

## Appearance of submicronic particles in the milk fat globule size distribution upon mechanical treatments

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**Abstract** – In this study, we show the appearance of particles with a diameter smaller than 400 nm in the fat globule size distribution of mechanically damaged raw milks, measured by Laser Light Scattering and Dynamic Light Scattering. These submicronic particles appeared upon homogenization above 3 MPa, upon ultrasonication, and upon pumping coupled with shear greater than  $50 \times 10^3 \text{ s}^{-1}$ . They were sometimes observed in raw milk received from the dairy plant. This may indicate bad handling or pumping, and caused the milk fat globule specific surface area to increase dramatically. Ultracentrifugal fractionation of homogenized milks revealed the presence of particles of around 100 and 400 nm that floated and sinking particles of 200–300 nm. The thickness of their surrounding layer, affected by urea treatment, was different. Hypotheses about the nature of the particles are given. The knowledge of such submicronic particles in mechanically damaged milk can bring new insights in the understanding of interactions between fat and the protein network in dairy products such as gels.

**milk fat globule / particle size distribution / laser light scattering / homogenization / shear stress / mechanical treatment / casein micelle**

**Résumé** – **Apparition de particules submicroniques dans la distribution de taille de globules gras du lait soumis à des traitements mécaniques.** Dans cette étude, nous montrons par granulométrie laser l'apparition de particules de diamètre inférieur à 400 nm dans la distribution de taille des globules gras de laits crus qui ont été endommagés mécaniquement. Ces particules submicroniques apparaissent lors d'une homogénéisation supérieure à 3 MPa, d'une exposition aux ultrasons et lors d'un pompage couplé à un cisaillement supérieur à  $50 \times 10^3 \text{ s}^{-1}$ . Elles apparaissent aussi parfois dans du lait cru de grand mélange reçu d'une laiterie industrielle : cela peut indiquer que le lait a subi des pompages et un transport inadaptés et entraîne une augmentation importante de la surface spécifique des globules gras du lait. Un fractionnement par ultracentrifugations de laits homogénéisés montre l'existence de particules autour de 100 et 400 nm qui crèment et des particules de 200–300 nm qui

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sédimentent. L'épaisseur de la membrane qui les entoure, sensible à l'urée, est différente. Des hypothèses sur la nature de ces particules sont données. La connaissance de telles particules dans le lait endommagé mécaniquement peut apporter de nouveaux éléments de compréhension des interactions entre matière grasse et réseau protéique dans des produits laitiers tels que les gels.

**globule gras du lait / distribution de taille de particule / granulométrie laser / homogénéisation / contrainte de cisaillement / traitement mécanique / micelle de caséine**

## 1. INTRODUCTION

The technological ability, and rheological and sensory properties of many dairy products depend greatly on the size distribution of fat globules and on the composition of their membrane, that affect their interactions with the protein matrix [3, 11, 27]. Milk fat is present in milk as droplets of a diameter in the range 1–10  $\mu\text{m}$ , with a maximum around 4  $\mu\text{m}$ , depending on cow breed and season [18]. Milk fat globules are covered with a natural membrane composed mainly of phospholipids and enzymes, that do not interact with the protein matrix during milk gelation. However, when milk is subjected to severe mechanical treatments such as homogenization, the fat globules are disrupted, due to shear and possibly cavitation, and their average diameter decreases dramatically, typically below 1  $\mu\text{m}$  [18, 23]. The increase of interfacial area that cannot be covered by the original milk fat globule membrane (MFGM) anymore is compensated by the adsorption of casein micelles and, in a minor proportion, whey proteins, on the newly formed surface [31]. This phenomenon has been clearly revealed by electron microscopy [8]. It also suggested the presence of particles spread on the globular surface, similar to casein micelle sub-units, possibly due to a surface free energy driving force [20]. Due to their new membrane composition upon homogenization, milk fat globules behave then as a structure promoter in acid milk gels [3, 27]. They have been reported to improve the consistency and texture of products such as Cheddar [15] or

yoghurt [13], whereas they increased hardness and lowered meltability of Mozzarella cheese [26]. The explanation for these results was that homogenized globules formed permanent crosslinks with the casein network [10], which is enhanced by their large surface area.

Recent techniques for particle size characterization give new insights into the size distribution of homogenized or mechanically damaged milk fat globules, by detecting more and more small particles. The exact knowledge of the importance of submicronic particles in a sample would bring new hypotheses about the interaction mechanisms between fat particles and the protein network. In recent years, new tools such as Laser Light Scattering (LLS) and Dynamic Light Scattering (DLS, or Photon Correlation Spectroscopy, PCS) have been the preferred techniques for the particle size determination of homogenized and microfluidized milks, since they allow the more precise characterization of small particles [14, 22, 24]. Typically, for a 20 MPa pressure using a regular homogenizer, the population of milk fat globules was found to be centered on a peak around 550 nm, and no smaller separate population was observed after casein micelle dissociation [14, 22]. Dalgleish, using DLS, has found that core particles from homogenized milk fractionated by centrifugation had a diameter greater than 300 nm [4]. Afterwards, using Integrated Light Scattering (ILS), the presence of particles with a diameter around 200 nm was detected in microfluidized milk [6, 25]. These have been supposed to be lipid-protein particles but no in-depth

investigation has been performed to characterize further their nature. Walstra [29], however, has shown that if no cavitation took place, homogenization could only have an effect on the larger casein micelles. The latter would rapidly reassociate, but maybe not completely in their original form. Fox [7] has revealed the presence of a fat-casein complex in homogenized milk, that sinks upon centrifugation. However, the size of these complexed particles has not been characterized.

The effect of other mechanical treatments appearing during industrial processes on the milk fat globule size distribution has to date not been found in the literature. Usually, the milk fat destabilization due to mechanical processes has been tracked by so-called free fat measurements or the presence of free fatty acids. The possible appearance of particles of smaller diameter has not been suggested. However, they could be of considerable importance concerning milk mouthfeel and gel properties.

