give the required protein content (typically 1.2–1.8% protein) in the final product. This protein level is higher than that of human milk (~0.9–1.1%), which is an intentional compensation to ensure the provision of sufficient quantities of essential amino acids, such as tryptophan, tyrosine and cysteine, which are present in higher concentrations in human milk than in bovine milk (De Wit, 1998; European Commission 2006/141/EC).

The whey protein fraction in bovine milk is dominated by β -lactoglobulin (β -lg), which accounts for ~50% of total whey

protein, while α -lactalbumin (α -lac) comprises 20% of total whey protein. However, β -lg is absent from human milk and α -lac predominates (Armaforte et al., 2010). In fact, the presence of β -lg in IMFs is believed to be one of the primary stimulators of allergenic responses in IMF-fed infants, which has resulted in the development of hydrolysed or "hypoallergenic" IMFs (Murphy et al., 2015). Another approach to reducing allergenicity involves the conjugation of β -lg with sugars to decrease its allergenicity to susceptible individuals (Böttger, Etzel, & Lucey, 2013; Taheri-Kafrani et al., 2009). In addition, methods to deplete β -lg from whey to produce α -lac-enriched ingredients for use in applications such as IMF formulation are well established (Lucena, Alvarez, Menéndez, & Alvarez, 2006; Pearce, 1995; Stack, Hennessy, Mulvihill, & O'Kennedy, 1999). Formulation of IMFs to contain higher α -lac: β -lg ratios can be achieved through fortification with these α -lac-enriched whey protein ingredients to yield a more humanised product, with scientifically-validated physiological benefits for the infant (Davis, Harris, Lien, Pramuk, & Trabulsi, 2007; Kuhlman, Lien, Weaber, & O'Callaghan, 2003; Sandström, Lonnerdal, Graverholt, & Hernell, 2008). Furthermore, enrichment with α -lac may allow a reduction in the protein content of IMFs to values closer to those found in human milk, as the amino acid requirements set forth in international regulations (European Commission 2006/141/EC) for IMFs would be satisfied to a greater degree (Kuhlman et al., 2003).

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It is important to investigate the implications of increasing α -lac: β -lg ratio on heat stability and heat-induced changes of IMFs, as IMFs are commonly subjected to several heating steps during their manufacture, which can include combinations of high-temperature short-time (HTST) heating, evaporation, ultra-high temperature (UHT) treatment and in-container sterilisation (Jiang & Guo, 2014). As whey protein, in particular β -lg, is one of the dominant contributors to heat-induced changes in milk (Ratnay & Jelen, 1997; Singh, 2004), any alteration to α -lac: β -lg ratio in IMFs is likely to alter their behaviour during thermal processing, which, in turn, may affect the quality of IMF products (Murphy, Fenelon, Roos, & Hogan, 2014).

In this study, model IMFs with a protein content of 5.5% (typical for IMFs after wet-mixing of ingredients and at the point of heat treatment), a constant whey protein:casein ratio of 60:40, and varying ratios of α -lac: β -lg were formulated. The role of α -lac: β -lg ratio in determining the extent of heat-induced changes in model IMFs was then determined in a series of experiments. Stability to heating at 140 °C and viscosity changes during lab-scale high-temperature short-time (HTST) were studied. Samples before and after HTST heating were the subject of further analysis, including particle size distribution (PSD) using dynamic-light scattering, physical stability using analytical centrifugation, and protein profile using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

2. Materials and methods

2.1. Materials

Milk protein concentrate 80 (MPC80) with a protein content of 82.8% (w/w) was supplied by NIZO food research (Zb Ede, The Netherlands). Whey protein isolates comprising α -lac (90% purity, 93.5% protein) and β -lg (90% purity, 88.7% protein) were obtained from Davisco Foods International (Le Sueur, MN, USA).

2.2. Preparation of model infant milk formula protein systems

Model IMF protein systems were formulated to contain 5.5% total protein, with MPC80 contributing 50% of protein, and the remaining 50% comprised of five different blends of α -lac and β -lg. All samples were reconstituted in simulated milk ultrafiltrate (SMUF; Jenness & Koops, 1962). Powders were reconstituted by magnetic stirring at 22 °C for 3 h, with whey protein powder being reconstituted first. Each sample was then adjusted to the desired pH using 0.5 M HCl or 0.5 M NaOH and stored at 4 °C for 18 h to facilitate complete rehydration. Before analysis, samples were stirred magnetically at 22 °C for 1 h and pH was re-adjusted if necessary.

The protein profiles of all model IMFs were determined by reversed-phase high-performance liquid chromatography (RP-HPLC) using the method of Crowley et al. (2015), and are shown in Fig. 1; for the remainder of this paper, the model IMFs will be referred to by their α -lac: β -lg ratios (Fig. 1). The whey protein:casein ratio, where total whey protein was considered to comprise α -lac and β -lg, was measured as $59 \pm 3:41 \pm 3$ for the model IMFs.

2.3. Compositional analysis

Total solids content was measured by oven drying (IDF, 1987). Total nitrogen was determined using the macro-Kjeldahl method (IDF, 1986), and a nitrogen–protein conversion factor of 6.38 was used to calculate total protein. Fat content was determined using the Gerber method (IDF, 1991). Lactose content was measured by

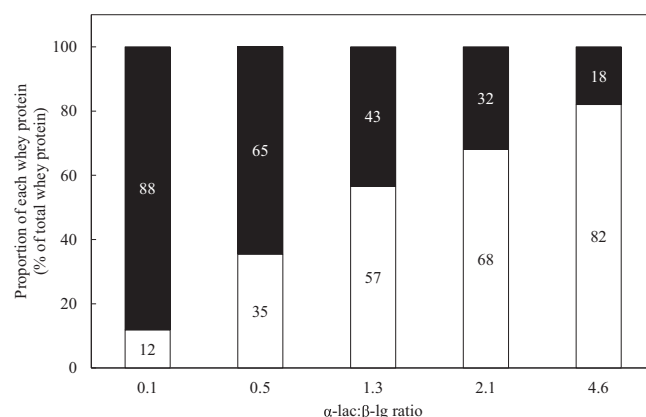


Fig. 1. Relative proportions of β -lactoglobulin, β -lg (■) and α -lactalbumin, α -lac (□) in model infant milk formula (IMF) samples with different α -lac: β -lg ratios. Data are from reversed-phase high-performance liquid chromatography analysis of at least two freshly prepared samples of each model IMF. “Total whey protein” refers to α -lac + β -lg.

