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The macrostructure of milk lipids: the fat globules

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Abstract: The aim of this review article is to summarize the information available related to milk fat globules (MFGs) in order to highlight their contribution to the nutritional and technological characteristics of milk and dairy products. The macrostructure of milk lipids is composed of globules made up of triglycerides with different melting points, enveloped by a biological membrane from the mammary epithelial cell**.** In milks of different animal species, there are different sized MFGs, ranging from diameters of less than 0.2 μm to a maximum of 15 μm. The average diameter and the number of globules are affected by endogenous, physiological and exogenous factors. The size of the globules in turn affects the qualitative characteristics of milk and cheese. In addition, the average diameter of the globules and their surface that is exposed to the digestive system affect fat digestibility in different ways. Finally, the components of the milk fat globule membranes have been shown to take part in the secretion process of globules and to have a beneficial effect on human health. In conclusion, by acting on factors influencing the dimensions of the fat globules and by increasing the content of the milk membrane could help adapt milk production to specific consumer targets and improve milk nutritional properties.

Keywords: milk fat globule; milk fat globule membrane; fatty acids; human health; milk and cheese quality.

1. Introduction

In the course of evolution, hypertrophy and specialization of skin glands led to the secretion of milk. Milk evolved as the main energy source for the development and immunological protection of offspring (Vorbach et al., 2006). In addition, milk is an optimal source of nutrients that have important effects on human health and bone metabolism (Caroli et al., 2011).

Of the components of milk, lipids are a class of molecules with different specialized functions and are an important source of energy for the newborn. However lipids are still the least understood cellular biomolecules (German, 2011).

The macrostructure of milk lipids is composed of globules made up of triglycerides with different melting points, enveloped by a biological membrane (Lopez et al., 2011).

The presence of fat globules (MFGs) in milk was first reported in 1674 by Van Leeuwenhoek (quoted by Wiking, 2005) who made the first microscopy analysis on milk placed in a capillary tube. Since then, the chemical, physical and colloidal characteristics of the MFGs have been investigated. The first studies focusing on bovine milk were carried out in the 1960s and 1970s (Walstra 1969; Mulder and Walstra, 1974). Recently there has been interest in the impact of specific foods and food components on health (Lock and Bauman, 2011), which led to the development of new research on milk fat globules.

This reviews summarizes the information available on MFGs in order to highlight their contribution to the nutritional and technological characteristics of milk and dairy products.

2. Methods for characterizing fat globules

Research on MFGs has followed various methods in order to determine the number, diameter, volume, surface area and distribution of the globules.

Indirect methods use the diffraction of the light from emulsions (dynamic light scattering, laser light scattering) and evaluate the number of MFGs and the average diameter through the surface-volume ratio ($d_{vs}\Sigma Nd^3/\Sigma d^2$) (Walstra, 1969; Michalski et al., 2001).

Other indirect techniques such as infrared spectroscopy, near-infrared (NIR) or Raman spectroscopies use the vibrations of the molecules excited by a light source (Argov et al., 2008; Cattaneo et al., 2009; Gallier et al., 2011).

Of vibrational techniques, Raman spectroscopy uses the inelastic scattering that the photons from a laser generate by interacting with the molecules (McGoverin, Rades, & Gordon, 2008). Raman spectroscopy provides information on the structures and their properties by the excitation of the vibro-rotational levels of the molecules (Nafie, 2001).

Many of the techniques based on indirect evaluations of MFGs produce complex primary data (Huppertz and Kelly, 2006). In addition some indirect methods (for example those using light scattering) generate high offset values and low absorptions for some samples (e.g. high-fat samples) due to the capacity of the milk components to scatter the light (Huppertz and Kelly, 2006). Therefore, to avoid interference with other constituents, indirect studies often carry out manipulations of milk, such as the dissociation of casein

micelles by calcium chelating agents (EDTA), or homogenization (Faquant et al., 2005; Cattaneo et al., 2009).

These techniques may alter the native MFGs. In fact intense mechanical treatments (e.g. homogenization and centrifugation) can totally or partially destroy the fat globule membranes (MFGMs) and destabilize the fat causing the casein adsorption by the MFGMs. New membrane structures are thus formed, which give the MFGs different physical and chemical properties (Michalski et al., 2005b).

Direct methods are based on microscopy and use image analysis systems (Scolozzi et al., 2003; Evers et al., 2008, Ong et al., 2010) which measure each visible globule in its *native* conditions. The MFGs are generally stained with fluorochrome substances (e.g. Acridine Orange, Red Nile) thus allowing observation by fluorescence microscopy. Immunohistochemistry techniques (Robenek et al., 2006) and confocal microscopy (Evers, 2004; Lopez et al., 2011) enable structural studies to be performed on MFGMs.

3. Origin and secretion of the milk fat globules

Although there have been several studies on the secretion of MFGs (Wooding, 1971; Heid and Keenan, 2005; Robenek et al., 2006), lipid assembly is not yet completely understood.

As highlighted in breast tissues in biochemical and electron microscopy studies (Mather and Keenan, 1998), triglycerides in the core of MFGs originate as lipid droplets in the double layer of the endoplasmic reticulum (ER), in the mammary epithelial lactating cell (Heid and Keenan, 2005). The lipid droplets are released into the cytoplasm of the breast cell enveloped by the outer membrane of the ER (Mather and Keenan, 1998). Some ER

trans membrane proteins whose domain is not on the side that surrounds the droplets are excluded from the droplets, while other proteins, including (adipophilin and TIP 47), spread from one side of the ER membrane to the other (McManaman et al., 2007). Subsequently, in experimental conditions it has been observed that smaller intracellular droplets (≤0.5 μm) increase in volume producing larger droplets (>1 μm) by fusion . Calcium and gangliosides would seem to regulate or take part in this process (Dylewsky et al., 1984; Vavullah et al., 1988; McManaman et al., 2007).