The aim of the present study was to highlight the appearance and importance of new populations of small particles in the size distribution of milk fat globules more or less mechanically damaged, using new light scattering apparatus. These results can give new insight into the role of milk fat in the structure of dairy gels made with homogenized or mechanically damaged milk fat globules.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Raw whole milk was collected from four sources: uncooled mixed milk from 8 cows of a local herd (Mellon, Pacé, France), cooled mixed milk (further called processed milk) from local dairy plants (Montauban-de-Bretagne, France and Triballat, Noyal-sur-Vilaine, France) and

cooled milk (unmixed) from four cows of our experimental farm, with different feed. Only the first two types of milk were subjected to mechanical treatments. Some milks were skimmed in the laboratory using a cream separator (Elecrem, Vanves, France) and skim milk was also purchased from the dairy plant. When necessary, fat content was determined using an infrared analyzer (Dairylab, Foss Electric, Nanterre, France) and by the Gerber method. EDTA (> 98%, disodium salt, 2 H<sub>2</sub>O, Prolabo, France) and urea (Merck, Darmstadt, Germany) were used as casein dissociating agents for particle size measurement [14] and SDS (sodium dodecyl sulfate, approx. 99% for Gas Chromatography, Sigma, Saint-Louis, USA) was used to dissociate fat globule clumps.

### 2.2. Mechanical treatments

Milk was subjected to various mechanical treatments in order to track the appearance of a new particle population:

- Homogenization was performed from 0 to 50 MPa in a two-stage homogenizer (nmGEN 7400H, Stansted, Essex, UK). At pressures below 10 MPa, only the second head was used (even though at 5 MPa a residual pressure of about 3 MPa could be observed on the first head). Above 10 MPa, the first head was used and the second head pressure was set at 20% of the first head's. Some experiments were performed on another two-stage homogenizer (Rannie Machine Works, Copenhagen, Denmark) to confirm that observed results were not dependent on the apparatus.
- Ultrasonication (VibraCell, Bioblock Scientific, Illkirch, France; 20 kHz) was performed on 20 mL of milk samples heated at 40 °C, at output 2 to 4, for 20 s to 5 min.
- Shear/pumping was performed in a model device, consisting of a closed rig with 2 chromatography volumetric

pumps and a 4 m capillary (0.25 mm diameter), especially designed to simulate damaging undergone by fat globules due to shear in pumps and pipes. Milk circulations were performed at 50 °C, varying the shear rate ( $6 \times 10^2$  to  $10^5 \text{ s}^{-1}$ ), the shear time (30 s to 15 min), and the milk fat content (40, 120 or 200  $\text{g}\cdot\text{kg}^{-1}$ ).

### 2.3. Fractionation of particles from homogenized milk

Homogenized milk (10 + 2 MPa) was fractionated by ultracentrifugation (UC) either once at 85 000  $g$  for 60 min at 4 °C (according to Strawbridge [25]) or 4 times successively at 20 °C: 9 800  $g$  for 25 min; 20 000  $g$  for 30 min; 50 000  $g$  for 1 h and 100 000  $g$  for 1 h (corresponding floating layers and pellets will be called fractions F1 to F4 and P1 to P4, respectively).

### 2.4. Size distribution measurements

#### 2.4.1. Laser light scattering

The milk fat globule size distribution was determined by LLS using a new apparatus, the Mastersizer 2000 (Malvern Instruments, Malvern, UK) that works with two different wavelengths (He/Ne laser: 633 nm; electroluminescent diode: 466 nm). This wavelength combination takes retrodiffusion effects into account and enhances sensitivity for small particles. This apparatus allows thus the measurement of particles with diameters down to 20 nm, whereas previous devices had a lower limit of  $\sim 300$  nm. This is of particular interest for the study of small milk fat globules. Each sample was measured by diluting (1:1 vol.) with 35  $\text{mmol}\cdot\text{L}^{-1}$  EDTA/NaOH, pH 7.0 buffer or (1:2 vol.) with 20  $\text{mmol}\cdot\text{L}^{-1}$  imidazole, 8  $\text{mol}\cdot\text{L}^{-1}$  urea, 5  $\text{mmol}\cdot\text{L}^{-1}$  EDTA, pH 7.0 buffer, to dissociate casein micelles and aggregates, then dispersing in a sample unit containing 100 mL of 0.1% SDS solution in mQ water. The absorption

coefficient of liquid milk fat at both wavelengths was measured to be  $k_a = 10^{-5}$  and the milk fat globule refractive index  $n_i = 1.460$  at 633 nm and  $n_i = 1.470$  at 466 nm [16]. However, because the refractive index of water is considered as constant in the software, corrected values were used for milk fat (1.458 at 633 nm and 1.46 at 466 nm), so that the difference between fat and water remains correct at both wavelengths [16]. With the size distribution calculated by the software (based on the Mie theory), the Sauter volume/surface diameter  $d_{3,2}$  and the mass specific surface area  $S_w = 6/(\rho \cdot d_{3,2})$  ( $\text{m}^2\cdot\text{g}^{-1}$ ) of milk fat globules were obtained. For unknown particles, however, specific surface area is preferred to be expressed as a volumic quantity,  $S$  ( $\text{m}^2\cdot\text{cm}^{-3}$ ). The size distributions were calculated using different  $n_i$  and  $k_a$  values, in order to confirm that the appearance of small particles was not an artefact due to the optical parameters. Moreover, it was checked that the particle volumic concentration calculated by the software corresponded to the concentration of natural milk fat globules that was actually circulating in the apparatus.

#### 2.4.2. Dynamic light scattering

The size distribution of particles in homogenized milk was measured at 25 °C by PCS using a Malvern Zetasizer 3000 HS (Malvern, UK), at 633 nm and a scattering angle of 90°. The samples were either measured after dilution in a protective buffer (20  $\text{mmol}\cdot\text{L}^{-1}$  imidazole, 5  $\text{mmol}\cdot\text{L}^{-1}$   $\text{CaCl}_2$ , 50  $\text{mmol}\cdot\text{L}^{-1}$  NaCl, pH 7.0) or in a buffer dissociating casein micelles as much as possible (20  $\text{mmol}\cdot\text{L}^{-1}$  imidazole, 8  $\text{mol}\cdot\text{L}^{-1}$  urea, 5  $\text{mmol}\cdot\text{L}^{-1}$  EDTA, pH 7.0). Hydrodynamic diameter was obtained from the Stokes-Einstein relationship and complete size distributions were obtained by the Contin algorithm. Pinhole size was 200  $\mu\text{m}$  and sample time was 0.5  $\mu\text{s}$ ; measurement duration was  $\sim 2$  min. Viscosity and refractive index were respectively 0.89 mPa.s and

1.33 for water and 1.17 MPa.s and 1.382 for the urea buffer [25].