HPLC as described by Indyck, Edwards, and Woollard (1996), except that the protein precipitation step was performed using tungstic acid. Mineral profiles were determined by inductively-coupled plasma mass spectrometry (ICP-MS) according to the method of Herwig, Stephan, Panné, Pritzkow, and Vogl (2011). Calcium (Ca)-ion concentration of model IMFs at 20 °C was measured using a polymer membrane Ca-ion-selective electrode (Metrohm Ltd., Ireland), as described by Crowley, Kelly, and O'Mahony (2014), except that the standard curve was prepared using standards containing 2, 4, 6, 8 and 10 mM L⁻¹ of Ca. Results showing the complete compositional profile of the model IMFs are presented in Table S1.

2.4. Heat stability at 140 °C as a function of pH

Samples were prepared as outlined in Section 2.2 and divided into 11 aliquots before adjustment of pH at 0.1 pH unit intervals in the range 6.2–7.2 using 0.5 M HCl or 0.5 M NaOH. Heat stability was measured at 140 °C by the method of Davies and White (1966) under the conditions specified by Crowley et al. (2014). In commercial UHT processing, the duration of the thermal treatment is much shorter than that in a typical heat stability test; however, tests in which heat coagulation time is measured are a very commonly used index of heat stability in milk protein systems, and are regarded as a reliable method of measuring inter-sample differences and trends in heat stability (Singh, 2004).

2.5. Simulated HTST treatment

The change in viscosity of model IMFs at pH 6.8 during a heating, holding and cooling cycle designed to simulate HTST treatment was measured using a TA Instruments AR-G2 controlled-stress rheometer (Crowley, West Sussex, UK) with an aluminium parallel plate (60 mm diameter) and TRIOS v8.32 software, as described by Crowley et al. (2014), except that samples were heated to 85 °C before cooling to 5 °C.

To increase the quantity of heated sample available for further analysis, the rheometer was equipped with a starch pasting cell geometry with 28 mL sample capacity. The same heating–holding–cooling regime detailed above for the parallel plate was applied, and the contents of the starch pasting cell were subjected to a constant shear rate of 15 s⁻¹. Before and after heat-treatment, samples were analysed as described in Sections 2.6–2.8.

2.6. Protein particle size distribution

Unheated and heated model IMFs were diluted 1:200 in SMUF and equilibrated for at least 1 h at 22 °C. Protein particle diameter and polydispersity index were measured at 25 °C, after 120 s of temperature equilibration, using dynamic light-scattering (Zetasizer Nano series HT, Malvern Instruments Ltd., Worcestershire, United Kingdom) and accompanying Malvern Zetasizer software v.7.02. The apparatus was equipped with a He–Ne laser emitting at 633 nm. A solvent viscosity of 0.8872 mPa s and solvent refractive index of 1.33 were used in particle size calculations. Intensity-weighted Z-average particle diameter and polydispersity index values are reported. A total of five measurements were taken for each individual replicate using a back-scattering configuration with a scattering angle of 173°. The term *protein particle size* is used in this manuscript as a general term which encompasses the size of native casein micelles, micelles with which whey proteins have complexed, and serum protein aggregates. Changes in protein particle size after heating were considered to have occurred due to a combination of whey protein–casein and whey protein–whey protein interactions.

2.7. Accelerated physical stability

The physical stability of unheated and heated model IMFs at 20 °C was measured using an analytical centrifuge (LUMiSizer, L.U.M. GmbH, Berlin, Germany) as described by Crowley et al. (2014), except that a shorter centrifugation time of 1 h was used. For calculating the change in transmission over time, integration limits were set at 109 and 127 mm in order to exclude the meniscus and sediment regions. A total of 60 profiles were collected over 1 h.

2.8. Protein profile analysis by SDS–PAGE

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed under reducing and non-reducing conditions as described by Laemmli (1970) on the model IMFs before and after heating. An AcquaTank mini gel unit (Acquascience, Bellbrook Industrial Estate, Uckfield, UK) was used for running the gels, which were pre-cast 4–20% acrylamide tris–glycine gels (Pierce Protein Research Products, Australia). The staining solution used was Bio-Safe Coomassie G-250 Stain (Bio-Rad Laboratories, Inc., USA) and gels were scanned using a desktop scanner (HP Scanjet G4010).

2.9. Statistical data analysis

One-way analysis of variance (ANOVA) with Tukey's HSD on selected data from at least two independent trials on freshly prepared model IMFs was carried out using MINITAB® v.16.2.4 (Minitab Ltd, Coventry, UK) statistical analysis package. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Composition

There were no significant differences ($P > 0.05$) in the composition of the model IMF protein systems in terms of total solids, crude protein, lactose or mineral profile (Table S1), with fat levels $< 0.20\%$ in all samples. Altered α -lac: β -lg ratios in model IMFs formulated with different proportions of α -lac- or β -lg-enriched WPIs were confirmed by HPLC (Fig. 1).

3.2. Heat stability at 140 °C as a function of pH

In model IMFs with α -lac: β -lg ratios of 0.1 and 0.5, samples showed very poor heat stability (HCT < 2 min) across the entire pH range (Fig. 2), with a very slight increase in stability as pH increased. In model IMFs with α -lac: β -lg ratios of 1.3, 2.1 and 4.6, broad areas of increased heat stability were observed at pH 6.6–6.9 (Fig. 2). Maximum HCT for these samples increased with increasing α -lac: β -lg and shifted to slightly lower pH values. Heat stability at pH < 6.6 and > 7.1 remained very low for all samples irrespective of α -lac: β -lg ratio (Fig. 2). Overall, increasing α -lac: β -lg ratio improved the heat stability of model IMF protein systems.

3.3. Viscosity changes during lab-scale HTST heating

No significant differences ($P > 0.05$) in viscosity were measured between the model IMFs during initial holding at 20 °C (Fig. 3). After HTST treatment and during holding at 5 °C, model IMFs with α -lac: β -lg ratios of 1.3 or 4.6 were significantly ($P < 0.05$) less viscous than samples with α -lac: β -lg ratios of 0.1 or 0.5 (Fig. 3). These results demonstrated that heat-induced increases in viscosity were reduced as α -lac: β -lg ratio increased.

