

## Composition, Properties and Nutritional Aspects of Milk Fat Globule Membrane – a Review

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In the last few years, knowledge on the composition and properties of the milk fat globule membrane (MFGM) increased significantly. It is now recognized that the MFGM is highly complex in structure and composed of different protein and lipid components with specific technological and nutritional properties. As such, MFGM materials have been isolated and characterized as valuable ingredients for incorporation into new food products. However, MFGM are also sensitive to modification during isolation and processing, and care should be taken to standardize the composition and characteristics of the membrane to maintain its unique properties during application in food products.

The MFGM is subject to changes in composition and structure from the moment the fat globule leaves the mammary secretory cell. Upon milk harvesting and further milk handling, further changes to the MFGM take place. Depending on the type and degree of treatment, this may involve different physico-chemical interactions between various membrane components, the loss of membrane components and/or adsorption of components from the milk plasma. However, the effects appear to be variable and dependent on physiological (animal) factors, and much remains to be learned about the phenomena on a molecular level.

### INTRODUCTION

Milk fat, composed mainly of triglycerides, is secreted as droplets of variable sizes. In the milk of cows, 99% or more of the total lipid is found in these droplets, which are called milk fat globules (MFG). These fat globules are formed throughout the mammary epithelial cell, grow in size as they move toward the apical cell membrane, and are extruded into the alveolar lumen [Buchheim, 1986; Keenan & Dylewski, 1985; Mulder & Walstra, 1974]. During the extrusion process, the globule is enveloped by portions of the cell membrane that becomes the milk fat globule and membrane (MFGM). This membrane, about 10–20 nm in cross-section, acts as an emulsifier and protects the globules from coalescence and enzymatic degradation.

MFGM is highly structured and contains unique polar lipids and membrane-specific proteins. Sphingolipids (highly bioactive molecules, mainly present in polar lipids from animal origin) account for up to one third of the MFGM polar lipid fraction. Scientific evidence on the nutritional benefits of these sphingolipids is accumulating. MFGM proteins represent only 1–4% of total milk protein content; nevertheless, the MFGM consists of a complex system of integral and peripheral proteins, enzymes, and lipids. MFGM proteins have been reported to play an important role in various cellular

processes and defense mechanisms in the newborn [Cavalletto *et al.*, 2006].

The stabilizing membrane acts as reactive sorts on the interface between the barriers of milk serum. As such, it can be globule and rate-controlling for a host of physical and of enzymes chemical interactions, *e.g.*, binding mace elements; controlled release of the polar materials products of lipolysis; transfer of milk serum; maintenance of emulsion into stability by prevention of globule fusion; availability of fatty acids and cholesterol for micellar absorption in the small intestine; and destabilization by creaming, clumping, churning, freeze-thaw, and heating, resulting in loosely bound substances into milk transfer serum. Worthy of notice is that the interactions are dynamic.

Moreover, it is assumed that the MFGM proteins also possess specific nutritional properties. As such, due to their origin, composition and structure, MFGM polar lipids and proteins could be used as an emulsifier or stabilizer, combining technological and nutritional functionality [Mulder & Walstra, 1974; Anderson & Cawston, 1975; Patton & Keenan, 1975; Patton & Jensen, 1975, 1976; Keenan *et al.*, 1983; Mather & Keenan, 1983; McPherson & Kitchen, 1983; Walstra & Jenness, 1984; Keenan & Dylewski, 1985, 1995; Buchheim, 1986; Mather, 1987; Keenan *et al.*, 1988; Keenan & Dylewski, 1995; Keenan & Patton, 1995; Mather & Keenan, 1998; Danthine *et al.*, 2000; Keenan, 2001; Keenan & Mather, 2002; Ollivier-Bousquet, 2002; Lopez *et al.*, 2006, 2007; Sanchez-Juanes *et al.*, 2009; Zamora *et al.*, 2009].

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This review focuses on the knowledge on the MFGM relating to the origin and formation, isolation and purification techniques from milk, composition, structure, the technological aspects and applications as well as finally, the nutritional aspects of MFGM lipids and proteins.

## FORMATION AND SECRETION OF MILK-FAT GLOBULES BY THE MAMMARY SECRETORY CELL

### Origin of milk-fat globules

The processes involved in the formation and secretion of fat globules have attracted considerable research attention over the last 50 years. The origin and the secretion of milk fat globules have been covered in many reviews [Bargmann & Knoop, 1959; Patton & Jensen, 1975; Franke *et al.*, 1981; Keenan *et al.*, 1983; Mather & Keenan, 1983, 1998; Mather, 1987; Keenan *et al.*, 1988; Aoki *et al.*, 1994; Keenan & Dylewski, 1995; Keenan & Patton, 1995; Keenan, 2001; Heid & Keenan, 2005].

Intracellular fat globule precursors appear to originate from the endoplasmic reticulum [Zaczek & Keenan, 1990] and to assemble into globules of various sizes ranging from less than 0.2  $\mu\text{m}$  to greater than 8  $\mu\text{m}$  [Mather & Keenan, 1998; Ollivier-Bousquet, 2002], which migrate through the mammary secretory cell to the apical plasma membrane by, as yet, unidentified mechanisms [Keenan, 2001; Ollivier-Bousquet, 2002]. The intracellular fat globule is surrounded by a diffuse interfacial layer, the composition of which includes phospholipids, glycosphingolipids, cholesterol and proteins [Keenan *et al.*, 1983; Kanno, 1990; Keenan & Dylewski, 1995]. The distribution of cholesterol between the fat globule core lipid and the fat globule interfacial layer has not been established [Keenan & Dylewski, 1995].

When the fat globule approaches the plasma membrane, a dense-staining layer of 10–20 nm between the fat globule and the plasma membrane is seen by electron microscopy [Wooding, 1971a]. This layer consists mainly of protein, including xanthine oxidase (XO), butyrophilin (BTN), adipophilin (ADPH) [Mather & Keenan, 1998] and possibly a class of low molecular mass guanosine triphosphate-binding proteins [Keenan & Dylewski, 1995; Ollivier-Bousquet, 2002]. The generally accepted mechanism for the excretion of the fat globule from the cell (a process that is sometimes called “budding”) is *via* progressive envelopment of the fat globule by the apical plasma membrane of the secretory cell. The latter is a true bilayer membrane.

Hence, the MFGM originates from several distinct layers with total thickness varying between approximately 10 and 20 nm [Walstra *et al.*, 1999]. As viewed from the lipid core outwards, there is first an inner surface-active layer that surrounds the intracellular fat droplet, then a dense proteinaceous coat located on the inner face of the bilayer membrane and finally a true bilayer membrane [Keenan & Mather, 2002]. In electron micrographs of globules that were in the process of being secreted by the cell [Wooding, 1971a], the dense coat and the innermost interfacial layer could not be distinguished from one another. Whether this was due to limitations in electron microscopy technology, to a merging of the two layers into one coat or to loss of membrane material is not clear [Keenan & Mather, 2002].

As the bilayer membrane of the MFGM is derived from the apical plasma membrane of the secretory cell. Some corroborating evidence for the applicability of the fluid mosaic model to the MFGM may be derived from nuclear magnetic resonance studies, which indicate that MFGM proteins have a highly ordered structure [Chandan *et al.*, 1972]. Further, the very low interfacial tension between the fat globule core and the milk plasma that results from the presence of the MFGM [Phipps & Temple, 1982] is indicative of a somewhat ordered membrane [van Boekel & Walstra, 1989].

The cream fraction of milk comprises droplets of triacylglycerol coated with cellular membranes. These droplets are formed and secreted from mammary epithelial cells during lactation. This secretory system is especially interesting because the assembled lipid droplets are secreted from the cytoplasm enveloped by cellular membranes. In other cells, such as hepatocytes and enterocytes, lipid is secreted by exocytosis from membrane-bounded compartments of the secretory pathway. Milk lipids originate as small droplets of triacylglycerol, synthesized in or on the surfaces of rough endoplasmic reticulum (ER) membranes. These droplets are released into the cytoplasm as microlipid droplets (MLDs) with a surface coat of protein and polar lipid. MLDs may fuse with each other to form larger cytoplasmic lipid droplets (CLDs). Droplets of varying size are transported to the apical cytoplasm by unknown mechanisms and are secreted from the cell coated with an outer bilayer membrane. CLDs may increase in size in all regions of the cell, especially at the plasma membrane during secretion. Two possible mechanisms for lipid secretion have been proposed: an apical mechanism, in which lipid droplets are enveloped with apical plasma membrane, and a secretory-vesicle mechanism, in which fat droplets are surrounded by secretory vesicles in the cytoplasm and are released from the surface by exocytosis from intracytoplasmic vacuoles. A combination of both mechanisms may be possible. Following secretion, a fraction of the membrane surrounding the globules may be shed from the droplets and give rise to membrane fragments in the skim milk phase. This process is summarized and illustrated in Figure 1 [Mather & Keenan, 1998].