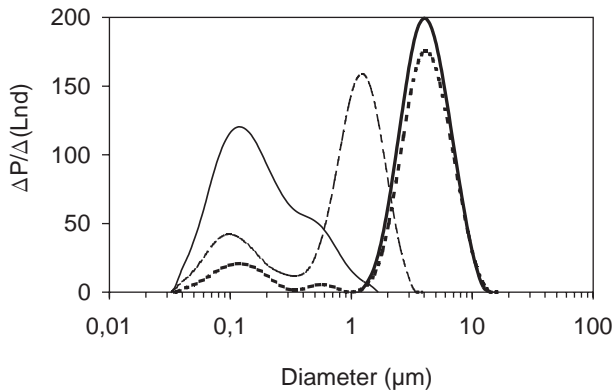
### 3. RESULTS

#### 3.1. Appearance of a new population of small particles upon mechanical treatments

The size distribution of fat globules in raw whole milk is presented in Figure 1. The curve for milking machine drawn milk is a characteristic distribution for most milks (> 90%) that were analyzed throughout one year (hand drawn, milking machine drawn, unpumped mixed milks). The specific surface area *S* of this type of milk fat globule population was typically 1.6 m<sup>2</sup>.cm<sup>-3</sup> (1.75 m<sup>2</sup>.g<sup>-1</sup>). The size distribution of skim milk, however, was found to present a small fat globule population centered at 100 nm, corresponding to *S* = 65 m<sup>2</sup>.g<sup>-1</sup> (the latter was also observed by PCS, results not shown). Since the residual fat content of

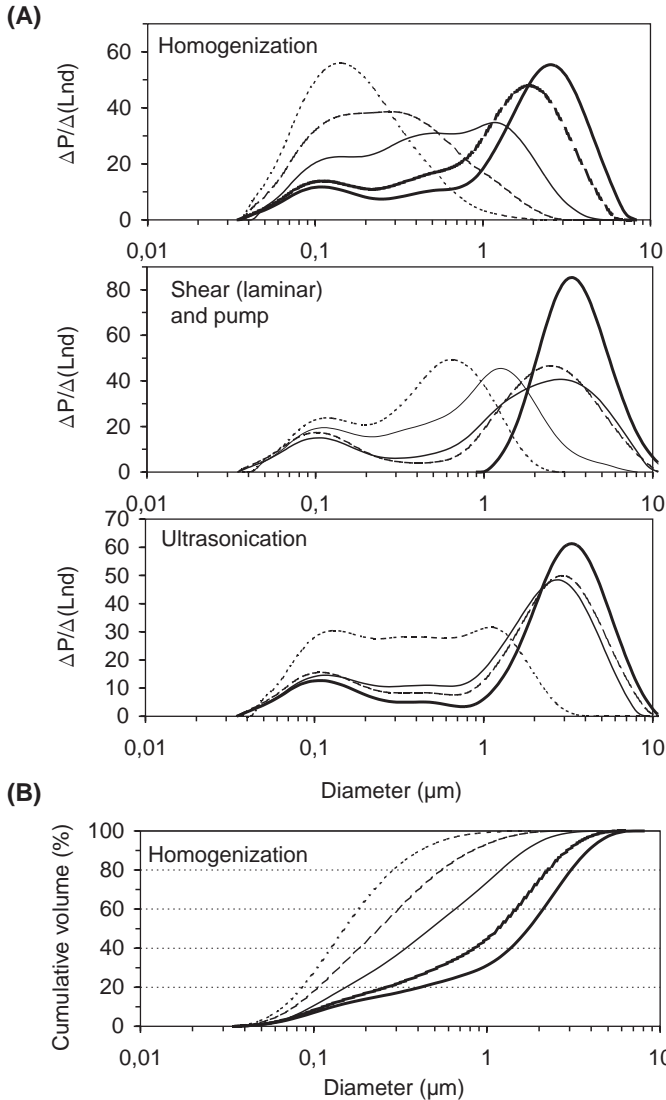
skim milk is typically 0.5 g.kg<sup>-1</sup>, this peak corresponds to less than 1% of the initial fat globule content in whole milk. The curve for pumped milk is an example sometimes observed when the mixed milk was purchased from a dairy plant, where it was pumped after transport. The striking point is the appearance of small particles (mode diameters around 120 and 500 nm), corresponding to 18% of fat volume (~7 g.kg<sup>-1</sup>), that were not originally present in raw whole milk. Since these populations remain after casein micelle dissociation with an appropriate buffer, and since milk fat is not sensitive to such a buffer, these particles are thus newly formed ones.

The origin of this new population in raw whole milk (no thermal treatment) was investigated by applying various mechanical treatments (namely homogenization, shear and ultrasonication). The appearance of such particles was subsequently tracked. Figure 2 shows that each type of mechanical treatment could lead to the formation of populations with a mode diameter around



**Figure 1.** The size distribution of natural milk fat globules from various sources (MasterSizer, after casein micelle dissociation). Thick line: milking machine-drawn milk – farm –; dotted line: cooled pumped mixed milk – dairy plant –; dashes: milk skimmed in the laboratory; thin line: milk skimmed at the dairy plant.

