

Emulsions stabilization by lactoferrin nano-particles under *in vitro* digestion conditions

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ABSTRACT

In recent years there has been a spur of interest in the utilization of nano and micro-particles to fabricate novel food-grade Pickering emulsions. Aligned with increased interest and efforts to promote health through food, this study aimed to extend the understanding of Pickering emulsions stabilized by lactoferrin (LF) nano-particles in respect to their stability and responsiveness to physiological conditions of the human mouth and stomach. Analytical centrifugation revealed that LF nano-particles did not alter mean droplet size of coarse emulsions but significantly ($p < 0.05$) reduced creaming rates by an order of magnitude. In fine emulsions produced through high pressure homogenization, the use of nano-particles increased mean droplet sizes. This resulted in noted ($p < 0.05$) differences in stability with emulsions stabilized by LF nano-particles and alginate showing poorest stability. Concomitantly, the use of i-carrageenan and LF nano-particles yielded emulsions with the most reduced creaming ($<1 \mu\text{m/s}$), even compared to emulsions stabilized by native LF. Interestingly, the use of alginate and i-carrageenan with LF nano-particles also altered emulsion stability to artificial saliva and modulated emulsion behavior under gastric conditions, which was linked to reduced rate of LF gastric proteolysis. Overall, this work establishes a new possibility to incorporate LF in emulsions and demonstrates how LF nano-particles could be harnessed to modulate emulsion destabilization and breakdown in the mouth and stomach.

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1. Introduction

Engineering foods for health and well-being has become a central effort for many food scientists and manufacturers. Aligned with such efforts various studies have focused on the development of various hydrocolloid delivery systems, their functionalization and adaptation to food (Benshitrit, Levi, Tal, Shimoni, & Lesmes, 2012; Lesmes & McClements, 2009; McClements, 2010; Singh, Ye, & Horne, 2009; Velikov & Pelan, 2008). In many cases extending the benefits of food beyond its nutritional value requires the incorporation of lipophilic bioactive compounds which can be achieved through emulsion-based systems (McClements, 2010; McClements & Li, 2010). Thus, various studies have established physical and chemical strategies of modifying droplet interfaces to affect emulsion physical and chemical properties as well as their digestive fate (Lesmes & McClements, 2012; Li, Hu, & McClements,

2011; McClements, 2010; McClements & Li, 2010; Oliver, Melton, & Stanley, 2006; Singh et al., 2009; Wooster & Augustin, 2006).

In spite of the great progress in emulsion science and technology, there is a constant need for ingredient and formulation innovations which cope with the shortcomings of current formulations. Recently, there is a growing interest in the utilization of nano and micro-particles to fabricate novel food-grade Pickering emulsions (Adelmann, Binks, & Mezzenga, 2012; Dickinson, 2010b, 2012; Gupta & Rousseau, 2012; Kargar, Fayazmanesh, Alavi, Spyropoulos, & Norton, 2012; Rayner, Timgren, Sjöo, & Dejmek, 2012; Tzoumaki, Moschakis, Kiosseoglou, & Biliaderis, 2011). Such emulsions were described in literature as early as the 1900s and are now being revisited by researchers due to their special features, namely physical stability, distinct interfacial properties and ease of fabrication.

Unlike emulsions stabilized by common food emulsifiers, e.g. whey proteins and lecithin, Pickering emulsions are stabilized by solid particles and the use of particles such as silica, chitin nanocrystals, cellulose nanocrystals, starch granules, solid lipid nanoparticles and proteinaceous fibrils have been recently reported (Adelmann et al., 2012; Dickinson, 2012; Gupta & Rousseau, 2012; Humblet-Hua, Scheltens, van der Linden, & Sagis, 2011; Rayner

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et al., 2012; Tzoumaki et al., 2011). Concomitantly, there has been significant progress into protein and protein–polysaccharide nano-particle formation and stability (Jones & McClements, 2011). Such studies have already established how thermal processing, ionic strength, biopolymer segregation, electrostatic layer by layer deposition, protein self-assembly and other intrinsic reactant characteristics affect particle formation, size, morphology, fine architecture and overall stability. In respect to emulsions, there have been various recent advances in the study of protein-stabilized emulsions and their responsiveness to the physiological and anatomical conditions of the human gastrointestinal tract (GIT) (Dickinson, 2008, 2010a; Shani-Levi, Levi-Tal, & Lesmes, 2013; Singh & Sarkar, 2011; Wilde & Chu, 2011). However, scarce data exists on the application of protein-based nano-particles to the formation and stabilization of emulsions, particularly as it pertains to emulsion behavior in the GIT.

To this end, the use of proteins in emulsion systems also entails potential health benefits which may arise for the consumption of bioactive proteins or the formation of bioactive peptides post-ingestion (Agvei & Danquah, 2012; Nagpal et al., 2011). In emulsions, milk proteins have been extensively applied and studied (Dickinson, 2010a; McClements, 2010; Singh & Sarkar, 2011), with certain recent reports demonstrating the specific benefits of using bovine lactoferrin to promote and extend emulsion physical and chemical stability (Lesmes, Baudot, & McClements, 2010; Lesmes, Sandra, Decker, & McClements, 2009; Sarkar, Horne, & Singh, 2010; Schmelz, Lesmes, Weiss, & McClements, 2011; Tokle, Lesmes, Decker, & McClements, 2012; Tokle, Lesmes, & McClements, 2010; Ye & Singh, 2007). This whey protein has drawn attention for its various implications to biological functions, e.g. antioxidant and antimicrobial activities, and as a source of bioactive peptides formed during digestion (Kuwata, Yip, Tomita, & Hutchens, 1998; Lesmes et al., 2009; Lonnerdal & Iyer, 1995; Madureira, Pereira, Gomes, Pintado, & Malcata, 2007; Nagpal et al., 2011; Tomita et al., 2009; Troost, Steijns, Saris, & Brummer, 2001; Wakabayashi, Yamauchi, & Takase, 2006).