### Changes in the milk fat globule membrane (MFGM) during and after secretion

At the present time, very few facts are available regarding changes that might occur in the MFGM post secretion from the mammary secretory cell. Reported observations are based mainly on microscopy techniques, although some biochemical data are also available. Electron microscopy observations indicate that MFGM isolated from the fat globules of harvested milk tends not to vesiculate, in contrast to isolated plasma membrane, which tends to vesiculate [Keenan *et al.*, 1970]. The former phenomenon is presumably due to the presence of the dense layer at the inner face of the phospholipid bilayer [McPherson & Kitchen, 1983]. This and other observed morphological differences, such as a lack of intramembranous particles (probably membrane proteins; [Peixoto de Menezes & Pinto da Silva, 1978; Hui & Boni, 1991] in the plasma membrane at locations where fat globules are budding [Peixoto de Menezes & Pinto da Silva,

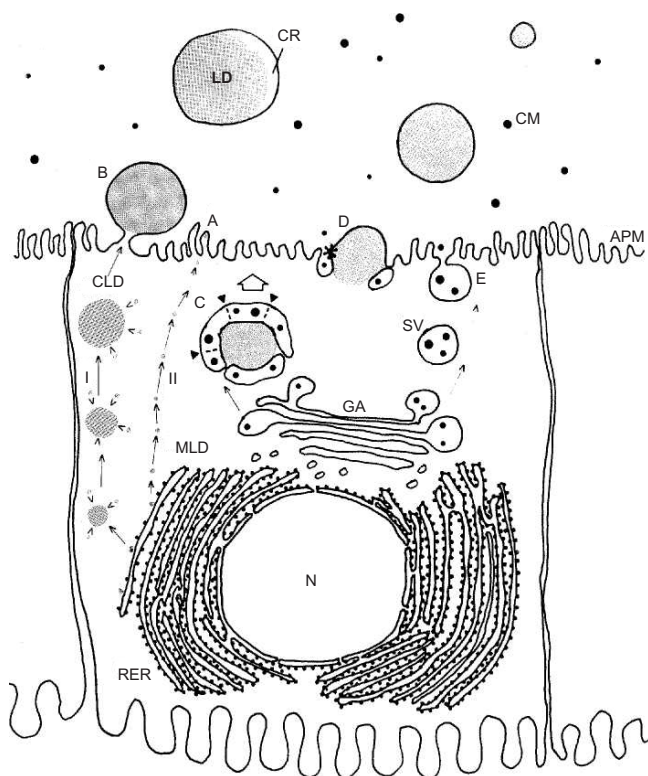


FIGURE 1. Summary of pathways for lipid droplet transit and secretion from mammary epithelial cells [Mather & Keenan, 1998].

- **(Pathway I)** Microlipid droplet (MLD) formed in the rough endoplasmic reticulum (RER) may fuse with each other and with larger cytoplasmic lipid droplet (CLD) as they are transported to the apical plasma membrane (APM).
- **(Pathway II)** Many MLDs transit to the apical plasma membrane directly without further accretion in size.
- Fat droplets may be secreted from the apical plasma membrane, either as MLDs (mechanism A), or CLDs (mechanism B). Fat droplets may be secreted after secretory vesicles surround CLDs and progressively fuse with each other to form intracytoplasmic vacuoles (mechanism C); dotted lines in the figure mark the former boundaries of fused secretory vesicles). These vacuoles are presumed to be transported to the apical surface (arrowhead, mechanism C) and the contents released by exocytosis (not shown). A combination of both apical and secretory vesicle routes may be possible (mechanism D) (asterisk marks secretory vesicle which has just fused with apical membrane). Cytoplasmic inclusions, "crescents," are trapped between the outer membrane layer and the lipid globule in some secreted fat droplets. Caseins and other milk proteins are processed through the secretory pathway and are secreted with the aqueous phase of milk by either compound (not shown) or simple exocytosis from secretory vesicles at the apical plasma membrane (mechanism E).
- Apical plasma membrane (APM); basal plasma membrane (BPM); cytoplasmic lipid droplet (CLD); casein micelle (CM); cytoplasmic crescent (CR); rough endoplasmic reticulum (RER); lipid droplet (LD); Golgi apparatus (GA); microlipid droplet (MLD); nucleus (N); secretory vesicle (SV).

1978], suggest that a re-arrangement of constituents within the apical plasma membrane/MFGM occurs upon secretion of the fat globule by the mammary secretory cell. The clearing of intramembranous particles in the membrane during budding may increase local membrane elasticity [Freudenstein *et al.*, 1979], but the phenomenon is not consistent, as the MFGM of some extra cellular fat globules does contain intramembranous particles, albeit at a reduced density [Banghart *et al.*, 1998]. The reason for the inconsistency is not known.

Similarly, the process of loss of material from the membrane after secretion of the fat globule by the secretory cell is unclear. Morphological observations of excreted fat globules suggest that gradual loss or restructuring of membrane material occurs during the sojourn of the globule in the secretory alveolus and its passage into expressed milk [Henson *et al.*, 1971; Wooding 1971a, b]. At least some loss appears to occur through either dissolution or vesiculation (*i.e.* the formation of small, micro some-like particles that are subsequently dislodged from the fat globule, a phenomenon called blebbing) [Wooding, 1971b].

In general, the sometimes conflicting opinions of authors [*e.g.* Patton, 1973; Shimizu *et al.*, 1979; Keenan *et al.*, 1983] and the paucity of reliable data regarding the MFGM composition and the structure of fat globules post secretion by the mammary secretory cell, as a function of time and other variables, make this area ripe for further study. Future research, using techniques such as confocal microscopy, atomic force microscopy and fluorescence methods, should shed new light on these aspects and contribute to settling existing controversies.

## COMPOSITION AND STRUCTURE

The MFGM is characterized by a complex mixture of proteins, phospholipids and glycoproteins, and acts as a natural emulsifier by covering the surface of the milk fat globule [McPherson & Kitchen, 1983]. The composition of MFGM has been reported by Goff & Hill [1993].

The majority of the MFGM comprises membrane-specific proteins, mainly glycoproteins, and phospho- and sphingolipids. Its gross composition is given in Table 1. Literature findings on the composition of the MFGM material are highly variable due to differences in isolation, purification and analytical techniques.

### Lipids of the milk fat globule membrane

The lipids of the MFGM are primarily polar lipids, although neutral lipids can also occur. The latter are triglycerides, diglycerides, monoglycerides, cholesterol and its esters.

TABLE 1. Estimated average composition of the milk fat globule membrane [Walstra *et al.*, 2006].

Component	mg/100 g fat globules	g/100 g MFGM dry matter
Protein	1800	70
Phospholipids	650	25
Cerebrosides	80	3
Cholesterol	40	2
Monoglycerides	+ <sup>a</sup>	-
Water	+	-
Carotenoids + Vit. A	0.04	0.0
Fe	0.3	0.0
Cu	0.01	0.0
Total	>2570	100

+<sup>a</sup>: present, but quantity unknown.

It was often mentioned that the MFGM contains a significant amount of high-melting triglycerides [Wooding & Kemp, 1975], although this must be rather attributed to the isolation methods of the MFGM-preparate [Walstra, 1974, 1985], as during isolation from milk, these MFGM-fragments can easily become contaminated with triglyceride crystals.

Asker, [1974] separated the fat globule membrane neutral and polar lipids from Egyptian buffalo and cow's milk, fractionated by TLC. He found that the lipid classes and composition of the FGM in both of species almost the same. While Shahin *et al.* [1987] isolated the fat globule membrane neutral lipids (FGM-NL) from Egyptian buffalo, goat and cow's milk, fractionated by TLC and individual fractions were quantitatively determined. Triglycerides were found to be the main fraction in FGM-NL from all species, followed by free fatty acids, 1, 2 diglycerides, 1, 3 diglycerides, monoglycerides and then cholesterol.

The polar lipids of the MFGM consist of phospho- and sphingolipids. These are amphiphilic molecules with a hydrophobic tail and a hydrophilic head group. The glycerophospholipids consist of a glycerol backbone on which two fatty acids (FA) are esterified. A phosphate residue with different organic groups (choline, serine, ethanolamine, *etc.*) may be linked on the third hydroxyl group. The characteristic structural unit of sphingolipids is the sphingoid base, a long-chain aliphatic amine, containing two or three hydroxyl groups. A ceramide is formed when the amino group of this sphingoid base is linked with a FA. On this ceramide unit, an organophosphate group can be bound to form a sphingophospholipid (*e.g.*, phosphocholine in the case of sphingomyelin, SM) or a saccharide to form the sphingoglycolipids (glycosylceramides) [Christie, 2003; Fong *et al.*, 2007; Newburg & Chaturvedi, 1992; Pfeuffer & Schrezenmeir, 2001; Vanhoutte *et al.*, 2004; Vesper *et al.*, 1999; Yang *et al.*, 2004].

The major types of polar lipids (PL) present in the membrane are phosphatidylcholine (PC), 35%; phosphatidylethanolamine (PE), 30%; sphingomyelin, (SM), 25%; phosphatidylinositol (PI), 5%; phosphatidylserine (PS), 3%. Glucosylceramide (GluCer), lactosylceramide (LacCer) and gangliosides (Gang) are present in trace amounts [Danthine *et al.*, 2000; Deeth, 1997].

