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INTERPRETATIVE SUMMARY

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D'Incecco, 2017.

Natural creaming, the first step of Grana Padano and Parmigiano Reggiano cheese making, can be carried out at varying temperatures to maximize skimming. This work shows how selected temperatures, or successive combinations of temperature, change the microstructure of fat globules, a factor known to affect final cheese microstructure. This study also identifies the immunoglobulin classes that mediate interactions between fat globules and bacteria, helping to explain the naturally occurring variations in milk debacterization during creaming and offering a route to optimize debacterization. Overall, this study will help cheesemakers optimize creaming and decrease late blowing defects in ripened cheese.

RUNNING HEAD: INTERACTIONS OF FAT GLOBULES IN RAW MILK CREAMING

Effect of temperature on the microstructure of fat globules and the immunoglobulin-mediated interactions between fat and bacteria in natural raw milk creaming

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ABSTRACT

Natural creaming of raw milk is the first step in both Grana Padano and Parmigiano Reggiano PDO cheese production. This process decreases the fat content and plays an important role in the removal of Clostridia species that may cause late blowing defects in ripened cheeses. Partial coalescence of fat globules that may influence fat behavior in cheese making and affect the microstructure of fat in the final cheese product could be observed at creaming temperatures higher than 22 °C by confocal laser scanning microscopy. The widespread practice of pre-heating of milk at 37 °C before creaming at 8 °C caused important changes to the size distribution of fat globules in raw milk, potentially also altering the ability of fat to entrap Clostridia spores. The role of the immunoglobulin classes in both the clustering of fat globules and agglutination of *Clostridium tyrobutyricum* to fat globules during creaming was investigated. Immunogold labelling carried out in combination with transmission electron microscopy showed IgA and IgM but not IgG to be involved in both clustering and agglutination. Both vegetative cells and spores were clearly demonstrated to agglutinate to fat droplets; a process that was suppressed by thermal denaturation of the immunoglobulins. The debacterization of raw milk through natural creaming was improved by the addition of purified immunoglobulins. Overall, these findings provide not only a better understanding of the phenomena occurring during the natural creaming but also provide practical insights into how the process of creaming may be optimized at cheese production plants.

Key words: fat globule coalescence, microstructure, *Clostridium tyrobutyricum*, immunoglobulin

INTRODUCTION

Natural creaming occurs when the fat globules present in unagitated raw milk rise to the surface (Ma and Barbano, 2000). This process not only skims the milk but also eliminates somatic cells and spore forming bacteria when the upper cream layer is removed (Dellaglio et al., 1969; Zacconi and Bottazzi, 1982; Geer and Barbano, 2014; D'Incecco et al., 2015). This purifying effect is important for cheeses like Grana Padano and Parmigiano-Reggiano, where natural creaming represents the first step of the manufacturing process, as outlined in the product specification (European Union, 2011a, b). The spores remaining in the vat milk, including those of *Clostridium tyrobutyricum*, can germinate in cheese during ripening causing a late blowing defect (LBD) that results in important economic losses (Bassi et al., 2009; D'Incecco et al., 2015).

Natural creaming is usually carried out within a broad temperature range from 8 °C to 20 °C, with the actual temperature chosen by the cheesemaker on an empirical basis, mostly based on observation, with consideration of the season. The creaming temperature is critical, however, as it affects the tendency of fat globules to adopt a specific supramolecular organization, i.e. to cluster or to coalesce (Fredrick et al., 2010). Clustering is thought to occur when two or more fat globules are in close contact for a substantial period of time, enabling the formation of stable aggregates. In contrast, coalescence involves the fusion of the membranes of two or more globules to form one larger unit, also arising as a consequence of contact between fat globules. When fat is partly solid, partial coalescence can also lead to the formation of fat clumps (Fredrick et al., 2010).

The clustering of fat globules is likely promoted by van der Waals forces, though studies have suggested that milk immunoglobulins may be involved in this phenomenon (Honkenen-Buzalski and Sandholm, 1981; Zacconi and Bottazzi, 1982; Euber and Brunner, 1984). Immunoglobulins and somatic cells also potentially contribute to the aggregation of fat globules with bacterial vegetative cells and spores during gravity separation. This moderation of bacterial and fat globule interactions by immunoglobulins has only been inferred indirectly, however, by the addition of colostrum as an immunoglobulin-enriched medium (Geer and Barbano, 2014).

Immunoglobulins, also known as antibodies, are synthesized by mammals in response to antigenic or immunogenic stimuli, such as bacteria and viruses, providing protection against infection (Lilius and Marnila, 2001). They are ~150 kDa in size, resulting from two identical heavy (~50 kDa) and two identical light (~25 kDa) polypeptide chains, linked by disulphide bonds (Hurley and Theil, 2011). The protective bioactivity of these molecules varies but most act via an initial binding event. Typically, milk and colostrum contain three major types of immunoglobulin, IgG, IgM and IgA. The most abundant immunoglobulin within bovine colostrum is IgG, which is present typically at ~44 g/L, whereas IgA and IgM are each present at much lower concentrations of ~4-5 g/L (Raducan, 2013).

This study sought to better understand the role of creaming in Grana Padano cheese making, in particular the role of creaming in the elimination of spores in raw milk. We applied a holistic approach to study the creaming process; examining the effect of temperature on the supramolecular structure of fat globules and the role of the main immunoglobulin classes in promoting interactions between fat globules and the spores and vegetative cells of *C. tyrobutyricum*. The temperatures selected are of interest for Grana Padano cheese making. In addition, a trial including a rapid pre-heating step at 37 °C was performed to simulate the so-called “cold milk reactivation” that is sometimes adopted by cheese factories before natural creaming.

MATERIALS AND METHODS

Milk samples

Raw bulk milk was collected at a dairy farm of 100 Holstein cows in the north of Italy (Lodi, Italy) at the morning milking. Aliquots of 500 mL of milk were taken before refrigeration and brought to the laboratory (University of Milan) within 2 h of milking and used for creaming trials with the addition of Ig. Colostrum for Ig purification was collected at the same dairy farm from heifers on the second day postpartum. Freshly produced raw microfiltered skimmed (0.3% fat) milk was taken

at an industrial plant (Tetrapack, Arhus, Denmark), immediately frozen and kept at -18 °C until use for creaming trials with addition of spore suspension.

Trials of natural creaming at different temperatures were carried out at the University of Melbourne. Raw bulk milk was collected from a local dairy manufacturer in Victoria (Australia). The bulk milk was pooled from different dairy farms from several cow breeds. The milk was collected and used within 1 day.

Purification of immunoglobulins from colostrum

Native immunoglobulins were purified from colostrum by ammonium sulfate precipitation (**Suppl. Fig. 1**). Briefly, the fat and caseins were removed from colostrum by centrifugation at 4,000 × *g* at 4 °C for 20 min after acidification to pH 4.6. Two protein fractions were successively precipitated from the skimmed whey colostrum with 45% and 80% $(NH_4)_2SO_4$. The respective precipitates were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel using a Mini Protean 3 apparatus (Bio-Rad, Hercules, CA) (Laemmli, 1970) to determine the optimal salt concentration for higher and more selective precipitation of immunoglobulins. SDS-PAGE was conducted under both reducing and non-reducing conditions, as described by Barbiroli et al., (2013). Molecular weight (MW) markers (Amersham Biosciences, Amersham, UK) were used for calibration. Concentrated immunoglobulins (33.4 mg protein/mL) were dialyzed using Spectra/Por® dialysis tube (20,000 Da MWCO, 24 mm flat width, 1.8 mL/cm volume/length, Spectrum Laboratories Inc. CA, USA) against 15 mM sodium phosphate buffer (PBS) pH 7.2, containing 150 mM NaCl and used for natural creaming trials.

Natural creaming trials

Three different natural creaming trials were carried out, at laboratory scale, to produce cream samples destined to Confocal Laser Scanning Microscopy (CLSM), immunogold labelling (IGL) for Transmission Electron Microscopy (TEM) and for an assessment of the spore count by the Most

Probable Number (MPN) method. In the first trial, four samples of 250 mL of raw milk were kept in graduated cylinders at 4 °C, 8 °C, 22 °C or 40 °C. An additional sample was preheated at 37 °C for 5 min in a thermostatic bath prior to creaming at 8 °C. The volume of cream that rose to the surface was visually evaluated using a graduated cylinders at 0.5 h, 1 h, 3 h, 6 h, 8 h and 24 h as described by Farah and Rüegg, (1991) and Franciosi et al., (2011), and the fat content of the 24 h samples was analyzed using the Babcock method, as described previously (Ong et al., 2012). This creaming trial was carried out in five replicate experiments, however, the results presented are from duplicate analyses where the shipping temperature of the milk from the farm to the lab was more closely controlled.

In the second trial, 25 mL aliquots of raw milk were transferred into 25 mL tubes, each with 1 mL of washed *C. tyrobutyricum* spore suspension and carefully mixed by gentle inversion. The spore suspension was prepared as described by D'Incecco et al. (2015) and contained predominantly spores, together with some vegetative cells. The tubes were kept at 8 °C for 12 h and then the bacteria-enriched cream layer gently removed using a spatula for inclusion within a resin and subsequent immunogold labelling. This creaming trial was performed in duplicate.