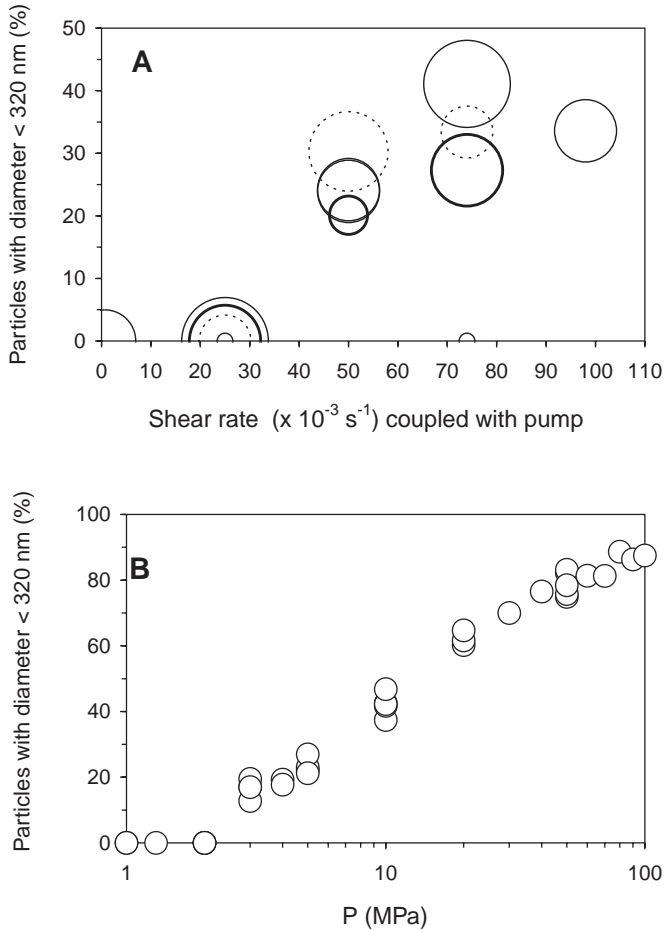
Log-normal distributions are presented as the volumic percentage of particles in a size class  $\Delta P$  (%) divided by the logarithmic width of the size class  $\Delta(\ln d) = \ln d_{i+1} - \ln d_i$ , vs the mean diameter of the size class ( $\mu\text{m}$ ) [2]. Please note that due to the logarithmic scale, the area under the curve below a given diameter does not represent the corresponding fat percentage.



**Figure 2. A** – The particle size distribution of mechanically damaged milk using homogenization, shear and pumping, or ultrasonication (25 kHz) (MasterSizer, after casein micelle dissociation). Please note that due to the logarithmic scale, the area under the curve below a given diameter does not represent the corresponding fat percentage.

- Homogenization: (—) 3 MPa; (---) 5 MPa; (···) 10 MPa; (- · -) 20 MPa; (····) 50 MPa.
- Shear and pump: (···) 25 × 10<sup>3</sup> s<sup>-1</sup> for 15 min, fat 120 g·kg<sup>-1</sup>; (····) 75 × 10<sup>3</sup> s<sup>-1</sup> for 15 min, fat 120 g·kg<sup>-1</sup>; (thin dashes) 50 × 10<sup>3</sup> s<sup>-1</sup> for 7 min, fat 40 g·kg<sup>-1</sup>; (—) 50 × 10<sup>3</sup> s<sup>-1</sup> for 3 min, fat 200 g·kg<sup>-1</sup>; (- · -) 25 × 10<sup>3</sup> s<sup>-1</sup> for 10 min, fat 200 g·kg<sup>-1</sup>.
- Ultrasonication: (···) output 3 for 30 s; (dash-dot) output 3 for 1 min; (dashed) output 2 for 3 min; (—) output 4 for 5 min.

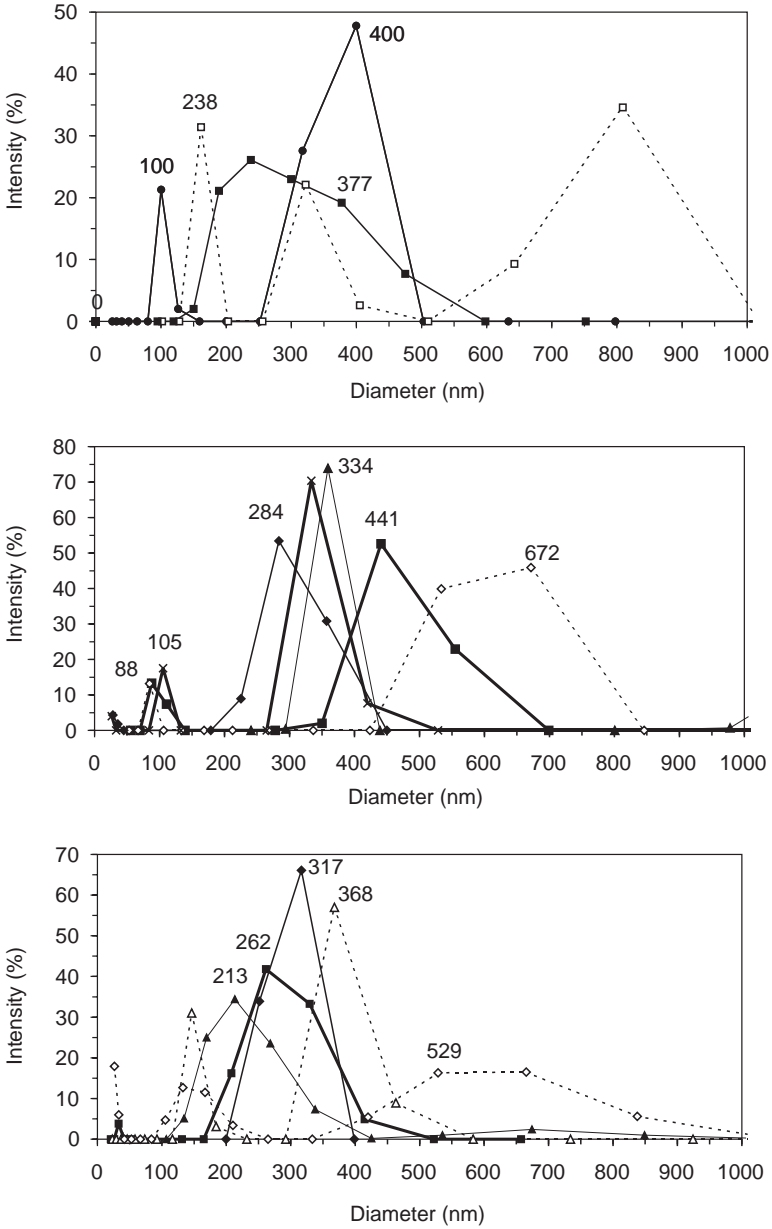
**B** – The cumulative volume of particles in homogenized milks (Mastersizer, after casein micelle dissociation). Captions as defined above.



**Figure 3.** Effect of the intensity of mechanical treatments (**A**: shear rate, **B**: homogenization pressure) on the percentage of particles with diameter smaller than 320 nm in the milk fat globule size distribution. **A**: circle diameter represents the residence time in the shearing and pumping device (from 30 s to 15 min), line aspect represents product fat content (dotted line: 40 g·kg<sup>-1</sup>, regular line: 120 g·kg<sup>-1</sup>, thick line: 200 g·kg<sup>-1</sup>).

