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Factors affecting vitamin degradation in oil-in-water nano-emulsions

Perla Relkin · Jin-Mi Jung · Michel Ollivon

Special Chapter dedicated to the memory of dr. Michel Ollivon

Abstract Fat fractions composed by different proportions of low (LMT) or high (HMT) melting temperature triacylglycerols were used, alone or in mixture with α-tocopherol for the preparation of oil-in-water protein stabilised nano-emulsions. Addition of α-tocopherol to the LMT or HMT fat fractions was accompanied by different changes in the emulsion characteristics such as fat droplet size distributions, under-cooling and polymorphic transitions, in parallel with different extent of α-tocopherol degradation reactions. Our results showed higher immobilisation pattern of α-tocopherol molecules and higher protection against degradation when incorporated in higher size fat droplets, which presented $2L_{\alpha} \rightarrow 2L_{\beta'}$ polymorphic transitions under cooling and re-heating cycles.

Keywords Antioxidant · DSC · Emulsion · Encapsulation · Fat droplet size · Fat crystallization · Fat polymorphism · Vitamin · XRD

Introduction

Lipid oxidation reactions cause the reduction of products shelf-life in terms of nutritive value and sensory quality [1]. Their inhibition or retardation in lipid-based foods and particularly in those containing polyunsaturated fats may be obtained by prevention of food contact with oxygen, use of low temperature and appropriate packaging, and also addition of antioxidants. Phenolic compounds play a role in the body for control of chronic and age-related disorders and they are also used for their antioxidant activity in lipid-based foods to prevent their oxidation [2]. Vitamin E (α_D-tocopherol) is a lipid compound which has received a great attention as an antioxidant acting agent to prevent free radical damage in tissues [3]. A high-dose of vitamin E supplementation is part of the therapy of those people who suffer from abetalipoproteinemia, due to extremely low levels of plasma cholesterol and triglycerides causing defective absorption of vitamin [4].

Research studies indicated that lipophilic bioactive molecules presenting low oral delivery, inability to cross cell membranes or low stability in gastrointestinal tract led to divergent results observed between antioxidant activities as evaluated either through in vitro or in vivo tests [5]. And, recently studies showed that emulsions containing lipid droplets may be used as drug matrix carriers with enhanced oral absorption and delivery properties to target sites [6, 7]. Among factors that appear to be critical determinants for the bioavailability and delivery properties to various sites, there are the particle size and carrier matrix type characteristics, in addition to triggering effects of environmental and storage conditions (pH, metal ions, oxygen or heat exposure or heat) on the drug release properties. Numerous studies that were performed on biomedical and pharmaceutical applications have highlighted the dramatically altered physicochemical properties and biological activity of sub-micron or nanoparticles, but there is still a much less emphasis on micro or nano-encapsulation technology in food applications such as protection of

P. Relkin (✉) · J.-M. Jung
Department of Science and Engineering for Food and Bioproducts (UMR 1145), AgroParisTech, 91300 Massy, France
e-mail: perla.relkin@agroparistech.fr

M. Ollivon
CNRS UMR 8612, Equipe de Physicochimie des Systèmes Polyphasés, 92296 Chatenay-Malabry, France

oxygen or heat-sensitive food molecules against damages [8–10].

Emulsification procedure and ingredients complexity have a dominant role in characteristics of fat droplets, such as particle average diameters and size distributions, composition and physical properties of surrounding surface layers, and also crystalline fat content and polymorphism [11–19]. Studies performed on fat thermal transitions, showed that crystallization temperature of dispersed fat droplets is lowered, compared to bulk fat, and the degree of super cooling temperature (needed to initiate fat crystallization in globules) was shown to differ depending on triacylglycerol (TAG) composition, mean droplet size and lipophilic or hydrophilic nature of emulsifiers. Those emulsifier's molecules are capable of partitioning between hydrophilic phases (continuous aqueous phase) or hydrophobic (interior of dispersed fat droplets) or interface and they have effects not only on the initial temperature of crystallization but also on the rise of solid fat content [11, 17, 18] Recently [20], we showed that vitamin E was protected against storage degradation when incorporated in fat droplets, in which $2L_{\alpha} \rightarrow 2L_{\beta'}$ polymorphic transitions were observed to be more impeded than in emulsions without added vitamin. In the present study, we compared the ability of fat droplets containing high melting temperature triacylgycerols (HMT TAG) to those containing LMT TAG for vitamin protection, taking into account the fat composition, droplet size and thermal behaviour.