3.4. Protein particle size before and after HTST treatment

Unheated model IMFs had mean protein particle diameters between 181 and 187 nm (Table 1), which is typical of values reported for casein micelles (Anema, Lowe, & Li, 2004; Crowley et al., 2014; Nair, Dalgleish, & Corredig, 2013). Heated samples with α -lac: β -lg ratios of 0.1, 0.5 or 1.3 exhibited significant ($P < 0.05$) increases in protein particle diameter compared to the samples before heating (Table 1) whereas, at higher α -lac: β -lg ratios (2.1 or 4.6), no significant changes in protein particle size were detected after heating. There was also a trend towards decreasing polydispersity after heating as α -lac: β -lg increased (Table 1). The PSD of the model IMF with the highest α -lac: β -lg (4.6) was monomodal, irrespective of whether it was heated or not, but displayed a shift to slightly smaller protein particle sizes after heating (Table 1; Fig. S1). A second population of small protein particles with diameters ranging from 40 to 100 nm was

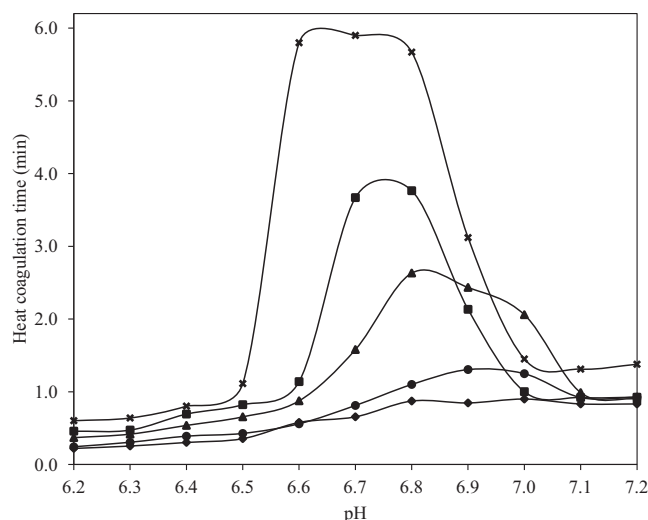


Fig. 2. pH-heat coagulation time (HCT) profiles for model infant milk formula samples with α -lactalbumin (α -lac): β -lactoglobulin (β -lg) ratios of 0.1 ($\blacklozenge$), 0.5 ($\bullet$), 1.3 ($\blacktriangle$), 2.1 ($\blacksquare$) or 4.6 ($\times$) in the pH range 6.2–7.2. Results are the means of data from at least three independent trials on freshly prepared samples.

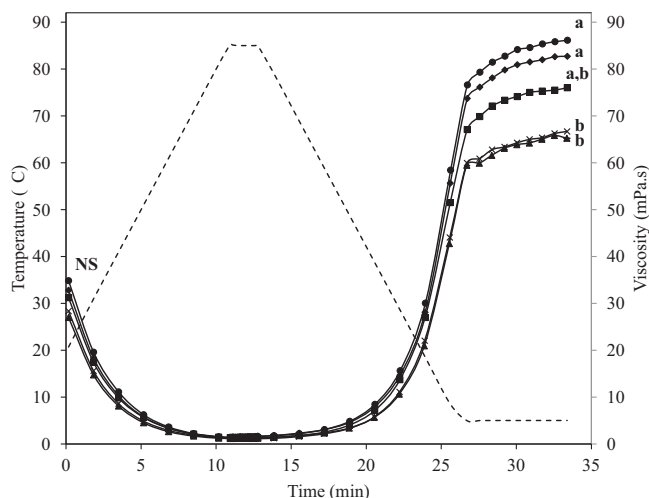


Fig. 3. Temperature (broken line) and viscosity (symbols) during lab-scale high-temperature short-time (HTST) treatment of model infant milk formula samples with α -lactalbumin (α -lac): β -lactoglobulin (β -lg) ratios of 0.1 (◆), 0.5 (●), 1.3 (▲), 2.1 (■) or 4.6 (×) at pH 6.8. Results are the means of data from at least three independent trials on freshly prepared samples. Different letters after the curve indicate that samples were significantly different ($P < 0.05$) at 5 °C; NS indicates no significant difference between samples at 20 °C.

Table 1

Protein particle size and polydispersity index values of model infant milk formula samples with different α -lactalbumin (α -lac): β -lg (β -lactoglobulin) ratios, before and after application of lab-scale high-temperature short-time heating. Results are the means $\pm$ standard deviations of data from three independent trials on freshly prepared samples.

α -lac: β -lg ratio	Protein particle size (nm)		Polydispersity index (–)	
	Unheated	Heated	Unheated	Heated
0.1	187 $\pm$ 2.07 ^a	319 $\pm$ 80.2 ^b	0.19 $\pm$ 0.02 ^a	0.34 $\pm$ 0.09 ^b
0.5	182 $\pm$ 2.08 ^a	249 $\pm$ 35.9 ^b	0.17 $\pm$ 0.01 ^a	0.26 $\pm$ 0.07 ^a
1.3	181 $\pm$ 4.88 ^a	252 $\pm$ 41.2 ^b	0.17 $\pm$ 0.01 ^a	0.22 $\pm$ 0.05 ^a
2.1	184 $\pm$ 3.62 ^a	233 $\pm$ 33.7 ^a	0.18 $\pm$ 0.03 ^a	0.17 $\pm$ 0.04 ^a
4.6	186 $\pm$ 4.73 ^a	165 $\pm$ 2.00 ^a	0.19 $\pm$ 0.02 ^a	0.10 $\pm$ 0.01 ^b

^{a,b}Means within rows are significantly ($P < 0.05$) different after treatment if they carry different superscript letters.

apparent in model IMFs with α -lac: β -lg ratios of 0.1 and 0.5 after heating, indicating the presence of serum protein aggregates (Donato and Guyomarc'h, 2009); in addition, micron-sized particles were formed in the sample with α -lac: β -lg of 0.1 (Fig. S1), suggesting that particularly extensive aggregation had occurred in this sample.