Individual PL species were separated by two-dimensional TLC, identified by co-migration with authentic standards, and quantified. The MFGM contained PE, PC, PS, PI, SM and the lyso-derivative forms of PE and PC [Sanchez-Juanes *et al.*, 2009]. Lyso-derivatives of PL have previously been reported in milk [Keenan & Patton, 1995], but several authors consider that they are probably artifacts caused by careless sample preparation, or that they could be due to lipolytic enzyme activity [Rombaut *et al.*, 2005]. Ceramide monohexoside (glucosylceramide) and ceramide-dihexoside (lactosylceramide) have often been mistakenly included by several authors as PL (Table 2).

The short and medium chain length FA (C<sub>4</sub>-C<sub>14</sub>), typically for milk fat, are virtually absent in the phospholipid fraction of milk. In particular, PE is highly unsaturated, followed by PI and PS. PC is rather saturated compared with the other glycerophospholipids. The FA pattern of SM is very uncommon. Although long-chain FAs occur, nearly all of them are saturated (97%). In addition, the occurrence of C<sub>23</sub> (>17%) is remarkable [Bitman & Wood, 1990; Jensen, 2002].

TABLE 2. Phospholipid content <sup>a</sup> of MFGM and fresh milk [Sanchez-Juanes *et al.*, 2009].

Phospholipid <sup>b</sup>	MFGM	Whole milk	MFGM (Literature) <sup>c</sup>	Whole milk (Literature) <sup>d</sup>
PC	27.4 ± 0.0	32.7 ± 1.6	33.6; 33.2; 32.1	35.1; 21.1
PE	33.0 ± 1.9	28.5 ± 1.5	22.3; 32.6; 36.4	19.8; 46.3
PS/PI	17.8 ± 2.2	14.1 ± 1.4	4.3; 12.9; 14.1	13.7; 12.7
SM	18.8 ± 1.1	23.0 ± 1.6	35.3; 21.3; 17.3	31.4; 19.8
LPE	1.4 ± 0.5	N.D.	–	–
LPC	1.6 ± 0.4	1.8 ± 0.5	–	–

<sup>a</sup>: Values given are percentages of the total phospholipids content and are means ± SD of three independent determinations; ND: not detected; an asterisk denotes significant difference  $p < 0.05$ . The total PL contents determined were 9750 ± 1146 µg/100 mg dry weight for MFGM and 168.8 ± 6.7 mg/L for fresh whole milk.

<sup>b</sup>: **Abbreviations are:**

PC: phosphatidylcholine; PE: phosphatidylethanolamine; PS: phosphatidylserine; PI: phosphatidylinositol; SM: sphingomyelin; LPE: lyso-phosphatidylethanolamine; LPC: lysophosphatidylcholine.

<sup>c</sup>: Values from Bracco *et al.* [1972], Fong *et al.* [2007]; Fauquant *et al.* [2007], respectively.

<sup>d</sup>: Values from Bitman & Wood [1990]; Rombaut *et al.* [2005], respectively.

### Proteins of the milk fat globule membrane

Depending on the source, 25–70% of the MFGM consists of proteins [Danthine *et al.*, 2000; Deeth, 1997; Fong *et al.*, 2007; Walstra *et al.*, 2006]. These membrane proteins are only present in very small amounts in other milk phases, and account for 1–2% of total milk protein [Ricci, 2004]. The reported composition is highly dependent on the isolation and analysis procedures used, since not all proteins are equally connected with the MFGM. Some are integral proteins, some are peripheral proteins, and others are believed to be only loosely attached. Upon separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the MFGM material is resolved into 7–8 major bands. However, several minor species are as yet unidentified. Despite the recent efforts undertaken to elucidate their structure and amino acid sequence, little is known about their specific concentration and function.

Major MFGM proteins (Table 3) such as mucin 1 (MUC1) [Pallesen *et al.*, 2001], xanthine dehydrogenase/oxidase (XDH/XO) [Berglund *et al.*, 1996b; Spitsberg *et al.*, 1995], CD36 [Berglund *et al.*, 1996a; Greenwalt, 1993; Rasmussen *et al.*, 1998], PAS 6/7 [Bash *et al.*, 1976; Hvarregaard *et al.*, 1996; Kim *et al.*, 1992], adidophilin (ADPH) and butyrophilin (BTN) [Nielsen *et al.*, 1999] have been purified and characterized. Furthermore, it is assumed by several authors that parts of the proteose peptone fraction like proteose peptone 3 (PP3), originate from the MFGM [Campagna *et al.*, 2001; Girardet *et al.*, 1995; Nejjar *et al.*, 1990; Sorensen & Petersen, 1993a, b; Sorensen *et al.*, 1997].

Bovine MFGM preparations contain many more proteins and enzymes than those discussed above. These components include enzymes, immunoglobulins, proteins derived from the cytoplasm of the secretory-epithelial cells, proteins from milk leukocytes and skim milk constituents. The majority are undoubtedly peripheral proteins loosely adsorbed to the MFGM. However, they could exert important biological

TABLE 3. Protein components of the MFGM [Keenan & Dylewski, 1995; Mather, 2000].

Proteins	Molecular weight (Da)
Mucin I (MUC1)	160 000 – 200 000
Xanthine oxidase (XO)	150 000
Periodic acid schiff III (PAS III)	95 000 – 100 000
Cluster of differentiation (CD36) or PAS IV	76 000 – 78 000
Butyrophilin (BTN)	67 000
Adipophilin (ADPH)	52 000
Periodic acid schiff 6/7 (PAS 6/7)	48 000 – 54 000
Fatty acid binding protein (FABP)	13 000
Breast cancer type 1 (BRCA1)	210 000

functions. A more exhaustive listing of possible protein components of bovine MFGM is given in works by McPherson & Kitchen [1983], Keenan *et al.* [1988], Mather, [2000], Reinhardt & Lippolis, [2006], and Fong *et al.* [2007].

Figure 2 illustrates the polypeptide patterns of MFGM, isolated from early, mid and late season cow milks, as determined by SDS-PAGE (15% acrylamide) under reducing conditions. About 37 protein bands were observed in the gel, in which there were 10 major bands, ranging in molecular weight from 47 to 200 kDa. No bands corresponding to casein and whey proteins were observed, indicating that these proteins were entirely removed by the washing procedure used [Ye *et al.*, 2002].

Milk fat globule membrane proteins of individual goats were characterized using one-dimensional SDS-PAGE analysis. Many differences were observed between bulked caprine and bovine milk samples. Goat sample showed much higher content of XO and of minor proteins compared with cow sample. Among individuals, a high heterogeneity could be observed; 32 bands were identified of which 19 were present in all caprine samples [Zamora *et al.*, 2009].

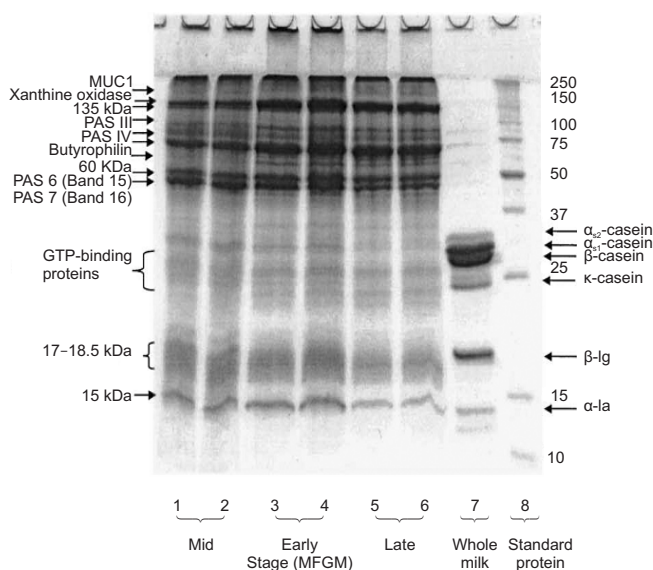


FIGURE 2. SDS-PAGE patterns (15% polyacrylamide) of MFGM material from fresh whole cow milk [Ye *et al.*, 2002].

### Structure of the milk fat globule membrane

As viewed from the lipid core outwards, the MFGM consists of an inner monolayer of polar lipids and proteins surrounding the intracellular fat droplet, an electron dense proteinaceous coat located on the inner face of the bilayer membrane and finally a true bilayer membrane of polar lipids and proteins (Figure 3). Cytoplasmatic material can be entrained between the inner coat and the outer double membrane layer resulting in ‘cytoplasmatic crescents’ [Danthine *et al.*, 2000; Evers, 2004a; Michalski *et al.*, 2002a; Rasmussen *et al.*, 2002].

As the greater part of the membrane of the MFGM is derived from the apical plasma membrane of the secretory cell, the most widely accepted model for this type of membrane would be the fluid mosaic model. This suggests that the phospholipid bilayer serves as a backbone of the membrane, which exists in a fluid state. Peripheral membrane proteins are partially embedded or loosely attached to the bilayer. Trans-membrane proteins extend through the lipid bilayer. Carbohydrate moieties from glycolipids and glycoproteins are orientated outwards, whilst cholesterol is present in the polar lipid bilayer.