Most recently this bioactive protein has been shown to form different nano-particles through heat treatment, pH adjustments and electrostatic interactions with food-grade polysaccharides (Bengoechea, Jones, Guerrero, & McClements, 2011; Bengoechea, Peinado, & McClements, 2011; Peinado, Lesmes, Andres, & McClements, 2010). Moreover, our recent work has demonstrated that the susceptibility of such nano-particles to gastro-duodenal digestion alters depending on the type of dietary fiber used (David-Birman, Mackie, & Lesmes, 2013). Thus, the present study focused on extending the application of lactoferrin nano-particles to form emulsions and stabilize them under some physiological conditions relevant to their potential oral consumption. Particularly, we sought to assess whether altered nano-particle susceptibility to gastric proteolysis could be extended to control emulsion stability under bio-relevant physiological conditions.

2. Materials and methods

2.1. Materials

Food-grade bovine lactoferrin (Vivinal lactoferrin FD, 95.6% protein) was kindly donated by DMV International (Delhi, NY, USA). The polysaccharides (PS) used were GENUGEL® carrageenan type Cj kindly donated by CPKelco. Alginate sodium salt from brown algae was purchased from Sigma–Aldrich (Rehovot, Israel). Olive oil was purchased from Shemen Industries Ltd. (Haifa, Israel). Pepsin from porcine gastric mucosa (≥ 250 units/mg) and α amylase from *Aspergillus oryzae* (1.7 units/mg) were purchased from Sigma–

Aldrich (Rehovot, Israel). All reagents and chemicals used were of analytical grade.

Artificial saliva was prepared according to previous work (Hur, Decker, & McClements, 2009). Practically, saliva was made by mixing 10 mL KCl solution (89.6 g/L), 10 mL KSCN solution (20 g/L), 10 mL NaH₂PO₄ solution (88.8 g/L), 1.7 mL NaCl solution (57 g/L), 20 mL NaHCO₃ solution (84.7 g/L) and 8 mL urea solution (25 g/L). Into this solution 290 mg α -amylase, 15 mg uric acid and 25 mg of mucin were added and the pH was adjusted to 6.8 before use. Saliva was freshly made each day of experimentation and stored refrigerated until use. Simulated gastric fluid (SGF) contained 2 g NaCl, 7 mL HCl, pepsin (2.4 units/mL) and pH was initially adjusted to 1.2 using HCl or NaOH 1 M, as previously described (Lesmes & McClements, 2012; Sarkar, Goh, Singh, & Singh, 2009).

2.2. Preparation of biopolymer solutions and nano-particle stabilized emulsions

LF-based nano-particles were fabricated based on the protocol described previously (David-Birman et al., 2013; Peinado et al., 2010) to form three types of nano-particles (np): a. LF nano-particles (LFnp), b. LF nano-particles with alginate (LFnp + ALG), c. LF nano-particles with i-carrageenan (LFnp + CAR). Native LF was used as control.

Pre-emulsions were prepared as follows: (olive oil 2%w/w; LF/np 0.2%w/w in DDW at pH 7) homogenization for 1 min at 25,000 RPM using a hand blender (PRO 200, BIOGEN series pro scientific, Oxford CT, USA). Fine emulsions were produced by passing coarse emulsions 4 times through a high pressure homogenizer (Micro DeBEE air operated, BEE International, MA, USA) at 15 kPa. For clarity, overall production scheme is illustrated in Fig. 1. All emulsions were stored at 4 °C prior to *in vitro* digestion for maximum of 72 h.

2.2.1. Characterization of emulsion properties

Characterization of fine emulsions focused on stability, size, charge and appearance, while coarse emulsions exhibited rapid separation limiting their analysis to direct observations. Physical stability to creaming and particle size of fine emulsions were evaluated using an analytical centrifugal analyzer (LUMISIZER, L.U.M. GmbH, Berlin, Germany). The LUMISIZER allows measurement of time and space-resolved transmission extinction profiles under analytical centrifugation. The data produced was then used to calculate the creaming rate of emulsions as well as their harmonic mean droplet size, as previously described (Detloff, Sobisch, & Lerche, 2006; Lerche & Sobisch, 2011). Practically, stability of non-diluted emulsions was determined at 23 °C under 2000 RPM for 2 h or 8.5 h for fine emulsions. Harmonic mean droplet sizes were determined based on analytical centrifugation at 23 °C for 45 min or 85 min for coarse or fine emulsion, respectively, under increasing speed between 500 and 4000 RPM using diluted (1:20 v/v) emulsion samples.

