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Lipid domains in the milk fat globule membrane: Dynamics investigated *in situ* in milk in relation to temperature and time

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ABSTRACT

The microstructure of the milk fat globule membrane (MFGM) is still poorly understood. The aim of this study was to investigate the dynamics of the MFGM at the surface of milk fat globules in relation to temperature and time, and in relation to the respective lipid compositions of the MFGM from bovine, goat and sheep milks. *In-situ* structural investigations were performed using confocal microscopy. Lipid domains were observed over a wide range of temperatures (4–60 °C). We demonstrated that rapid cooling of milk enhances the mechanisms of nucleation and that extended storage induces lipid reorganization within the MFGM with growth, leading to circular lipid domains. Diffusion of the lipid domains, coalescence and reduction in domain size were observed upon heating. Different MFGM features could be related to the respective cholesterol/sphingomyelin molar ratio in the three milk species. These structural changes may affect the interfacial properties of the MFGM, with consequences for the functional properties of fat globules and the mechanisms of their digestion.

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1. Introduction

Milk fat globules are the biological assembly of lipids secreted by female mammals to provide the dietary energy (about 50% in human milk) and bioactive molecules that are essential for the growth and health of suckling neonates. They are also involved in the manufacture of many dairy products (e.g. cream, butter, cheese) that are consumed by children and adults (Lopez, Cauty, & Guyomarc'h, 2015). Milk fat globules have a core that is rich in triacylglycerols (TAG) and are enveloped by a biological membrane known as the milk fat globule membrane (MFGM). The relationship between the lipid composition of the MFGM and its organization remain poorly understood despite its importance in dairy technology and food quality and in the mechanisms of milk lipid digestion in the gastrointestinal tract, and despite its possible implications for biological functions, human nutrition and health.

The MFGM is composed of a range of polar lipids, cholesterol, and membrane-specific proteins (Dewettinck et al., 2008; Jensen & Newburg, 1995). The main polar lipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (milk-SM; main milk sphingolipid), while anionic phosphatidylserine (PS) and phosphatidylinositol (PI) are quantitatively minor polar lipids (Dewettinck et al., 2008; Lopez, 2011). From a structural point of view, the MFGM is thought to be organized as a trilayer of polar lipids, with a monolayer in contact with TAG originating from the endoplasmic reticulum and a bilayer originating from specialized regions of the apical plasma membrane of mammary epithelial cells (Heid & Keenan, 2005). The lateral arrangement of polar lipids in the MFGM has long been considered to be homogeneous (Dewettinck et al., 2008). Using confocal microscopy and various fluorescent lipophilic dyes, Evers et al. (2008) showed heterogeneities in the MFGM. Approaches using CLSM with Rhodamine-DOPE dye have in recent years revealed non-homogeneous distribution of polar lipids with non-fluorescent areas in the outer bilayer of the bovine MFGM, interpreted as the formation of SM-rich domains (Gallier, Gragson, Jiménez-Flores, & Everett, 2010; Lopez, Madec, & Jiménez-Flores, 2010; Zou et al., 2015). Milk-SM differs from the other MFGM polar lipids in that it contains long-chain saturated fatty acids which contribute to its high phase transition temperature Tm ~ 35 °C (Murthy, Guyomarc'h, Paboeuf, Vié, & Lopez, 2015). Other saturated polar lipids with high Tm, such as DPPC and POPE, are present in the MFGM to lesser extents and contribute in the wide melting range of MFGM polar lipids with Tm = 36.4 °C (Murthy et al., 2015). The presence of lipid domains has also been reported in the MFGM surrounding fat globules in human milk (Gallier et al., 2015; Lopez & Ménard, 2011; Zou et al., 2012) and buffalo milk (Nguyen et al., 2015). In these studies, differences in the sizes and patterns of the SM-rich domains have been reported between mammal

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species (bovine, buffalo, human). Little is currently known of the respective effects of the chemical composition of the MFGM (e.g. relative proportions of polar lipids, cholesterol and cholesterol/milk-SM ratio) and/or of the thermodynamic history of the milk samples on the microstructure of the MFGM.

Most studies to date have investigated the microstructure of the MFGM *in situ* at room temperature after a poorly monitored thermodynamic history of the milks (Gallier et al., 2010; Lopez et al., 2010; Nguyen et al., 2015). However, after their secretion from the mammary gland at the physiological temperature of ~38 °C (Wooding & Kemp, 1975), milk fat globules are submitted to thermal kinetics and changes in temperature, e.g. during storage (4–7 °C), churning of cream (10–12 °C) and heat treatment (temperature above 60 °C, e.g. pasteurization). The few authors that have considered the effects of temperature characterized the MFGM in milks after storage at 4 °C and rewarming at physiological temperature (Lopez & Ménard, 2011; Zou et al., 2015). They reported that the domains became larger and more numerous as temperature decreased to 4 °C while they became smaller when temperature increased to 37 °C (Zou et al., 2015, 2012). A recent study investigated the structural characteristics of lipid domains in the buffalo MFGM up to 60 °C (i.e. above the Tm of all saturated polar lipids of the MFGM; Murthy et al., 2015) and reported that the temperature and rates of cooling affect the number, size and morphology of the SM-rich domains at the surface of buffalo milk fat globules (Nguyen et al., 2016). A way to improve understanding of the morphology and properties of the lipid domains in the MFGM is i) to change the temperature to reach temperatures above the phase transition temperature of the saturated polar lipids located in the MFGM (Tm ~ 35 °C for milk-SM; Tm ~ 41 °C for DPPC), ii) to investigate different thermal histories of the milk samples, and iii) to consider the specific lipid composition of the MFGM by comparing milks from various mammal species e.g. buffalo, goat, sheep, bovine, human milk.