Experimental

Materials and samples

Vitamin E (D_L-α-tocopherol) and chemicals were purchased from Sigma Aldrich (Sfeinheim, Suisse). The sodium caseinate and milk fat samples were provided by Lactalis-France. We used two milk fat samples (HMT or LMT), which were dry-fractionated from anhydrous milk fat. Their melting temperatures and their composition in the main saturated and unsaturated TAGs is reported in Table 1.

Table 1 Fatty acid composition (wt%) of high-melting temperature (HMT) and low-melting temperature (LMT) fat fractions used for the emulsions' preparation and their melting temperature as determined by DSC (0.5 °C min^{-1})

Fat fraction	C16:0	C18:0	C18:1	C18:2	Melting point (°C)
HMT	40	32	9	0.7	44.5 °C
LMT	23	9.5	28	2.1	7.9 °C

Emulsions' preparation

We prepared four emulsions, where the lipophilic phase (40 wt%, relative to final emulsion) was composed either by the HMT or LMT milk fat sample alone, or in presence of α-tocopherol (4 wt%, relative to the total emulsion weight). The aqueous phase was prepared by dispersing sodium caseinate in phosphate buffered saline solution (50 mM NaH_2PO_4 at pH = 6.6) at 3.6 wt% protein relative to final emulsions. The lipid phases were melted at 65 °C, mixed to the protein solution and pre-homogenized by using an ultra-disperser (UltraTurrax, IKA- Staufen, Germany) at 10,000 rpm for 10 min. The coarse emulsions were then passed six times through a high pressure homogeniser (APV Gaulin, Lübeck, Germany) at 600 bar, and the resulting fine-emulsions were cooled to 20 °C before characterisation.

The fat droplet size distributions in the emulsions were evaluated using integrated light scattering measurements (Malvern Mastersizer 2000 Instruments-Orsay-France) after dilution in distilled water (22 °C) and gentle stirring for 30 min before sampling.

Thermal analysis

Calorimetric parameters of fat droplet crystallisation and melting in the emulsions were monitored using a DSC 7 Perkin-Elmer associated to Pyris software (Perkin-Elmer, Norwalk, USA), as described in reference [14]. Fat polymorphism was monitored using X-ray scattering measurements at small (SAXS) and wide angles (WAXS), using MICROCALIX a lab-scale equipment developed by Dr Ollivon and his colleagues [20]. Microcalix is composed of a long fine-focus sealed X-ray source (ENRAF NONIUS, CU anode) a multilayer mirror (OSMIC), collimation slits and two position-sensitive linear gas detectors (HECUS, Austria) working at $0 < q < 0.45$ $Å^{-1}$ (SAXS) and $1.1 < q < 2.1$ $Å^{-1}$ (WAXS). For X-ray and DSC measurements we used similar sample masses (around 30 µL), scanning mode (0.5 °C min^{-1}] and cooling temperature ranges at which the emulsions aqueous phase did not crystallise [14, 20]. The samples were loaded in stainless steel pans (DSC) or glass capillaries (GLAS, Muller, Berlin, Germany for SAXS and WAXS measurements), heated and cooled from 50 to −7 °C. Crystallisation and fusion calorimetric parameters (peak temperatures and enthalpy changes) were extracted from exothermic and endothermic DSC traces, respectively [14]. Short and long spacing were deduced from X-ray peaks using Bragg's equation [20–22]. To determine the variation of the X-ray scattering peak's area as a function of temperature the X-ray scattering patterns were fitted by a Gauss-Lorentz function using Peakfit Software (Jandel Scientific, Erkrath, Germany).

Vitamin stability

Vitamin E assay was conducted by UV-spectrophotometry and spectrofluorimetry as described elsewhere [20]. Briefly, the vitamin content in the emulsions' lipid phases was extracted after dispersion in ethanol (5% vol), vortex shaking (10 min) and centrifugation (15,000 rpm for 7 min at 25 °C). The vitamin content in the resulting supernatant was determined by spectrofluorimetry at 290 and 327 nm for excitation and emission maximum wavelengths, respectively, by using a calibration curve (FI = 28,481 × [Vitamine], $R^2 = 0.9987$) obtained from freshly prepared solution of vitamin E in ethanol at concentrations ranging from 0.01 to 0.25 weight ratio of vitamin-to-ethanol).