The electrical charge (ζ -potential) of the oil droplets was determined by measuring their electrophoretic mobility using an automated capillary electrophoresis device (Zetasizer Nano ZS series, Malvern Instruments, Worcestershire, U.K.). Emulsion samples were diluted in double distilled water (DDW) in pH 7 at a ratio of 1:50 (v/v) and then filled into the test cell. After 60 s of equilibration in the instrument, the data was collected from at least 10 sequential readings per sample and the ζ -potential was calculated by the instrument using the Smoluchowski model, as previous work (Lesmes et al., 2010; Lesmes & McClements, 2012; Schmelz et al., 2011). Emulsions were visually inspected using a Cell Observer-Zeiss Axiovert 200 inverted microscope. Images were processed by the AxioVision (Zeiss) image analysis software for acquisition and

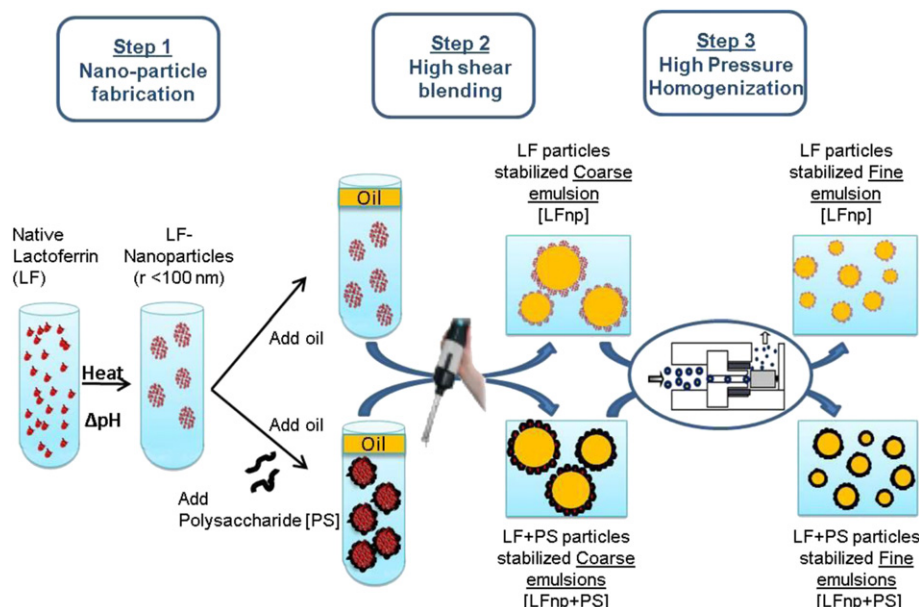


Fig. 1. Production LF nano-particles and fabrication of coarse and fine emulsion stabilized by LF, LFnp, LFnp + CAR or LFnp + ALG.

image processing. Additionally, coarse emulsions were directly inspected and photographed using a Nikon camera.

2.3. Evaluation of emulsion stability to saliva

Protein-stabilized emulsions have been reported to be destabilized by saliva and consequently affect emulsions' sensorial properties (Aken, Vingerhoeds, & Wijk, 2011; Sarkar, Goh, & Singh, 2009; Vingerhoeds, Silletti, de Groot, Schipper, & van Aken, 2009; van Vliet, van Aken, de Jongh, & Hamer, 2009). This study sought to assess the potential oral behavior of the emulsion samples using artificial saliva, as previously described by others (Hur et al., 2009; Sarkar, Goh, & Singh, 2009). In these experiments samples were mixed at 37 °C with artificial saliva (1:1 v/v) before being loaded onto the analytical centrifuge to measure their time and space-resolved extinction profiles (as mentioned in Section 2.2.1). The data collected was then analyzed to deduce emulsion stability and creaming rate.

2.4. Evaluation of emulsion behavior during *in vitro* gastric digestion

Gastric proteolysis has been demonstrated to be detrimental to the stability of protein-stabilized emulsions (Sarkar, Goh, Singh, & Singh, 2009; Singh & Sarkar, 2011). Thus, we studied the physical stability of emulsion samples using a dynamic gastric model mirroring gastric pH profiles of an adult's stomach, as recently described (Shani-Levi et al., 2013). For this purpose, a water heated jacketed reactor was maintained at 37 °C and continuously stirred (230 RPM) while controlled by an auto titration unit (Titrand 902, Metrohm, Switzerland) which gradually varied reactor pH using "TIAMO 2.3" software (Metrohm, Switzerland), based on previous reports (Blanquet et al., 2004; Chatterton, Rasmussen, Heegaard, Sorensen, & Petersen, 2004; Mason, 1962; Yoo & Chen, 2006).

Accounting for the serial events of digestion, emulsions were first mixed with artificial saliva (1:1 v/v) for 2 s and then introduced into SGF (reaching final ratio of 2:3 emulsion:SGF). Reactor pH was rapidly increased to 4.5 using 1 M NaOH then TIAMO software was instructed to begin the gastric pH gradient reaching a final pH of 1.7.

Samples were aspirated after 10, 60 and 120 min and rapidly neutralized to pH = 7 using freshly prepared NaHCO₃ 1 M to inactivate pepsin before any further analysis. Control samples were produced from mixtures of emulsion:saliva:SGF before initiation of the TIAMO software.

2.5. Characterization of gastric proteolysis by SDS-PAGE

Differences in emulsifier degradation of LF and LF np as well as the formation of peptides during the *in vitro* gastric digestion of emulsions were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was run in a Tris/Glycine/SDS buffer (BioRad, Rishon le Zion, Israel) in pre-cast 4–15% gradient gels (Bio-Rad, USA) which were stained by Instant blue stain (Bio Consult, Israel).

2.6. Experimental design and analysis

All experiments were carried out in triplicates with each sample measured thrice, and results are presented as the calculated mean and standard deviation. Statistical analyses were performed using Microsoft Excel 2010 data analysis toolpack and mainly relied on *t*-tests assuming equal variances and ANOVA single factor.