The lipid droplets therefore move towards the apical region of the cell. The migration occurs through the involvement of actin or cytoskeletal elements (Dylewsky et al., 1984), except for the microtubules (Patton et al., 1977). Once in the apical portion of the cell membrane, the release process consisting in molecular interactions between the droplets and the apical membrane that envelope the droplets (McManaman et al., 2007). MFGs are released by interacting with the membrane proteins (Robenek et al., 2006) and also by the hormones prolactin and oxytocin (Ollivier-Bousquet, 2002).

In fact, *in vitro* studies report that prolactin stimulates the release and metabolism of arachidonic acid (Daudet et al, 1981). The lipoxygenase metabolites of arachidonic acid seem to be involved in the interactions and fusions between cell membranes (Blachier et al., 1988), such as those that occur during the envelopment and the release of the globules. The increased metabolism of arachidonic acid induced by prolactin may affect or regulate the release of MFGs. Besides causing contractions of the epithelial cells that allow milk ejection, oxytocin acts by stimulating the release of triglycerides by the RE in rat mammary cells (Da Costa et al., 1995).

During the envelopment and release of MFGs by the mammary cell membrane, it seems that the cell membrane is rearranged and some proteins and lipids are excluded from forming MFGMs (Evers, 2004, Heid and Keenan, 2005).

4. Endogenous and physiological factors influencing the size and distribution of globules: species, breed, parity, stage of lactation, amount of fat secreted.

In milks of different animal species the MFGs are of various sizes, ranging from a diameter smaller than 0.2 μm to a maximum of 15 μm (Walstra, 1969), with an average diameter between 2 μm and 5 μm in the main livestock species (Walstra 1969; Mulder and Walstra, 1974; Mehaia, 1995; Martini et al., 2010).

It has been suggested that bovine MFGs are made up of three populations (Walstra, 1969; Michalski et al., 2001):

a) one subpopulation includes MFGs with a diameter smaller than 1 μm. These are not easily measurable by the techniques most frequently used and to date have rarely been investigated (Argov et al., 2008; Gallier et al., 2011). It has been estimated that in bovine this subpopulation represents 80% of MFGs, but only about 5% of the milk fat volume (Walstra, 1969). In ruminant milk, globules smaller than 1 μm are generally not included in the casein network during the cheese making process, thus they remain in the residual whey (Vanderghem et al., 2010). Evaluations of this category of globule on human and bovine milk by Raman spectroscopy, show that the MFGs smaller than 1 μm have almost no triglyceride core and are rich in unsaturated fatty acids (Argov et al., 2008; Gallier et al., 2011).

b) a population of medium globules (ranging from 1 to 8 μm) that includes approximately 94% of the fat volume (Walstra, 1969).

c) the remaining 1-2% of fat volume consists of globules with a diameter greater than 8 μm.

Although it is not understood why the fat is excreted as globules of different diameters (Walstra 1969; Walstra and Mulder, 1974), the distribution of the measured MFGs and the average diameter vary as a function of endogenous (species, breed), physiological (parity, stage of lactation) and exogenous factors (feeding) (Mehaia, 1995, Martini et al., 2004; Couvreur et al., 2006, Martini et al., 2009; Salari and Martini, 2009).

Other studies such as in cows describe the average diameter as 3.5-5.5 μm (Mulder and Walstra, 1974; Martini et al., 2003), while the range of average diameters in buffalo species varies between 2.96 (Schafberg et al., 2007) and 5 μm (Menard et al., 2010). In camel milk, the average diameter ranges from 2.66 μm to 4.40 μm (Karray et al., 2004; Mehaia, 1995, respectively).

The range of diameter in ewes is between 2.79 μm (Martini et al., 2012a) and 4.95 μm (Mehaia,1995); while in goats the average diameter of MFGs is smaller than in cows and sheep, and is between 2.2 μm and 2.5-2.8 μm (Attaie and Richer, 2000; Martini et al., 2009; Martini et al., 2010b).

The smaller average diameter found in the caprine species is linked to a lower percentage of larger globules, since 90% of measurable MFGs are smaller than 5μm (Attaie and Richer, 2000; Martini et al., 2009). Similar to findings observed in goats, a percentage of about 90% of MFGs smaller than 4.5 microns has been detected in camels (Mehaia, 1995). In sheep on

the other hand, about 80% of the measured MFGs have been found to be smaller than 5 μm (Mehaia, 1995; Martini et al., 2012a) and in cows 90% of the measured MFGs were smaller than 6.42 microns (Attaie and Richter, 2000). These data show that the average diameter of MFGs follow an ascending order for camels, goats, sheep, and cows.

Differences in the morphometric characteristics of MFGs (number and average diameter) were found in the different breeds of sheep and cows (Mulder and Walstra, 1974; Martini et al., 2006a). A study on Comisana, Sopravissana, Sarda, Massese sheep and crossbreeds showed that breeds more selected for dairy traits (Sarda, Massese) exhibited a higher rate of small MFGs, and a greater number of globules per mL of milk (Bianchi et al., 2004; Martini et al., 2006a).

Research on cows show that the Jersey breed has a larger MFG diameter than Friesian and Brown Swiss cows (Mulder and Walstra, 1974; Martini et al., 2003; Caroll et al., 2006). The average diameter follows a descending order in Jersey, German Holstein and Italian Holstein Friesian cows (Martini et al., 2003). In contrast, a higher average number of MFGs per mLhas been reported for Friesian cows (4.33×10^9 for Italian Holstein Friesian, 4.19×10^9 for German Friesian, and 3.55×10^9 for Jersey) (Martini et al., 2003).