Vitamin E content in bulk and emulsified fat systems was determined just after preparation (t0), and after storage at room temperature during 8 weeks followed by a heating step (60 °C for 180 min). The analysis was repeated three times for each emulsion.

Results and discussion

Effect of vitamin on fat droplet size characteristics

Partial replacement of fat fraction by vitamin E had higher effects on the fat droplet size distribution in the HMT emulsion than in LMT one, as shown in the curves reported in Fig. 1. The volume average median diameter (D_{50}) and the percentage of fat droplets higher than 200 nm, %D (>200 nm) and lower than 1 µm, %D (<1 µm) are presented in Table 2. It is seen that addition of vitamin was accompanied by changes in the fat droplet size characteristics for HMT emulsion, whereas both D_{50}, $\%D$ (<200 nm) and $\%D$ (<1 µm) where hardly affected upon addition of vitamin for LMT emulsion.

Fat crystallisation and melting behavior

Examples of DSC curves observed from the HMT in the absence or presence of vitamin are shown in Fig. 2. ΔT values, calculated from differences between the temperature of initial fat crystallization (T_{onset}) and melting completion (T_{end}) were used for evaluation of under cooling ($\Delta T = T_{end} - T_{onset}$). They correspond to the decrease in the temperature needed for formation of fat crystals upon cooling from melted fat samples. Due to the higher proportion of polyunsaturated TAG in LMT, the DSC signals observed from LMT emulsions were weak (not shown), however they indicated clearly that addition of vitamin was accompanied by a decrease in peak temperatures and an

Table 2 Fat droplet size characteristics observed from emulsions without and with α- tocopherol (vitamin E), after dilution in distilled water

Emulsions	D_{50} in nm	$\%D$ (<200)	$\%D$ (<1 µm)
HMT fat	255 ± 1	27	100
HMT fat + α-tocopherol	416 ± 14	13	71
LMT fat	304 ± 3	18	100
LMT fat + α-tocopherol	293 ± 8	22	96

D_{50} is the volume average median diameter; % (D < 200 nm) and % D(< 1 µm) are the percentage of droplets with size lower than 200 nm, or higher than 1 µm

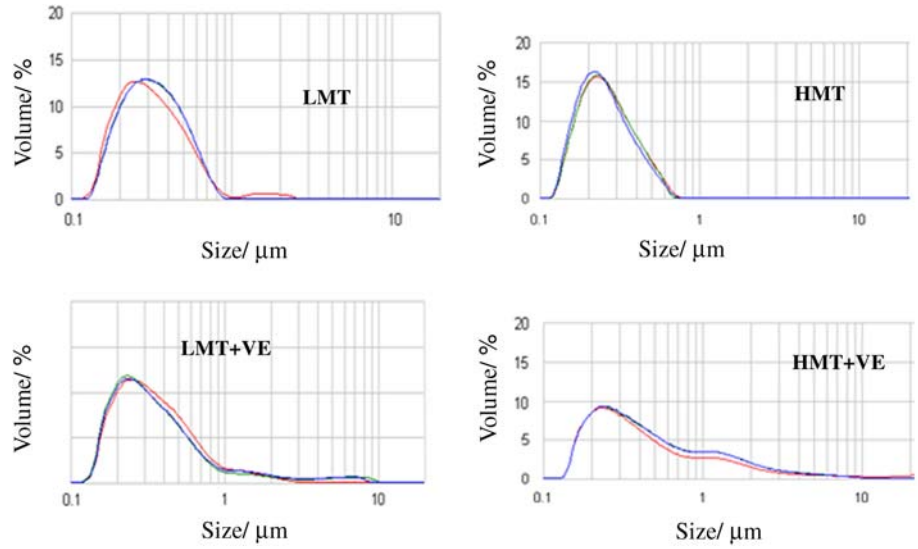

Fig. 1 Fat droplet size distributions observed from emulsions containing HMT (high-melting temperature) and LMT (low-melting temperature), without and with added α-tocopherol (VE)