3.5. Physical stability before and after HTST treatment

Physical stability was measured using an analytical centrifuge. The transmission of near-infrared light through the sample cell at the beginning and end of centrifugation was $\sim 30\%$ and 65% , respectively, for all unheated model IMF samples (Fig. 4). Under these conditions, this increase in transmission during centrifugation can be attributed to the sedimentation of large particles such as casein micelles (Crowley et al., 2014; Tobin, Fitzsimons, Kelly, & Fenelon, 2011) and presumably large whey protein aggregates. All samples were more physically stable after heating. For heated systems with α -lac: β -lg ratios of 0.1–2.1, the transmission at the beginning of centrifugation decreased to between 10% and 15% compared to unheated samples (Fig. 4), due to the increased light-scattering by the large protein particles which were formed

(Table 1), while a much smaller decrease in transmission was observed for the 4.1 α -lac: β -lg sample due to negligible protein aggregation (Table 1). Fig. 4 shows that the value for transmission after 60 min of centrifugation increased with increasing α -lac: β -lg ratio, indicating decreased physical stability. The model IMF with an α -lac: β -lg ratio of 0.1 was the most stable to protein sedimentation on centrifugation after heating (Fig. 4). This result seems counterintuitive, as this sample had the largest average protein particle diameter and may, thus, have been expected to sediment more rapidly (Guyomarc'h, Nono, Nicolai, & Durand, 2009). It is not clear why samples with larger protein particles were more physically stable, but the markedly increased polydispersity of these systems (Table 1) indicates that populations of particles in these systems were very complex, likely comprising both casein micelle-whey protein complexes and serum protein aggregates (Fig. S1). Possible explanations for reduced sedimentation in these systems include: (1) that the formation of a large range of aggregate sizes, including small serum protein aggregates, resulted in increased inter-aggregate proximity with a concomitant increase in stabilising interactions between aggregates during centrifugation; (2) that a significant portion of the particle population was comprised of aggregates with higher hydration or lower density after heating, with, for example, Guyomarc'h et al. (2009) demonstrating that complexes formed between whey proteins were more dense than complexes formed between κ -casein and whey proteins.

3.6. Protein profile before and after HTST treatment

After heating, the intensity of both β -lg and α -lac bands decreased strongly under non-reducing conditions at α -lac: β -lg ratios between 0.1 and 1.3 (Fig. 5a). A substantial proportion of α -lac did not form covalent associations with other proteins during heating in samples with α -lac: β -lg ratios of 2.1 and 4.6, as indicated by the markedly higher band intensity in non-reducing SDS-PAGE gels (Fig. 5a). These results indicate that both β -lg and α -lac readily formed covalently-linked complexes with casein and/or themselves at low α -lac concentrations; the dissociation of these complexes under reducing conditions, indicated by the presence of distinct, intense bands (Fig. 5b), suggests that the protein-protein interactions were predominately covalent in nature (i.e., linked by disulphide bonds).

An increase in the levels of high molecular weight (MW) material remaining in the wells of the non-reducing gel as α -lac: β -lg ratio increased was also observed (Fig. 5a); in addition, there was noticeable streaking in the region between the BSA band and the loading well in samples with α -lac: β -lg ratios of 1.3, 2.1 and 4.6. The high MW material was also covalently linked, as evidenced by its dissociation under reducing conditions (Fig. 5b). The sample with the highest α -lac: β -lg ratio (4.6) had the greatest quantity of material remaining in the loading wells of the non-reducing SDS-PAGE gel (Fig. 5a); however, the same sample underwent the least significant changes in particle size distribution (Table 1) and viscosity (Fig. 3) during HTST treatment, and exhibited the highest heat stability at 140 °C (Fig. 2). The intensity of the BSA band decreased in heated IMFs as α -lac: β -lg increased, particularly after the high MW complexes had been dissociated (Fig. 5b). The high MW BSA has a free thiol group and, hence, can form complexes with α -lac (Wijayanti, Bansal, & Deeth, 2014). Thus, it is possible that, as α -lac: β -lg ratio is increased, BSA begins to play a more prominent role in influencing protein-protein interactions while, under normal conditions, β -lg is present in sufficient quantities to mask the influence of BSA. Although the level of covalently-linked high MW material remaining in wells was observed to increase in non-reducing SDS-PAGE gels as α -lac: β -lg ratio increased (Fig. 5a), this appeared to be insufficient