120 nm and 500 nm. These populations were observed whatever the software calculation mode and refractive indexes used, so that we could not consider them to be an artefact. Figure 3 shows that the smallest peak at 120 nm appeared above a threshold intensity of the mechanical treatments. For shear in a capillary and pumps, the product had to be processed at a shear stress of  $\geq 50 \times 10^3 \text{ s}^{-1}$  for a circulation time of

$\geq 2.5$  min for the new peak to appear; for homogenized milk, the population appeared at a pressure of  $\geq 3$  MPa. Moreover, the volumic percentage of this population in the distribution increased with the intensity of mechanical treatments. We should point out that such particles of diameter  $\sim 100$  nm can be seen on electron micrographs [5, 8]. However, these particles were never identified as a separate population



**Figure 4.** The size distribution (PCS) of particles from milks homogenized at 10 MPa:  
– Open symbols, dotted lines: undissociated samples. Full symbols: samples after casein micelle dissociation by EDTA+urea.  
– (□, ■) Homogenized milk 1 (next fractionated once); (●) homogenized milk 2 (next fractionated 4 times).  
– (–■–) Fractions of homogenized milk 1; fractions of homogenized milk 2: (▲, △) F1 and P1, (◆, ◇) F3 and P3, (×) F4.

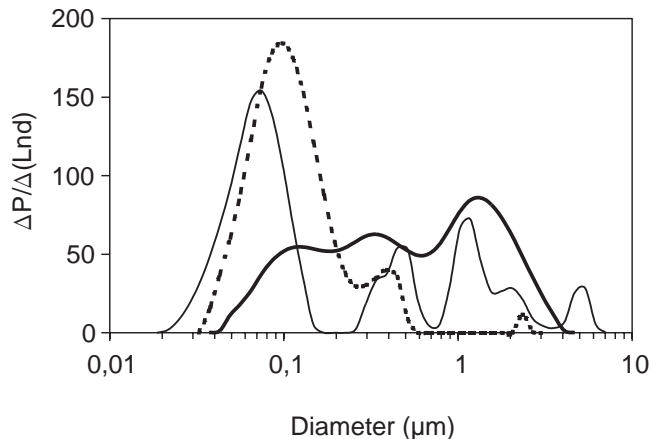


(instead of the lower end of the main population), certainly because they were too numerous to calculate their size distribution. Until now, they were not studied by light scattering and their importance was not highlighted. However, the smallest particles are the most numerous: it is therefore important to characterize them.

### 3.2. Preliminary characterization of the small population

It was first checked that the small population could not entirely be explained by the presence of tiny globules visible in skim milk (Fig. 1), due to volumic percentage considerations. A preliminary characterization of the new small population was thus performed by ultracentrifugal fractionation of homogenized milk (10 MPa) and particle size measurements by PCS. Results presented in Figure 4 show the intensity-weighted size distribution for milks and fractions dissociated in urea/EDTA buffer, and some of the corresponding undissociated ones (not all of the latter are shown for the sake of clarity). The first homogenized milk presented, after casein micelle dissociation, a broad peak at 240 nm with an elbow at 380 nm. Upon one UC at 85 000 g for 60 min, a floating fraction was collected, with populations at 90 and 440 nm,

and a pellet, with a single peak at 260 nm. The second dissociated homogenized milk presented two populations at 100 nm and 400 nm. For the corresponding floating layers, fraction F1 presented a peak at 330 nm, fraction F2 at 300 nm (not shown), fraction F3 at 280 nm and fraction F4 at 100 nm and 300 nm. For the pellets fraction P1 had a population at 210 nm, fraction P2 at 220 nm (not shown) and fraction P3 at 320 nm (fraction P4 was too negligible to be collected). Therefore, homogenized milk at 10 MPa roughly presented particles of diameter 100 nm and 300–400 nm that floated and particles of 200–300 nm that sank. The same particle size range was also found by fractionating milk with 0.45  $\mu\text{m}$  and 0.2  $\mu\text{m}$  filters instead of centrifuging (results not shown). Small discrepancies between experiments are due to the poor precision when reproducing low pressures at the second head of the homogenizer. We should point out that the multimodal mode of the MasterSizer software led to size distributions more similar to those obtained by PCS between 0.1 and 1  $\mu\text{m}$  than the general mode (Fig. 5). However, the MasterSizer generally underestimates the smallest population size. This can arise from a difference between the refractive index of milk fat globules and that of the smallest population.



**Figure 5.** The particle size distribution (MasterSizer, after casein micelle dissociation) of milk homogenized at 10 MPa and fractionated 4 times (thin line: multimodal mode of the software, thick line: general mode) and its fraction P1 (dotted line, multimodal mode).

## 4. DISCUSSION

### 4.1. Raw milk fat globules

The raw milk fat globule size distribution is consistent with Walstra [28], with a “tail” at the largest sizes, even though it did not present any fat globule in the range 0.5–1  $\mu\text{m}$ . However, it is known that milk contains numerous fat globules smaller than 1  $\mu\text{m}$ , even though they represent a negligible fraction of total fat [28, 30]. The smallest milk fat globules (< 1% of total fat) were observed using skim milks (Fig. 1). Size distributions presented in the literature showed a single peak with a mode diameter around 0.6–0.7  $\mu\text{m}$  [18], but precise characterization of smaller particles was difficult due to the high number of tiny globules to be counted on electron micrographs. Using LLS, we found that these small globules from skim milk have a mode diameter of 105 nm ( $d_{32} = 98$  nm) and a specific surface area of  $\sim 65$   $\text{m}^2\cdot\text{g}^{-1}$  fat (Fig. 1). Milk skimmed using our cream separator had a main population (around 1  $\mu\text{m}$ ) consistent with Mulder and Walstra [18], residual fat globules being larger than in milk from the dairy plant for which separation was more efficient. Since the smallest population, around 100 nm, comprises less than 1% of the initial fat content, the true specific surface area of milk fat globules in whole milk, including the small population that is not usually detected, should be about  $2.5$   $\text{m}^2\cdot\text{g}^{-1}$  instead of  $1.85$   $\text{m}^2\cdot\text{g}^{-1}$ . Overall, these results confirm that the milk fat globule size distribution is composed of three sub-populations. The smallest one is hidden by the larger ones during LLS measurements, because its volume fraction is negligible, but it contributes greatly to globular surface area.