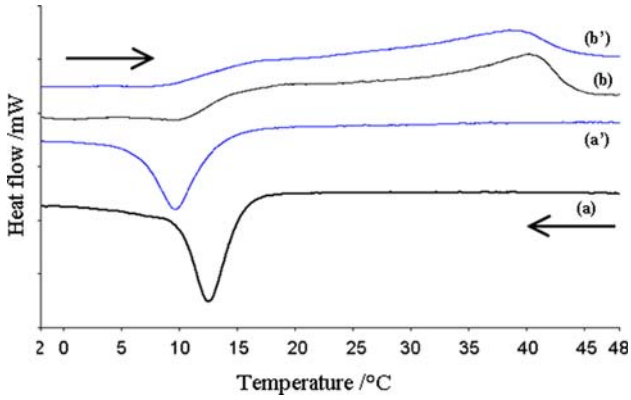

Fig. 2 Cooling and re-heating curves obtained by differential scanning calorimetry (0.5 °C min^{-1}) from HMT in the absence of added α-tocopherol (a and b, respectively) or in the presence of added α-tocopherol (a′ and b′, respectively)

increase in under-cooling, as shown in Fig. 3a. Enthalpy changes of fat crystallization and fusion (Fig. 3b) were also affected by vitamin incorporation, but with a higher extent for the melting reaction of HMT fat globules, where $\Delta_{fusion}H$ decreased by approx 25%, instead of 10% due to partial replacement of crystalline fat by vitamin. Physical state transformations (crystallization, melting and polymorphisms) in fat samples are accompanied by energy release or absorption at different temperatures, depending on factors such as fat composition (TAG chain length, saturation/unsaturation degree), fat droplet size and adsorbed additives [11–19]. Following the hypothesis of heterogeneous nucleation [23], larger droplets containing a higher number of catalytic impurities are assumed to crystallize first and, as crystallization proceeds smaller droplets containing fewer impurities could crystallize at lower temperatures. In our experimental conditions, 100% fat droplets were lower than 1 μm in HMT and LMT emulsions without added vitamin, and slightly less in LMT emulsions in the presence of added vitamin; but in HMT emulsion addition of vitamin was accompanied by formation of a approx 30% fat droplets higher than 1 μm (%D (>1 μm)) ∼ 30%), (Table 2). In addition, results in Fig. 3a and b showed a larger decrease in crystallisation peak temperature, under-cooling and melting energy in HMT emulsion than in LMT emulsion. Thus addition of vitamin, a guest lipophilic molecule, inside HMT fat droplets seemed to counterbalance effects of high size fat droplets on the calorimetric parameters of fat crystallisation and re-fusion.

The curves in Fig. 4 show SAXS and WAXS patterns recorded on HMT emulsion in the absence of vitamin, using MICROCALIX which also recorded the DSC signals (midled). It is seen that the cooling step was accompanied by formation of an exothermic DSC single peak (T_{peak} ∼ 13 °C) and a WAXS single peak observed at wide (q = 1.4793 $Å^{-1}$ or 4.24 Å) and SAXS single peak at small (q = 0.1675 $Å^{-1}$ or 38.1 Å) angles, respectively. These results indicated that under cooling TAG molecules crystallize in the $2L_\alpha$ polymorphic form (α lateral packing and $2L$ longitudinal stacking). After cooling up to −7 °C, the re-heating cycle led to a broad DSC endothermic peak lying from around 9 to 45 °C, and to SAXS and WAXS patterns indicating the development of a newly formed phase characterised by peaks located at 3.94 and 4.37 Å (WAXS) and at 38.1 Å (SAXS). This behaviour is characteristic of a heat-induced polymorphic transition from $2L_\alpha \rightarrow 2L_{\beta'}$ forms. Using integration of each single WAXS and SAXS pattern, as functions of cooling and re-heating temperatures we deduced the variation of the corresponding peak intensity, as presented in Fig. 5a (SAXS) and b (WAXS). SAXS and WAXS measurements on the other emulsions indicated also the formation of $2L_\alpha$ crystals upon cooling, and presence of $2L_\alpha \rightarrow 2L_{\beta'}$ polymorphic transition upon re-heating, but their corresponding DRX/DSC signals were less intense, indicating more impeded polymorphic transitions in HMT emulsions containing vitamin. In LMT emulsions we observed much weaker peak patterns (not shown) corresponding to $2L_\alpha$ crystals, but without $2L_\alpha \rightarrow 2L_{\beta'}$ transitions. All these results indicated that addition of vitamin affects differently the fat droplet characteristics depending on the TAG composition.