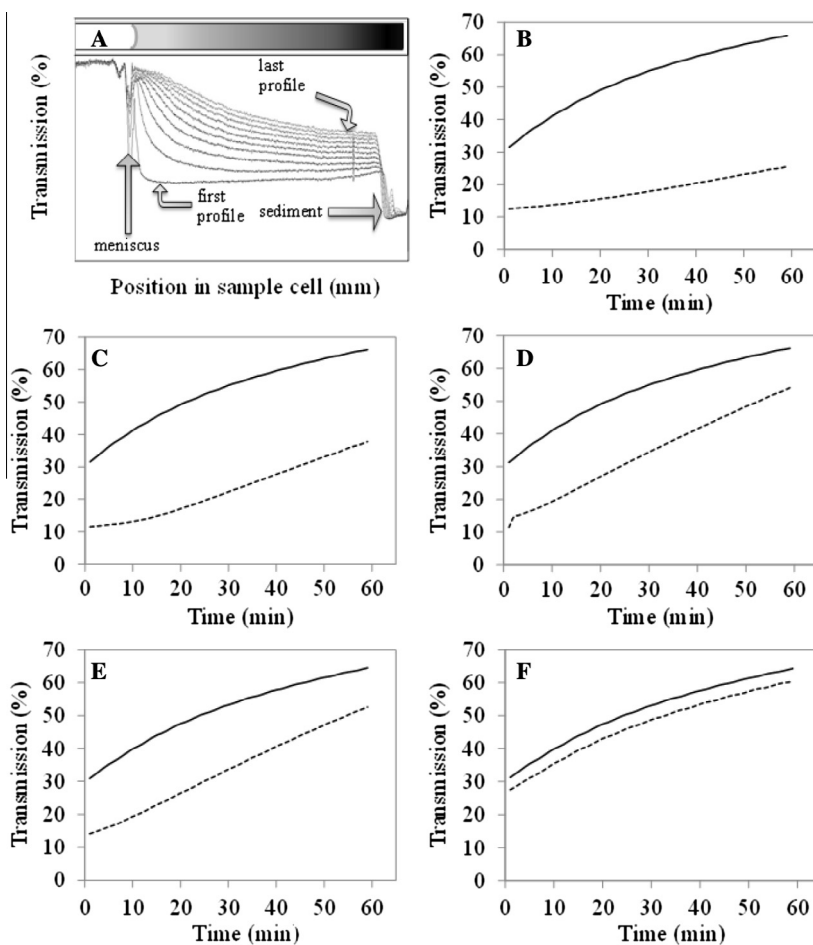


Fig. 4. Clarification of model infant milk formula samples during centrifugation (2300g; 20 °C). Increasing transmission was used as an index of clarification due to protein sedimentation, and was calculated by integrating raw profiles showing the transmission of near-infrared light across the length of a sample during centrifugation. An example raw profile is shown in (A); integration limits were set between the meniscus and sediment (region of clarification). Changes in transmission are shown for model infant milk formula samples with α -lactalbumin (α -lac): β -lactoglobulin (β -lg) ratios of (B) 0.1, (C) 0.5, (D) 1.3, (E), 2.1 or (F) 4.6 before (—) and after (---) heating. Results are the means of data from at least three independent trials on freshly prepared samples.

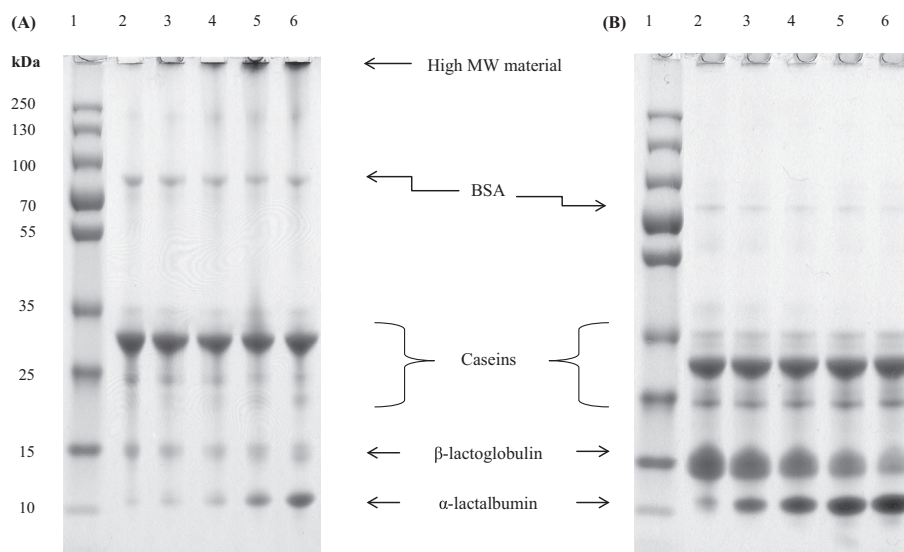


Fig. 5. Sodium dodecyl sulphate–polyacrylamide gel electrophoretograms (SDS–PAGE) under (A) non-reducing and (B) reducing conditions for heated model infant milk formula (IMF) samples. Wells were loaded with molecular weight marker (lane 1), and heated model IMF samples with α -lactalbumin (α -lac): β -lactoglobulin (β -lg) ratios of 0.1 (lane 2), 0.5 (lane 3), 1.3 (lane 4), 2.1 (lane 5) or 4.6 (lane 6).

to induce significant physicochemical changes of relevance to processing.

4. Discussion

This study conclusively demonstrated that modulating the α -lac: β -lg ratio of model IMF protein systems significantly affects their heat stability. Differences in the heat stability of these model IMFs can be primarily attributed to β -lg being more heat-labile than α -lac; hence, when α -lac: β -lg ratio was increased the extent of protein–protein interactions decreased, which resulted in increased heat stability. In addition to protein–protein interactions, multiple other compositional factors can influence the heat stability of milk (e.g., lactose content and Ca-ion activity); however, in this study, these factors were constant across the model IMFs (Table S1), and their contribution to inter-sample differences were therefore considered to be negligible. Model IMFs with high α -lac: β -lg ratios were the most resistant to heat-induced coagulation (Fig. 2). In high α -lac: β -lg ratio systems, protein–protein interactions were less pronounced, as evidenced by the less extensive increases in protein particle size (Table 1), viscosity (Fig. 3) and covalent interactions between proteins (Fig. 5) after heating. These data demonstrate that increasing α -lac: β -lg ratio decrease the protein–protein interactions that negatively affect heat stability. This is consistent with the report of Rattray and Jelen (1997), that blends (3.4% protein) of β -lg/skim milk were generally much less heat-stable than those of α -lac/skim milk at various casein:–whey protein ratios and pH values. It is clear that, in the pH range 6.6–6.9, increasing α -lac: β -lg ratio increased the heat stability of model IMFs at 140 °C (Fig. 2). Outside of this pH range, a higher Ca-ion activity at pH < 6.6 and increased dissociation of κ -casein at pH > 6.9 (Crowley et al., 2014; Singh, 2004) may have limited the otherwise strong stabilising effect of increased α -lac: β -lg.

Protein–protein interactions occurring in milk during heating can have both stabilising and destabilising influences on milk proteins. When milk is heated, the formation of complexes between whey proteins themselves and between whey proteins and casein can occur. Where reported, increases in protein particle size shown in Table 1 for model IMFs are likely due to the formation of both types of protein complexes. Whey proteins, and in particular β -lg, have a strong influence on the pH-dependant stability of milk to heating, and contribute to high heat stability in unconcentrated milk when complexed with casein micelles (Singh, 2004). In concentrated milks, substantial dissociation of κ -casein occurs throughout the pH–HCT profile, which is promoted by interactions with β -lg (Pearce, 1979; Singh & Creamer, 1991). In systems containing no casein, the β -lg is far more prone to heat-induced coagulation than α -lac; hence, increasing the level of β -lg in milk systems considerably reduces maxima values for heat stability, while increasing the levels of α -lac has a negligible or even positive effect on heat stability (Rattray & Jelen, 1997). In mixed systems containing both casein and whey proteins, association of whey proteins with casein micelles occurs during heating, but α -la only participates in these reactions if in the presence of sufficient quantities of β -lg and to a much more limited extent compared to β -lg (Oldfield, Singh, & Taylor, 1998; Oldfield, Singh, Taylor, & Pearce, 2000; Vasbinder & de Kruif, 2003). Thus, of the principal whey proteins, β -lg has the dominant influence on heat stability, whether positive or negative. The results of the present study indicate that the destabilising effects of β -lg (i.e., increased whey protein aggregation and/or increased κ -casein dissociation) had a stronger influence on the heat stability of model IMFs than the stabilising effects (i.e., formation of complexes at micellar surfaces), and that this factor was responsible for the positive effect of increased α -lac: β -lg ratio on heat stability. Thus, altering α -lac: β -lg ratio in the

production of humanised IMFs may affect performance during thermal processes such as UHT.