The third trial was designed to study the effect of immunoglobulins on the creaming of fat without the interference of other milk components. Purified immunoglobulins, washed fat globules and washed *C. tyrobutyricum* spore suspensions were added to either raw microfiltered skimmed milk (MMilk) or 0.01 M PBS in 10 mL tubes prior to natural creaming at 8 °C, as shown in **Suppl. Fig. 1**. Washed fat globules were prepared from cream separated from raw milk by centrifugation at 2,000 *g* at 10 °C for 15 min. Cream was diluted five times in 0.01 M PBS, gently stirred for 10 min and centrifuged under the same conditions. The washing was done three times and a final concentration of fat equal to 35 g/100 mL was obtained in the washed cream. The spore enriched suspension was washed twice in sterile 0.01 M PBS by centrifugation at 3,000 *g* for 10 min at room temperature. The prepared components were added to either MMilk or 0.01 M PBS to reach a final concentration of 3.5 g fat/100 mL, 0.5 g immunoglobulins/100 mL and 4 x 10^6 spores/100 mL,

according to the experimental plan shown in Tables 1 and 2. A parallel set of samples was prepared using the same milk heated at 80 °C for 10 min to inactivate the constitutive immunoglobulins; these immunoglobulins are referred to as heated immunoglobulins (Table 1). The most probable number (MPN) of *C. tyrobutyricum* spores in the top cream layer was evaluated using a previously described method (D'Incecco et al., 2015) together with the volume of cream that had risen after natural creaming at 8 °C for 8 hours. These creaming trials were performed in triplicate.

Confocal Laser Scanning Microscopy of cream samples

Cream samples were stained with Nile red (Sigma-Aldrich, St. Louis, USA) to observe the triacylglycerol core of fat globules. The Nile red solution (1 mg/mL) was prepared in dimethyl sulfoxide (DMSO, Chem Supply, Gillman, Australia) and kept protected from light until use. Cream samples were initially diluted 1:5 with water at room temperature. Then 900 µL of sample was mixed with 100 µL of Nile red solution and incubated for 15 min at room temperature. An inverted confocal laser scanning microscope (CLSM) from Leica Microsystem (Heidelberg, Germany) was used, adopting the conditions described by Ong et al. (2010). Nile red was excited at 488 nm using an argon laser and the emission filter was set at 520-590 nm.

Transmission Electron Microscopy

Cream samples and a culture of *C. tyrobutyricum* were embedded in resin for TEM. A 0.5 mL aliquot of sample was mixed with 1.5 mL of fixative solution (glutaraldehyde 1%, paraformaldehyde 4% in Na cacodylate buffer, w/v - Agar Scientific, Stansted, UK). The mixture was kept for 2 h at room temperature before mixing with 2 mL of low-temperature gelling agarose (2% in water, melted at 35-40 °C, VWR, Milan, Italy). The suspension was layered onto a microscope slide, allowed to set and then cut into 1 mm^3 cubes. The cubes were further fixed in the same fixative solution for 30 min at 4 °C, then washed with 0.1 M Na cacodylate buffer for 1 h and post-fixed in osmium tetroxide (EMS, Hatfield, USA) (1% w/v in water) for 2 h. Dehydration was

carried out in a series of ethanol solutions, then samples were embedded in Spurr resin (EMS) and cured at 60 °C for 24 h. Ultrathin sections, 50-60 nm thick, were cut and stained with uranyl acetate and lead citrate (EMS), both 0.2% in water (w/v) and examined with a Philips E208 transmission electron microscope (Philips Technology, Aachen, Germany).

Immunogold labelling

Thin sections prepared for TEM were incubated at room temperature for 5 min in 50 mmol PBS containing 1% bovine serum albumin (BSA) and 0.01% Tween 20 (both AURION, Wageningen, The Netherlands) before staining. The sections were then incubated at 4 °C for 12 h or 24 h in the primary anti-serum to either cow IgG, IgM or IgA that had been raised in rabbit (Thermo Fisher Scientific, Massachusetts, USA); these antisera were diluted 1:100 or 1:1000 in 50 mmol PBS. After careful washing with PBS, the sections were then incubated at room temperature for 2 h in a 15 nm colloidal-gold labelled secondary anti-rabbit serum that had been raised in goat (Thermo Fisher Scientific).

Image analysis

Three dimensional (3D) CLSM images of cream samples were reconstructed using Imaris image analysis software (Bitplane, South Windsor, CT, USA). Each 3D image consists of 40 layers of 512 x 512 pixels that are stacked together with a distance between layers set at 0.25 µm. The volume of the fat globules in the cream was calculated using Image J software (Research Services Branch, National Institute of Health and Medicine, Maryland, USA). Microsoft Excel was used to calculate the relative volume distribution of the fat globules.

Statistical analysis

Statistical treatment of data was performed by SPSS Win 12.0 program (SPSS Inc., Chicago, IL, USA). A *t-test* was used to separate the mean values of spores in pairs of different creams.

Regression analysis was performed where the temperature was assigned as the independent variable and the fat content or the cream volume as dependent variables. A $P < 0.05$ was used as the significance limit.

RESULTS AND DISCUSSION

Effect of temperature on natural creaming of milk

Natural creaming trials were carried out over a 24 h time period at temperatures relevant to Grana Padano (GP) production. As expected, the initial creaming rate was faster at higher temperatures (Walstra, 1995) (Fig. 1a). A cream layer was visible after 30 min in the sample preheated at 37 °C and kept at 8 °C or for samples kept at 22 °C or 40 °C, consistent with observations in the literature of rapid creaming after milking (Fox et al., 2015). In contrast, samples kept at 4 °C or 8 °C developed a cream layer more slowly after incubation for more than an hour (Fig. 1a).

Temperature also affected the volume of the cream layer and an increase in creaming temperature caused a reduction in the volume of cream collected after 24 h (Fig. 1b). The fat content on a % w/w basis increased with temperature, however, due to the formation of a surface oil layer of melted triglycerides (Mortensen, 1983) that lead to a greater exclusion of the aqueous phase (Fig.1b). This free oil layer is expected in raw milk after extensive fat globule coalescence has occurred (Walstra et al., 2005). The thermal history of the samples is also important to the process of creaming and a significantly ($P < 0.05$) larger volume of cream was observed in the sample preheated at 37 °C prior to creaming at 8 °C, compared to the sample just creamed at 8 °C.

Changes in the supramolecular organization of the fat during creaming at different temperatures were investigated by CLSM (Fig. 2). Prior to creaming, distinct individual fat globules were observed in raw milk (Fig. 2a). Only a small volume of the fat appeared in clusters or floccules and these aggregates were dispersed easily by shaking (see also **Suppl. Fig. 2**). The fat globules in samples creamed at 4 °C or 8 °C appeared more concentrated than in the initial milk (Figs. 2b and 2c) and the fat globule density was highest at a higher temperature (Fig. 2c), consistent with the

trend for the fat content (% w/w) obtained from the chemical analysis of the cream layer (Fig. 1b). Pre-treatment of the milk at 37 °C prior to creaming altered the supramolecular organization of the fat; the number of distinct individual fat globules decreased and extent of fat coalescence increased. Higher creaming temperatures also increased coalescence and this effect was most notable at 40 °C, where large clumps of intact globules appeared entrapped in the free oil (Fig. 1f). Changes in the fat supramolecular organization were more extensive in the samples preheated at 37 °C for 5 min before creaming at 8 °C than in the samples creamed at 22 °C with no preheating (Fig. 1d vs Fig. 1e). This means that such thermal treatments, often performed by cheese makers, can damage the milk fat globules, although it should be noted that cheese makers typically apply heat in a continuous process while batch heating was applied here.

The clustering of fat droplets was also clearly visible in the rendered surfaces of the fat globules obtained from image analysis of the 3D CLSM images (**Suppl. Fig. 3**). Greater coalescence was visible in the cream layer obtained after preheating at 37 °C and creaming at 8 °C or after creaming at 40 °C without preheating than in all other samples (**Suppl. Fig. 3**).

The size distribution of fat globules, obtained by analysing ~1000 fat globules per sample, is shown in Figure 3. A large population of native fat globules was present in raw milk but this population decreased as the creaming temperature increased and fat globules of increasing size were observed (Fig. 3). A new population of coalesced fat with an average size of ~28 µm was observed in samples preheated at 37 °C and creamed at 8 °C or samples creamed at 40 °C. This population represented 20% and 39% of the total fat volume in these samples respectively. Another separate small population (10% by fat volume) of fat ~17 µm in size was also observed in samples creamed at 40 °C. The supramolecular organization of milk fat observed is likely the result of several factors including: i) the composition of the fat, in terms of fatty acids and triglycerides (TAGs); ii) the composition and structural organization of the milk fat globule membrane (MFGM); iii) the size of the fat globules and the physical or thermal process history of the samples (Michalski et al., 2004; Lopez, 2011; Murthy et al., 2016; Et-Thakafy et al., 2017). Each of these factors leads to a different

ratio between liquid and solid fat. In these experiments, however, much of this variability has been excluded by using the same source of milk in all trials. Overall, this investigation shows that the different temperatures adopted during natural creaming can induce a different supramolecular organization of milk fat globules, independently from the other cited factors. Structural changes are also promoted because of the extended period of contact and close proximity between fat globules within the creamed layer compared to within milk (Fredrick et al., 2010).

Our observations of little coalescence occurring in creams containing 17 % and 19 % fat content here during creaming at 4 °C or 8 °C are consistent with those of Boode (1992), who showed that oil-water emulsions with a fat content lower than 25% are not prone to fat coalescence. The same study showed that tempering cycles from 5 °C to 30 °C increased the tendency of emulsions to partial coalescence, because newly formed fat crystals tend to reposition within the fat globule protruding into the aqueous phase. Our study also showed high levels of partial coalescence between fat globules and fat clumping in samples preheated at 37 °C before creaming at 8 °C. More recently, Moens et al. (2016) demonstrated by light microscopy that commercial cream showed little or no aggregation of fat globules after tempering at 20 °C, while some fat clusters occurred at 30 °C, consistent with our observations.