Wiking et al. (2004) measured the daily fat secreted from Danish Holstein cows and found that it was positively related to the average diameter of the MFGs. The relationship between daily fat yield and a larger diameter of the globules was also confirmed by Caroll et al., 2006. In a study of 15 cows from three different breeds (Holstein, Jersey and Brown Swiss) these authors observed that the average diameter of the globules tended to increase in all the breeds linked to the increase in fat secreted, as a result of increasing amounts of fat in the

diet (0 to 45 g / kg of fat included in feed). However, differences in diameter among the breeds were also observed, related to genetic factors. In fact, although Jersey cows had a larger diameter, the daily fat yield secreted was lower than the Holstein with all levels of fat inclusion in the diet.

Similarly, individual variations in diameter (more than 1 μm) have been reported in cows (Mulder and Walstra, 1974). There has been little research on the genetic variability of the characteristics of MFGs, with no studies on the determination of the genetic quota of the morphometric characteristics of MFGs on farm animals.

Concerning the relationship between fat secretion and the diameter of MFGs, Argov et al. (2008) hypothesized that since the secretion of fat involves a loss of resources due to the fact that part of the membrane of the mammary epithelial cell is sacrificed to envelop the globule, larger MFGs may be secreted to reduce the amount of membrane lost by the cell per unit volume of fat (Ollivier-Bousquet, 2002).

Unlike with cows, Martini et al. (2013a) found that during lactation in sheep, MFGs of a smaller average diameter are secreted, as a result of the increase in the amount of secreted fat, which is probably due to energy metabolism. In fact it has been hypothesized that during the first stage of lactation, due to the energy deficit common to dairy animals, the availability of the membrane to envelope the MFGs is reduced, thus leading to the secretion of larger sized globules. On the other hand, with the advance of lactation as the energy balance becomes positive, the mammary gland has greater energy for MFGM synthesis thus causing an increase in globules of a lower diameter (Martini et al., 2013a).

The differences observed between cows and sheep (Wiking et al., 2004; Martini 2013a) may be due to effects on the mammary metabolism of the energy balance and the availability of nutrients (including endocrine changes) which vary between species, thus leading to different responses in the secretions of fat, lactose and proteins (Chilliard et al., 2003). In fact, the effects of energy balance on the metabolism of the mammary gland are not yet fully known (German, 2011).

The increase in the number of MFGs and the decrease in diameter with advancing lactation has been reported in several studies (Wiking, 2004; Salari and Martini, 2009) and opposing correlations between the number of MFGs and the diameter have been observed both in sheep and in cows (Martini et al., 2006d; 2010a; 2012a).

During lactation the average diameter of MFGs decreases from 4.4 to 2.9 μm in cows' milk (Walstra, 1995), and from 3.17 to 2.47 μm in sheep's (Martini et al., 2013a).

A decreasing diameter during lactation has been reported in buffalo, in which the average diameter of MFGs changes by a maximum of 5.8 μm, during the first two months, to 4.7 μm in the final stage of lactation (Scafberg et al., 2007).

5. Exogenous factors affecting the size and distribution of the fat globules

Of the factors affecting the diameter and the number of MFGs in small ruminants, the geographical location of the farms also has a significant effect, linked to different environmental conditions (Martini et al., 2008a).

Studies in cattle have shown that spring pasture (Lolium perenne and Trifolium repens) supplemented with cereals, compared to a diet of equal energy levels consisting of corn

silage with soybean meal supplement, causes a slight decrease in the average diameter of MFG (-0.3 μm) (Couvreur et al., 2007). Decreases in diameter (0.3-0.5 μm of decrease) have also been observed in cows of the same breed as a result of replacing corn silage with increasing amounts of fresh grass (Couvreur et al., 2006), or as a result of the addition on silage corn diet with supplements rumen protected of fish meal (Avramis et al., 2003). In all these studies, the diets did not influence ingestion, but led to a reduction in the amount of milk fat.

A higher forage: concentrate (40:60 vs. 60:40) ratio in isoenergetic and isoproteic rations has been shown to increase fat percentages in sheep and to significantly decrease the percentage of MFGs between 2 and 5 μm (-17.32%), but did not affect the average diameter and the number per mL of MFGs (Martini et al., 2010b). In a study by Caroll et al. (2006), an increase in the diet energy level of dairy cows caused an increase in the diameter of the MFGs.

A similar observation was also made by Wiking et al. (2003), who compared three different diets in terms of fatty acid composition and fat level. They noted that milk form Danish Holstein cows fed a diet consisting of high-fat concentrates and rich in saturated fat, contained more and larger globules.

Season can also affect the average diameter of MFGs and the fatty acid composition of ruminant milk (Asker et al., 1978; Abeni et al., 2005; Martini et al., 2008a). A larger average diameter was measured in bulk ewe milk in spring and summer compared to autumn-winter (Martini et al., 2008a). A seasonal effect on the characteristics of MFGs was also reported in buffalo milk (Asker et al., 1978). Although they did not assess the

average diameter and distribution of MFGs they found an increase in the amount of MFGMs (g/100g fat) during the summer months. Since the increase in the amount of MFGMs occurred in the late lactation, a decrease in diameter was suggested.

6. Fat globules and milk quality

The differences related to the diameter and number of MFGs are of interest with regard to the chemical-physical and nutritional properties of milk and milk products (Michalski et al., 2003; Fauquant et al., 2005).

Martini et al. (2008b) found in sheep that the MFGs that were larger than 5 μm were linked to lower percentages of proteins, dry matter, caseins, lactose, solids not fat, calcium, phosphorus and ash, while smaller MFGs (between 2 to 5 μm) showed opposite relationships, but a higher casein / fat ratio.