In the manufacture of an IMF by a “wet-mixing” approach, the liquid IMF at ~4–7% total protein and pH 6.6–7.0 is typically subjected to HTST treatment followed by refrigerated storage, during which refrigerated storage heat-labile ingredients are often added before further processing. In this study, such a HTST-cooling cycle was simulated at lab-scale. Heating milk at 75–100 °C increases its viscosity, with the extent of this increase commonly linked with the size and volume fraction of casein micelles, which are increased through their interactions with denatured whey proteins (Anema et al., 2004). Analysis of the model IMFs post-HTST heating generally indicated that the extent of the increase in viscosity decreased with increasing α -lac: β -lg (Fig. 3), due to less extensive protein–protein interactions (Table 1, Fig. 5). It is evident from the results of simulated HTST treatment that increasing α -lac: β -lg ratio alters the physicochemical properties of IMFs (e.g., reduces viscosity) after heating. This may have important implications for manufacturers of IMFs in powder and liquid formats. For example, in IMF powder manufacture, differences in feed solution viscosity after HTST (Fig. 3) and vacuum evaporation would be expected to change certain physical properties of spray-dried IMFs, such as bulk density (Keogh, Murray, & O’Kennedy, 2003; Murphy et al., 2014). In addition, analytical centrifugation experiments indicated that physical stability after HTST heating decreased with increasing α -lac: β -lg (Fig. 4). In liquid IMF manufacture, where more intense heat treatments such as UHT are applied, stabiliser addition may be required to limit the more rapid sedimentation of protein particles in high α -lac: β -lg samples during storage (Tobin et al., 2011).

5. Conclusion

Increasing α -lac: β -lg ratio conferred improved heat stability on model IMF protein systems by limiting the extent of whey protein–casein interactions. Protein particle size in high α -lac: β -lg IMFs did not increase to the same extent as that in samples with low α -lac: β -lg ratio during HTST-heating, resulting in less extensive heat-induced viscosity increases in the former. Observed differences in heat stability and post-heating viscosity between IMFs with different α -lac: β -lg ratios may have practical implications for the manufacture of humanised IMF liquids and powders, in terms of destabilisation during thermal treatment or storage, and the physical properties of IMF powders, respectively.

Acknowledgments

The authors would like to acknowledge the financial support of the Food Institutional Research Measure (FIRM) initiative of the Irish Department of Agriculture, Food and the Marine, and the insightful discussions on whey protein denaturation/aggregation with Dr André Brodkorb and Ms. Sophie Gaspard of Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2015.07.077>.

References

- Anema, S. G., Lowe, E. K., & Li, Y. (2004). Effect of pH on the viscosity of heated reconstituted skim milk. *International Dairy Journal*, 14, 541–548.
- Armaforte, E., Curran, E., Huppertz, T., Ryan, C. A., Caboni, M. F., O’Connor, P. M., et al. (2010). Proteins and proteolysis in pre-term and term human milk and