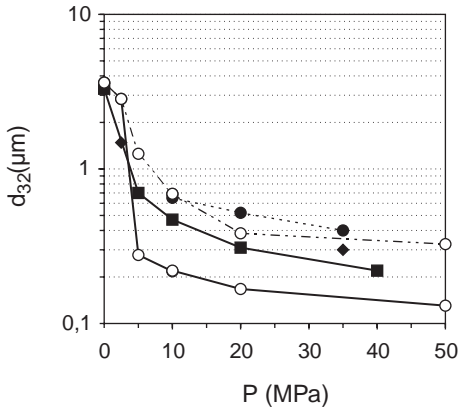
The new particles appearing upon globule disruption in processed milk from the dairy plant dramatically enhanced S (up to  $9$   $\text{m}^2\cdot\text{cm}^{-3}$ ), since each peak at 120 and 500 nm represented an area of 60 and

$11$   $\text{m}^2\cdot\text{cm}^{-3}$ , respectively. We excluded the idea that these peaks could be exactly those observed in skim milk, because they represent a much higher volume fraction (18% of total particles) and appeared in pumped milks only. These particles are thus newly formed ones. They induce a dramatic increase in particle surface area, that is likely to affect the milk technological properties through the interactions the small particles can undergo with proteins [25, 27, 32]. Therefore, unsuitable milk handling can lead not only to coalescence, as already mentioned in the literature, but also to small particles potentially affecting milk protein aggregation and gelling properties [32]. This can be beneficial or undesired, depending on the technological applications.

### 4.2. Homogenized milk particles: comparison with the literature

A pellet was found from UC fractionation of homogenized milk, undissociated by the casein-dissociating buffer (Fig. 4). It was suggested by Walstra [18] that homogenized milk fat globules present different protein loads, so that some are likely to be heavier than the continuous phase. The sinking particles are consistent with Fox [7], who identified a sedimenting fat-casein complex in homogenized milks, but did not measure its size distribution. Strawbridge [25], however, could not find such particles by ILS. At 10 MPa, particles with a diameter below 350 nm (comprising mostly sinking ones) represent 35% of the total distribution. The same order of magnitude was found by Fox [7], with sinking particles comprising 30% of total fat.

Figure 6 and Table I compare our results with those of the literature (using various techniques) about the effect of the homogenizing pressure on the average diameter of the milk fat globule size distribution. Diameters measured by LLS, transmission electron microscopy (TEM) or spectroturbidimetry (ST) are volume/surface



**Figure 6.** Effect of the homogenizing pressure on the particle diameter ( $d_{32}$ ) in dissociated milks, using different methods: comparison of our results (open symbols) with the literature (full symbols). (—○—) Homogenized milk; (---○---) Homogenized milk, excluding the small populations in the calculation – “main” fat globules only –; (—■—,◆) Mulder and Walstra [18] from different spectroturbidimetric experiments; (····●····) McCrae and Lepoetre [14] from LLS experiments.

average diameters  $d_{32}$ , while diameters measured by PCS are either number or -intensity-weighted diameters ( $d_{nb}$ ,  $d_{int}$ ). The hydrodynamic diameter  $d_{int}$  is larger than volume/surface or volume diameters. The milk fat globule  $d_{32}$  calculated from the LLS measurement without taking the small population into account (i.e., considering only the “main” milk fat globule population, whose membrane is composed of the natural MFGM together with casein micelles) is very similar to that of the literature, using the same technique with older apparatus [14, 22] (Fig. 6). This shows that our procedure and milk fat globule refractive indexes for both wavelengths are consistent. The distribution obtained in this study by taking the new small population into account leads to a much smaller  $d_{32}$ , since small particles greatly increase the specific surface area. Indeed,  $d_{32} = 220$  nm, against 690 nm in this study and 650 nm in [14] without the

small population (Tab. I). The  $d_{32}$  obtained by TEM and ST [18] is smaller than that obtained by LLS without the new small population, but still much larger (470 nm) than that found in this study. This arises from the difficulty of considering the huge number of small particles with these techniques. Additionally, comparing the order of magnitude of  $d_{32}$  measured by LLS, TEM and ST and the average diameters obtained by PCS is interesting. For pressures above 20 MPa, our results by LLS provide the  $d_{32}$  values the most similar to the  $d_{nb}$  obtained by PCS, for floating layers and pellets of homogenized and microfluidized milks [25]. In the present study,  $d_{32}$  is similar to  $d_{int}$  for homogenized milk and UC pellet, and  $d_{32}$  is in-between the peaks at 100 and 400 nm for UC floating layers. Therefore, the LLS with two laser sources seems to be the best LLS technique to estimate the entire particle distribution.

Concerning the fractions obtained from homogenized milk (20 MPa) after centrifugation, Dalglish found that floating fractions presented particles between 300–500 nm and 1000 nm before casein micelle dissociation [4, 5] and 400 to 800 nm after dissociation [4]. These results are larger than in the present study. Even if dissociation was performed without urea in the latter results, this does not explain the difference in size. However, we should point out that the present results correspond to precise mode diameters, whereas the above-mentioned are average diameters that cannot highlight multimodality and polydispersity.

### 4.3. Homogenized milk fractions behavior upon casein micelle dissociation

In the literature, fractions found by Strawbridge et al. [25] after microfluidization (around 150 nm) presented a slight diameter increase (20–30 nm) after casein micelle dissociation. The author

**Table I.** Effect of the homogenizing pressure on the particle diameter in dissociated milks and corresponding fractions from ultracentrifugations, using different methods: comparison of our results with the literature.  $d_{32}$ : Sauter diameter,  $d_{int}$ : intensity-weighted diameter (maximum of a distribution). H: regularly homogenized milk, MF: microfluidized milk.

P (MPa)	Homogenized milk				Floating layer		Pellet	
	$d_{32}$ LLS		$d_{int}$ PCS		$d_{int}$ PCS		$d_{int}$ PCS	
	Study (H)	Lit. (H)	Study (H)	Lit.	Study (H)	Lit. (MF)	Study (H)	Lit. (MF)
5	0.28 (1.25)*	0.7 <sup>a</sup>						
10	0.22 (0.69)	0.47 <sup>a</sup> 0.65 <sup>b</sup>	(i) 0.1** (ii) 0.24–0.28 (iii) 0.38–0.4		(i) 0.1, 0.11 (ii) 0.34, 0.44		0.22 0.26	
20	0.17 (0.38)	0.31 <sup>a</sup> , 0.62 <sup>b</sup>		0.54 <sup>c</sup> (H)				
40		0.22 <sup>a</sup> , 0.4 <sup>b</sup>		0.27 <sup>d</sup> (MF) 0.24 <sup>e</sup> (MF)		0.19 <sup>e</sup>		0.22 <sup>e</sup>
50	0.13 (0.33)							

\* Values in parentheses are  $d_{32}$  values calculated for the main fat globule distribution only, excluding the small populations from the calculation.