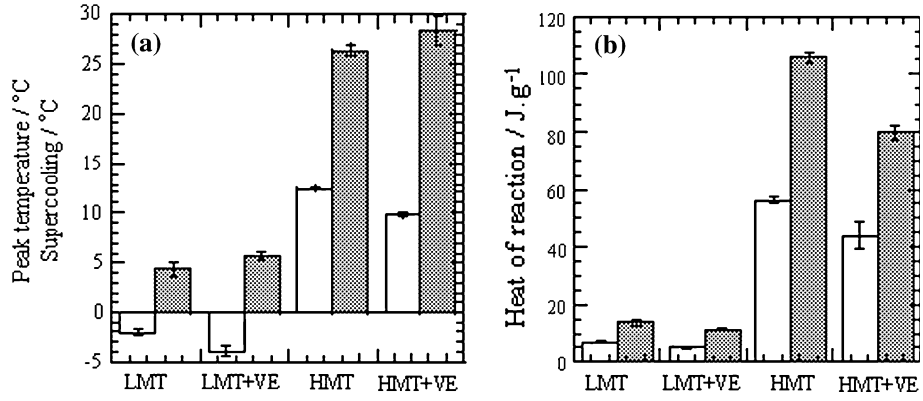

Fig. 3 **a** Values of crystallisation peak temperatures (*white color*) and under-cooling (*dark colour*) observed from HMT and LMT emulsions without and with added α-tocopherol (VE). **b** Values of melting and crystallisation reactions observed from HMT and LMT emulsions without and with added α-tocopherol (VE)

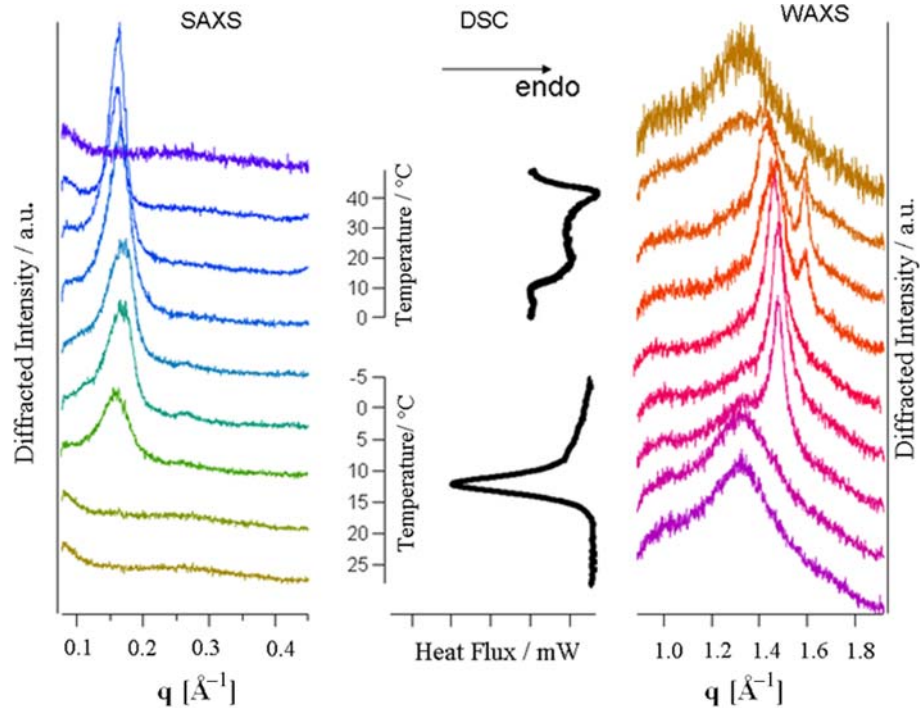

Fig. 4 DSC curves (middle) and X-ray diffraction patterns observed with small- (SAXS) and wide- (WAXS) from HMT emulsion, during cooling and re-heating (0.5 °C min^{-1})