3. Results and discussion

Recent advances in the field of food emulsions have focused on understanding the underlying principles for their design as oral delivery vehicles (McClements, 2010; McClements & Li, 2010; Singh et al., 2009). Among the different strategies studied, there is an upsurge of interest in the application of food colloids and hydrocolloids to form and stabilize emulsions (Dickinson, 2010b, 2012; McClements, 2010; Singh et al., 2009). This study aimed to extend the understanding of Pickering emulsions in respect to their stability and responsiveness to physiological conditions of the human mouth and stomach. Specifically, the application of lactoferrin-polysaccharide nano-particles to the fabrication and stabilization of oil/water (O/W) emulsions was investigated.

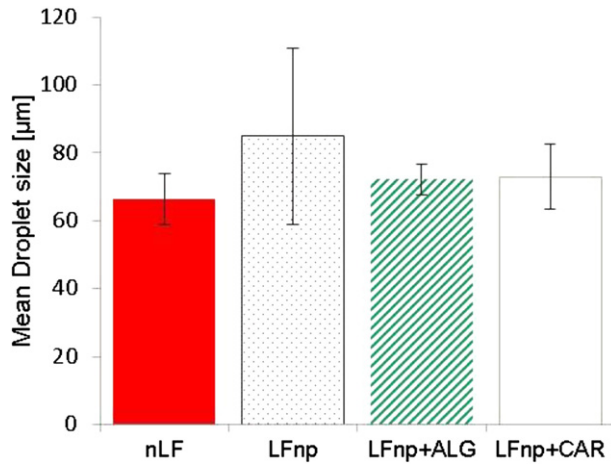


Fig. 2. Harmonic mean droplet size of LF and np stabilized coarse emulsions.

3.1. Characterization of coarse and fine emulsions

Initially, evaluation of the ability of different lactoferrin-based nano-particles to form coarse emulsions (Fig. 1) was performed. Based on a previously described method (Peinado et al., 2010), lactoferrin nano-particles were formed and used to fabricate coarse emulsions which were characterized for their size and overall

appearance using analytical centrifugation (Fig. 2). As can be seen, irrespective of the type of nano-particle used all coarse emulsions had similar droplet sizes (Fig. 2) and overall appearance. Droplet interfacial properties affect emulsion stability, including in the case of colloid stabilized emulsions (Dickinson, 2012; McClements, 2005). Thus, the physical stability of these emulsions was studied under analytical centrifugation and the time and space-resolved transmission extinction profiles were recorded (Fig. 3). In spite of the similar droplet sizes (Fig. 2), differences were noted in the progression of the transmission extinction profiles (highlighted by arrows). For example, the coarse emulsion stabilized by LFnp + ALG showed the most rapid changes in transmission profiles while LFnp + CAR showed the least changes. Such changes in the transmission profiles have been linked to colloid instability phenomena, such as sedimentation and creaming (Lerche & Sobisch, 2011). In this case, the clarification along the test tubes and away from the centrifugal axis can be attributed to creaming. This is due to the fact that the lipid droplets migrate toward the center of the rotor and cease to occupy the lower parts of the test tube; hence an increase in transmission in the lower parts of the test tube which are farther away from the centrifugation axis. Based on these observations, the creaming rate for each sample was calculated (Fig. 4) and statistical analyses revealed two main differences. The first was that emulsions stabilized by LFnp had significantly ($p < 0.05$) reduced creaming rate compared to emulsions stabilized by native LF. The second difference was that emulsions stabilized by LFnp and polysaccharides (alginate or carrageenan) exhibited enhanced