- possible implications for infant formulae. *International Dairy Journal*, 20, 715–723.
- Böttger, F. H., Etzel, M. R., & Lucey, J. A. (2013). In vitro infant digestion of whey protein-dextran glyicates. *Food Digestion*, 4, 76–84.
- Crowley, S. V., Caldeo, V., McCarthy, N. A., Fenelon, M. A., Kelly, A. L., & O'Mahony, J. A. (2015). Processing and protein enrichment characteristics of different polymeric membranes during filtration of skim milk at refrigeration temperatures. *International Dairy Journal*, 48, 23–30.
- Crowley, S. V., Kelly, A. L., & O'Mahony, J. A. (2014). Fortification of reconstituted skim milk powder with different calcium salts: impact of physicochemical changes on heat- and physical-stability. *International Journal of Dairy Technology*, 67, 474–482.
- Crowley, S. V., Megemont, M., Gazi, I., Kelly, A. L., Huppertz, T., & O'Mahony, J. A. (2014). Heat stability of reconstituted milk protein concentrate powders. *International Dairy Journal*, 37, 104–110.
- Davies, D. T., & White, J. C. D. (1966). The stability of milk protein to heat. I. Subjective measurement of heat stability of milk. *Journal of Dairy Science*, 33, 67–81.
- Davis, A. M., Harris, B. J., Lien, E. L., Pramuk, K., & Trabulsi, J. (2007). A-Lactalbumin-rich infant formula fed to healthy term infants in a multicenter study: Plasma essential amino acids and gastrointestinal tolerance. *European Journal of Clinical Nutrition*, 62, 1294–1301.
- De Wit, J. N. (1998). Nutritional and functional characteristics of whey proteins in food products. *Journal of Dairy Science*, 81, 597–608.
- Donato, L., & Guyomarc'h, F. (2009). Formation and properties of the whey protein/ κ -casein complexes in heated skim milk – A review. *Dairy Science and Technology*, 89, 3–29.
- European Commission (2006). Commission Directive 2006/141/EC of 26 December 2006 on infant formulae and follow-on formulae. Official Journal of European Union.
- Fomon, S. J. (2001). Infant feeding in the 20th century: Formula and Beikost. *The Journal of Nutrition*, 131, 409–420.
- Guyomarc'h, F., Nono, M., Nicolai, T., & Durand, D. (2009). Heat-induced aggregation of whey proteins in the presence of κ -casein or sodium caseinate. *Food Hydrocolloids*, 2, 1103–1110.
- Herwig, N., Stephan, K., Panne, U., Pritzkow, W., & Vogl, J. (2011). Multi-element screening in milk and feed by SF-ICP-MS. *Food Chemistry*, 124, 1223–1230.
- IDF (1986). *International IDF Standard 20A. Milk. Determination of nitrogen content (Kjeldahl method) and calculation of crude protein content*. Brussels, Belgium: International Dairy Federation.
- IDF (1987). *International IDF Standard 21B. Milk, cream and evaporated milk – determination of total solids content*. Brussels, Belgium: International Dairy Federation.
- IDF (1991). *International IDF Standard 52. Milk and milk products – fat content. General guidance on butyrometric methods*. Brussels, Belgium: International Dairy Federation.
- Indyck, H. E., Edwards, M. J., & Woollard, D. C. (1996). High performance liquid chromatographic analysis of lactose-hydrolysed milk. *Food Chemistry*, 57, 575–580.
- Jenness, R., & Koops, J. (1962). Preparation and properties of a salt solution which simulates milk ultrafiltrate. *Netherlands Milk and Dairy Journal*, 16, 153–164.
- Jiang, Y. J., & Guo, M. (2014). Processing technology for infant formula. In M. Guo (Ed.), *Human milk biochemistry and infant formula manufacturing technology* (pp. 211–229). Cambridge, UK: Woodhead Publishing Ltd.
- Keogh, M. K., Murray, C. A., & O'Kennedy, B. T. (2003). Effects of ultrafiltration of whole milk on some properties of spray-dried milk powders. *International Dairy Journal*, 13, 995–1002.
- Kuhlman, C. F., Lien, E., Weaber, J., & O'Callaghan, D. (2003). Infant formula compositions comprising increased amounts of alpha-lactalbumin. United States of America Patent Application Publication No.: US 2003/0124237 A1.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685.
- Lucena, M. E., Alvarez, S., Menéndez, R. F. A., & Alvarez, R. (2006). Beta-lactoglobulin removal from whey protein concentrates: Production of milk derivatives as a base for infant formulas. *Separation and Purification Technology*, 52, 310–316.
- Murphy, E. G., Fenelon, M. A., Roos, Y. H., & Hogan, S. A. (2014). Decoupling macronutrient interactions during heating of model infant milk formulas. *Journal of Agricultural and Food Chemistry*, 62, 10585–10593.
- Murphy, E. G., Roos, Y. H., Hogan, S. A., Maher, P. G., Flynn, C. G., & Fenelon, M. A. (2015). Physical stability of infant milk formula made with selectively hydrolysed whey proteins. *International Dairy Journal*, 62, 10585–10593.
- Nair, P. K., Dalglish, D. G., & Corredig, M. (2013). Colloidal properties of concentrated heated milk. *Soft Matter*, 9, 3815–3824.
- Oldfield, D. J., Singh, H., & Taylor, M. W. (1998). Association of β -lactoglobulin and α -lactalbumin with the casein micelles in skim milk heated in an ultra-high temperature plant. *International Dairy Journal*, 8, 765–770.
- Oldfield, D. J., Singh, H., Taylor, M. W., & Pearce, K. N. (2000). Heat-induced interactions of β -lactoglobulin and α -lactalbumin with the casein micelle in pH-adjusted skim milk. *International Dairy Journal*, 10, 509–518.
- Pearce, R. J. (1979). Heat stability in concentrated and non-concentrated milks – the effect of urea and β -lactoglobulin levels and the influence of preheating. *Journal of Dairy Research*, 46, 385–386.
- Pearce, R. J. (1995). Enriched whey protein fractions and method for the production thereof. United States of America Patent, US005455331A.
- Rattray, W., & Jelen, P. (1997). Thermal stability of skim milk/whey protein solution blends. *Food Research International*, 30, 327–334.
- Sandström, O., Lönnerdal, B., Graverholt, G., & Hernell, O. (2008). Effects of α -lactalbumin-enriched formula containing different concentrations of glycomacropptide on infant nutrition. *The American Journal of Clinical Nutrition*, 87, 921–928.
- Singh, H. (2004). Heat stability of milk. *International Journal of Dairy Technology*, 57, 111–119.
- Singh, H., & Creamer, L. K. (1991). Influence of concentration of milk solids on the dissociation of micellar κ -casein on heating reconstituted milk at 120 C. *Journal of Dairy Research*, 58, 99–105.
- Stack, F. M., Hennessy, M., Mulvihill, D., & O'Kennedy, B. T. (1999). Process for the fractionation of whey constituents. European Patent, EP0 765 125 B1.
- Taheri-Kafrani, A., Gaudin, J. C., Rabesona, H., Nioi, C., Agarwal, D., Drouet, M., et al. (2009). Effects of heating and glycation of β -lactoglobulin on its recognition by IgE of sera from cow milk allergy patients. *Journal of Agricultural and Food Chemistry*, 57, 4974–4982.
- Tobin, J. T., Fitzsimons, S. M., Kelly, A. L., & Fenelon, M. A. (2011). The effect of native and modified konjac on the physical attributes of pasteurized and UHT-treated skim milk. *International Dairy Journal*, 21, 790–797.
- Vasbinder, A. J., & de Kruij, C. G. (2003). Casein–whey interactions in heated milk: Influence of pH. *International Dairy Journal*, 13, 669–677.
- Wijayanti, H. B., Bansal, N., & Deeth, H. C. (2014). Stability of whey proteins during thermal processing: A review. *Comprehensive Reviews in Food Science and Food Safety*, 13, 1235–1251.

Table. S1. Composition of model infant milk formulae. Results are means $\pm$ standard deviations of data from at least three independent trials on freshly prepared samples.