The proteins of MFGM are arranged asymmetrically. Adipophilin (ADPH), which has a very high affinity for triglycerides, is located in the inner polar lipid monolayer. XDH/XO is exposed on the inner face of the monolayer, and is closely connected with BTN, which is a transmembrane protein of the outer layer, and with ADPH. As such, these proteins act as anchorpoints, thereby forming a supramolecular complex that interconnects the inner and outer membrane [Mather & Keenan, 1998]. Together with ADPH and XOR/XO, BTN plays an important role in the assembly and the stabilization of the MFGM [Mather, 2000]. Other proteins, like PAS 6/7, are located at the outer part of the membrane. Some MFGM proteins, like MUC1, are heavily glycosylated. Carbohydrate moieties appear to be uniformly distributed over the external membrane surface [Danthine *et al.*, 2000; Evers, 2004; Harrison, 2002; Mather, 2000].

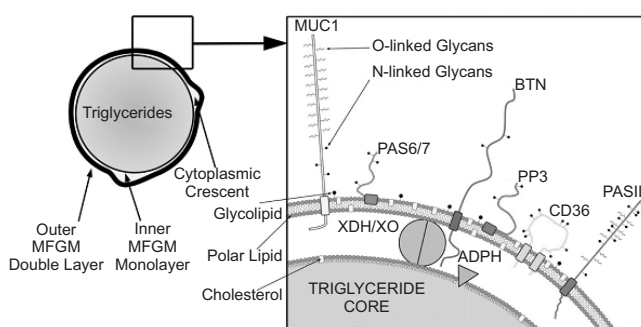


FIGURE 3. Structure of the fat globule with detailed arrangement of the main MFGM proteins. The drawing is highly schematic and sizes are not proportional. A double layer of polar lipids is placed on an inner monolayer of polar lipids. Membrane-specific proteins are distributed along the membrane. ADPH is located in the inner polar lipid layer, XDH/XO is located in between both layers. MUC1, BTN, CD36 and PASIII are located in the outer layer. PAS6/7 and PP3 are only loosely attached at the outside of the MFGM. The choline-containing phospholipids, PC and SM, and the glycolipids, cerebrosides and gangliosides, are largely located on the outside of the membrane, while PE, PS and PI are mainly concentrated on the inner surface of the membrane [Dewettinck *et al.*, 2008].

The surface of MFGM varies among species, in human and mare milk, filaments extend as far as 1 mm from the globule surface [Buchheim *et al.*, 1988a], whereas cow, goat, and sheep MFG have a smooth surface devoid of such filaments [Buchheim *et al.*, 1988b]. These filaments have been shown to contain the mucin MUC1. MFGM contain a number of glycoproteins that have been well characterized and have been shown to confer protection against bacteria and viruses. Among these are mucin (MUC1) [Peterson *et al.*, 1998a], which binds fimbriated *Escherichia coli* [Schroten *et al.*, 1992, 1993] and lactadherin that protects against rotavirus diarrhea in infants [Coonrod & Yoneda, 1983].

The lipids are, like the proteins, asymmetrically arranged. The choline-containing phospholipids, PC and SM, and the glycolipids, cerebrosides and Gang are largely located on the outside of the membrane, while PE, PS and PI are mainly concentrated on the inner surface of the membrane [Deeth, 1997].

## ISOLATION AND PURIFICATION

### Isolation of MFGM from milk

A typical isolation method can be divided into four steps [Mather, 2000; Singh, 2006]: fat globule separation, cream washing, release of MFGM from the globules and collection of the MFGM material. First, cream can be separated from milk by a laboratory centrifuge or in a large scale, bench-top cream separator. Next, the separated cream is washed two [Ye *et al.*, 2002], three [Fong *et al.*, 2007; Kanno & Kim, 1990] or more number of times [Asker, 1974; Mangino & Brunner, 1975] in 3–15 fold volumes of distilled or deionized water [Kanno & Kim, 1990; Newman & Harrison, 1973], sucrose-saline solution with [Erickson *et al.*, 1964; Mather *et al.*, 1977; Nejjar *et al.*, 1986; Snow *et al.*, 1977] or without [Dowben *et al.*, 1967] pH buffering, pH buffered sucrose solution [Khodaparast-Sharifi & Snow, 1989], isotonic phosphate buffer solution [Nielsen & Bjerrum, 1977], phosphate-saline buffer [Innocente *et al.*, 1997], or simulated milk ultrafiltrate [Ye *et al.*, 2004]. In some cases, detergents [Diaz-Maurino & Nieto, 1976; Mather *et al.*, 1977] or dissociating agents [Ye *et al.*, 2002] are added to facilitate the washing. Recently, skim milk ultrafiltrate has also been used as washing solution [Morin *et al.*, 2007]. The number of washes can be reduced if milk is suspended in washing solution prior to separation of the fat globules [Politis *et al.*, 1992].

After washing the cream, the MFGM is released from the triglyceride fat core into the aqueous phase by churning [Diaz-Maurino & Nieto, 1976; Dowben *et al.*, 1967; Fong *et al.*, 2007; Mather *et al.*, 1977; Nielsen & Bjerrum, 1977], agitation [Harrison *et al.*, 1975] at reduced temperatures or applying cycles of freezing-thawing [Dowben *et al.*, 1967; Khodaparast-Sharifi & Snow, 1989; Snow *et al.*, 1977, 1980]. Alternatively, MFGM can directly be released from washed cream by the use of polar aprotic solvents [Bingham & Malin, 1992; Dapper *et al.*, 1987], bile salts [Erickson *et al.*, 1964; Snow *et al.*, 1980], or nonionic detergents [Patton, 1982]; in addition these authors reported that direct extraction normally results in a lower yield, and a certain difference in composition depending on the concentration of the applied chemicals, the time and temperatures of extraction.

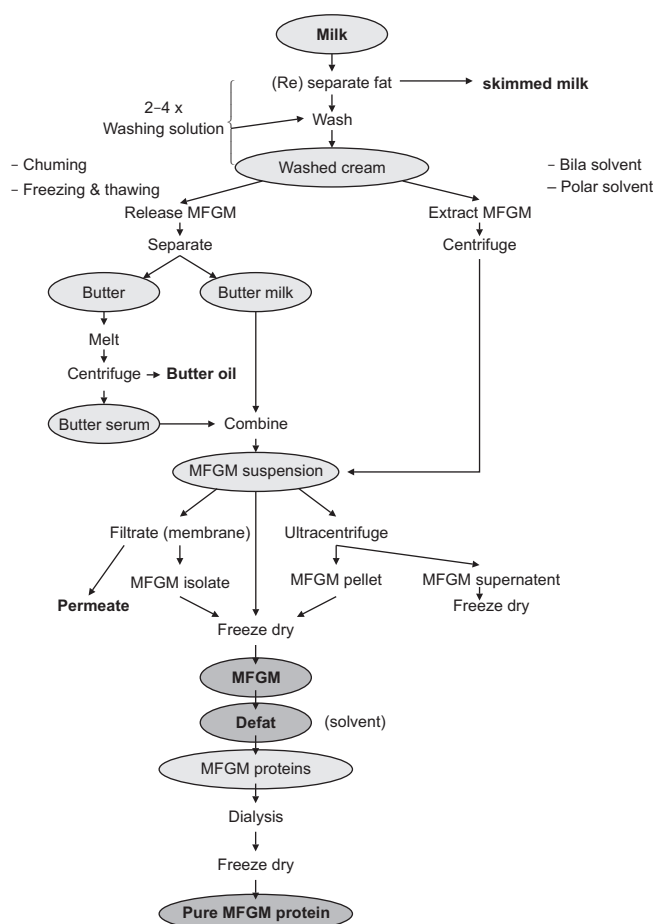


FIGURE 4. Summary of isolation methods of MFGM.

Finally, the released MFGM material from buttermilk and/or butter serum is collected by ultracentrifugation [Anderson & Brooker, 1975; Snow *et al.*, 1977], freeze-drying [Rombaut *et al.*, 2006] or microfiltration [Morin *et al.*, 2007]. Two fractions, the soluble supernatant and the MFGM pellet, are obtained by ultracentrifugation. Precipitation of MFGM fragments at low pH [Fong *et al.*, 2007; Kanno & Kim, 1990] or by 'salting out' with ammonium sulfate [Kanno & Kim, 1990; Nielsen & Bjerrum, 1977] may be applied to MFGM suspensions, after which the MFGM material is separated by centrifugation. All the above reviewed methods are laboratory applications for the isolation of MFGM material from untreated milk. They are summarized in Figure 4.