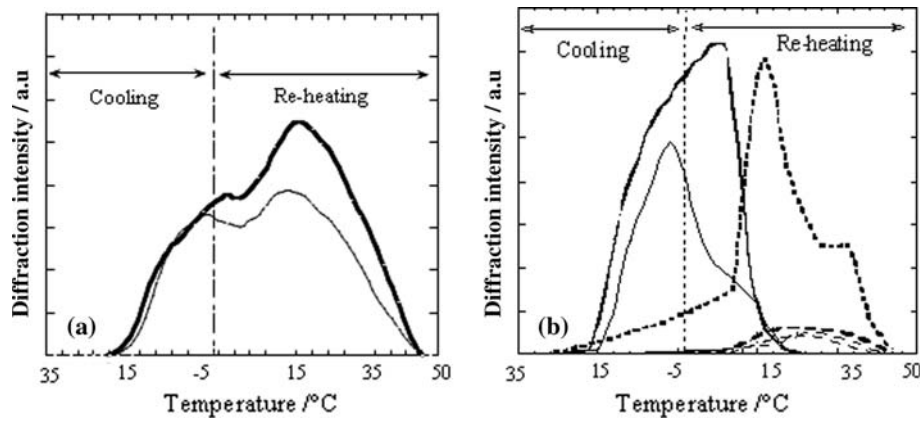

Fig. 5 Variation of X-ray diffraction intensity recorded at small- (SAXS) angles (**a**) and wide- (WAXS) angles (**b**) from emulsions without (*bold curves*) and with (*thin curves*) α-tocopherol, as function of temperatures during the cooling and re-heating cycles (0.5 °C min^{-1})

On the ability of protein-stabilised nanoemulsions for vitamin protection

Mixture samples of vitamin and fat samples in bulk phase, or emulsions containing vitamin were submitted to evaluation of their vitamin content, just after their preparation and after storage during 8 weeks at room temperature. The results reported in Table 3 indicated around 60% and 80% loss of vitamin in HMT and LMT bulk fat/vitamin samples, just after their preparation, but similar tests performed indicated less than 25% and more than 50% loss of vitamin in HMT and LMT emulsions after storage and light exposure at 22 °C for 8 weeks, and around 50% and 70% loss of vitamin after storage and light exposure at 22 °C for 8 weeks followed by heating at 60 °C for 180 min. It is noteworthy to indicate that around 50% of fat globules in

Table 3 Variation of α-tocopherol content in the bulk and emulsified samples, as evaluated from the specified conditions (see text)

Sample	Storage conditions	Fluorescence intensity (u.a)	% tocopherol
Bulk sample			
HMT	25 °C, t0	5.7 ± 0.6	
	60 °C (3 h), t0	2.2 ± 1.0	39
LMT	25 °C, t0	5.7 ± 0.5	
	+60 °C (3 h)	1.3 ± 0.4	22
Emulsion sample			
HMT	25 °C, t0	5.2 ± 0.6	
	25 °C, 8 weeks	3.6 ± 0.5	77
	+60 °C (3 h)	2.4 ± 0.1	47
LMT	25 °C, t0	5.1 ± 0.8	
	25 °C, 8 weeks	2.8 ± 0.5	46
	+60 °C (3 h)	1.7 ± 0.1	27

the HMT emulsion, which seemed to be the most efficient for vitamin protection are higher than 1 μm, whereas more than 20% fat droplets were lower than 200 nm in the LMT emulsion where loss of vitamin was much higher.

Conclusions

Thus, addition of vitamin, a guest lipophilic molecule, inside HMT fat droplets seemed to counterbalance effects of high size fat droplets on heat-induced calorimetric parameters and X-ray scattering parameters upon fat crystallisation and re-fusion. In addition, our results showed that protein-stabilised nanoemulsion containing fat droplets with a higher size seemed to be more able to protect vitamin against structure degradation throughout 8 weeks light exposure and heating. These results indicated that the degree to which lipid nanoparticles are able to protect bioactive molecules are more affected by their TAG composition, crystalline fat content and polymorphisms, rather than by their size. The increase in fat droplet size in HMT emulsion containing vitamin, as evaluated by laser light scattering measurements, could be due to interfacial bridging of HMT fat crystals leading to more crystallization-induced fat droplet aggregation/coalescence contrary to LMT emulsion. To confirm or infirm this hypothesis, further studies on emulsions containing HMT or LMT fat samples and presenting similar fat globule size distributions are carried on in our laboratory.

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