Studies on goat's milk (Neveu et al., 2002) found a link between the size of the MFGs and the genotype of the αs1 casein. In fact smaller MFGs seems to be related to a higher frequency of zero alleles (OO) compared to strong alleles (AA), contributing to the hypoallergenic characteristics of goat's milk (Park et al., 2007).

Furthermore, in sheep a higher amount of small globules has been found to be linked to a higher frequency of some alleles of αs1-casein (AC vs. DC) and of β-lactoglobulin (AB vs. AA) (Martini et al., 2006b).

In the Italian Holstein cow, the MFG size distribution appears to be linked to different genotypes of κ caseins, in particular BB genotypes seem to have higher percentages of smaller globules than AB (Martini, et al, 2006c).

7. Milk fat globules and milk fatty acid composition

The diameter of MFGs may affect milk fatty acid composition (Timmen and Patton 1988; Wiking et al., 2004; Martini et al., 2012a). Differences have been detected in the milk fatty acid composition in cows assigned to two groups on the basis of the MFG diameter: the first group included subjects that secreted small MFGs (average diameter of 3.44 μm), the second group included subjects that secreted larger MFGs (average diameter of 4.53 μm) (Couvreur, et al., 2007). The differences in fatty acid composition observed between the groups were found to be due to the diameter of the MFGs.

In bovine milk a higher proportion of polyunsaturated fatty acids (PUFA) in smaller globules than in larger globules has been found (Lopez et al., 2011), but with no differences in the total content of unsaturated and saturated fats. In contrast, positive correlations between PUFA and large globules (> 5 μm) have been reported for ovine milk (Martini et al., 2012a).

Lopez et al. (2011) separated globules of different diameters and reported higher percentages of polyunsaturated fatty acids (PUFAs) in smaller than in larger bovine globules, while no differences were observed in the total content of saturated and unsaturated fatty acids. In contrast, positive correlations between PUFAs and large globules (> 5 μm) were found for ovine milk (Martini et al., 2012a).

The ratio of linoleic acid / linolenic acid in cows is significantly higher in larger globules (Lopez et al., 2011) and positive correlations between linoleic acid / linolenic acid and the percentages of large globules have also been observed in sheep milk (Martini et al. 2012a). Lopez et al. (2011) found that in bovine milk, beyond the ratios, the individual content of

linoleic and linoleinic acids does not vary significantly with MFG diameter (); whereas Briard et al. (2003) reported a higher content of linoleic acid in large globules .

A positive relationship between the content of omega 6, of which linoleic acid is the precursor, and the percentage of large globules has been reported for ovine milk and opposing relationships have been described between omega 6 and percentages of small globules (Martini et al. 2012a).

Short chains are contained in higher quantities in milk characterized by large sized fat globules, as was revealed from an analysis of correlations between the diameter of MFGs and fatty acids in sheep, and as also observed in cow's milk (Timmen and Patton, 1988; Martini et al., 2012a).

Contrary to what has been shown by the correlations in sheep's milk (Martini et al., 2012a), greater amounts of lauric acid have been detected in small MFGs (1.6 μm) separated from bovine milk (Lopez et al., 2011). Moreover, in cows, the amounts of myristic and palmitic acids have been shown to decrease with the decrease in size of the MFGs (Lopez et al., 2011; Briard et al., 2003).

Small MFGs in cows also contain less stearic acid (Timmen and Patton 1988; Lopez et al., 2011) compared to the large globules, and larger amounts of unsaturated fatty acids with 18 carbon atoms, such as oleic, vaccenic, and rumenic acids (Timmen and Patton 1988; Lopez et al., 2011). However, other studies on the same species have reported a higher content of oleic acid in large globules (Briard et al., 2003). Different results have been highlighted in sheep, where large globules (greater than 5 μm) have been found to be positively correlated to the content of vaccenic acid (Martini et al., 2012a).

A greater ratio C14:1/C14:0 and C18:1/C18:0, which is indicative of the activity of the enzyme delta-9 desaturase, has also been detected in small MFGs in cows. This suggests that the different activity of delta-9 desaturase could be one of the physiological mechanisms underlying the secretion of globules of different sizes (Lopez et al., 2011). Furthermore, the differences observed between cows and sheep support the existence of different mechanisms of secretion between the two species (Lopez et al., 2011; Martini et al., 2013a).

Some authors have speculated that the differences in the fatty acid composition between MFGs of different sizes in cows are primarily due to the core of MFGs that contain most of the lipids in milk (Briard et al., 2003; Faquant et al., 2005). In fact Faquant et al. (2007) did not find any significant differences in the fatty acid composition of the membranes extracted from the different sized MFGs obtained by microfiltration, however they reported changes in the concentration of fatty acids with more than C 14 in the core with increasing sizes of the MFGs. According to Faquant et al. (2005), the core of the small MFGs contains more C12:0 (14.1%), C14:0 (15%), C14:1 (128%), C16:0 (13.3%), C16:1 (120.2%), C21:0 (116%), C20:3 n3 (18.6%) and less C18:0 (220.5%) and C20:5 n3 (25%) compared to large globules.

Membrane lipids also contribute to the characterization of the lipid fraction of milk (Jensen and Nielsen, 1996), albeit to a lesser extent. In fact, the MFGM fatty acid composition is different compared to the core (Faquant et al., 2005; Martini et al. 2012b) and the different composition of milks with MFGs of different diameters could also be due to the core:membrane ratio (Martini et al., 2013a).