Prior to measuring the activity of MFGM enzymes, extra washing steps of the pellet are often applied to remove most of the contaminating whey protein [Diaz-Maurino & Nieto, 1976; Khodaparast-Sharifi & Snow, 1989; Nielsen & Bjerrum, 1977; Snow *et al.*, 1977, 1980]. Solutes used in the washing solution can affect the membrane enzyme activity [Diaz-Maurino & Nieto, 1976; McPherson *et al.*, 1984; Snow *et al.*, 1980] and dialysis does not always remove the solutes completely [Bash *et al.*, 1976]. The selectivity and amount of losses during washing and desorption into buttermilk depends on the affinity for the suspension solution used [Walstra, 1985]. Lipid content, protein composition, and enzyme activities of MFGM pellets are different with different collect-

ing methods used [Kanno & Kim, 1990]. Using membrane filtration to collect MFGM fragments from buttermilk may result in a loss of small MFGM fragments in the permeate [Morin *et al.*, 2007]. Therefore, collecting the membrane material from both buttermilk and butter serum without separating the supernatant and the pellet – *e.g.*, by lyophilization of the combined solution [Rombaut *et al.*, 2006] – is necessary to have a representative evaluation of MFGM characteristics. Three washing steps are sufficient to remove virtually all milk serum components [Nejjar *et al.*, 1986; Ye *et al.*, 2004]. However, three washes already cause a loss of MFGM components [Anderson & Brooker, 1975; Nejjar *et al.*, 1986].

Membrane proteins are highly vulnerable to losses during isolation, especially the loosely bound proteins. Only 4% of phospholipids compared with 16% of the MFGM proteins were lost during the washing process [Anderson & Brooker, 1975]. Washing also causes losses of tocopherol, an antioxidant in the MFGM [Erickson *et al.*, 1964].

Bovine serum albumin (BSA) was still detected in isolated MFGM in spite of successive washings [Nejjar *et al.*, 1986]. On SDS-PAGE gels, casein,  $\beta$ -lactoglobulin, BSA and lactoferrin were still visible in MFGM material after three washes in three volumes of deionized water [Fong *et al.*, 2007]. It was suggested by Morin *et al.*, [2007] that skim milk proteins may interact strongly with MFGM even before milk is collected. However, examination by transmission electron microscopy (TEM) showed that casein micelles were uniformly distributed and did not increase in concentration at the MFGM [Lee & Morr, 1992].

A lower yield of the membrane proteins was seen at a washing temperature of 45°C compared with 20°C [Ye *et al.*, 2002]. Washing can be done at lower temperatures in laboratory centrifuges than in cream separators. The latter method, through repeated washing and separation of the cream, tends to produce small butter granules at temperatures lower than 40°C [Fong *et al.*, 2007].

MFGM material from milk can be obtained in a shorter time by the method of Patton & Huston [1986], where milk is added with sucrose (5 g/100 mL) and fat globules are, by a light centrifugation, passed through an above-situated phosphate-buffered salt solution. The method gave quite comparable results in phospholipid and cholesterol content compared with the washing method and was considered to give less damage to MFGM [Patton & Huston, 1986]. Making use of a density gradient, MFGM fragments can be collected as a layer separated from other components after centrifugation of unwashed cream, buttermilk or butter serum against a concentrated sucrose solution. This unwashed method gave a lower yield of MFGM material but similar protein composition compared to the washing method. The method was found suitable for extraction of MFGM material from milk products, *e.g.*, pasteurized cream and milk [McPherson *et al.*, 1984].

### Isolation of MFGM from industrial sources

The modern food processing industry is focused on utilizing natural components that improve the nutritional value and create specific functionalities for food products [Innocente *et al.*, 1997]. Rich sources of MFGM material, *e.g.*, butter-

milk and butter serum, are still considered as low value by-products originating from dairy processing. Many attempts have been performed to isolate MFGM from buttermilk. By using micro- and ultrafiltration, the transmission of components through the membrane was found to depend on filtration conditions such as temperature, pH, pore size and type of membrane material and the type of buttermilk [Morin *et al.*, 2004, 2006; Rombaut *et al.*, 2007].

The isolation is based on the selective removal of casein, whey proteins, lactose and minerals from the concentrate. The similarity in size of casein micelles and MFGM fragments was reported to be the major obstacle during isolation [Sachdeva & Buchheim, 1997]. The latter authors used renneting and acid coagulation (citric acid and fermentation by lactic acid bacteria) to remove caseins prior to concentration of MFGM by a combination of microfiltration and ultrafiltration. Of the total phospholipids in buttermilk, 70–77% was recovered depending on methods applied. Renneting coagulation was found to be the most efficient [Sachdeva & Buchheim, 1997]. Using another approach [Corredig *et al.*, 2003; Roesch *et al.*, 2004] used citrate to dissociate casein micelles followed by microfiltration to collect MFGM material in the retentate. Increasing the number of diafiltration steps with deionized water from 2 to 6 reduced the casein contamination in the retentate from 30% to 6% of total proteins [Corredig *et al.*, 2003]. However, this application also causes loss of MFGM material [Rombaut *et al.*, 2006]. Here citrate addition was applied to butter serum. These authors reported that 44% of polar lipids were lost during filtration due to blocking and fouling of the filter membrane with MFGM particles. Addition of sodium citrate agent causes dispersion of not only casein micelles but also MFGM fragments [Rombaut *et al.*, 2006]. Whey buttermilk [Morin *et al.*, 2006], the aqueous fraction obtained by churning of whey cream which is separated from cheese whey, and acid buttermilk whey [Rombaut *et al.*, 2007], the aqueous fraction obtained by acidification of sweet-cream buttermilk, were considered favorable for MFGM isolation by filtration due to absence of casein micelles. As expected, transmission of MFGM proteins through the membrane was lower when using whey buttermilk compared with regular buttermilk [Morin *et al.*, 2006]. At optimized conditions, 98% of the polar lipids from the acid buttermilk whey were recovered in the retentate. Thermocalcic aggregation of the whey before filtering aids to clarify the whey, but also results in low permeate fluxes and high retention of ash and whey proteins [Rombaut *et al.*, 2007].

Another strategy for removal of skim milk proteins has been explored, by washing cream with skim milk ultrafiltrate before churning has been studied. Compared with the filtering of buttermilk from unwashed cream, this method caused losses of MFGM material, but improved the permeation flux and gave an isolate with higher MFGM content and lower contamination of skim milk proteins [Morin *et al.*, 2007]. This method, however, may be difficult to apply in industry.

Selective removal of neutral lipids by supercritical fluid extraction [Astaire *et al.*, 2003] or precipitation of polar lipids with acetone after solvent extraction [Baumy *et al.*, 1990] are two of the vast number of methods used to further purify dairy phospholipids from a MFGM isolate.

More purified phospholipids can be separated into two application categories, namely for oil-in-water or water-in-oil emulsions based on their hydrophilic-lipophilic balance (HLB) [Boyd *et al.*, 1999].

### FACTORS AFFECTING THE STABILITY OF MILK FAT GLOBULES MEMBRANE

After milk secretion and milking, compositional and structural changes in the MFGM occur, and membrane material is shed into the skimmed milk phase. Factors like age of the cow, bacteriological quality of the milk, stage of lactation and season have an influence on these changes, but are rather insignificant compared with the effects of processing on the MFGM composition. Cold storage leads to specific migration of PL and proteins towards the serum phase, pumping and air inclusion induces serious MFGM damage and losses, a heat treatment causes denaturation of MFGM proteins and a further complexation of BTN and XO [Ye *et al.*, 2002], whilst homogenization leads to a newly formed membrane, mainly consisting of caseins and whey proteins.

Apart from MFGM fragments, secretory cell fragments (microvilli, cytoplasm and membrane particles) can be secreted into the lumen. This material, which sediments upon centrifugation, comprises only 4% of total milk lipids, is rich in polar lipids and has a similar composition to the MFGM material [Deeth, 1997; Keenan *et al.*, 1988].

Changes in the MFGM during and after milk harvesting (secretion) are affected by a number of factors, which may be divided into physiological, physical/mechanical and environmental. Such changes may manifest themselves as loss of membrane components, adsorption of milk plasma components and chemical or enzymic reactions, which, in turn, may affect the stability of the fat globule [Walstra & Jenness, 1984].

### PHYSIOLOGICAL (ANIMAL) FACTORS

The diet of the cow, breed, fat globule size and stage of lactation, have been claimed to be the factors affecting the stability of the fat globule [Anderson & Cawston, 1975; Te Whaiti & Fryer, 1975; McPherson & Kitchen, 1983; Deeth, 1997; Walstra *et al.*, 1999].

#### Breed

Little is known about breed variation. There has been no systematic investigation of breed effects on overall MFGM composition, although values for MFGM composition in the literature include different breeds [Chandan *et al.*, 1971; Huang & Kuksis, 1967; Newman & Harrison, 1973; Swope & Brunner, 1970]. It is possible that the major effect of breed on MFGM composition could be in the yields of membrane material due to variation in fat globule sizes [McPherson & Kitchen, 1983].