Although the study of the different supramolecular organizations of fat was not the main aim of the current study, our results clearly indicate that high creaming temperatures and tempering prior to creaming at low temperatures, caused significant changes to the organization of fat globules. These changes, in turn, can affect GP cheese making, as they can affect debacterization, which occurs as a result of fat globule aggregation and the removal of the cream layer prior to cheese production. Spores remaining in the skimmed milk might spoil the cheese causing the late blowing defect. Furthermore, the reorganization of fat observed in the cream layer, is also expected to take place in the partly skimmed milk at the end of creaming prior to GP production. This reorganized fat could affect the structure of the cheese and promote undesired lipolysis.

Role of immunoglobulins in natural creaming of milk

Next, immunoglobulins were purified from colostrum and a series of creaming trials performed using the purified proteins to investigate the role of immunoglobulins in the aggregation of fat globules and association with bacterial cells and spores during gravity separation. TEM and immunogold labelling (IGL) were used to specifically investigate the role of single immunoglobulin classes in fat globule clustering and bacterial adhesion.

Purification of immunoglobulins from colostrum

The immunoglobulins used in this study were purified from colostrum collected on the second day postpartum, when the viscosity of this product decreases, allowing operations such as centrifugation or filtration (Tsioulpas et al., 2007). Two consecutive precipitations of protein using $(NH_4)_2SO_4$ (45% and 80%) were applied with the aim of maximizing the immunoglobulin yield, which was assessed by SDS-PAGE. Protein bands were identified based on molecular weight (MW) and their behavior under both reducing and non-reducing conditions. In the reducing SDS-PAGE gel (Fig. 4a), both milk (M) and milk colostrum (C) contained the typical bands of casein (α-Cn, β-Cn, k-Cn) as well as those of β-lactoglobulin (β-Lg) and α-lactalbumin (α-La). Such caseins were mostly absent in the colostrum whey (CW).

Under reducing conditions the immunoglobulins are present in two different bands corresponding to heavy (Ig HC) and light (Ig LC) chains, as the disulphide bonds linking these molecules are reduced (Fig. 4a). The yield of immunoglobulins was highest and bands darkest after precipitation with 45% $(NH_4)_2SO_4$. The heavy chains of the immunoglobulins migrated close to the 67 kDa marker, similar to previous reports of a band at ~ 66 kDa (Patel et al., 2005). The light chains migrated slightly higher than previously reported at ~ 23 kDa (Patel et al., 2005). The MW of Ig HC and LC may vary slightly with both glycosylation level and the presence of complement factors (Korhonen et al., 2000) consistent with these observations. The corresponding bands were absent in the non-reducing

SDS-PAGE gel where a single band with a molecular weight higher than 100 kDa appeared, corresponding to whole Ig (Fig. 4b) (Farrell et al., 2004; Fuquay et al., 2011). Based on the electrophoretic patterns, precipitation at 45% $(NH_4)_2SO_4$ lead to the highest yield of immunoglobulins. Whilst some casein was present, the level of α-lactalbumin and β-lactoglobulin was lowest under these conditions. The addition of 80% $(NH_4)_2SO_4$ precipitated the whey proteins, especially α-lactalbumin and β-lactoglobulin, without any further precipitation of the desired immunoglobulins (Fig. 4). Consequently, the pellet obtained after the addition of 45% $(NH_4)_2SO_4$ was used for further experiments.

In the last two decades, a number of different techniques have been proposed to isolate immunoglobulins from colostrum or milk. The focus of these techniques, however, has been to separate Ig molecules, particularly in the case of column chromatography (El-Loly, 2007; Ayyar et al., 2012). Furthermore, some of these methods involve the exposure of immunoglobulins to either heat, acid or high pressure, which may negatively affect the molecular conformation and binding activity of the Ig molecules. The conditions adopted here allow immunoglobulins to be harvested in their native state, as shown by Figure 4b, potentially retaining the activity they would demonstrate in milk during creaming.

Effects of immunoglobulin addition on natural creaming

Purified immunoglobulins, washed fat globules and washed spore supension were added separately to either microfiltered milk (MMilk) (Table 1) or sterile PBS (Table 2) to assess the interaction between these components during creaming. The volume of the cream layer and number of *C. tyrobutyricum* spores were recorded after natural creaming for 8 h at 8 °C. In a set of parallel control experiments, milk samples were also heated to 80 °C for 10 min to inactivate the Ig molecules present.

A greater volume of cream was observed for MMilk samples with added fat globules than for the corresponding samples made of MMilk that had been preheated at 80 °C for 10 min before the addition of fat globules (Table 1). In addition, no cream layer was observed within the time frame of the experiment when the immunoglobulins naturally present in MMilk and those experimentally added were both thermally inactivated.

The number of spores in the cream layer was strongly dependent on whether the milk was heat treated prior to the natural creaming experiment. Table 1 shows that a significantly ($P < 0.05$) lower number of spores was found in the cream layer of the MMilk sample with prior heat treatment than in unheated MMilk. The addition of immunoglobulins to unheated MMilk caused a significant ($P < 0.05$) increase in the number of spores captured in the cream layer compared to the same sample where immunoglobulins were not added. The heat inactivation of both milk and added immunoglobulins also reduced the number of spores. Our results are consistent with Caplan et al., (2013), who showed that heat treatment at ≥ 76.9 °C for 25 s impaired the separation or rising of spores and fat globules, possibly because of the denaturation of native immunoglobulins or the effect of heat treatment on the structure of the fat globules. The denaturation temperature for bovine milk immunoglobulins is reported to range from 62 °C to 81°C, when immunoglobulins are heated for few seconds (Mainer et al., 1997), consistent with the reduction of spores in the cream layer observed here.

A second set of experiments was carried out in a simpler model system using PBS in the absence of other soluble components naturally present in milk and the effect of native immunoglobulins on the volume of cream and presence of spores also measured.

The immunoglobulins appeared to mediate spore association with the fat, as the number of spores in the cream layer increased after the addition of immunoglobulins when compared to the sample without immunoglobulin addition (Table 2). The volume of cream did not appear to significantly change, similar to the milk system (Table 1) and as already observed by Euber and Brunner, (1984) in raw milk. Considering that 60-99% of bacteria concentrate in the cream layer during the process

of creaming (Abo-Elnaga et al., 1981; D'Incecco et al., 2015), the significant ($P < 0.05$) approximately two fold increase in spores within the cream from 213 ± 5.7 to 527 ± 90.7 MPN/mL observed in samples with and without immunoglobulins here represents a significant enrichment that suggests immunoglobulins are responsible for spore association (Table 2). Interestingly, when the amount of added immunoglobulins was increased three fold the number of spores in the cream layer did not increase significantly. This observation suggests an efficient association of Ig with fat at physiologically relevant concentrations and addition at free antigenic sites may not be available for further immunoglobulin binding at higher concentrations. It is worth noting that a significant number of spores (MPN/mL) were also found in the cream where immunoglobulins were not added, indicating that physical entrapment of spores within clusters of rising fat globules may also play an important role in milk desporification.

In the absence of fat globules, the sample taken on top of the liquid milk contained a small number of spores, 83 ± 6.0 MPN/mL, not significantly different ($P < 0.05$) from that counted in the same sample where immunoglobulins were added. These experiments showed that fat globules are essential for spores to reach the top of the milk, because when they were not present, spores sedimented to the bottom layer of the milk due to their high density (~1.2 kg/dm^3) (Beaman et al., 1982) resulting in a high spore count of 327 ± 15 in this layer.

The immunoglobulins added to the MMmilk and PBS samples appeared to facilitate interactions between bacterial spores and fat globules, as higher numbers of spores were detected in the top cream layer in the presence of immunoglobulins. Previous studies have indirectly shown that immunoglobulins are responsible for fat globule association, the tendency of some immunoglobulins to undergo cold-induced aggregation has also lead to them being considered cryoglobulins (Walstra, 1995). This supports our observation that fat globules appear more clustered at lower temperatures (**Suppl. Fig. 4**), likely facilitated by the association of immunoglobulin molecules. The ability of fat globules to stick together after the addition of serum components, likely antibodies, is well known (Babcock, 1889), as well as the need for the presence

of immunoglobulins for effective gravity-induced separation of fat globules (Euber and Brunner, 1984). Indeed, ~ 7% of endogenous IgM is estimated as necessary for fat globule clustering. Here we have shown for the first time that the addition of purified immunoglobulins improves spore capture during creaming, a significant observation given the need to remove spores naturally in some cheese processes, such as GP production.

Identification of immunoglobulin classes by immunogold labelling

Immunogold labelling experiments were conducted next to further understand the role of Ig molecules in the clustering of fat globules and the agglutination of *C. tyrobutyricum*. An experiment was designed to determine whether all milk immunoglobulin classes, i.e. IgG, IgM and IgA, are equally involved in promoting either fat globule clustering or the agglutination of bacteria to fat globules via association with immunoglobulins. Creaming trials were conducted in duplicate for each immunoglobulin class and immunogold labelling was performed on ultrathin sections of resin-embedded samples of either cream obtained from raw milk with and without the addition of *C. tyrobutyricum* or pure cultures of *C. tyrobutyricum* as a control.