The membrane lipids contribute more as the MFG diameter decreases because the surface area per unit of volume is greater for smaller globules (Timmen and Patton, 1988). In fact in sheep membranes, lipids increase by 33% as a result of the decrease of 0.4 μm in the diameter of MFGs after 30 days of lactation (changing from 1.44 mg / mL of milk to 1.92 mg / mL) (Martini et al., 2013a).

In addition, cow MFGs with a diameter of 1.6 μm have a surface area equal to 4.4 m^2 /g of fat, whereas the surface area of the MFGs measuring 6.6 μm is 1 m^2 /g of fat (Lopez et al., 2011).

8. Globules and fat digestibility

The diameter of the MFGs has a different effect on the way in which fat is digested and metabolized (Michalsky et al., 2005b).

In fact, the MFGM maintains the emulsion of the MFGs, prevents aggregation and protects fat from oxidation (Walstra and Jennes, 1984). In addition when ingested, the MFGM interacts with the gastrointestinal system (Lopez et al., 2011), and some interactions relate to anti-infectious and anti-adhesive effects in the gut (Vesper et al., 1999).

Firstly , however, the MFGM is exposed to the attack of digestive enzymes, thus the different amount and quality of the surface exposed to the enzymes affect the digestive kinetics in different ways (Michalsky et al ., 2005b).

Some studies on lipid metabolism in rats have used breath tests. In a study by (Michalsky et al., 2005b), the increase in 13C during and after digestion of a meal enriched with 13C- was measured. The test showed that digestion was slower when an anhydrous fat emulsion

was given to animals, in which lipid droplets were covered by casein, compared to lipid droplets coated by phospholipids and non-emulsified fat.

Other studies reported a faster metabolism and gastric emptying in human adults with the ingestion of large native MFGs (Armand et al., 1999). Given the presence of large MFGs in ewe colostrum, it would seem that MFGs are better and more easily digested (Martini et al., 2012b), suggesting that the secretion is adapted to the immature digestive system of the offspring (Michalski et al, 2005a). However, the data in the literature regarding the effects of the MFG diameter on digestibility are conflicting. For example Armand et al. (1996) did not find an effect of the type of diet on the rate of gastric emptying. However in children they found a more efficient gastric digestion of human milk fat compared to infant formulas. Cavell (1981) on the other hand, showed a slower gastric emptying with infant formulas. In contrast the homogenization of pasteurized human milk seems to improve intestinal absorption in very low birth weight children (Pimenteira Thomaz et al., 1999).

Discrepancies between the different studies seem to be due to the different composition of the interface of MFGs, in fact the membranes that have absorbed caseins remain trapped in the clot in the stomach (Michalski et al., 2005a); thus, the native MFGMs reduce the emptying of the stomach. In fact, it seems that lipid droplets enveloped by the native membrane do not interact with caseins and can be more easily drained (Michalski et al., 2005a).

According to other authors, the smaller native MFGs may have the best digestive parameters, as has been reported for goat's milk compared to cow's milk (Ljutovac-Raynal

et al., 2008). The smaller sized globules in goat have been found to have a beneficial effect on fat and energy assimilation in malnourished children (Raynal-Ljutovac et al., 2008).

Given the lack of studies comparing the digestibility of different sized MFG in vivo and the presence in the milk of several components that interact with the digestive system, further research is needed to better define how the characteristics of fat affect digestion and metabolism.

9. Effects of the diameter of milk fat globules on the characteristics of dairy products

MFGs are responsible and / or contribute to some properties and phenomena in milk and dairy products. In fact, MFGs influence milk viscosity and stability of the emulsions, the creaming capacity of milk, the rheological, the chemical and the organoleptic properties of cheese and butter (Huppertz and Kelly, 2006).

In cheese, MFGs remain entrapped in the casein network during the formation of the clot, and protein-protein and protein-fat globule interactions affect the structure of the curd and the ability to retain water (Goudédreanche et al., 2000; Lopez and Dufour, 2001; Michalski et al., 2003).

In ewes, a greater percentage of MFGs with a diameter of between 2 and 5 μm seems to favor a better cheese yield at 24 h and to reduce weight loss during ripening (Martini et al., 2008b). This could be due to the larger membrane surface, which is able to bind more water, and similar results were found in cow's milk (Fannema and Powrie quoted by Goudédreanche et al., 2000; Wiking et al. 2004).

Studies carried out in sheep have shown that MFGs larger than 5 microns make rheological parameters worse, due to negative correlations with the casein / fat ratio and with the

percentage of casein, calcium and phosphorus, which in turn influence the consistency of the clot and the cheese yield (Martini et al., 2008b).

Furthermore, native MFGs of different sizes determine dairy products with different sensory characteristics (Michalski et al., 2003). The differences in the diameter of MFGs between cows and goats contribute to the softer texture of goat's cheese (Silanikove et al., 2010).

Several authors have selected smaller MFGs for cheese-making by milk skimming or tangential microfiltration (Xiong and Kinsella, 1991; Michalski et al., 2003, 2004a; O'Mahony et al., 2005). Smaller globules produce less rigid, softer, more elastic cheeses, with higher humidity, greater proteolysis, lower lipolysis (Michalski et al., 2004a, 2004b) and a more yellow color. It seems that larger MFGs may be more sensitive to the mechanical effects, and therefore more susceptible to lipolysis and to the formation of specific aromas (Carroll et al., 2006).

Furthermore, different product characteristics are obtained depending on whether or not MFGs have undergone homogenization. In fact, by virtue of the composition of their interface (native or non-native MFGMs), MFGs interact differently with the casein network (Michalski et al., 2006), for example the Emmental obtained from homogenized MFGs showed a higher lipolysis (Michalski et al., 2004b).