#### Diet

The composition of MFGM, especially the fatty acid composition of the lipid components, varies with the dietary

changes between winter and summer feeds [Huang & Kuksis, 1967]. Changes in the fatty acid composition of the neutral lipids of the MFGM were also observed after feeding cows protected or unprotected coconut oil [Anderson, 1974]. Fatty acid composition of the MFGM phospholipids and the polypeptide components largely unaffected by changes in the unsaturated lipid content of protected feeds [Sleigh *et al.*, 1976]. However, other scientists [Smith *et al.*, 1977] have observed that MFGM phospholipids obtained from cows fed protected sunflower/soya bean oil supplements contained higher properties of linoleic acid than the phospholipids from normal milks. Low fiber diets resulted in an increase in the PE content of the MFGM, while the levels of PC and SM decreased [Kinsella & Houghton, 1975].

#### Fat globule size

The fat globule size distribution depends on the breed of cow, the stage of lactation, *etc.* For instance, fat globules from the milk of Jersey cows (average size approximately 4.5  $\mu\text{m}$ ) are normally larger than fat globules from the milk of Friesian cows (average size approximately 3.5  $\mu\text{m}$ ), although there are also considerable variations between cows of the same breed. There is a decrease in the average fat globule size as lactation progresses [Singh, 2006].

#### Stage of lactation and season

From a study on bovine milk from 3 to 180 days post partum, Bitman & Wood, [1990] concluded that the relative amounts of the five major phospholipid classes (phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl inositol, phosphatidyl serine and sphingomyelin) remained constant. This constancy contrasts with results obtained for buffalo milk, which showed significant changes in relative levels of phospholipids during lactation. Sphingomyelin was found to be the predominant phospholipid in early lactation [Hofi *et al.*, 1977]. Furthermore, during the last two months of lactation, phosphatidyl choline and sphingomyelin were reported to decrease significantly in bovine milk [Kinsella, 1970].

In buffalo milk, major differences in the carbohydrate contents of the MFGM were observed when comparing colostrum and mid-, early- and late-lactation milk. Sialic acid, hexose and hexosamine levels tended to be lower in colostrum milk than in the other milks. SDS-PAGE showed eight major protein bands in colostrum milk compared with six bands in the other milks. Total phospholipid levels did not change significantly from the day of parturition to the final day of lactation.

Also, in buffalo milk, the total membrane material (g/100 g fat) increased during summer months and reached a maximum in September [Asker *et al.*, 1978]. As this coincided with late lactation, the increased membrane material may have been due to an increase in smaller fat globules, which have relatively more membrane material per unit volume of core fat than larger globules [Hofi *et al.*, 1977; Asker *et al.*, 1978]. However, the fat globule size distribution was not measured in these studies. Nevertheless, it is known that the average fat globule diameter is affected by the stage of lactation. According to Mulder & Walstra, [1974], the fat globule



diameter, being at a maximum in early lactation, decreases throughout lactation. Furthermore, recent results suggest that no significant change in the fat globule size distribution occurs after mid-lactation [Ye *et al.*, 2002]. As the latter study involved only three sampling points during lactation, confirmation of the observations on the fat globule size distribution is required, including comparisons between different breeds. In contrast to the fat content, the fat globule size distribution does not appear to change significantly during milking [Guinard-Flament *et al.*, 2001].

When considered together, the above results suggest that the quantity of membrane material is lower in mid lactation than in either early or late lactation. This could indicate that the fat globules in mid-lactation are less stable than those at either the beginning or the end of lactation [Kinsella, 1970].

Generally, it is difficult to positively identify the factors responsible for variation in the composition of the MFGM when comparing different studies. In most studies, the experimental design did not allow for an evaluation of the separate effects of stage of lactation and season. Hence, it is difficult to discern which of these variables are responsible for the observed results and to what degree. Future experiments would require a controlled feeding regime, as factors other than stage of lactation, such as feed (the quality of which can be season dependent), may have confounded the results obtained in previous studies. Furthermore, the results of the study on buffalo milk suggest that mammary gland development plays a significant role [Singh & Ganguli, 1976], particularly in the days immediately post partum. On the basis of the fact that large compositional changes occur in the first few days post partum [Anderson & Cheeseman, 1975], it is postulated that significant changes in fat globule stability take place during this period.

#### **SOME PHYSICAL AND MECHANICAL FACTORS AFFECTING THE STABILITY OF FAT GLOBULES MEMBRANE**

It arises in milk handling during and after milk harvesting. Pre-factory milk handling involves air inclusion, agitation of the milk (pumping and stirring), changes in temperature and changes in time (ageing of the milk). Handling of the milk at the factory involves ageing, agitation, air inclusion and temperature changes; deliberately applied treatments or processes include separation, heat treatments (*e.g.* pasteurisation), homogenisation and changes in water content [McPherson & Kitchen, 1983].

In many studies, MFGM material was obtained from milk by either physical separation techniques (*e.g.* churning, repeated freezing and thawing) or chemical techniques, such as those using surfactants to destabilize the fat globules, or washing techniques using water, sucrose solutions and/or buffers followed by a separation technique (usually centrifugation). Different results were obtained depending on the isolation techniques and conditions used [Wooding, 1971a, b; Anderson & Brooker, 1975; Bhavadasan & Ganguli, 1976, 1977; Mather *et al.*, 1977; Yamauchi *et al.*, 1978; McPherson & Kitchen, 1983; Walstra, 1985; Keenan *et al.*, 1988; Keenan

& Patton, 1995; Walstra *et al.*, 1999; Danthine *et al.*, 2000; Morin *et al.*, 2007].

#### **Air inclusion**

Air is incorporated into milk at various points during milk handling and processing. Sometimes, the presence of air is deliberate and wanted (*e.g.* in buttermaking), but usually it is deemed to be undesirable. Mixing of milk or cream with air, or any gas [Stannard, 1975; Tolle & Heeschen, 1975], can significantly reduce the stability of the fat globules [Te Whaiti & Fryer, 1975]. In this process, it is envisaged that, when a milkfat globule and an air bubble come in contact with each other, the MFGM is ruptured. Consequently, the membrane material and (part of) the core fat will spread over the air/milk plasma interface and will be released into the milk plasma when air bubbles collapse or coalesce [Walstra & Jenness, 1984; van Boekel & Walstra, 1989].

Significant changes in the MFGM caused by mixing milk and air can occur readily on the farm. Most of these changes occur in milking machines in which air is used as the transport medium for the milk [*e.g.* Salvatierra *et al.*, 1978; Evers & Palfreyman, 2001]. Rough treatment of the milk may be evident visibly by the presence of foam on top of the milk in the farm bulk tank [Deeth & Fitz-Gerald, 1976; Fluckiger, 1987]. Although indicative of MFGM damage, this does not necessarily result in significantly increased concentrations of free fatty acids (FFA) by lipolysis [Evers & Palfreyman, 2001], because this is also dependent on other variables, such as the activity of the native lipase [Evers, 2004b].

#### **Agitation**

The effect of agitation (stirring and pumping) is dependent on other factors such as temperature, the presence of air and the fat content. At temperatures below 40°C, fat crystals start to form in the fat core of the globule. Upon deformation of the fat globule, such fat crystals can cause local structural changes to the membrane, for example by piercing it. This can lead to fat globule aggregation and partial coalescence [Walstra *et al.*, 1999]. During handling and storage of milk, agitation of the milk by pumping from the farm bulk tank to the milk tanker can, in principle, cause further damage to the fat globules. But, as no experimental data appear to be available in the literature, the extent of these changes is unknown.

#### **Temperature and ageing**

In the dairy industry, manipulation of the temperature of milk is employed to safeguard the quality of milk and to influence the properties of processed milk products. For example, to improve its keeping quality, milk is cooled at the farm and heat treated at the factory. Further heat treatment at the factory is performed to aid processing and to manufacture products having certain water content. However, heating, cooling and ageing of milk can effect physical changes in the MFGM and fat globules, and these are discussed below.

#### **Temperature effects on adsorption**

The composition of the MFGM can change by adsorption of surface-active milk plasma constituents and selective, or

non-selective, desorption of membrane components [Anderson *et al.*, 1972; Buchheim, 1986; van Boekel & Walstra, 1989; Houlihan, 1992]. In turn, this may affect other properties of the fat globule such as the electrokinetic, or zeta ( $\zeta$ ), potential [Walstra, 1983] and stability.

Pasteurization of cream increased the  $\beta$ -lactoglobulin content of the MFGM, which is not surprising as the surface hydrophobicity of  $\beta$ -lactoglobulin is known to transiently increase when heated even at temperatures lower than those required for its insolubilisation or for its association with other milk components [Iametti *et al.*, 1997; Macej *et al.*, 2002]. Also, at 60°C, a temperature lower than the denaturation temperatures of the whey proteins, BTN and XO started to aggregate, probably by forming intermolecular disulphide bonds [Ye *et al.*, 2002]. At 65°C, serum proteins were reported not to interact with the MFGM in one study [Dalglish & Banks, 1991], but it was demonstrated that they did in another study [Corredig & Dalglish, 1996]. At higher temperatures (70–90°C), significant association of serum proteins, particularly  $\beta$ -lactoglobulin, occurred [Dalglish & Banks, 1991; Corredig & Dalglish, 1996]. For heating at 80°C similar results were found in other studies [Koops & Tarassuk, 1959; Houlihan, 1992; Lee & Sherbon, 2002]. Furthermore, heating at 80°C resulted in the total loss of PAS-6 and partial loss of PAS-7 protein from the MFGM, as determined by SDS-PAGE, but had no effect on the size and surface area of the fat globules [Lee & Sherbon, 2002].