TEM was employed, as it not only allows an assessment of immunolabelling but also allows vegetative cells to be examined, unlike MPN counts. IgM was rarely observed in the interactions among fat globules and between globules and bacteria when observed by TEM, IgG was also absent from these sections (**Suppl. Fig. 5**). In contrast, IgA was consistently present in the material associated with either the fat globules (Fig. 5), between vegetative cells and fat globules (Fig. 6), as well as between spores and fat globules (Fig. 7).

The material associated with the IgA molecules appeared to have low electron density (Fig. 5a, 6a, and 7, as indicated by the arrows), as previously observed by D'Incecco et al., (2015). The material was also labelled by high numbers of gold particles (Fig. 5b and 6b, indicated by the arrows), confirming the presence of IgA molecules.

Overall, these results show that immunoglobulin class IgA is involved in both the association of fat globules and the agglutination of vegetative cells and spores to fat globules. These observations are consistent with the hypothesis made by Caplan et al., (2013) that the surface of a fat globule may associate with another fat globule. In the presence of bacterial spores, these spores may also bind replacing the direct interaction between fat globules resulting in the entrapment of spores during creaming.

The TEM ultrathin sections of immunogold labelled *C. tyrobutyricum* culture confirmed that IgA but not IgM or IgG immunogold labelled probes directly associated with the cell walls of *C. tyrobutyricum*, indicating a high affinity between IgA and this bacterium (Fig. 8).

It is important to emphasise the difference between the agglutination of bacteria to each other in the presence of serum (IgA) and the association of a bacterium (vegetative cells or spores) to fat globules taking place during cream rising. The former is a biological event that should be expected, as it is part of the natural response of the immune system towards an antigen, thus not requiring any fat globule to be present. In fact, Hurley and Theil, (2011) reported IgA as the major immunoglobulin class found in mucosal secretions that prevents infection by agglutinating microbes. Antimicrobial properties, such as the agglutination of microbes, were also reported for sIgM (Brandtzaeg and Johansen, 2007). This natural agglutinating activity was demonstrated either in bovine colostrum preparations against a variety of pathogenic bacteria (Stephan et al., 1990; Loimaranta et al., 1998) or in normal milk and colostrum against *Lactic streptococci* (Choemon *et al*., 1976; Todd *et al*., 1989). Although, IgM activity appears less important than IgA in the experiments reported here.

The capture of bacteria during creaming is a poorly understood phenomenon that involves two steps: 1) the agglutination of bacteria to fat globules via association with IgA molecules and 2) the subsequent rising of the two associated particles. The agglutination observed here implies that there is a specific association between Ig and the surface antigens of *C. tyrobutyricum* and that the ability of *C. tyrobutyricum* to agglutinate to fat globules is positively influenced by the presence of

antigens on the surface of bacterial walls that act as ligands for IgA and to a lesser extent IgM molecules. Consequently, two further factors will influence the degree of agglutination: 1) the extent of antigen expression on the *C. tyrobutyricum* surface and 2) the concentration of IgA and IgM in the milk.

Very few studies have focused directly or indirectly, on the agglutination of bacteria to fat globules during the natural creaming of milk. Franciosi et al., (2011) showed that psychotropic bacteria in milk intended for cheese making were mainly concentrated in the cream layer after natural creaming. Geer and Barbano (2014) showed that the addition of colostrum to skim milk was able to partially restore the natural rising of *Clostridium's* spores in raw milk previously pasteurized at 76 °C for 7 min, suggesting the role of immunoglobulins. To our best knowledge, however, only Honkenen-Buzalski and Sandholm, (1981) have shown different immunoglobulin classes interact with milk bacteria. Consistent with our results, their study showed that *Staphylococcus aureus* appears to associate with the fat globules within cream in the presence of IgA and IgM. No clustering and no bacterial agglutination were observed in the presence of IgG, as observed here for *C. tyrobutyricum*. Stadhouders and Hup (1970) also showed that the addition of an antibody-enriched preparation to a suspension of fat globules clearly increased the binding of *Streptococcus cremoris* but no creaming step was performed.

Our findings indicate that not all classes of milk immunoglobulins are involved in the association of fat globules and agglutination of *C. tyrobutyricum* to fat globules. An examination of the characteristics reported for immunoglobulins in the literature identified some possible explanations. IgA and IgM differ from IgG in both their physiological and structural features. IgA and IgM are found in bovine milk and colostrum in the form of secretory IgA and IgM, namely sIgA and sIgM that are produced predominantly by plasma cells in the mammary tissue (Hurley and Theil, 2011). The secretory component of sIgA and sIgM is a highly glycosylated polypeptide that plays a protective role within the immune system (Phalipon et al., 2002). IgA and IgM are also the only polymeric immunoglobulins; forming dimers and pentamers, respectively in both milk and

colostrum. In these polymeric structures, the monomeric units are covalently bound through a joining (J) chain (Mix et al., 2006; Kaetzel, 2007). This J chain also results in additional features, such as high valency of antigen-binding sites, allowing these molecules to effectively agglutinate bacteria. This high valency is likely also important in the role these Ig molecules play, facilitating interactions between fat globules and bacteria in creaming. Being monomeric, IgG lacks these characteristics. This may explain why IgG is not involved in any agglutinating or clustering phenomena observed during the natural creaming of milk.

CONCLUSIONS

Natural creaming of raw milk is the first step of Grana Padano cheese making. This study showed that natural creaming carried out at temperatures of 22 °C or 40 °C induces coalescence, compromising the integrity of the native fat globule structure. This coalescence, in turn, potentially decreases the availability of the native MFGM surface for interfacial mechanisms during the natural creaming of milk. Such changes were also observed in milk that was preheated at 37 °C prior to creaming at 8 °C, indicating that the practice of cold milk reactivation may alter fat globules and change the creaming process, potentially also reducing the capacity of fat globules to entrap spores. Compared to cold agglutination, partial coalescence promotes formation of larger fat structures that cream faster. However, the rate of creaming will depend on the magnitude of the temperature induced changes to bulk properties (density, viscosity) compared to magnitude of the temperature induced changes to individual fat globules from agglutination, clustering or coalescence.

An efficient method for Ig purification was presented and we demonstrated that IgA and, to a lesser extent, IgM were among the components responsible for the clustering of fat globules and agglutination of *C. tyrobutyricum* to fat globules during the natural creaming of milk. In contrast, IgG which is monomeric rather than polyvalent, was not involved. *C. tyrobutyricum* showed a high affinity to IgA molecules, indicating that the natural variation in IgA and Ig concentration in milk could contribute to the variability in LBD. The addition of immunoglobulins to raw milk prior to

natural creaming could also improve desporification, reducing the risk of LBD in GP or similar cheeses.

ACKNOWLEDGEMENTS

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TABLES

Table 1. Number of *C. tyrobutyricum* spores (MPN) determined in cream that rose during natural creaming of fat globules in microfilterd milk (MMilk) where spores and immunoglobulins (Ig) were added. In control samples the milk was heated to 80 °C for 10 min to inactivate Ig molecules (Heated MMilk). The spore data presented are the mean ± the standard deviation based on duplicate analysis of three different creaming trials (n = 3).

Sample	Cream (*mL*)	Spores (*MPN/mL*)
MMilk + fat globules	1.5 ± 0.1	na
Heated MMilk + fat globules	1.1 ± 0.1	na
MMilk + fat globules + spores	1.5 ± 0.1	317 ± 12 [a]
Heated MMilk + fat globules + spores	1.1 ± 0.2	87 ± 6.0 [b]
MMilk + fat globules + spores + Ig	1.5 ± 0.1	537 ± 80 [c]
Heated MMilk + fat globules + spores + heated Ig	0.0 ± 0.0	83 ± 6.0 [b]

[a,b,c,d,] Means in the same column with different letters are significantly different ($P < 0.05$).

na = not applicable.

Table 2. Number of *C. tyrobutyricum* spores (MPN) counted at the top or bottom of a model system where fat globules, spores and immunoglobulins (Ig) were added in different combinations to 0.01 M PBS in the absence of other milk components. The spore data presented are the mean ± the standard deviation of the mean based on duplicate analysis of three different creaming trials (n = 3).

Sample	Cream *(mL)*	Spores *(MPN/mL)*
PBS + fat globules	0.4 ± 0.1	na
PBS + fat globules + spores	0.4 ± 0.1	213 ± 6.0 [a]
PBS + fat globules + spores + Ig	0.5 ± 0.1	527 ± 91 [b]
PBS + fat globules + spores + Ig x 3	0.6 ± 0.1	537 ± 81 [b]
PBS + spores	na	83 ± 6.0 [c]
PBS + spores + Ig	na	87 ± 6.0 [c]
PBS + spores + Ig (B)	na	327 ± 15 [d]

[a,b,c,d,] Means in the same column with different letters are significantly different ($P < 0.05$).

Ig x 3 = 3 fold higher concentration of Ig

(B) = sampled at the bottom of the liquid sample.

na = not applicable.

FIGURES

Figure 1. Volume (mL) of cream collected at various temperatures (●, 40 °C; ○, 22 °C; Δ, preheated to 37 °C then creamed at 8 °C; ♦, 8 °C; or □, 4 °C) during the first three hours of natural creaming (a). The data are the average of two separate creaming trials.

(b) Volume (■) and fat content (●) of the cream layers obtained after 24 h of creaming. □, Sample preheated at 37 °C then ○, creamed at 8 °C, (volume and fat content, respectively). The data are the average of two separate creaming trials but are representative of a larger set of five replicate experiments. The error bars are the standard deviation of the mean. The regression lines were calculated for the samples exposed to a single temperature excluding preheated samples (□, ○).