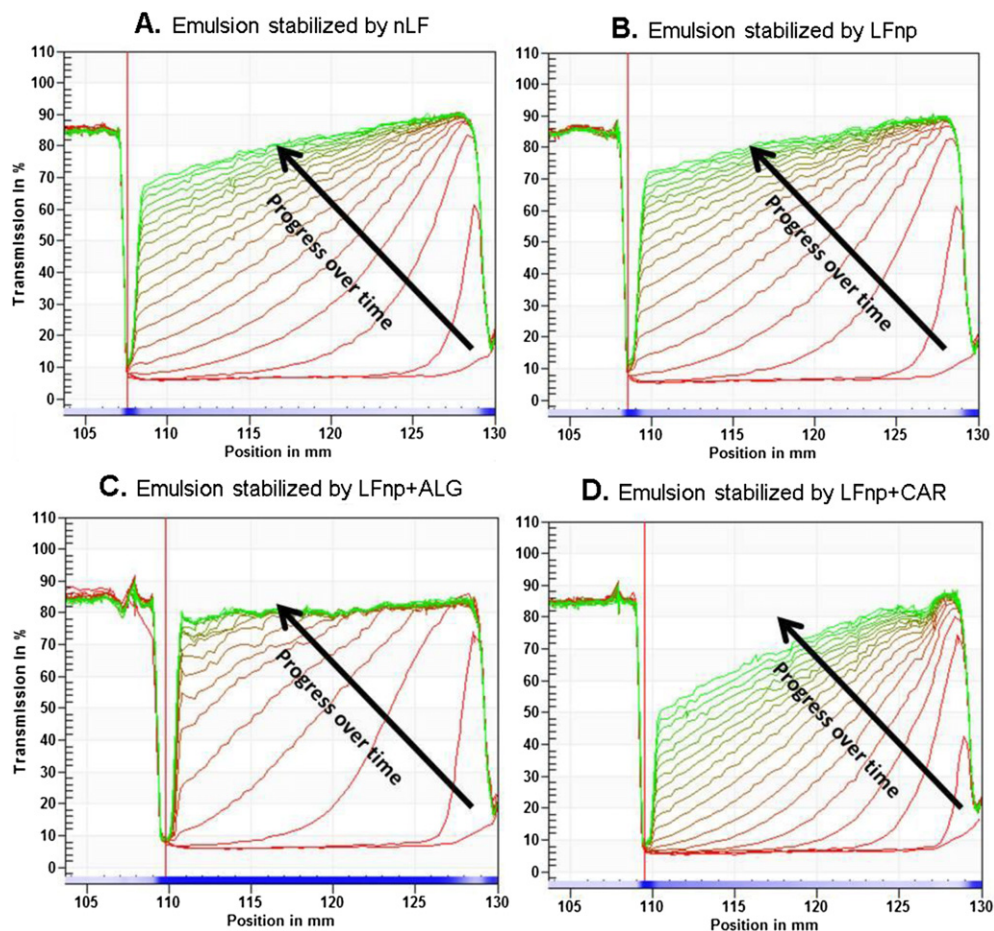


Fig. 3. Time and space profiles of coarse emulsions under analytical centrifugation. [A] Emulsion stabilized by native lactoferrin (nLF), [B] emulsion stabilized lactoferrin nano-particles (LFnp), [C] emulsion stabilized lactoferrin nano-particles and alginate (LFnp + ALG), [D] emulsion stabilized lactoferrin nano-particles and iota-carrageenan (LFnp + CAR).

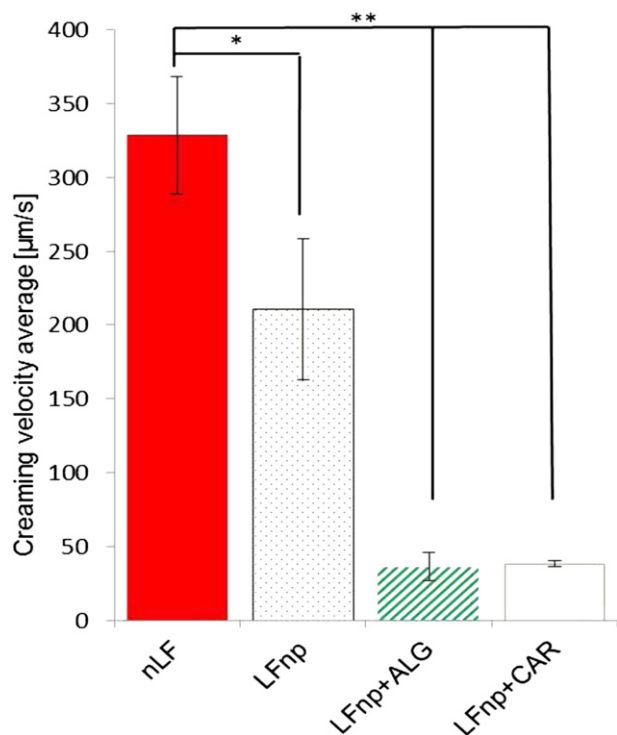


Fig. 4. Average creaming velocities of coarse emulsions under analytical centrifugation. Statistically different samples denoted * $p < 0.05$, ** $p < 0.01$.

stability to creaming and significantly ($p < 0.01$) reduced creaming rates.

In light of the marked improvement in emulsion stability when using LFnp, additional experiments focused on manufacturing corresponding fine emulsions through high pressure homogenization (as described in Fig. 1). Results of the characterization of these emulsions in terms of size and electrokinetic charge (ζ -potential) are given in Fig. 5. In contrast to the observation made for coarse emulsions, marked differences were noted in the mean droplet sizes (Fig. 5A) with all LFnp stabilized emulsions. These revealed LFnp stabilized emulsions had increased sizes compared to native LF-stabilized emulsions. Moreover, only emulsions stabilized by LFnp + CAR showed a significant ($p < 0.06$) difference in ζ -potential values compared to native LF-stabilized emulsions

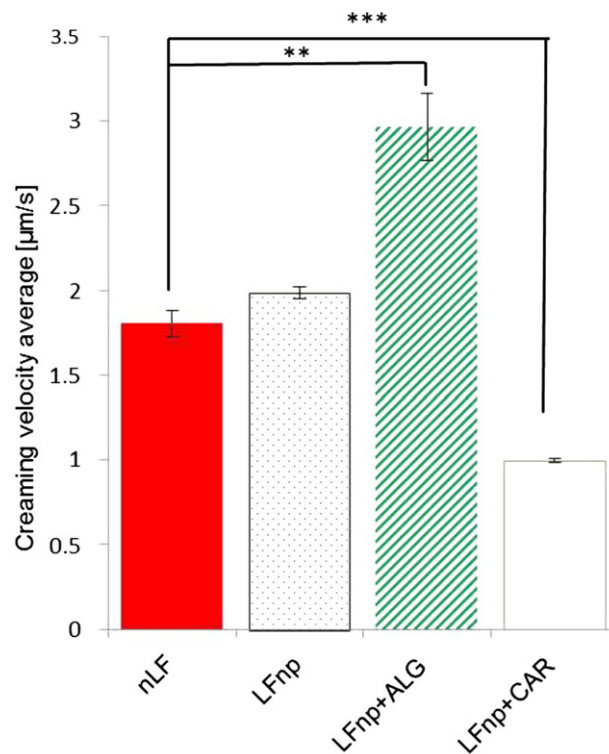


Fig. 6. Velocity average of fine emulsions under analytical centrifugation demonstrates creaming rates. Statistically different samples denoted ** $p < 0.01$, *** $p < 0.001$.