Cold storage of fresh raw milk at 8°C resulted in about 10% loss of phospholipids from the MFGM over 96 h, but no loss of 5'-nucleotidase or adenosine triphosphatase activity was detected [Baumrucker & Keenan, 1973]. The ageing of bovine milk at 2–4°C for 24 h caused, on average, no change in the cholesterol content of the skim milk [Patton *et al.*, 1980]. These authors concluded that this did not prove that on average no cholesterol was lost from the MFGM, because there could have been equilibrium between membrane cholesterol and milk plasma cholesterol. Although there are indications that cooling changes the structure and composition of the MFGM, from these studies it is not clear which of the two factors (cooling or ageing) has the greater effect.

Conflicting results have been reported regarding the effect of heating on phospholipids. Heating milk at 80°C for 20 min resulted in statistically significant losses of triacylglycerol, but not of phospholipid, from the MFGM [Houlihan *et al.*, 1992]. This contrasts with earlier studies where heating at 80°C for 15 min, followed by cooling and separation, was reported to result in the loss of about 20% of phospholipids; at 90°C for 15 s, the figure was about 14% [Koops & Tarassuk, 1959]. These results agree with the conclusion of Greenbank & Pallansch [1961] that loss of phospholipids is both temperature and time dependent. However, there is no agreement as to what degree phospholipids are lost from the MFGM upon either heating or cooling.

Few morphological results on the effect of the temperature and the ageing of milk on the structure of the MFGM have been reported in the literature. However, electron microscopy observations suggested that heat treatment, including ultra-high temperature (UHT) treatment, did not result

in the release of the native membrane from the fat globule, which was contrary to the effects observed for cooling and stirring [Buchheim, 1986].

Generally, losses of glycoproteins and/or glycosphingolipids appear to occur upon cooling, heating or pressurizing milk, as these treatments resulted in a significant decrease in total carbohydrate in the MFGM of buffalo milk with large reductions (46–80%, depending on the treatment) being observed in sialic acid content [Bandyopadhyay & Ganguli, 1975].

#### Temperature and stability of fat globules

Cooling of milk can have a pronounced effect on the stability of the fat globules [Anderson *et al.*, 1972; Ismail *et al.*, 1972; Anderson & Cheeseman, 1975; Deeth & Fitz-Gerald, 1978]. However, in most studies reported in the literature, the process of cooling involved ageing as well as some form of agitation. The effects of these additional factors could have confounded the results and make it very difficult to establish the true effects of cooling on the MFGM.

Furthermore, increased FFA levels upon cold storage of raw milk [Evers, 2003] are indicative of a changing MFGM. A reduced stability of the MFGM upon cold storage may be caused by the partially selective loss of certain proteins from the MFGM [Anderson & Cheeseman, 1975].

Heat-induced changes can either improve or impair the stability of the fat globules [van Boekel & Walstra, 1989], but results are not always in agreement. Fink & Kessler [1985a, b], using 30% unhomogenised cream, concluded that the MFGM became more permeable after UHT treatment at 115–135°C. However, van Boekel & Folkerts, [1991], using milk (4% fat) and two creams (of approximately 30% and 38% fat), could not reproduce results of Fink & Kessler's and claimed that natural fat globules are remarkably stable against coalescence during UHT heating.

#### Temperature and fat globule size distribution

The effect of heating on the fat globule size distribution appears not to have been studied systematically. Fink & Kessler, [1985a] reported that the fat globule size distribution changed when cream was heated above 90°C, but van Boekel & Folkerts, [1991] claimed that heating did not affect the fat globule size distribution. Agitation is a confounding factor [van Boekel & Walstra, 1989] and the results of the study of Corredig & Dalglish [1996], comparing indirect heating and direct steam injection, suggest that agitation has a greater effect than temperature.

#### Homogenization

Homogenization of whole milk before and after heating causes major physical changes in the size and structure of the fat globules. When whole milk is heated before homogenization, the whey proteins are deaturated and interact with both the  $\kappa$ -casein of the casein micelle and the native FGM [Dalglish & Banks, 1991]. During subsequent homogenization, the micellar complex of casein and whey proteins will absorb on the newly formed fat surfaces. On the other hand, if milk is homogenized before it is heated, the caseins, either as semi-intact micelles or as micellar fragments, cover the

newly formed surface of the fat globules [Walstra & Oortwijn, 1982], and no whey proteins are present on the membrane [Sharma & Dalglish, 1993].

Homogenization is achieved by artificially increasing the number and net surface area of MFG. At the end of the homogenization process, the large amount of the new milk fat globules surface area created by homogenization must be covered by protein. Cano-Ruiz & Richter [1997] estimated that only 10% of the total MFGM after homogenization is covered by the original MFGM material. The newly formed fat globules exhibit different physical and chemical properties. In addition, the MFG special structure is disrupted and interactions between different milk constituents like caseins and whey proteins occur, which in turn will influence the dairy products properties [Michalski *et al.*, 2002a]. Therefore, the development of adequate protocols and nondestructive methods which will facilitate naturally MFG usage would enable a new dimension of milk functionality to be explored, leading to dairy products with altered functional, nutritional and physical properties.

From a nutritional prospective, it has been previously shown that some fractions of the MFGM may be shed into the skim milk [Singh, 2006] and spontaneously assemble into liposomes, or those liposomes might be artificially formed due to homogenization. Through disruption of the native macrostructure by homogenization, some bioactivities or at least bioactive molecules might be carried over to the skim milk. Hence, the possible effects on digestion, absorption and plasma lipid distribution should be assessed in relation to the small milk fat globule macrostructure and skim milk liposomes.

### Copper and oxidation

Cooling of milk appears to induce the migration of copper from the MFGM to the milk plasma, whereas heating of milk has the reverse effect [Mulder & Walstra, 1974]. The latter phenomenon may contribute to the oxidation of unsaturated fatty acids of membrane phospholipids [McPherson

& Kitchen, 1983; van Boekel & Walstra, 1995]. This appears to be so particularly for phosphatidyl ethanolamine, which contains 40–60% unsaturated fatty acids, of which about one-third is polyunsaturated, and which strongly binds copper [Allen & Humphries, 1977; Deeth, 1997]. Oxidation of MFGM constituents may thus be expected to affect the fluidity and stability of the MFGM. However, considering that all processing equipment in modern dairy factories is made of stainless steel, rather than copper, it is questionable whether the effect of copper is a significant factor in changing the MFGM.

## ENVIRONMENTAL FACTORS

### Bacteriological quality and mastitis

Secreted milk contains bacteria from both the cow's udder and the external environment. Bacteria in milk may produce enzymes such as lipases, phospholipases, proteinases and glycosidic hydrolyses. These could affect the properties and composition of the MFGM [Shimizu *et al.*, 1980; McPherson & Kitchen, 1983], and may lead to flavour defects such as rancid milk or bitty cream [Deeth & Fitz-Gerald, 1995]. It may be assumed that significant changes in the MFGM occur only when such bacteria are present in sufficiently high numbers (*i.e.* in poor quality milk).

Mastitis may also result in the production of enzymes; mastitic milk has been found to have higher levels of acid hydrolyses, which could change the surface charge of the MFGM. Furthermore, the MFGM of fat globules in mastitic milk appears to contain less phospholipid, more protein and two additional protein components compared with milk from healthy udders [Anderson & Cawston, 1975]. Nevertheless, the effects of mastitis on the MFGM are not well known as little research has been carried out in this area [McPherson & Kitchen, 1983].

In conclusion, the various factors and their effects on the bovine MFGM after the milk leaves the udder are summarized in Table 4 and Figure 5.

TABLE 4. Summary of various factors and their effects on the bovine MFGM after the milk leaves the udder [Evers, 2004].