The diameter of MFGs influences the rate of milk creaming (Couvreur et al., 2006), affecting both the butter processing, and cheese production such as Parmesan production, in which evening milk is skimmed. The creaming increases with the increase in the MFG diameter, although the formation of clusters between the smaller MFGs and the lower

temperature increases the potential for creaming (Huppertz and Kelly, 2006). In this regard, cow MFGs, unlike other types of milk, are more susceptible to cold agglutination (Huppertz and Kelly, 2006).

In butter there is a multiphase emulsion that includes MFGs, crystalline fat and an aqueous phase dispersed in a continuous fat phase (Huppertz and Kelly, 2006).

During the transformation and churning, the MFGs break, coalescence occurs and the fat forms a continuous phase (Wright et al., 2001), which contains aggregates of crystals between intact and damaged MFGs (Kalab, 1985). The smaller MFGs negatively influence the churning (Alais, 1984), and also tend to remain in the serum after centrifugation (Karray et al., 2004). To confirm this, the smaller diameter of MFGs in camels and goats (Silanikove et al., 2010) make the manufacturing of butter difficult in these species.

Furthermore, the smaller the diameter of the MFGs, the lower the temperature required for the crystallization of the fat (Lopez et al., 2002). This process, by influencing the extent of solidification and the solid-liquid fat ratio, has an effect on the product's consistency (Rohm and Weidinger, 1993). Poor crystallization adversely affects the butter yield, viscosity and elasticity. These characteristics are linked to the networks of fat crystals associated with the continuous phase. Furthermore, crystallization takes place in a more disordered way and with the formation of smaller fat crystals when smaller MFGs are present (Huppertz and Kelly, 2006). The different surfaces of different sized MFGs and the different MFG interfaces also influence the presence of water in the butter. In fact, the small water drops increase the rigidity of the system (Prentice 1992).

10. The milk fat globule membrane

The MFGM is a triple membrane resulting from the mammary secretory cell that surrounds a core of triglycerides distributed in a lamellar way (Heid and Keenan, 2005). The MFGM is about 10-20 nm thick and accounts for 2-6% of the globule mass (Heid and Keenan, 2005). MFGM consists of different classes of lipids (phospholipids, triglycerides and cholesterol) and of several proteins and enzymes (Evers, 2004; Lopez et al., 2011).

Besides conveying fat in an aqueous environment, MFGM is a dietary source of functional substances and is considered as a nutraceutical (Dewettinck et al., 2008).

The functionality of the MFGM is provided by its content of phospholipids, sphingolipids, fatty acids and proteins with an antibacterial effect (such as xanthine oxidoreductase and mucins) and / or health benefits (Dewettinck, et al., 2008).

10.1 Lipid components of milk fat globules

Given the beneficial properties of the MFGM, its content in milk is important from a nutritional point of view (Martini et al., 2013a) and there are more MFGM lipids in ewe's milk than in cow's (1.64 vs. 0:36 mg / g of milk), in fact the fat content differs between the two species (Fong, et al., 2007; Martini et al., 2013a).

The polar lipids from the MFGM mainly include phosphatidylcholine (PC; 19.2-37.3%), phosphatidyl ethanolamine (PE; 19.8-42%), sphingomyelin (SM; 18.0-34.1%), phosphatidyl inositol (PI; 0.6-13.6%) and phosphatidylserine (PS; 1.9-16%) (Rombaut and Dewettinck, 2006; Ménard et al., 2010). In cow membranes small amounts of glucosyl lattosil and cerebrosides have also been found (Fong et al., 2007). Glycerophospholipids are rich in unsaturated fatty acids (C18: 1, C18: 2, C18: 3), and regulate the fluidity of the

membrane given that they have a low melting point. Sphingolipids, above all sphingomyelin, are characterized by a sphingoid base, which contains high melting point saturated fatty acids (C16: 0, C22: 0, C23: 0, C24: 0) (Fong et al., 2007).

Given the bioactivity of sphingolipids, researchers have attempted to enrich milk in sphingolipids through nutritional strategies and animal management, but without success (Graves, et al., 2007). One strategy of sphingolipid enrichment could be the concentration of the membranes from buttermilk, or using ultrafiltration, to select milk characterized by smaller MFGs with a greater surface (membrane) area per unit of fat volume.

Differences have been found in the MFGM in the content of individual classes of polar lipids, and the differences depend on the size of the MFGs (Lopez et al., 2010). The amounts of PE, PI, PS, PC and SM per g of fat are higher in smaller MFGs (Lopez et al., 2011). In addition, in whole milk and in larger MFGs, choline-containing lipids (PC and SM) are mainly present, while in the smaller MFGs the most important lipid is PE (Lopez et al., 2011).

These differences may arise either through secretion (Lopez et al., 2011) or by post secretory rearrangements of the MFGM (Evers, 2004).

In fact, the secretion of small MFGs may perhaps induce changes in the double layer of the mammary cell membrane, leading to a loss of material (Lopez et al., 2011). PC and SM, which are arranged more externally in the membrane (Lopez et al., 2011), might be lost because of a greater curvature of the surface (Deeth, 1997; Lopez et al 2011). Alternatively, it has been suggested that some rearrangements may lead to the loss of PC

and SM by vesciculation and by the formation of microsomal-like particles, which are subsequently removed from the globules (Evers, 2004).

In confirmation of the second hypothesis, Deeth (1997) reported that polar lipids are not uniformly arranged in the MFGM , but PE, PS and PI are located in the inner face of the MFGM, while PC, SM and glycolipids are in the outer double layer. Confocal microscopy studies confirm this heterogeneity (Lopez et al., 2010).