(Fig. 5B). These emulsions were also studied for their physical stability to creaming using analytical centrifugation. The calculated creaming rates are summarized in Fig. 6. This analysis revealed that emulsions stabilized by LF or LFnp did not vary in their stability to creaming while emulsions stabilized by LFnp + ALG or LFnp + CAR had pronounced alterations in creaming rates. Specifically, the use of LFnp + ALG led to the formation of emulsions with significantly ($p < 0.01$) reduced stability to creaming. This reduced physical stability can be attributed to the noted elevated droplet sizes (Fig. 5A) without any substantial ζ -potentials (Fig. 5B) to provide electrostatic stabilization. Contrary, the use of LFnp + CAR led to the formation of emulsions with significantly ($p < 0.001$) improved stability. This improved stability may stem from the elevated ζ -

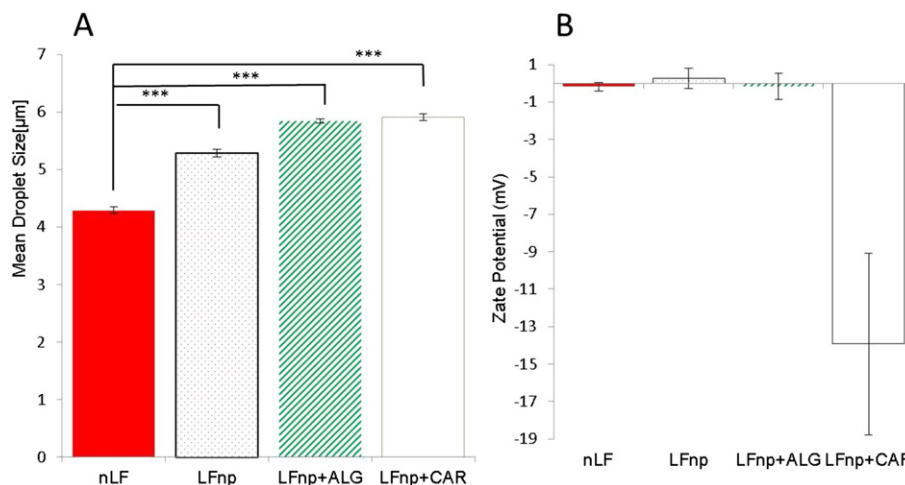


Fig. 5. Mean droplet size of LF and np stabilized fine emulsions [A] and averages of ζ -potential [B]. Statistically different samples denoted *** $p < 0.001$.

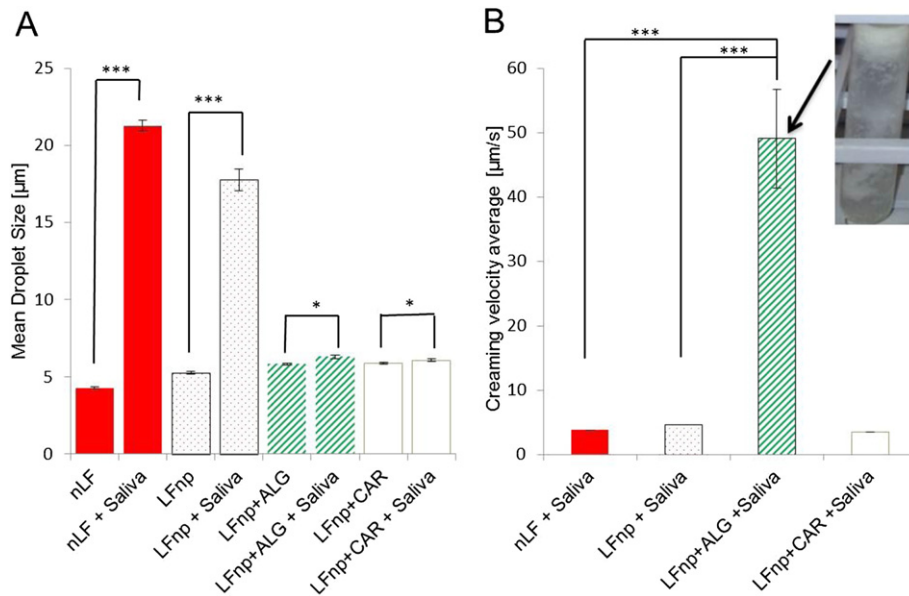


Fig. 7. Harmonic mean droplet size [A] and velocity average [B] of LF and np stabilized fine emulsions before and after adding saliva. Insert image – LFnp + saliva. Statistically different samples denoted * $p < 0.05$, *** $p < 0.001$.

potential of the sample that may increase droplet–droplet repulsion thereby limiting creaming. Alternatively, it is possible that high pressure homogenization may break up the nano-particles and hence alter their emulsifying properties, as recently described for proteinaceous particles (Dickinson, 2009, 2012).

Altogether, coarse and fine emulsions were produced and were found to have altered properties and physical stability. Having human health and pleasure in mind, it was hypothesized that these emulsions might exhibit modulated responsiveness to the

physiological conditions of the mouth and stomach. Thus, further experiments focused on evaluating how emulsifier type and/or composition could be linked to emulsion behavior under *in vitro* digestive conditions.