\*\* Marks (i), (ii)... correspond to different peaks of maximum diffusion intensity observed for a single sample.

<sup>a</sup> Mulder and Walstra [18], from different experiments by spectroturbidimetry.

<sup>b</sup> McCrae and Lepoetre [14].

<sup>c</sup> Pouliot et al. [22].

<sup>d</sup> Sharma and Dalgleish (1993) cited in [25].

<sup>e</sup> Strawbridge et al. [25].

considered this was due to an uncertainty in the particle refractive index (taken as 1.455 for fat globules). In this study, conversely, the particle diameter decreased in the dissociating buffer acting on Ca-bridges and H-bonds. In fraction F3, the diameter difference with and without dissociation of casein micelles was 400 nm. This means either (i) that the core particle has a diameter of 280 nm and is surrounded by other particles of diameter 200 nm that are dissociated (e.g., large casein micelles), or (ii) that core particles form clusters of 2 or 3 particles, bridged by casein micelles. In fraction P1, the diameter difference after dissociation was 160 nm (the core particle can be covered by casein fractions of 80 nm). The diameter difference after dissociation was always 300–500 nm for the floating layers against 150–300 nm for the

pellets (results not shown). This suggests the different nature of both types of particles. However, it is not possible to know at this point if particles are completely covered by proteins and what their conformation is.

#### 4.4. Hypotheses about particle formation

The way these particles are formed will be interesting to investigate further. Considering the effect of shear in turbulent eddies formed in the homogenizing valve, the Kolmogorov scale for isotropic turbulences estimates that, at 10 MPa, particles smaller than 240 nm should not be disrupted [18]. It is striking that this size corresponds roughly to the particles found in the UC

pellets. However, smaller particles were also observed, especially in the floating layers. These are likely to be, at least partly, the tiny globules observed in skim milk (Fig. 1). Moreover, local anisotropic turbulences could also appear in the homogenizer, for which the Kolmogorov scale would not apply anymore and other smaller particles could be obtained.

Additionally, the apparition of the new small particles upon ultrasonication suggests that they can be a consequence of cavitation. Cavitation causes the collapse of small vapor bubbles instantaneously formed, inducing great localized mechanical stress [9] and vibrations at resonant frequency that can cause the particle to shatter [23]. Because cavitation occurs in two-stage homogenizers to enhance disruption and can appear in pilot rigs with pumps, it can be a major cause for such new particles to appear. Similar results were found in the literature [2], where polydispersity was explained by the wide distribution of cavitation intensity.

More puzzling is the extensive homogenization that also occurred in the model capillary rig under laminar simple shear flow conditions, with a decrease of the main population diameter and the appearance of new small particles. For breakup in shear to happen, the Capillary number (Ca, ratio of viscous to surface forces) should be higher than a critical value. This critical value depends on the viscosity ratio of the droplets

over the continuous phase and cannot be calculated simply from hydrodynamic considerations. Standard curves are available to estimate  $Ca_{crit}$  [12]. With  $Ca = d \cdot \dot{\gamma} \cdot \eta_c / 2\gamma_i$  ( $d$ : droplet diameter;  $\dot{\gamma}$ : shear rate;  $\eta_c$ : continuous phase viscosity;  $\gamma_i$ : fat globule interfacial tension), the critical diameter  $d_{crit}$  below which fat globules cannot theoretically be disrupted can be calculated. According to [21],  $\gamma_i = 1.15 \pm 0.06 \text{ mN}\cdot\text{m}^{-1}$  at 40 °C, and  $\eta_c \sim 0.85 \text{ mPa}\cdot\text{s}$  at 50 °C [18]. Since the surface free energy decreases as temperature increases [1],  $\gamma_i$  was considered to be  $\sim 1 \text{ mN}\cdot\text{m}^{-1}$  at 50 °C. The droplet breakup theory holds if  $\gamma_i$  is constant, which was hypothesized for native milk fat globules. Values of  $d_{crit}$  were calculated (Tab. II) [30], using  $Ca_{crit} = 2$ , that is likely for simple shear flow at a viscosity ratio  $\tau_\eta = \eta/\eta_c = 28$  according to Lucassen-Reynders [12]. Moreover, another way could be to estimate  $d_{crit}$  from the globule distortion, that must be greater than 0.5 for the droplet to burst [18]:

$$d_{crit} = 16 \cdot \gamma_i \cdot \eta (1 + \tau_\eta^{-1}) / [\dot{\gamma} \cdot \eta^2 \cdot \tau_\eta^{-1} \cdot (19 + 16 \cdot \tau_\eta^{-1})]$$

(this equation being usually valid for  $\tau_\eta < 4$ ). From Table II,  $d_{crit}$  values calculated from globule distortion suggest that globules larger than 10  $\mu\text{m}$  would be breakable at  $\dot{\gamma} \geq 100 \times 10^3 \text{ s}^{-1}$ . However, this fat globule size concerns only 2 to 3% of the fat (Fig. 1), which is lower than the actual percentage of fat that was actually disrupted (Fig. 2) and cannot explain our results. On the other hand,  $d_{crit}$  values calculated from

**Table II.** Critical fat globule diameter  $d_{crit}$  above which breakup can occur in shear, according to capillary number (Ca) and droplet deformation (Def) calculations, and critical diameters  $d_m$  and  $d_i$  actually observed.

Shear rate ( $\text{s}^{-1}$ )	$d_{crit}$ ( $\mu\text{m}$ ) from $Ca_{crit} = 2$	$d_{crit}$ ( $\mu\text{m}$ ) from Def	$d_m^*$ ( $\mu\text{m}$ )	$d_i^{**}$ ( $\mu\text{m}$ )
$25 \times 10^3$	160	41	12	3
$50 \times 10^3$	80	21	8.5	2.5
$75 \times 10^3$	53	14	3	2
$100 \times 10^3$	40	10	3	2

\* Maximum diameter of the population at a given shear rate, above which all globules from the original milk have been disrupted.