In fact, Lopez et al. (2010) observed that the glycolipids and glycoproteins are heterogeneously distributed in a liquid phase, and that the glycoproteins protrude in the aqueous phase to form a glycocalyx that surrounds the MFGs, while the SM and cholesterol form ordered domains. SM domains are dynamic structures and are mobile on the plane of the double-layer liquid (Lopez et al., 2011). The heterogeneous distribution of both glycolipids and glycol phospholipids could partly be caused by the change in the MFG budding from alveoli in different physiological states in the course of lactation (Molenaar et al., 1992).

Some studies evaluated the fatty acid composition the MFGM compared to the core. They compared, the same weight of the MFGM and the core isolated from the same milk. It was found that the membranes of cow and sheep milk are characterized by higher contents of long chains compared to the core (Jensen and Nielsen, 1996; Faquant et al., 2005, Martini et al., 2013a). The ewe MFGM is also richer in PUFA (+48.66%) (Martini et al., 2013a). In addition, considering the unsaturated / saturated ratio, in sheep compared to cows, this ratio is greater in the core than in the MFGM (0.437 vs 0.386) (Martini et al., 2013a; Jensen and Nielsen, 1996).

The lower unsaturated / saturated ratio in ewe MFGM is due to the fact that MFGM is richer in saturated fatty acids (72.89% vs 70.85% in the core), especially C16:0 (+21.5%) and C18: 0 (+67.64%). A high content of C18:0 has also been detected in cow membranes (Faquant et al., 2005). In cow MFGM, there are also higher contents of some unsaturated fatty acids such as C18:1 cis-9 and C18:2 cis 9.12. In contrast, these fatty acids have been found in higher percentages in the core from ewe's milk (Jensen and Nielsen, 1996).

Greater amounts of essential and beneficial fatty acids have been found both in ewe's and cow's milk membranes compared to the core such as: CLA cis-9 trans-11, C18: 3 n3 and C22: 6 (Jensen and Nielsen, 1996; Martini et al., 2013a). The ewe MFGM also contains higher quantities of the essential fatty acid C20: 4 (+89.19%) and C24: 0 (+ 1 475%). The latter results from the membrane sphingomyelin (Lopez et al., 2011; Martini et al., 2013a). In addition, in the MFGM a higher n3/n6 ratio has been found than in the core (0.163 vs. 0.075) (Martini et al., 2013a). The higher n3/n6 ratio in the MFGM could contribute to the positive effects of dairy products on human health, since it is advisable to reduce the intake of n6 in the human diet (EFSA, 2010).

10.2 Major proteins of the milk fat globule membrane

Many of the MFGM proteins are known to be involved in membrane trafficking, in the synthesis and transport of proteins, in the metabolism and transport of fat, in enzyme activities and in the transmission of cellular signals, in immune functions and apoptosis

(Pisanu et al., 2011). Some can be considered as indicators of patho-physiological states of the epithelial cells (Bianchi et al., 2009).

MFGM proteins represent a small percentage of milk nitrogen components, approximately the 0.48% of the average value of milk proteins in sheep (Martini, unpublished data).

Xanthine oxidoreductase (XOR), butyrophilin (BTN) and adipophilin (ADPH) make up more than 70% of MFGM proteins (Reinhardt and Lippolis, 2008, Bianchi et al., 2009) and are involved in the secretion of fat globules (Heid and Keenan, 2005; McManaman, et al., 2007). BTN is a transmembrane glycoprotein that seems to have a direct role in the assembly of the droplets, in the transport and in the interaction between the cell membrane and the lipid droplet (Franke et al., 1981). Since BTN and XOR are present in a constant molar ratio in the membrane of the same species, and this ratio is 4:1 in cows (Mondy and Keenan, 1993) and 1.44:1 in sheep (Pisanu et al., 2011), it has been hypothesized that these two proteins form complexes with a high molecular weight (Ye, et al., 2002).

It has been suggested that BTN binds the XOR (Mather and Jack, 1993), which leads to the formation of dimers of the BTN in the mammary epithelial cell membrane. These BTN dimers form complexes that cause deformations in the membrane and the envelopment of the lipid droplet (Mk Manaman et al., 2007). It has been suggested that oligomers of BTN interact with ADPH, XOR or other proteins (Manaman et al., 2007). Alternatively BTN may mediate the secretion of fat globules by indirectly interacting with XOR, working as a receptor of reactive oxygen species generated by XOR (Jack and Mather, 1990; Oggs et al., 2004).

The increased activity of XOR detected when smaller MFGs are secreted (Martini et al., 2013b), may confirm that XOR is involved in the secretion of the globule.

Another hypothesis results from the observation of divergent distributions of XOR, BTN and ADPH proteins in the MFGM. It has thus been suggested that BTN acts as a single mediator secretion through the BTN molecules in the single layer of the droplet and those in the double layer of the cell membrane (Robenek et al., 2006). It is not known why the BTN, which is an integral membrane protein, is found in the single layer of the lipid droplet. It has been suggested that the BTN present in a soluble form in the cytoplasm or transported by vesicles may accumulate on the surface of the droplet or between the droplet and the plasma membrane (Mather and Jack, 1993).

Although some authors maintain that BTN modulates the response of encephalitogenic T cells and is involved in autoimmune diseases (Riccio, 2004), others have suggested that BTN prevents or suppresses the clinical manifestations of experimental allergic encephalomyelitis in mice (Dewettinck et al., 2008).

XOR is a protein derived from cytoplasm and associated with the inner face of the apical membrane (Bianchi et al., 2009). Several inactive forms of the enzyme XOR have been reported in milk (Harrison, 2006). It has also been suggested that this enzyme is affected by activation and deactivation in the course of lactation (Benboubetra et al., 2004).