3.2. Responsiveness to saliva

Emulsion behavior under oral conditions is strongly linked to emulsion sensorial perception and the sequential digestive events

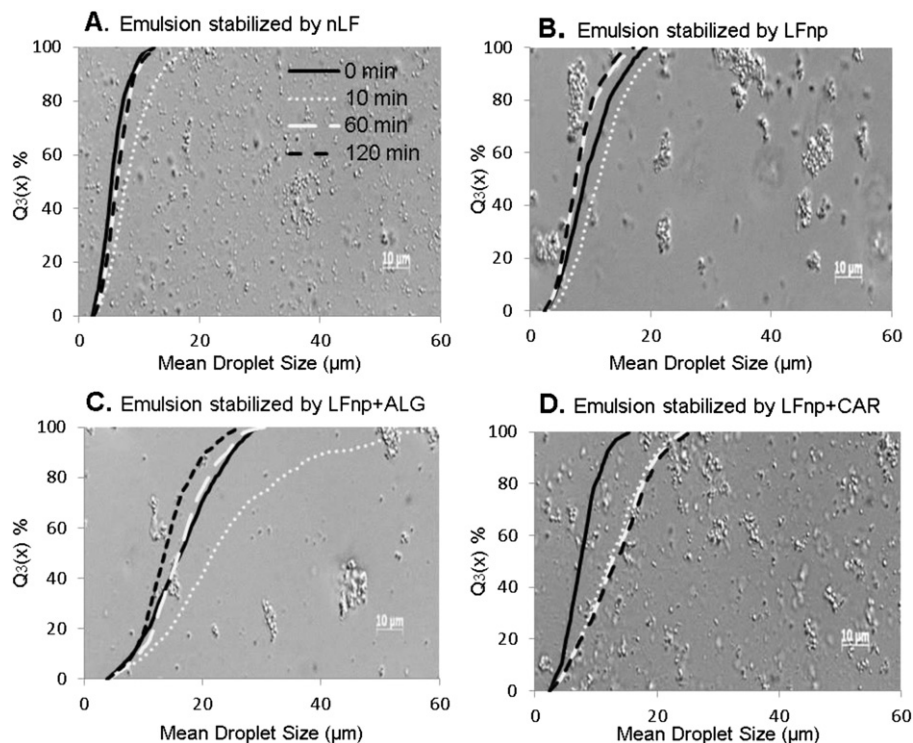


Fig. 8. Cumulative volume weighted particle size distribution of emulsions during gastric digestion ($n = 6$). Background: light microscopy images (magnification $\times 60$) of emulsions after 10 min of gastric digestion. [A] nLF-stabilized emulsion. [B] LFnp stabilized emulsion. [C] LFnp + ALG stabilized emulsion. [D] LFnp + CAR stabilized emulsion.

(Singh & Sarkar, 2011; Vingerhoeds et al., 2009; van Vliet et al., 2009). Subjecting the coarse emulsions to oral conditions and mixing with artificial saliva revealed that only an emulsion stabilized by native LF was destabilized. However, no reliable data could be produced for these samples under analytical centrifugation. In contrast, fine emulsions did enable analytical centrifugation, sample mean size and creaming rates were calculated (Fig. 7). These findings demonstrate that emulsions stabilized by either native LF or LFnp showed significant ($p < 0.001$) increase in size upon exposure to saliva (Fig. 7A) and similar creaming rates (Fig. 7B). Emulsions stabilized by LFnp + ALG showed only a slight increase in size (Fig. 7A) accompanied by a significant ($p < 0.01$) increase in creaming rate (Fig. 7B). This instability may arise from either alginate–alginate physical interactions mediated by divalent ions (e.g. phosphate ions) present in the saliva or *via* mucin bridging, as described by others (Sarkar, Goh, & Singh, 2009). This could lead to increased droplet–droplet interactions and consequently flocculation and accelerated creaming, which concurs with the flocks directly observed (insert in Fig. 7B). Interestingly, emulsions stabilized by LFnp + CAR showed increase in size ($p < 0.01$) (Fig. 7A) but no marked increase in creaming rate (Fig. 7B). This may arise from the demonstrated ability of carrageenan to form gels as a result of polysaccharide interactions (Campo, Kawano, da Silva, & Carvalho, 2009) and not mediated by divalent ions or mucin present in the saliva. This modulation in emulsion responsiveness to saliva could have implication not only to emulsion sensorial perception and product acceptability but

also on emulsion subsequent behavior under gastric conditions and overall digestive fate.

3.3. Responsiveness to *in vitro* gastric digestion

Gastric acidity and pH timed-profiles, proteolysis by pepsin and mechanical agitation are key parameters affecting the destabilization of protein-stabilized emulsions (Kong & Singh, 2010; McClements & Li, 2010; Singh & Sarkar, 2011). Some studies have shown modification of droplet interfacial structure and composition may modulate emulsion digestibility and that emulsification alters protein digestibility (Lesmes & McClements, 2012; Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009; McClements & Li, 2010; Singh et al., 2009). Recently, electrostatic deposition of dietary fibers was shown to alter the stability of LF-stabilized emulsions to gastro-duodenal conditions (Tokle et al., 2012). Moreover, electrostatic deposition of alginate and carrageenan onto LF nano-particles was demonstrated to enable a proportion of lactoferrin to resist up to 1 h of simulated gastric digestion (David-Birman et al., 2013). Thus, it was postulated that such a protective effect could also extend to emulsions stabilized by such nano-particles and result in enhancing emulsion stability to gastric conditions and altering LF gastric proteolysis. This hypothesis was studied by subjecting all emulsions to *in vitro* gastric digestion according to a recently described protocol (Shani-Levi et al., 2013). Digestion experiments revealed that coarse emulsions had very poor stability and complete separation occurred

