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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 production of Grana Padano and Parmigiano Reggiano Protected Denomination of Origin cheeses. 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—was observed at creaming temperatures higher than 22°C by confocal laser scanning microscopy. The widespread practice of heating of milk at 37°C before creaming at 8°C resulted in important changes in the size distribution of fat globules in raw milk, potentially altering the ability of fat to entrap clostridia spores. We investigated the role of immunoglobulin classes in both the clustering of fat globules and the agglutination of *Clostridium tyrobutyricum* to fat globules during creaming. Immunogold labeling and transmission electron microscopy showed that IgA and IgM but not IgG were involved in both clustering and agglutination. Both vegetative cells and spores were clearly shown 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 practical insights into how the process of creaming may be optimized in 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 such as 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 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, because it affects the tendency of fat globules to adopt a specific supramolecular organization; that is, to cluster or to coalesce (Fredrick et al., 2010). Clustering is thought to occur when 2 or more fat globules are in close contact for a substantial period, enabling the formation of stable aggregates. In contrast, coalescence involves the fusion of the membranes of 2 or more globules to form one larger unit, also arising because 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, although studies have suggested that milk immunoglobulins may be involved in this phenomenon (Honkanen-Buzalski and Sandholm, 1981; Zacconi and Bottazzi, 1982; Euber and Brunner, 1984).

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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, and provide protection against infection (Lilius and Marnila, 2001). They are ~150 kDa in size and comprise 2 identical heavy (~50 kDa) and 2 identical light (~25 kDa) polypeptide chains linked by disulfide 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 3 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 to 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 preheating 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, brought to the laboratory (University of Milan) within 2 h of milking, and used for creaming trials with the addition of immunoglobulins. Colostrum for immunoglobulin purification was collected at the same dairy farm from heifers on d 2 postpartum. Freshly produced, raw, microfiltered, skimmed (0.3% fat) milk was taken at an industrial plant (Tetrapack, Aarhus, 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 d.

Purification of Immunoglobulins from Colostrum

Native immunoglobulins were purified from colostrum by ammonium sulfate precipitation (Supplemental Figure S1; https://doi.org/10.3168/jds.2017-13580). 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 analyzed by 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. The SDS-PAGE was conducted under both reducing and nonreducing 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 of protein/mL) were dialyzed using Spectra/Por dialysis tube [20,000 Da MW cut-off, 24 mm flat width, 1.8 mL/cm (volume/length); Spectrum Laboratories Inc., Rancho Dominguez, CA] against 15 m*M* sodium phosphate buffer (PBS), pH 7.2, containing 150 m*M* NaCl and used for natural creaming trials.

Natural Creaming Trials

Three natural creaming trials were carried out, at the laboratory scale, to produce cream samples destined to confocal laser scanning microscopy (**CLSM**), immunogold labeling (**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, 4 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 before creaming at 8°C. The volume of cream that rose to the surface was visually evaluated using graduated cylinders at 0.5, 1, 3, 6, 8, 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., 2010). This creaming