Some authors have described anti-inflammatory and bactericidal actions of the XOR (Harrison, 2006), either by the inhibition of bacterial adhesion to the enterocytes (Ito et al.,

1993), or by its enzyme activity. In fact, it seems that the enzyme catalyses reactions (Hancock et al., 2002) that lead to the production of peroxynitrite and nitric oxide, both chemical compounds with bactericidal actions.

However, the absence of the oxidase activity of XOR in ewe colostrum (Martini et al., 2013b) suggests that the enzyme is not expressed in early lactation (Liao et al., 2011) or, if present, it probably does not have an antibacterial role, at least in the initial stages of lactation. In fact as is known, the protective role of the newborn is mainly linked to other components, primarily immunoglobulins.

The dehydrogenase activity of XOR does not seem to vary significantly during lactation, either in ewe's milk (Martini et al., 2013b) or in cow's (Heinz and Reckel, 1983). In fact it seems that the enzyme XOR is present mainly in the form of oxidase, both in the mammary gland and in milk. (Heinz and Reckel, 1983).

ADPH is another of the main MFGM proteins. It is localized in the inner face of the monolayer of polar lipids, and is closely associated with the membrane (Heid and Keenan, 2005). This protein has a high affinity for triglycerides and is acylated with myristic, palmitic, stearic and oleic acids (Quaranta et al., 2001) the main fatty acids present in the ewe's MFGM (Martini et al., 2013a). ADPH seems to be involved in the transport of fatty acids, and is present in other tissues that accumulate lipid droplets, as well as in the breast tissue (Gao and Serrero, 1999; Riccio, 2004).

10.3 Other MFGM proteins

Mucin-1 (MUC1) is a high molecular weight protein, consisting of hydrophobic peptites and highly glycosylated peptides which enable it to bind more water (Fong and Norris, 2009). The number and position of MUC1 glycosylation sites are well conserved in sheep (Rasero et al., 2007). The MUC1 has a strong allelic polymorphism in humans, in which 30 different alleles have been reported, compared to only 4 alleles in sheep (Rasero et al., 2007).

It has been reported that MUC1 is upregulated on the seventh day of lactation rather than the first (Reinhard and Lippolis, 2006). Since MUC1 seems to have antibacterial and antiviral actions (Kvistgaard et al., 2004), downregulation of MUC1 in colostrum may indicate that it does not play an antiinfective role in the initial mammary secretion. This finding is confirmed by Martini et al. (2013b) for the XOR.

Another MFGM protein with enzymatic activity is the alkaline phosphatase (AP). This enzyme is present in milk in part dispersed in the milk aqueous phase and resulting from the mammary myoepithelial cell, and in part associated with MFGM and acquired at the intercellular level (Rankin et al., 2010). A positive correlation has been observed in sheep between the activity of the AP and the number of globules per mL (Martini et al., 2010a), thus confirming the link between AP and the MFGM, where there are also significant quantities of phosphatidylethanolamine, a substrate of AP (Whyte, 1994). It is well known that AP is a stable enzyme at slightly higher temperatures than those necessary to kill the pathogens in milk and this enzyme is the most important indicator of adequate pasteurization and the hygienic safety of milk (Rankin et al. 2010). In any case the legal

limit for the negative test for AP activity (350 mU / L of milk) is currently only defined by European legislation for bovine milk (EC Regulation 1664/2006). Sheep's milk, however, presents higher values of AP activity than bovine (Rankin et al., 2010), although the AP in sheep may be more sensitive to thermal inactivation (Anifantakis and Rosakis, 1983). Rankin et al. (2010) described three AP isoenzymes (α, β and γ); the one associated with the outer face of the MFGM being responsible for the reactivation of AP activity after heat treatment. The same authors also reported that AP may have an effect on cheeses maturation, as it seems to dephosphorylate caseins.

Glycoprotein ecto 5 'nucleotidase (5'-N) is an integral MFGM protein that has not yet been sufficiently characterized, with few data regarding 5'N activity in milk (Snow et al., 1980). 5'-N plays a major role in the catabolism of mononucleotides (AMP) and dinucleotides (NAD) (Fini et al., 1986). The activity of 5'-N shows a tendency to increase during lactation (Martini et al., 2013b).

γ-glutammiltranspeptidasi (γ-GT) is a MFGM protein known to be one of the many enzymes of colostrum (Swaisgood, 1995). In sheep γ-GT activity has been positively correlated with immunoglobulin content. γ-GT activity is higher at the beginning of lactation in both sheep and cows (Martini et al. 2012d; Grün et al., 1992), therefore, it is considered indicative of the quality of this initial secretion. The plasma levels of γ-GT in newborn animals are used as indicators of the intake of colostrum (Baumrucker et al., 1994). It also been hypothesized that γ-GT is involved in the synthesis of colostrum, in particular in the transport of amino acids (Johnston et al., 2004).

Another MFGM protein is PAS 6/7 (lactadherin in humans or MGF-E8 in mice). It is acylated or phosphorylated (Mather, 2000) and seems to have a role in the secretion of fat, in the budding or shedding of the cytoplasmic membrane (Oshima et al., 2002). PAS 6/7 also could inhibit the infectivity of pathogens (Bojsen et al., 2007).

11. Conclusions

Several methods have been used to study the physical characteristics of milk fat globules. An inverse relationship between the number of globules and the diameter has been found, and both the number of globules and the diameter are influenced by endogenous, physiological and exogenous factors. The characteristics of the fat globules affect milk quality and the digestive parameters. In addition, the size of the globules affects the cheese-making aptitude, the organoleptic characteristics and the quality of cheeses. The fat globule membranes are beneficial for human health, in terms of the lipid and protein components. In conclusion, acting on the factors that influence the dimensions of the fat globules and increasing the milk membrane content, could help adapt milk production to specific consumer's targets and thus improve the nutritional properties of the milk.

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