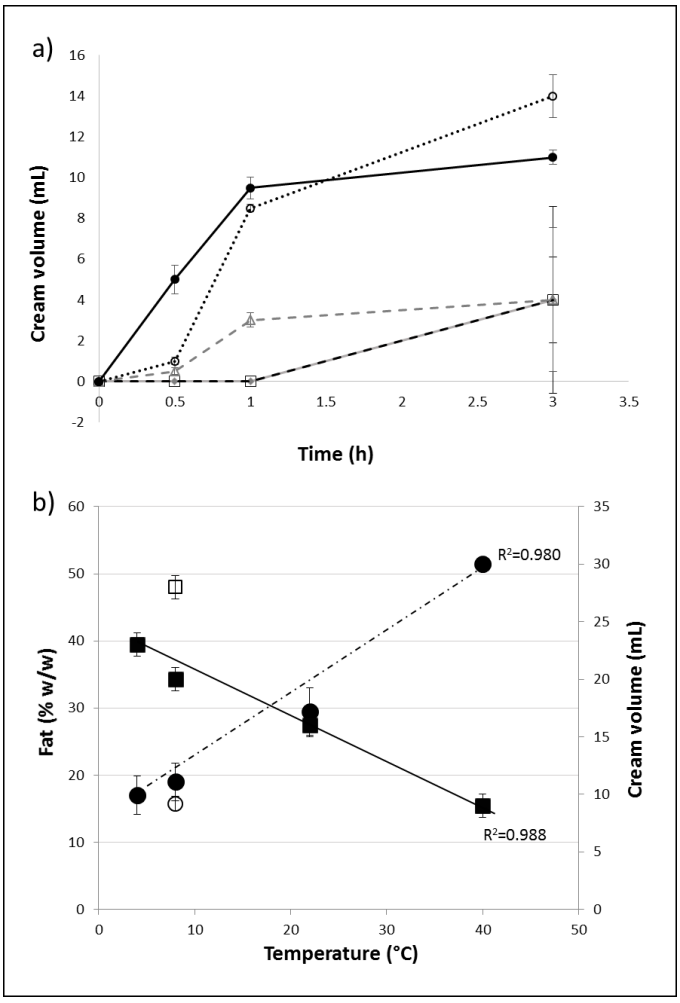


Figure 2. The microstructure of the fat within raw milk (a) and the surface cream layers collected after natural creaming at 4 °C (b), 8 °C (c), milk that was preheated at 37 °C and creamed at 8 °C (d) or creamed at 22 °C (e) or 40 °C (f). The Nile red stained fat globules appear red. The scale bars are 20 µm in length.

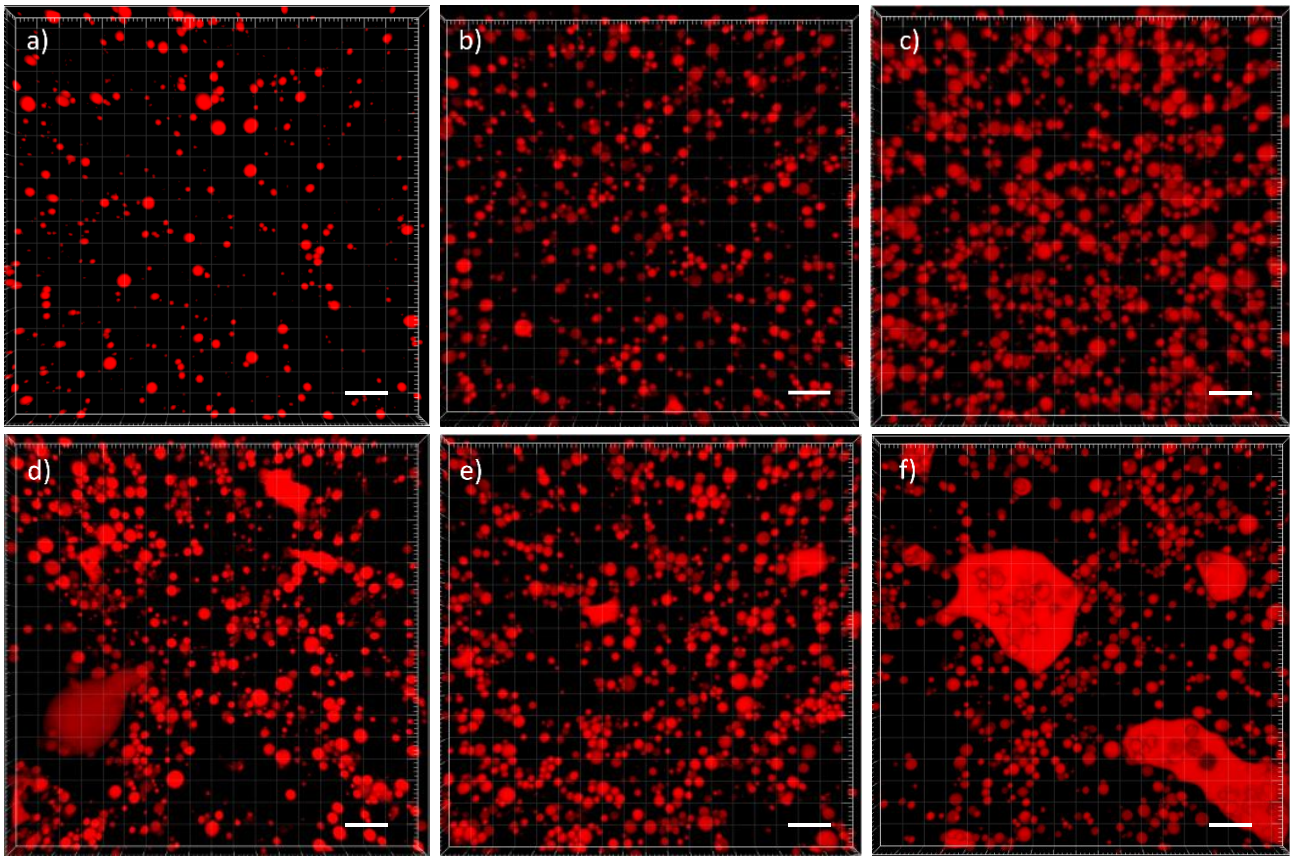


Figure 3. Volume distribution of fat globules obtained from image analysis of the 3D CLSM images (**Suppl. Fig. 3**) of raw milk (——), cream samples separated at 4 °C (− − −), 8 °C (• • • •), 8 °C and pre-heated 37 °C (——), 22 °C (− − −) or 40 °C (• • • •).

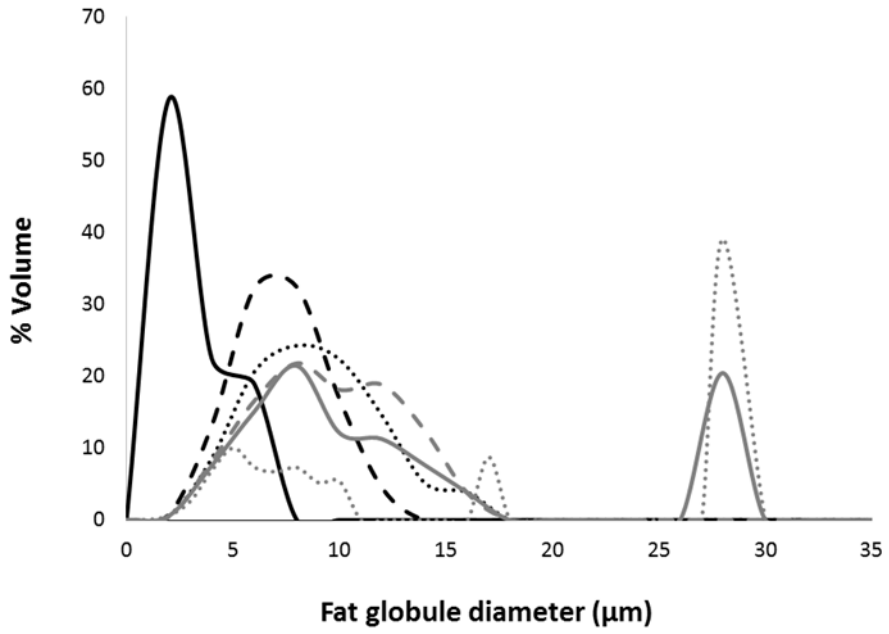


Figure 4. SDS-PAGE of purified immunoglobulin proteins under reducing (a) and non-reducing (b) conditions. M = milk; C = colostrum; CW = colostrum whey; 45% = protein fraction precipitated with 45% $(NH_4)_2SO_4$; 80% = protein fraction precipitated with 80% $(NH_4)_2SO_4$ after an initial precipitation with 45% $(NH_4)_2SO_4$; MW = molecular weight markers. Major bands are identified as: α-Cn = α-casein; β-Cn = β-casein; k-Cn = k-casein; β-Lg = β-lactoglobulin; α-La = α–lactalbumin; Ig = immunoglobulins; Ig HC = immunoglobulin heavy chains; Ig LC = immunoglobulin light chains.