\*\* Diameter above which part of the globules from the original milk have been disrupted.

Ca are much larger than the maximum milk fat globule size (Fig. 1) and indicate that no disruption should appear in simple shear flow (Poiseuille flow). The viscosity ratio of fat globules over the continuous phase is high ( $\tau_{\eta} \sim 28$ ), which is likely to prevent breakup in simple shear flow, since the maximum viscosity ratio allowing breakup is theoretically 4 [18]. Moreover, Rees [23] found that shear in a capillary caused insignificant damages, even at  $\dot{\gamma}$  as high as  $10^6 \text{ s}^{-1}$ . The maximum diameter  $d_m$  of milk fat globules present in the different sheared and pumped products, and the diameter  $d_i$  corresponding to the intersection of the sheared globule distribution with that of the native milk, are presented in Table II. For one sheared and pumped product, all globules larger than  $d_m$  and part of the globules larger than  $d_i$  have been disrupted. Values of  $d_m$  and  $d_i$  can only be consistent with  $Ca_{crit} < 0.2$ , which would be possible for elongational flow only [12] and is not possible for simple laminar shear flow.

Consequently, the globule disruption and appearance of new submicronic particles in this study cannot be due to shear alone. These phenomena can arise from the positive pumping device of the apparatus. Indeed, positive rotary pumps are more likely to induce disruption than centrifugal ones, as soon as no air is included [18]. In a pilot rig with a centrifugal pump, no disruption was observed at  $\dot{\gamma} \sim 10^5 \text{ s}^{-1}$  (results not shown). Disruption in the positive pump can be due to cavitation. Indeed, cavitation is known to occur at the end of a long pipeline leading to a suction pump [18]. Moreover, turbulence may appear when the rapid stream impacts at the pump entrance. It is also possible to invoke the combination of cavitation with orthokinetic casein aggregation. It has been determined that the stress applied to a micelle spreading at the aqueous phase/fat interface was of the order of  $10^5 \text{ Pa}$  [31]. Since the water/air interfacial free energy  $\gamma$  is  $72.8 \text{ mN}\cdot\text{m}^{-1}$ , and  $\gamma$  of milk aqueous phase/air is around  $50 \text{ mN}\cdot\text{m}^{-1}$ , an interfacial spreading pressure

of 20 to  $25 \text{ mN}\cdot\text{m}^{-1}$  is to be expected. For a casein micelle, this leads to a spreading stress larger than  $10^5 \text{ Pa}$ , which should be sufficient to cause micellar destabilization and the formation of new particles after the liquid/air interface collapse. Finally, if air inclusion occurs, fat spreads over air bubbles and forms tiny droplets upon bubble disruption. These possibilities would have to be considered in-depth.

#### 4.5. Hypotheses about particle nature

Whether the submicronic particles can be considered as newly formed milk fat globules should be discussed. The tiniest native milk fat globules ( $\leq 100 \text{ nm}$ ), representing less than 1% of total fat, seem to remain in mechanically damaged milks (Fig. 4) due to their size smaller than the Kolmogorov scale. However, the total volume of submicronic particles around 200 and 400 nm and their different behavior in centrifugal fields show that there are also newly formed particles. Comparing their radius of curvature to casein micelles, they would rather be considered as lipid-protein complexes or maybe complexed protein particles. Moreover, their population is always well distinct from that of the standard mechanically damaged milk fat globules, that can be characterized by their own average diameter and fraction of membrane covered by caseins [17]. Conversely, it is unlikely that part of the new population is covered by MFGM phospholipids. Indeed, the latter represent a potential surface coverage of  $1.6 \text{ m}^2\cdot\text{cm}^{-3}$ , while that of the small population is at least  $65 \text{ m}^2\cdot\text{cm}^{-3}$ . However, since the MFGM is damaged during the homogenizing process, some phospholipids might take part in the complex of small particles, where hydrophobic links are likely to be involved. Roughly, considering a protein load of  $10 \text{ mg}\cdot\text{m}^{-2}$  in homogenized milk fat in skim milk [19], this would correspond to  $\sim 700 \text{ mg}$  of proteins to cover  $1 \text{ cm}^3$  of small particles.



Since the core particles resist EDTA and urea treatments, it means that they are stabilized neither by calcium bridges, nor by hydrogen bonds. A hypothesis can be the association of casein hydrophobic regions with lipid particles and possibly whey proteins. Since  $\alpha_{s2}$ - and  $\kappa$ -caseins are the least dissociated by EDTA and urea, they may be involved in this complex. Fox [7], after homogenizing mineral oil in skim milk, suggested that van der Waals forces are implied in the casein-fat interactions for these particles. Moreover, the concentration of milk fat globules calculated by the MasterSizer overestimates by only 10% the real fat concentration that was injected, and the volume percentage of small particles in homogenized milk corresponds roughly to that of the large globules that disappeared. This means that even if the small population is not only composed of fat, neither is it solely proteic.

## 5. CONCLUSION

According to LLS and PCS measurements, homogenized milk was found to be composed of three types of particles: (i) regular milk fat globules (disrupted globules from the main population, whose surface fraction covered by casein micelles can be calculated from their increase in specific surface area), (ii) tiny native milk fat globules around 100 nm (that were originally present in milk as a separate population and should not be affected due to their small size), and (iii) small newly formed lipid-protein complexes having a new membrane, presumably mainly composed of caseins. These complexes were also observed when milk was subjected to ultrasonication or positive pumping in a simple shear device, probably because of cavitation as may appear in dairy plants and technological rigs.

The smallest populations have an area  $S > 65 \text{ m}^2\text{-cm}^{-3}$ , according to LLS. There-

fore, such particles appearing unintentionally in a product (because of bad handling with air inclusion or pumping) provide a great surface area. The latter can lead to interactions with other proteins, fat globules and water, that can change the product's characteristics. This should be of particular importance concerning gelling and foaming properties, and could be either undesirable or sought-after. Moreover, pointing out the presence of smaller particles in homogenized milk could allow the development of new hypotheses concerning mechanisms of gel formation. The new small particles that are composed mainly of caseins can interact as a structure promoter. Conversely, the remaining tiny globule population covered by the native milk fat globule membrane, and possible new particles covered by whey proteins, can act as an inert filler or a structure breaker in acid gels, for example [3]. Experiments are being carried out in our laboratory to characterize these small particles and their impact on milk gel properties.

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