The aims of this study were therefore to characterize the lipid composition of the MFGM from bovine, goat and sheep milks, and to investigate the molecular arrangement of MFGM polar lipids located at the surface of fat globules, in relation to the temperature and thermal history of the milks. Temperature-controlled CLSM with Rh-DOPE dye was used to characterize *in situ* the microstructure of the MFGM in bovine, goat and sheep milks.

2. Materials and methods

2.1. Milk samples

All the milks used in this study corresponded to bulk tank raw whole milks collected at mid-lactation. The milks were produced in the Brittany region of France and were provided by local dairy plants. Bovine milk was provided by Entremont (Montauban de Bretagne, France). Sheep and goat milks were provided by Triballat (Noyal, France). Sodium azide (NaN_3, 0.02% w/v) was added to the milks to prevent the growth of bacteria. Milk samples were stored at room temperature (19 ± 1 °C) before particle size measurements and confocal microscopy experiments. Milk samples were lyophilized and then stored at −20 °C before chemical analysis.

2.2. Chemical analysis

The fat content in the fluid milks was determined using the Gerber method (IDF, 2008). In the lyophilized milks, the total fat content was determined as previously described by Lopez, Briard-Bion, and Ménard (2014). The quantification of total polar lipids, e.g. glycerophospholipids and milk-SM, and determination of the polar lipid classes were performed using HPLC combined with an evaporative light scattering detector according to a method already detailed (Lopez et al., 2014). The sum of glycerophospholipids (PE, PI, PS, PC) and milk-SM concentration was regarded as the total polar lipid concentration in the milks. The amount of cholesterol in the lyophilized milk was determined by gas chromatography (NF EN ISO 12228-1).

2.3. Measurement of milk fat globule size

Laser light scattering experiments were performed to determine the fat globule size distributions in the bovine, goat and sheep milks. The apparatus used (Mastersizer 2000, Malvern Instruments, Malvern, U.K.) was equipped with a He/Ne laser (λ = 633 nm) and an electroluminescent diode (λ = 466 nm). The refractive indices of milk fat were set at 1.46 (at 466 nm) and 1.458 (at 633 nm). The refractive index was set at 1.33 for water. Whole milk samples were introduced into the measurement cell of the apparatus in order to reach the optimal condition of 7% obscuration. A solution of 35 mM EDTA/NaOH pH 7.0 buffer (>98% disodium salt dihydrate, Prolabo, Fortenay-sous-Bois, France) was added to the measurement cell to disrupt the casein micelles. All analyses were performed in triplicate. The specific surface area and volume-weighted average diameter d_{43}, defined as $\Sigma n_i d_i^4/\Sigma n_i d_i^3$, where n_i is the number of fat globules of diameter d_i, were calculated by the Malvern software.

2.4. Microstructural analysis

Microstructural analysis was performed using an inverted microscope NIKON Eclipse-TE2000-C1si (NIKON, Champigny sur Marne, France). Confocal laser scanning microscopy (CLSM) was used with a He-Ne laser operating at 543 nm wavelength excitation and emission detection between 565 nm and 615 nm. A ×100 (numerical aperture NA 1.4) oil immersion objective was used for the microstructure observations. The neutral milk lipids (i.e. triacylglycerols) were stained with the lipid-soluble Nile Red fluorescent probe (5H-benzo-alpha-phenoxazine-5-one,9-diethyla mino; supplied by Sigma Aldrich, St Louis, USA; 100 μg/mL in propanediol). About 10 μL of Nile Red was added to 1 mL of each type of milk. The exogenous head-labelled phospholipid fluorescent probe N-(Lissamine rhodamine B sulfonyl) dioleoylphosphati dylethanolamine (Rh-DOPE, Avanti Polar lipids Inc., Birmingham, England) was used at a concentration of 1 mg/mL in chloroform to label the phospholipids in the membrane surrounding the milk fat globules. For observation of the MFGM phospholipids, 20 μL of the Rh-DOPE solution was added per glass vial and the chloroform was evaporated under nitrogen, to avoid the possible artefacts caused by this organic solvent. Then 1 mL of each milk was introduced into the vials containing the Rh-DOPE fluorescent dye and the samples were kept at room temperature for at least 30 min prior to observation by CLSM. The stained samples of sheep, goat and bovine milk were divided over several vials to monitor their temperature and thermal history. Several protocols were used to investigate the microstructure of the MFGM in a range of temperatures between 4 °C and 60 °C, using a temperature-regulated stage (Linkam Scientific Instruments Ltd., Tadworth Surrey, England). In the first set of experiments, the milks were heated at a given initial temperature T_i (i.e. 60 °C or 20 °C) and quenched at the final temperature T_f (i.e. 20 °C or 4 °C; cooling rate dT/dt > 100 °C/min). The milk samples were then either observed directly under the microscope at T_f or stored overnight (~16 h) at T_f and observed on the following day at the same T_f. In the second set of experiments, the milks were stored at 4 °C and then heated under the microscope using the temperature-regulated stage from 4 °C to 60 °C, allowing tracking of changes in the microstructure according to increasing temperature, with

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