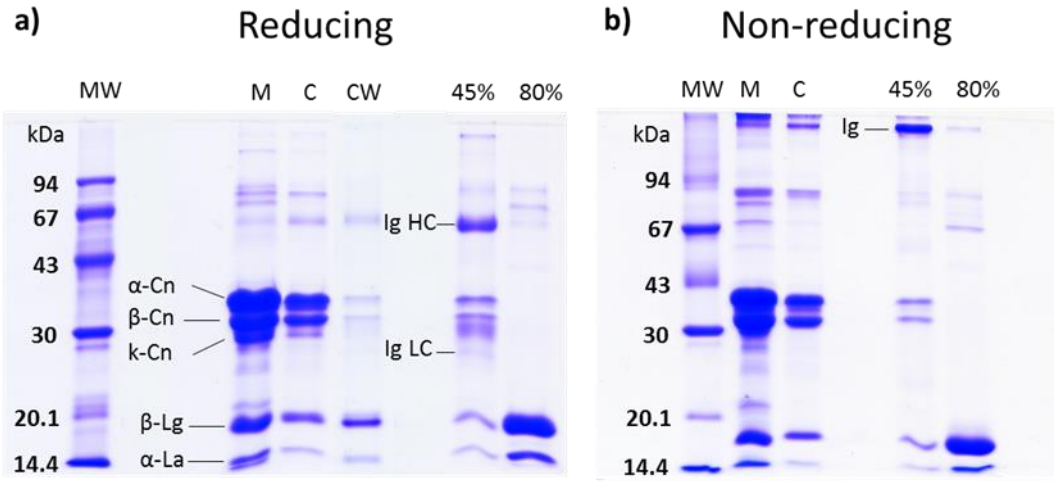


Figure 5. TEM micrographs of an immunolabelled ultrathin section of fat globule clusters showing the presence of IgA in the amorphous material present between the fat globules (visible as black dots, indicated by the arrows). A higher magnification of the area framed in (a) is shown in (b) where the 15 nm gold label of the IgA is clearly visible (as indicated by the arrows). FG = fat globule; C = casein micelle. The scale bars are 200 nm in length. This image is representative of three different experiments.

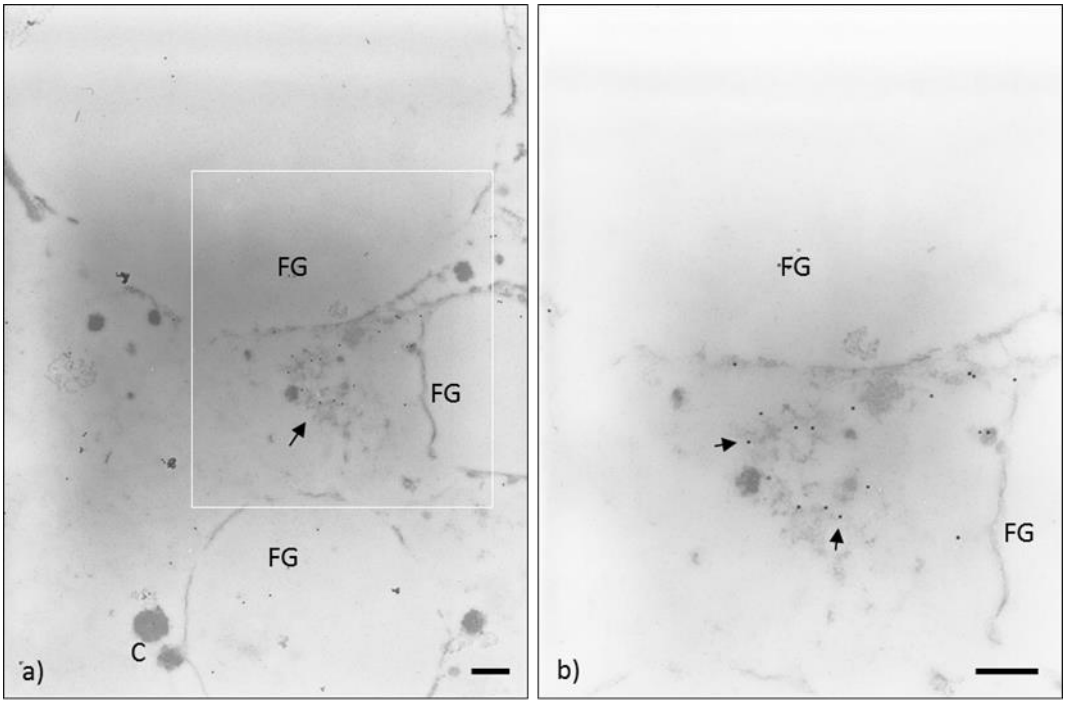


Figure 6. TEM micrographs of an immunolabelled ultrathin section of a vegetative cell (VC) associated with a fat globule (FG) via amorphous material (white arrows (a)). This material is labelled by 15 nm gold labelled IgA antibodies (visible as black dots, indicated by the arrows) (b), is an enlarged framed area of (a) where the IgA antibodies are visible. C = casein micelle. The scale bars are 300 nm and 100 nm in length in (a) and (b), respectively. This image is representative of three different experiments.

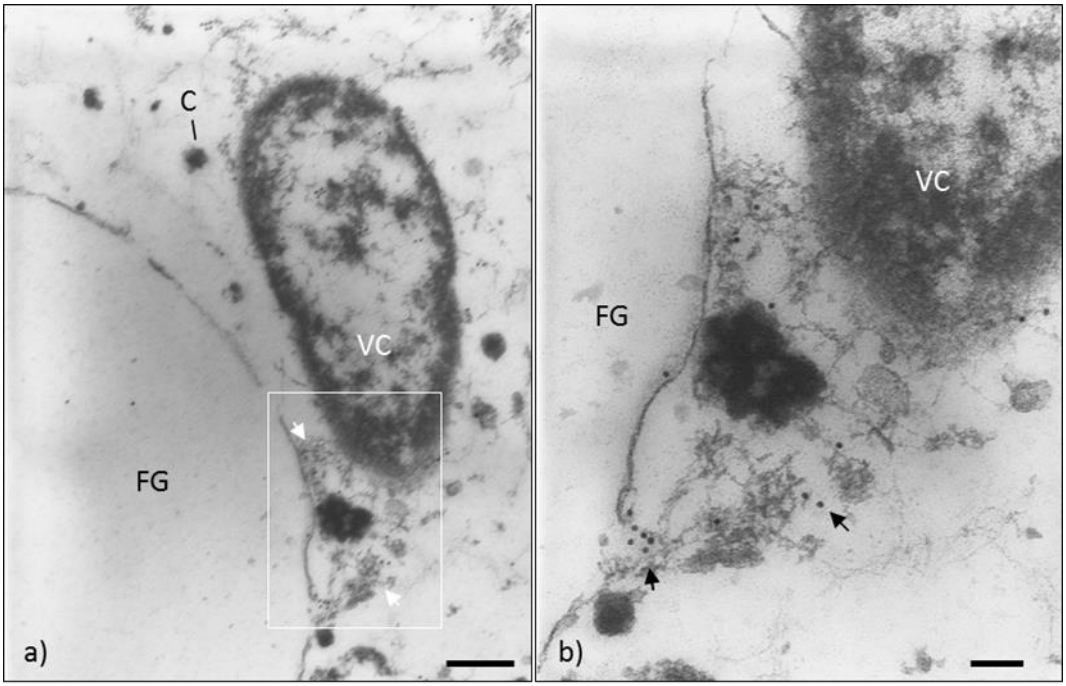


Figure 7. TEM micrographs of an immunolabelled ultrathin section of a spore (S) and vegetative cell (VC) interacting with a fat globule (FG) via amorphous material (arrows (a)). Gold labelled IgA antibodies (black dots) on the amorphous material within the dashed lines are enlarged in panels (b) and (c). The scale bars are 300 nm in length in (a) and 100 nm in length in (b) and (c). This image is representative of three different experiments.

