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The dynamics of the biological membrane surrounding the buffalo milk fat globule investigated as a function of temperature

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ABSTRACT

The biological membrane surrounding fat globules in milk (the MFGM) is poorly understood, despite its importance in digestion and in determining the properties of fat globules. In this study, *in situ* structural investigations of buffalo MFGM were performed as a function of temperature (4–60 °C), using confocal microscopy. We demonstrate that temperature and rate of temperature change affected the lipid domains formed in the MFGM with the lateral segregation (i) of high Tm lipids and cholesterol in a Lo phase for both $T < Tm$ and $T > Tm$ and (ii) of high Tm lipids in a gel phase for $T < Tm$. Rapid cooling favours nucleation, while slow cooling favours growth, leading to the formation of small and large lipid domains, respectively. Changes in the interfacial properties of the MFGM, as a function of temperature, could modulate the functions of fat globules during processing and digestion.

1. Introduction

Milk is a natural oil-in-water emulsion in which the organisation of lipids is complex and specific to this biological fluid (Lopez, 2011). Milk lipids are organised as colloidal assemblies called milk fat globules, the core of which is mainly composed of triacylglycerols (TAG, 98% of milk lipids) covered by a biological membrane that governs all the interfacial phenomena (e.g. hydrolysis of TAG by the digestive enzymes). These biological entities are secreted by all mammal females to deliver lipids and bioactive molecules to the gastrointestinal tract of newborns (Lopez, 2011). Milk fat globules are also functional elements within many dairy products consumed by infants and human adults (e.g. creams, cheeses). Despite the importance of the biological membrane surrounding milk fat globules for determining the nutrition and the properties of many dairy products, this membrane is not well understood.

The milk fat globule membrane (MFGM) is thought to be comprised of three layers of polar lipids and proteins as a result of the mechanisms involved in milk fat globule secretion from the epithelial cells of the mammary gland (Heid & Keenan, 2005; Lopez, 2011). The first layer originates from the endoplasmic reticulum, while the outer bilayer results from the envelopment of milk fat globules in the apical plasma membrane of the epithelial cells during their secretion. The MFGM contains membrane-specific proteins and different classes of lipids, such as the glycerophospholipids (namely phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylinositol, PI; and phosphatidylserine, PS), the sphingolipids (mainly sphingomyelin, SM) and cholesterol (Heid & Keenan, 2005; Le, Van Camp, & Dewettinck, 2014; Lopez, 2011; Ménard et al., 2010). Previous studies have reported that PE, PI and PS are mainly concentrated on the inner surface of the MFGM while SM, PC and the glycolipids are mainly located in the outer bilayer of the MFGM (Deeth, 1997). The packing of the lipids and proteins located in the outer bilayer of the MFGM has been recently further characterised using confocal microscopy with fluorescent dyes. Structural analysis of the MFGM have revealed heterogeneities (i) in the lateral organisation of the components (Evers, 2008), (ii) in the localisation of membrane proteins (Lopez, Madec, & Jimenez-Flores, 2010; Lopez & Ménard, 2011; Nguyen et al., 2015) and (iii) in the lateral packing of polar lipids with the occurrence of SM-rich domains at the surface of

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fat globules from bovine milks (Gallier, Gragson, Jimenez-Flores, & Everett, 2010; Lopez et al., 2010), breast milks (Lopez & Ménard, 2011; Zou et al., 2012) and buffalo milks (Nguyen et al., 2015). These lipid domains correspond to phase separation, where the non-fluorescent areas may be liquid ordered (Lo) phase or gel phase domains while the fluid liquid disordered (Ld) phase domains form the surrounding fluorescent matrix (Gallier et al., 2010; Lopez et al., 2010). The hypothesis is that the Lo phase domains of the MFGM are rich in polar lipids with high phase transition temperature (mainly SM that accounts for about 25% of polar lipids in the MFGM (Lopez, 2011), but also saturated phospholipids and cholesterol), while the Ld phase is mainly comprised of unsaturated glycerophospholipids (PC, PE, PI, PS) (Lopez & Ménard, 2011; Lopez et al., 2010). SM contains long chain saturated fatty acids (Sanchez-Juanes, Alonso, Jancada, & Hueso, 2009) responsible for its high phase transition temperature (Tm = 34.3–35 °C, Malmsten, Bergentahl, Nyberg, & Odham, 1994; Murthy, Guyomarc'h, Paboeuf, Vié, & Lopez, 2015). Dipalmitoyl phosphatidylcholine (DPPC) is also characterised by a high Tm (Tm = 41.1–41.7 °C, Benesch & McElhaney, 2014; Murthy, Guyomarc'h, Paboeuf, Vié, & Lopez, 2015). Polar lipids containing one unsaturated fatty acid chain, such as POPE, also have a Tm above room temperature (Murthy, Guyomarc'h, Paboeuf, Vié, & Lopez, 2015; P = palmitic acid, O = oleic acid). These high Tm lipids could pack in the MFGM and segregate from the fluid Ld phase of unsaturated polar lipids to form domains in the gel phase, as recently shown in a model membrane with milk-SM (Guyomarc'h et al., 2014) and in monolayers of MFGM lipid extracts (Murthy, Guyomarc'h, Paboeuf, Vié, & Lopez, 2015). The domains formed by milk SM in the absence of cholesterol (gel phase) have different shapes, and different nanomechanical properties and protrude from the fluid phase of the membrane with a higher dimension than the domains formed in the presence of cholesterol (Lo phase) (Guyomarc'h et al., 2014).