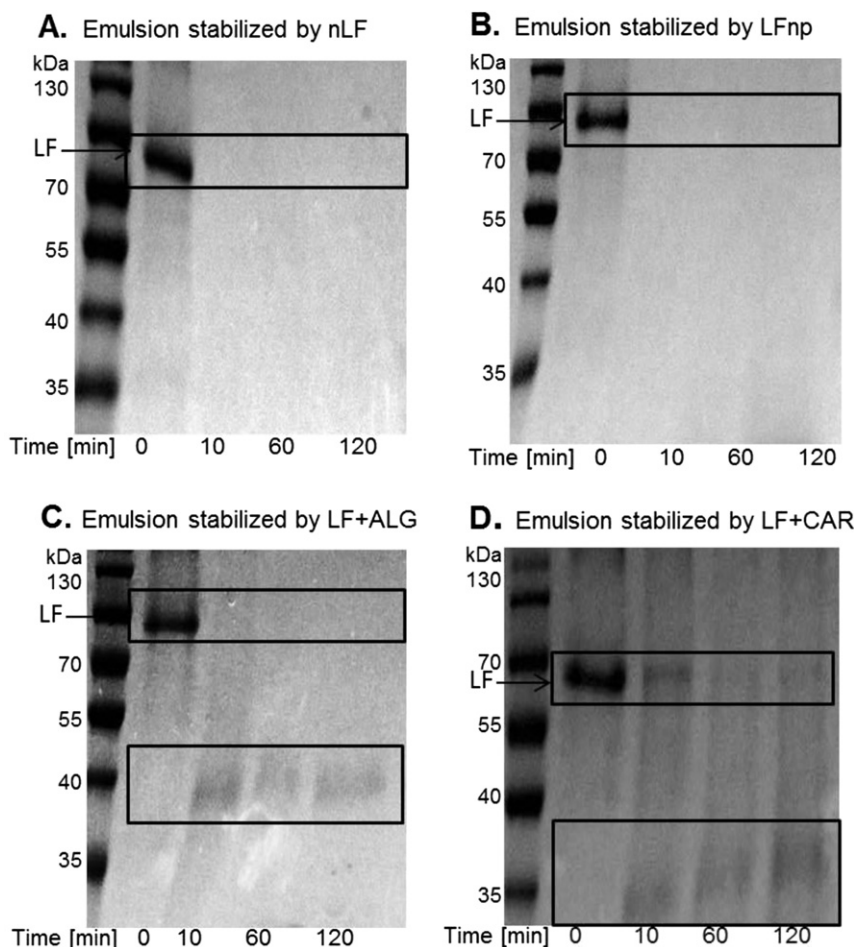


Fig. 9. SDS-PAGE of fine emulsions stabilized by nLF [A], LFnp [B], LFnp + ALG or LFnp + CAR [C and D respectively] following *in vitro* gastric digestion.

within minutes, limiting its physiological relevance. Thus, only the digestion of fine emulsions was analytically monitored by assessing emulsion organization and droplet size using light microscopy and analytical centrifugation (Fig. 8). Emulsions stabilized by native LF (Fig. 8A) exhibited little instability to the experimental conditions which is in agreement with a previous report (Tokle et al., 2012). A similar behavior was observed for LFnp stabilized emulsions (Fig. 8B). In contrast, fine emulsions stabilized by LFnp + ALG or LFnp + CAR (Fig. 8C and D) showed noteworthy fluctuations in droplet sizes and microscopy images indicated emulsion flocculation was the governing instability phenomena. As electrostatic interactions between LF and the polysaccharides were elemental to the formation of the nanoparticles, one can stipulate that during the breakdown of the protein nano-particles on the droplet interfaces some bridging between droplets may occur. If so, this could result in bridging flocculation and explain our observations. However, further investigation is required to establish the mechanisms behind these observations.

In light of the possible ramifications of emulsification to proteolysis (Macierzanka et al., 2009), SDS-PAGE analyses were performed on digesta samples collected during *in vitro* gastric digestion of all fine emulsions (Fig. 9). Previous studies have shown that LF is highly susceptible to gastric proteolysis and generate bioactive peptides in the human stomach (Kuwata et al., 1998; Troost et al., 2001). Fig. 9A and B reveals that emulsification did not significantly affect the rapid proteolysis of LF both when using native LF or LFnp as emulsifiers. On the contrary, the use of LFnp + ALG as emulsifier (Fig. 9C) enabled a fraction of LF to resist at least 10 min of gastric digestion. This protective effect was significantly more pronounced when LFnp + CAR was used as an emulsifier (Fig. 9D) enabling some fractions of intact LF to evade even 120 min of gastric digestion. Moreover, different peptide fractions and quantities were noted to form during these gastric digestion experiments. These findings require further investigations into their potential implications to health, as LF and LF-derived peptides carry miscellaneous biological activities (Conesa et al., 2010; Lonnerdal & Iyer, 1995; Madureira et al., 2007; Nagpal et al., 2011; Wakabayashi et al., 2006).

4. Conclusions

Overall, this work sought to explore the applicability of lactoferrin-based nano-particles to form and stabilize emulsions, including the assessment of behavior under the bio-relevant physiological conditions. Physical stability tests as well as droplet sizing revealed that the use of protein nano-particles increased the stability of coarse emulsions but not of fine emulsions produced by high pressure homogenization. This is believed to stem from the size of the nano-particles which limit their ability to support the formation and stability of fine droplets of the same order of size magnitude, in line with previous work (Dickinson, 2012). Interestingly, the use of alginate and i-carrageenan not only affected emulsion properties and physical stability but also improved emulsion stability to saliva and modulated emulsion behavior under gastric conditions. In spite of LF high susceptibility to gastric proteolysis, the incorporation of alginate and carrageenan into the emulsion formulations also delayed LF proteolysis with some LF escaping even 2 h of gastric digestion. Taking into account the bioactive nature of LF as well as emulsion functionality as a potential delivery vehicle, this work raises the possibility that LF-based nano-particles could be harnessed to modulate emulsion and protein digestion to promote human health with limited impact to food sensorial properties. Further work could provide the underlying principles for the rational design of such emulsion

formulations which is held to be attainable through various structure–function studies.

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