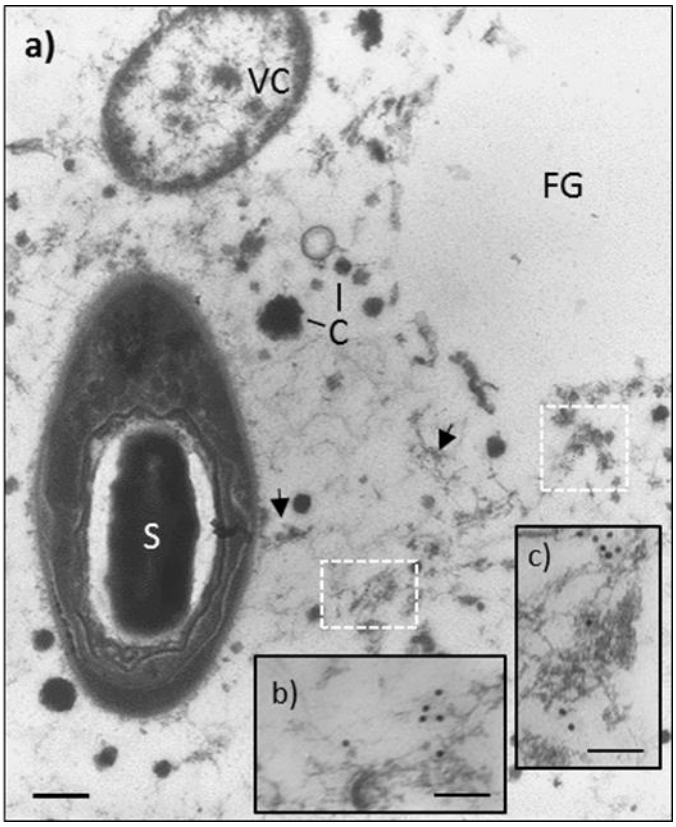
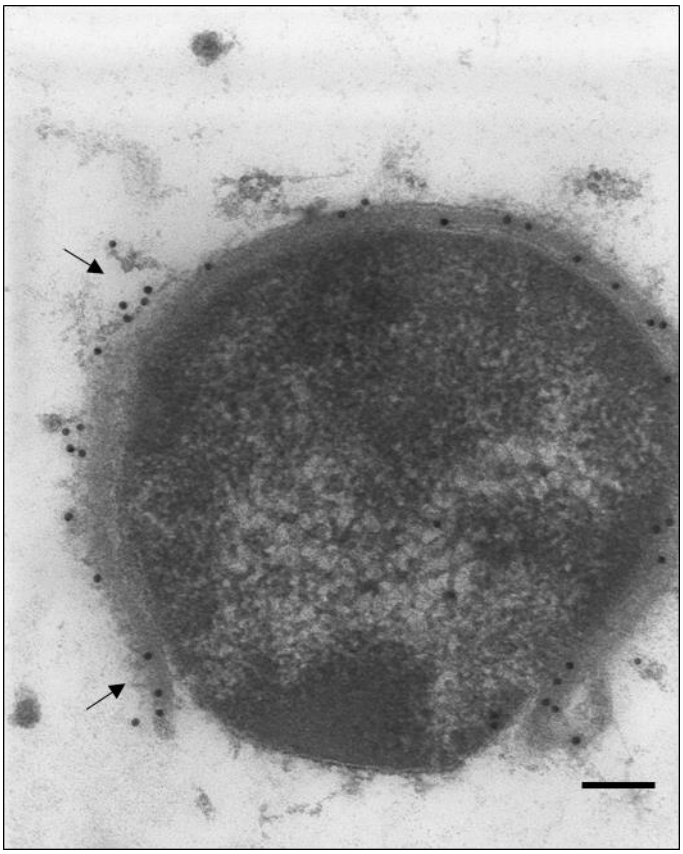
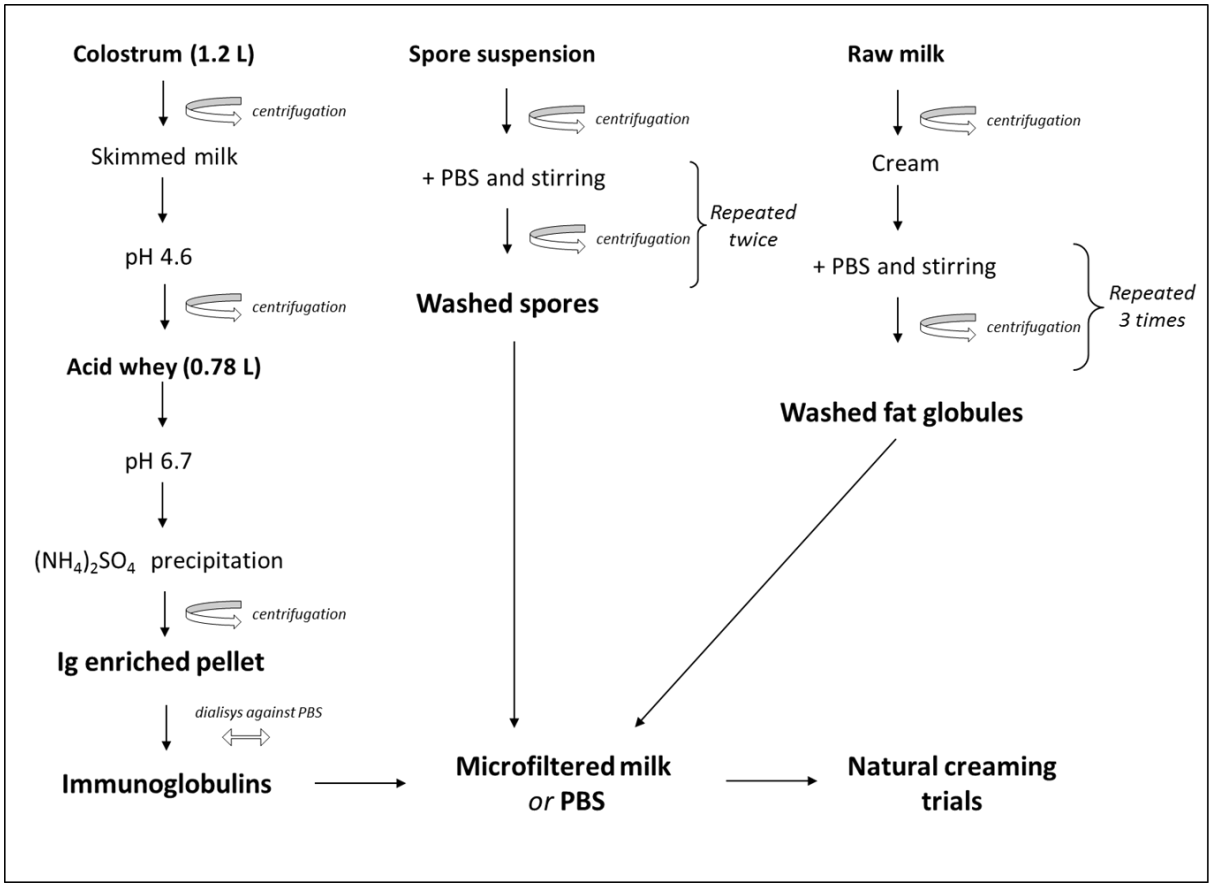


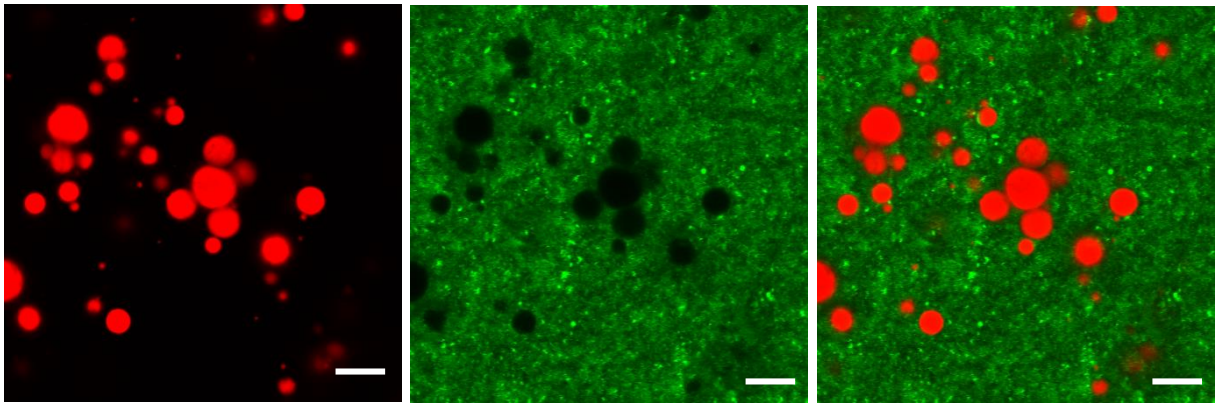
Figure 8. TEM micrograph of immunolabelled ultrathin section of *C. tyrobutyricum* vegetative cell. Immunogold labels 15 nm in diameter are shown in association with the IgA on the cell walls of *C. tyrobutyricum* (as indicated by the arrows). The scale bars are 100 nm in length. This image is representative of three different experiments.