Component	α -lactalbumin: β -lactoglobulin ratio				
	0.1	0.5	1.3	2.1	4.6
Total solids (% w/w)	6.72 $\pm$ 0.07 ^a	6.36 $\pm$ 0.08 ^a	6.44 $\pm$ 0.05 ^a	6.44 $\pm$ 0.11 ^a	6.57 $\pm$ 0.10 ^a
Protein (% w/w)	5.53 $\pm$ 0.09 ^a	5.59 $\pm$ 0.13 ^a	5.47 $\pm$ 0.02 ^a	5.49 $\pm$ 0.07 ^a	5.44 $\pm$ 0.14 ^a
Lactose (% w/w)	0.20 $\pm$ 0.00 ^a	0.18 $\pm$ 0.01 ^a	0.19 $\pm$ 0.01 ^a	0.21 $\pm$ 0.03 ^a	0.19 $\pm$ 0.00 ^a
Ash (% w/w)	0.81 $\pm$ 0.16 ^a	0.79 $\pm$ 0.05 ^a	0.79 $\pm$ 0.04 ^a	0.75 $\pm$ 0.02 ^a	0.82 $\pm$ 0.06 ^a
Ionic calcium (mM L ⁻¹)	2.61 $\pm$ 0.03 ^a	2.76 $\pm$ 0.02 ^a	2.71 $\pm$ 0.06 ^a	2.51 $\pm$ 0.11 ^a	2.16 $\pm$ 0.23 ^a
Calcium (mg L ⁻¹)	1225 $\pm$ 17.3 ^a	1198 $\pm$ 40.0 ^a	1220 $\pm$ 149 ^a	1119 $\pm$ 11 ^a	1141 $\pm$ 217 ^a
Phosphorus (mg L ⁻¹)	913 $\pm$ 21.1 ^a	870 $\pm$ 16.9 ^a	896 $\pm$ 128 ^a	864 $\pm$ 64 ^a	823 $\pm$ 175 ^a
Magnesium (mg L ⁻¹)	114 $\pm$ 6.9 ^a	112 $\pm$ 10.6 ^a	112 $\pm$ 6.0 ^a	100 $\pm$ 19 ^a	104 $\pm$ 15.8 ^a
Potassium (mg L ⁻¹)	1681 $\pm$ 35.8 ^a	1689 $\pm$ 35.7 ^a	1704 $\pm$ 216 ^a	1468 $\pm$ 366 ^a	1604 $\pm$ 321 ^a
Sodium (mg L ⁻¹)	672 $\pm$ 14.4 ^a	658 $\pm$ 9.4 ^a	673 $\pm$ 84.5 ^a	643 $\pm$ 102 ^a	642 $\pm$ 116 ^a

^a Values within a row with the same superscripts are not significantly different ($P > 0.05$).

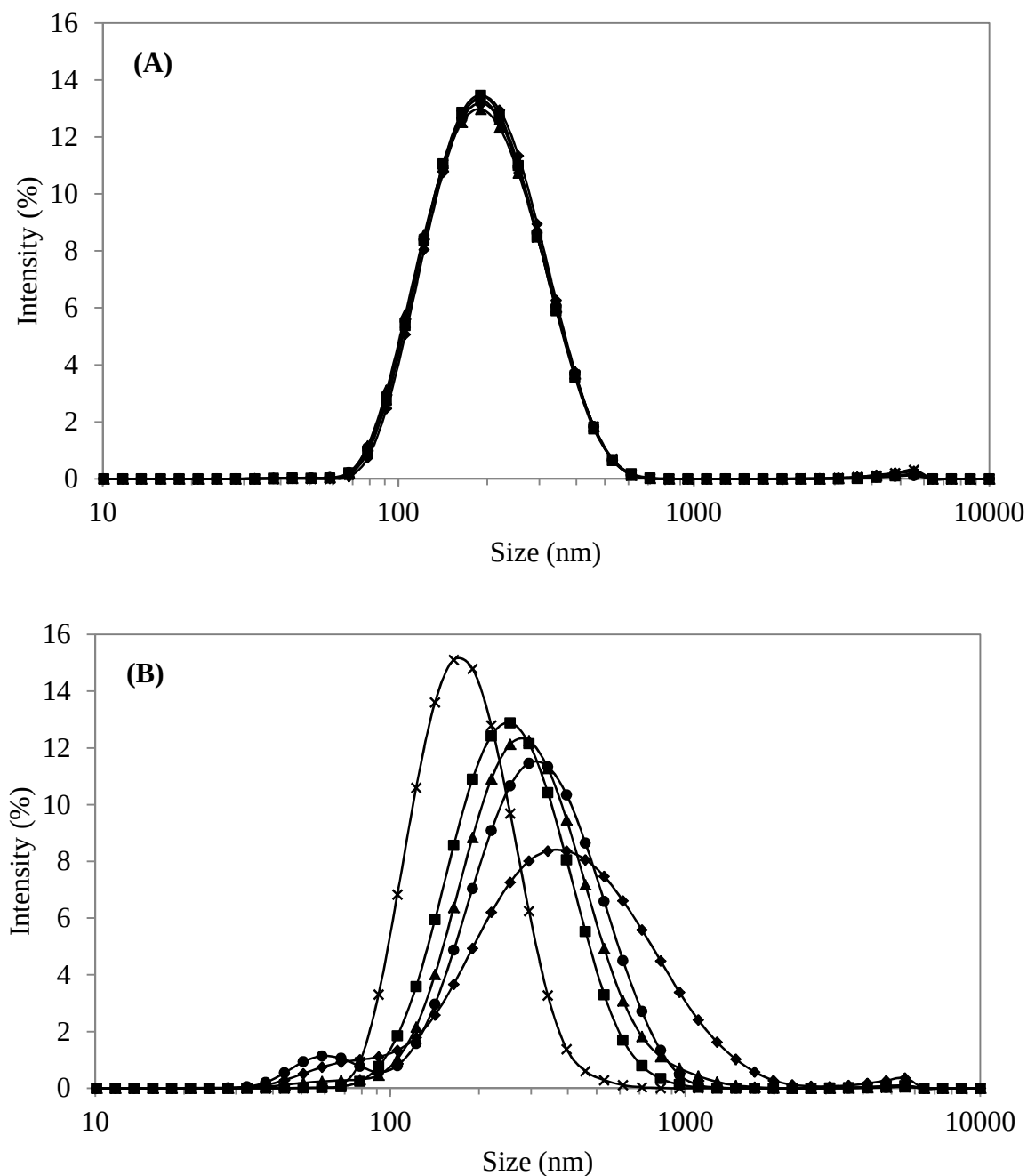


Fig. S1. Particle size distribution (A) before and (B) after simulated high-temperature short-time (HTST) treatment for model infant milk formulae at pH 6.8 with α -lactalbumin: β -lactoglobulin ratios of 0.1 (♦), 0.5 (●), 1.3 (▲), 2.1 (■) or 4.6 (×). Results are the means of data from at least three independent trials on freshly prepared samples.