The characteristics of the lipid domains (e.g. number, size, shape, lipid phase, nanomechanical properties) could be affected by temperature. Consequently changes induced by fluctuations in temperature could have consequences for the mechanisms involved in the absorption of lipids in the gastrointestinal tract (37 °C) and in dairy processing where storage can be at 4 °C and heat treatments can occur at 60 °C or higher, potentially altering the mechanical properties of the fat globule. The SM-rich domains in the human MFGM have been found to be responsive to temperature, with a decrease in domain size observed when the temperature increases (Zou et al., 2012). The effect of temperature on the microstructure of the MFGM of other species, such as the temperature sensitivity of the lipid domains, needs to be further investigated to gain a better understanding of the factors affecting the organisation of the MFGM and the species differences of these observations. Specifically, the change in lipid domains in the outer bilayer of the MFGM, as a function of temperature, will be useful, not only to better understand the function of these globules in the gastrointestinal tract, but also for understanding the potential impact of changes occurring during dairy processing.

Information about buffalo milk and the microstructure of the buffalo MFGM is scarce compared to other milks from cows and humans. Yet buffalo milk comprises approximately 13% of total world milk production (about 97 million tons per year) (IDF, 2009). Previous studies have demonstrated that the physicochemical properties of buffalo fat globules (e.g. size, zeta potential, composition of the MFGM) differ from those of bovine fat globules (Ménard et al., 2010). The amount of polar lipids (2.6 mg/g fat and about 190 mg/l) in buffalo milk, is 28% higher than that in bovine milk (Ménard et al., 2010). The relative proportion of SM, which is assumed to be the major component of the lipid domains found in the MFGM (Lopez & Ménard, 2011; Lopez et al., 2010; Zou et al., 2012), is lower in buffalo MFGM than in bovine MFGM (24.8% vs. 26.9% of polar lipids) (Ménard et al., 2010). Also, the amount of cholesterol is lower in buffalo milk than in bovine milk (7.0–10.2 mg/100 ml vs. 10.5–19.8 mg/100 ml) (Strzalkowska, Jozwik, Baghnicka, Krzyzewski, & Horbanczuk, 2009; Talpur, Memom, & Bhanger, 2007; Zotos & Bampidis, 2014). These differences in lipid composition may provide structural specificities to the buffalo MFGM that require further investigation. In a first paper (Nguyen et al., 2015), we performed *in situ* structural investigations of the buffalo milk fat globules, showed the presence of cytoplasmic remnants, and characterised the heterogeneous distribution of proteins and lipids in the outer bilayer of the MFGM. All these experiments have been conducted at room temperature.

The objective of this work was to investigate the microstructure of the buffalo MFGM as a function of temperature and rate of temperature change (slow vs. rapid temperature gradients). The MFGM was characterised using confocal laser scanning microscopy (CLSM) under well-controlled temperatures ranging from 60 °C to 4 °C. This range of temperatures is pertinent to the technological processes used in the dairy industry and for the consumption and digestion of milk and dairy products.

2. Materials and methods

2.1. Buffalo milk samples

The buffalo milks used in this study were provided by Coopérative de Bufflonnes (Maurs, Cantal region, France). These milks corresponded to a mixture of the individual milks produced by 30 buffaloes of the Mediterranean breed *Bubalus bubalis* and collected from evening and morning milking. The growth of bacteria was prevented by adding NaN_3 (0.02% w/v) to the milks after their collection. The milk samples were stored at ambient temperature before microstructural analysis.

2.2. Microstructural analysis

An inverted NIKON Eclipse-TE2000-C1si microscope (NIKON, Champigny sur Marne, France) was used for the confocal laser scanning microscopy (CLSM) experiments, with a He–Ne laser operating at 543 nm wavelength excitation and emission detected between 565 nm and 615 nm. The observations were performed using a ×100 (numerical aperture NA 1.4) oil immersion objective. The staining protocols followed previously described methods for the investigation of the lateral packing of lipids in the outer bilayer of the MFGM (Lopez & Ménard, 2011; Lopez et al., 2010). Briefly, N-(lissamine rhodamine B sulfonyl) dioleoylphosphatidyl ethanolamine (Rh-DOPE, concentration of 1 mg/ml in chloroform; Avanti polar lipids Inc., Birmingham, England) was used to label the phospholipids in the MFGM, *in situ* in the buffalo milks. Wheat germ agglutinin Alexa fluor 488 (WGA-488, Invitrogen, Cergy Pontoise, France) was prepared at a concentration of 1 mg/ml in phosphate saline buffer and used to label the glycosylated molecules in the membrane, i.e. carbohydrate moieties containing N-acetylglucosamine and N-acetyl neuraminic acid (sialic acid) residues. A volume of 40 µl of the Rh-DOPE solution was placed in a glass vial and the chloroform was evaporated under nitrogen, to avoid possible artefacts caused by this organic solvent. For dual staining of the MFGM polar lipids and carbohydrate moieties, 10 µl of the WGA-488 solution were also put into the vial containing Rh-DOPE. Then 1 ml of buffalo milk sample was placed in the vial. The stained milk samples were kept at room temperature for at least 1 h prior to observation by CLSM. Optical microscopy using differential interference contrast (DIC), also called Nomarski microscopy, (Cogswell & Sheppard, 1992) was also used to characterise buffalo milk fat globules. DIC images were superimposed on the flu-