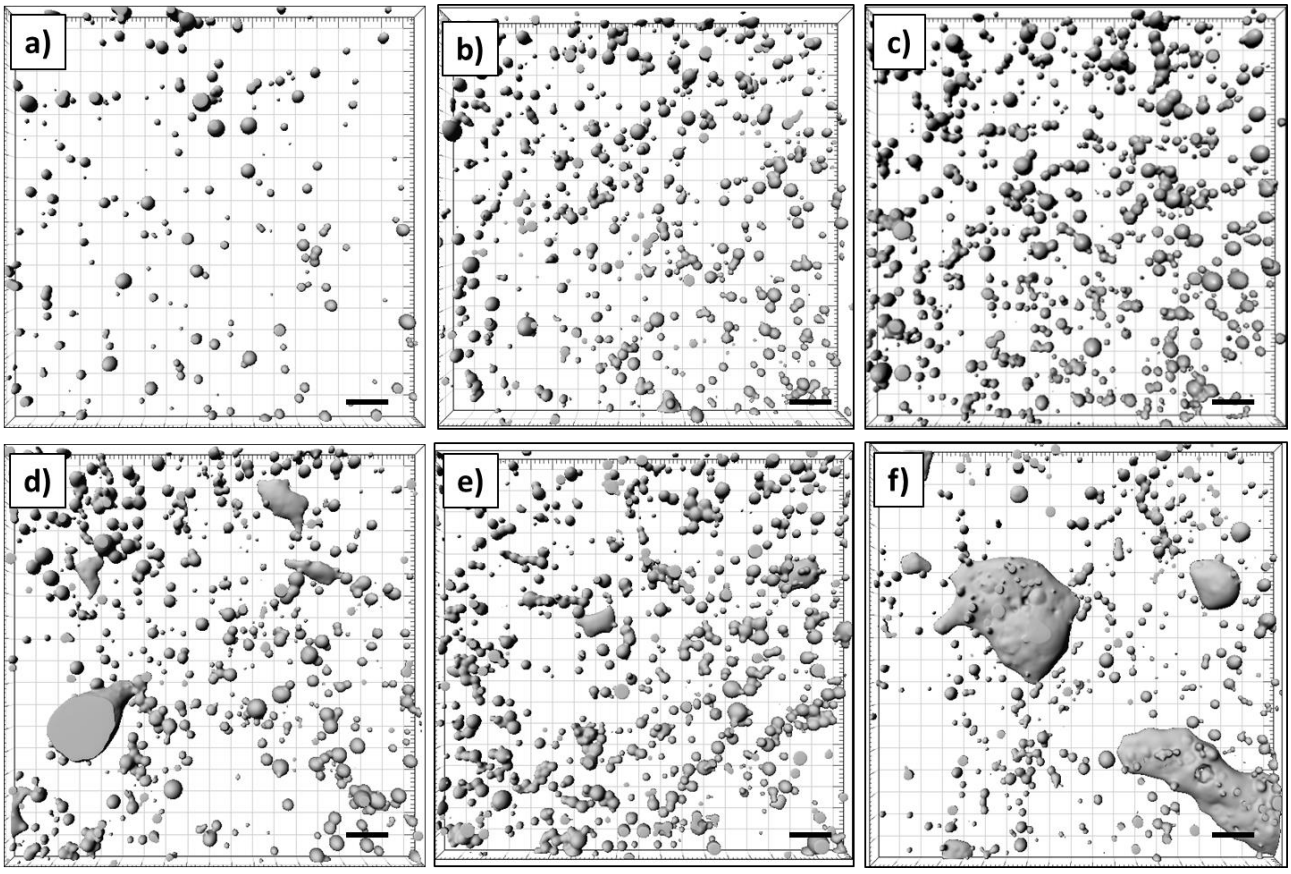
SUPPLEMENTARY FIGURES



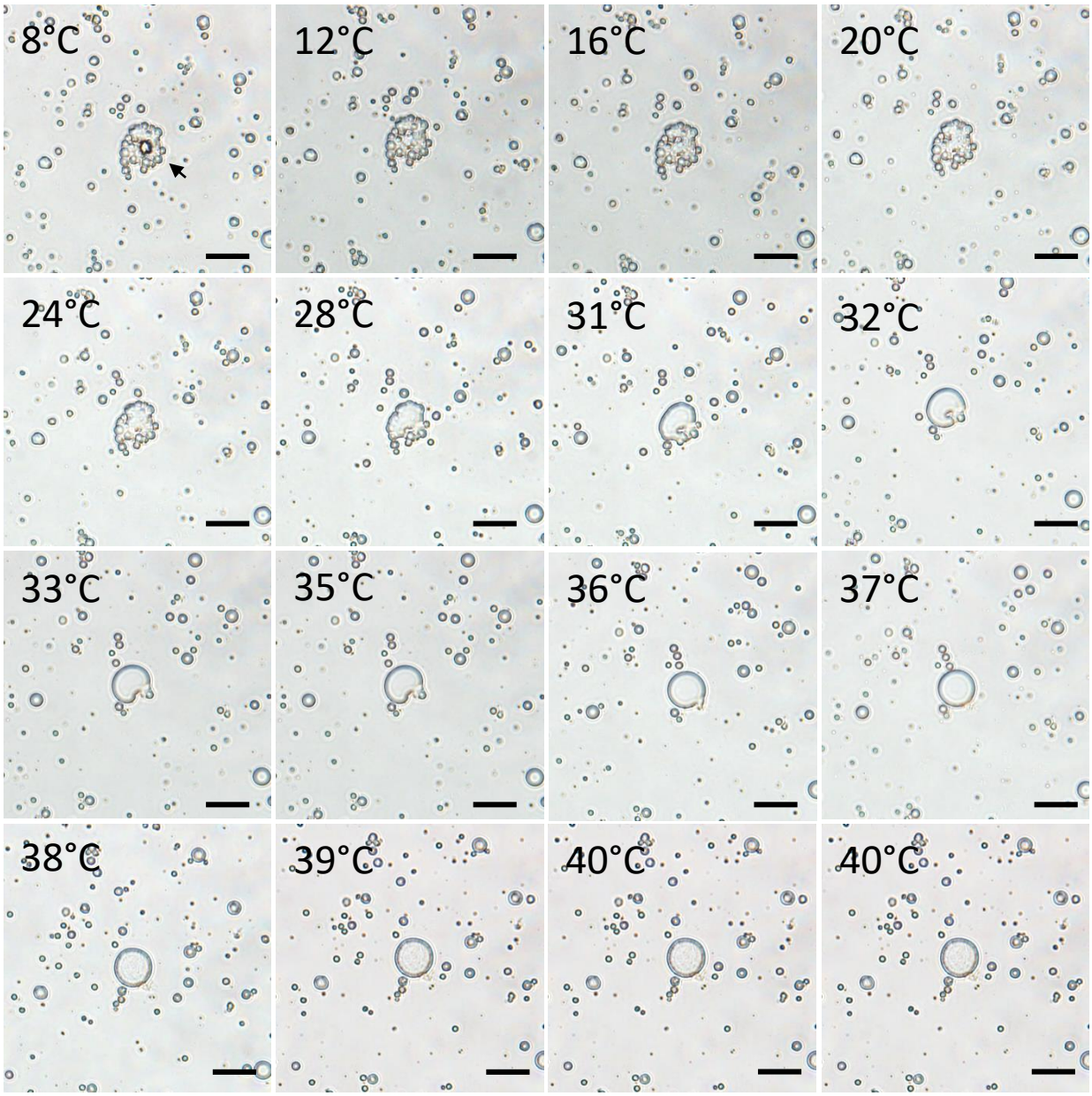
Suppl. Fig. 1. Experimental design applied to reproduce natural creaming using immunoglobulins precipitated from colostrum, spores washed and isolated from broth and fat globules washed and isolated from raw milk.



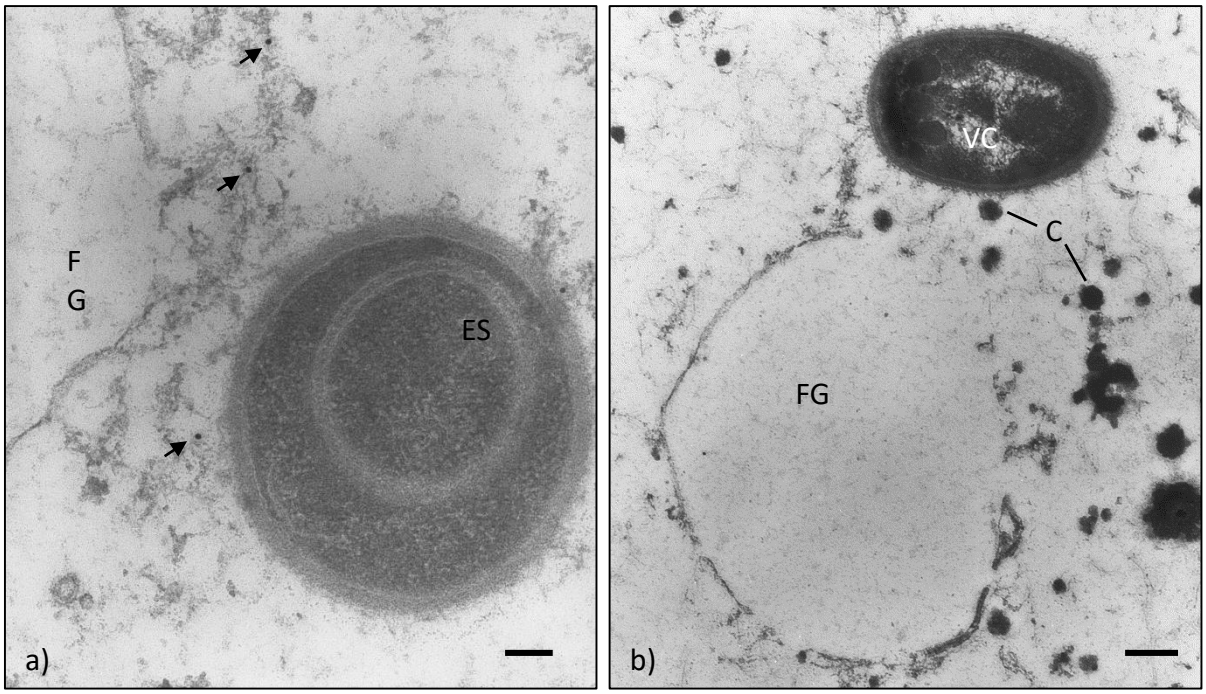
Suppl. Fig. 2. Clusters of fat globules naturally present in raw milk. The image on the left shows the fat channel, the centre shows the protein channel and the right shows the superimposed channel of both fat and protein. Nile red stained fat globules appear red. Fast green stained protein appears green. The scale bars are 10 µm in length.



Suppl. Fig. 3. The microstructure of the fat in cream samples as a function of temperature. The rendered fat obtained using image analysis software appears grey. These rendered surfaces correspond to the CLSM images in Figure 2. The raw milk was examined prior to creaming (a), after creaming at 4 °C (b) or 8 °C (c) or for sample preheated at 37 °C then creamed at 8 °C (d). Samples were also examined after creaming at 22 °C (e) or 40 °C (f). The scale bars are 20 µm in length. The microstructure presented is representative of two separate trials.



Suppl. Fig. 4. Real time light microscopy images of a fat globule clusters (indicated by the arrow) present in raw milk during heating from 8 °C to 40 °C over a period of 160 min. The temperature was increased by 1 °C every 5 min interval. The cluster (indicated by the arrow) remained unchanged up to 24 °C, at this temperature the cluster started to break up and single fat globules were free to move within the milk sample. The scale bars are 10 µm in length in all images.



Suppl. Fig. 5. TEM micrographs of an immunolabelled ultrathin section of **a)** an endospore (ES) associated with a fat globule (FG) via amorphous material, where there are few IgM immunogold labels (black arrows); **b**) Section of a vegetative cell (VC) associated with a fat globule via amorphous material showing no IgG immunogold labels. C = casein micelle. The scale bars are 100 nm and 300 nm in length in (a) and (b), respectively.