Factor	Effect	Comments / References
Air bubbles	Substantial loss of membrane material	Walstra & Jenness [1984]
Cooling	Loss of copper Loss of phospholipids	Mulder & Walstra [1974] Baumrucker & Keenan [1973]; Patton <i>et al.</i> [1980]
Heating	Adsorption of copper Adsorption of whey proteins Aggregation of BTN and XO Loss of PAS 6/7 Loss of phospholipids	Mulder & Walstra [1974] Iametti <i>et al.</i> [1997] Ye <i>et al.</i> [2002] Lee & Sherbon [2002] Koops & Tarassuk [1959]; Greenbank & Pallansch [1961]; Houlihan <i>et al.</i> [1992]
Ageing	The effects depend on temperature	Little is known about how the MFGM is affected by ageing as a function of temperature
Agitation	Depends on the degree of air incorporation. High shear forces are required to change the MFGM in the absence of air	Mulder & Walstra [1974]; Stannard [1975]; Te Whaiti & Fryer [1975]; Miller & Puhon [1986]
Bacterial growth	Production of lipases, Phospholipases, Proteinases and glycosidic hydrolyses may change the MFGM	McPherson & Kitchen [1983]
Stage of lactation – season	Affect the amount of membrane material FG size distribution	Few controlled studies investigating the effects of stage of lactation, season and other factors have been reported in the literature

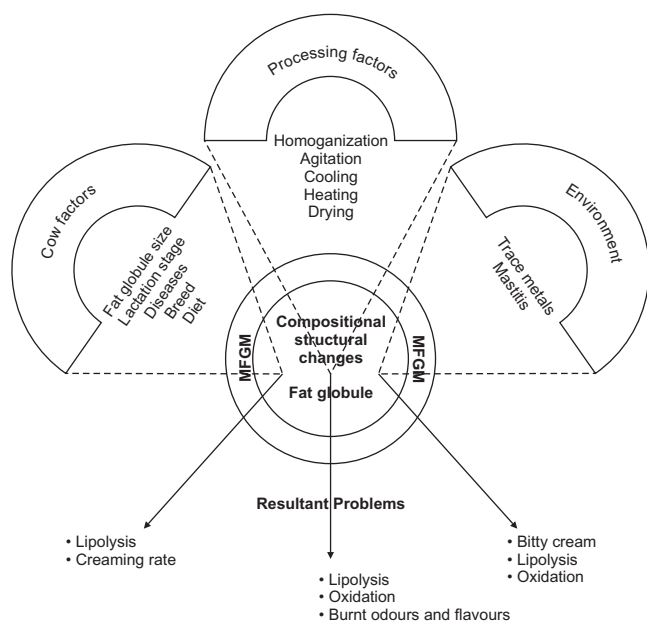


FIGURE 5. Summary of various factors affecting the milk fat globule membrane [McPherson & Kitchen, 1983].

### CONTENT OF THE MFGM IN DAIRY PRODUCTS

Polar lipids in milk, which comprise phospholipids and sphingolipids, are mainly (60–70%) situated in the MFGM. When milk is processed, this biological membrane is disrupted and as such is no longer associated with the fat globules. Table 5 shows that during processing polar lipids are preferentially distributed to aqueous phases such as buttermilk and butter serum. In the MFGM, polar lipids and proteins are closely associated so they will probably co-migrate during dairy processing. As seen in Table 5 and figures in various references [Corredig *et al.*, 2003; Roesch *et al.*, 2004; Rombaut *et al.*, 2006, 2007], dairy products rich in polar lipids are also enriched in MFGM proteins. As such, buttermilk and butter serum are suitable as sources for the isolation of

TABLE 5. Polar lipid content of various dairy products during processing [Rombaut & Dewettinck, 2006; Rombaut *et al.*, 2007].

Product	On products (g/100 g)	On dry matter (g/100 g)
Raw milk	0.03–0.04	0.23–0.32
Skim milk	0.02	0.28
Cream	0.19	0.40
Pasteurized cream	0.14	0.31
Butter	0.14–0.23	0.17–0.26
Buttermilk	0.16	2.03
Butterserum	1.25	11.54
Fresh acid buttermilk quarg	0.31	1.86
Acid buttermilk whey	0.10	1.84
Cheddar cheese	0.15	0.25
Cheddar cheese whey	0.02	0.26

MFGM material; the latter is the richest source of MFGM material on dry basis.

In a milk-based gel, interactions between fat and milk proteins occur *via* the MFGM. Changes in membrane composition, decrease of the MFG size and disruption of the MFGM along with the formation of a new modified composition occur through processing treatments such as heat, homogenization, and applied stress. These changes will alter the interaction, hence also the functional properties of the final products. A native fat globule may act as an inert filler (structure breaker) in milk-based gels [Michalski *et al.*, 2002a], while the newly formed MFGM (mainly casein and some serum proteins) in homogenized fresh milk or recombined milk would cross-link (structure promoter) with the protein network and reinforce it in both rennet and acid gels [Lopez & Dufour, 2001; Lucey *et al.*, 1998; Michalski *et al.*, 2002b]. Heat treatment induces disulfide bridges formation between  $\kappa$ -casein and  $\beta$ -lactoglobulin [Dagleish, 1990] which is in turn absorbed to MFGM [Cano-Ruiz & Richter 1997; Houlihan *et al.*, 1992; Ye *et al.*, 2004].

The microstructure of the MFG is of great importance to the texture of ripened cheeses. Depending on resistance of MFG to disruption caused by processing, fat can be present as small fat globules surrounded by the MFGM, clusters of fat globules with partly disrupted MFGM or pools of TAG filling voids in the protein matrix [Lopez *et al.*, 2007; Michalski *et al.*, 2007].

The MFGM possesses a high water-holding capacity [Goudedranche *et al.*, 2000]. This explains why Emmental produced from native small MFG ( $\sim 3 \mu\text{m}$ ) had 5.0% more moisture on non-fat basis than the cheese made from large MFG ( $\sim 6 \mu\text{m}$ ) after 52 days of ripening, and 2.2% more moisture in the case of Camembert cheese after 40 days of ripening [Michalski *et al.*, 2003, 2004]. The binding of  $\beta$ -lactoglobulin to the MFGM caused by heat treatment is also another reason for the increase in cheese yield [Molina *et al.*, 2000]. Lysophospholipids, which are released from the MFGM by phospholipase treatment before the pressing of the curds, act as surface-active agents and help to emulsify water and fat during processing, leading to their increased retention [Lilbaek *et al.*, 2006]. Serum (moisture) captured by the MFGM can serve as a reservoir where enzymes can act and enhance flavor development.

Hydrolyzed MFGM components may be a source of carbon for some lactic acid bacteria, residing at the interface region of the para-casein matrix and the fat globule surface, which is suggested to interact with the MFGM in Cheddar cheese during ripening [Laloy *et al.*, 1996; Lopez *et al.*, 2007]. Proteolysis caused by starter proteases and proteolysis and lipolysis by MFGM enzymes may lead to a richer and more intense flavour in cheeses with higher MFGM contents [Laloy *et al.*, 1996; Lopez *et al.*, 2007; Ma & Barbano, 2000; Michalski *et al.*, 2003]. The larger fat globule surface area is likely to enhance aroma perception due to a greater contact surface of fat in the mouth [Michalski *et al.*, 2003]. Due to the high amount of unsaturated FAs, MFGM phospholipids are susceptible to oxidation and may provoke a soapy-rancid flavor [Erickson *et al.*, 1964; Lopez *et al.*, 2007].

## FUNCTIONALITIES AND APPLICATIONS OF MFGM

Because of their amphiphilic nature and original function in stabilizing the fat globules in whole milk, MFGM fragments are considered to be efficient, natural emulsifiers [Corredig & Dalgleish, 1997; Corredig & Dalgleish, 1998c; Kanno *et al.*, 1991]. Kanno *et al.* [1991] investigated the emulsifying properties (foam and emulsion stability, emulsion capability and whippability) of MFGM isolates by reconstituting the milk fat globules (MFG). The amounts of MFGM material (20–80 mg MFGM material g/fat) significantly affected the properties of the reconstituted emulsions. A more or less linear decrease in average globule diameters with increasing membrane concentration was observed [Kanno, 1989; Kanno *et al.*, 1991]. The stability of an emulsion against aggregation and coalescence of pure milk fat (25%) and MFGM material (2%) was found to be similar to that of natural milk cream. However, commercial buttermilk was found to have an emulsifying capacity and stability inferior to that of non-fat dried milk, which does contain very little MFGM [Wong & Kitts, 2003]. Similar findings were reported by Corredig & Dalgleish, [1997], where even more concentrated MFGM isolates, prepared by adding citrate followed by high-speed centrifugation to collect the membrane material, were reported to be inferior in emulsifying properties compared with industrial buttermilk, from which they were prepared.

The stability of oil-in-water emulsions with MFGM material depends on the heat treatment of the cream [Corredig & Dalgleish, 1998b] and that MFGM material isolated from raw cream, which did not undergo any heat treatment, had a good emulsifying capacity [Corredig & Dalgleish, 1998b]. The emulsions stabilized by this isolate were stable and the absorbed MFGM material at the interface could not be displaced by surfactants or caseins and  $\beta$ -lactoglobulin added to the emulsions. Both the heat treatment and the churning process used in the industrial manufacture of buttermilk significantly affects the behaviour of the membrane, due to extensive denaturation of the membrane proteins and the association of whey proteins ( $\beta$ -lactoglobulin) with the MFGM [Houlihan *et al.*, 1992]. The pasteurization temperature had no effect on the emulsifying properties of the whole buttermilk, while temperatures higher than 65°C resulted in loss of emulsifying capacity of the MFGM isolate, as the amount of membrane associated  $\beta$ -lactoglobulin augments with an increasing temperature, especially if it exceeds 65°C [Corredig & Dalgleish, 1998a].

Sodini *et al.* [2006] reported that commercial sweet, sour, and whey buttermilks have better emulsifying properties and a lower foaming capacity compared to milk and whey. Furthermore, among the three, whey buttermilk was found to have the best emulsifying properties and the lowest foaming capacity, possibly due to a higher ratio of phospholipids to protein in whey buttermilk compared with the other two.

Different studies on the functionality of MFGM material may have inconsistent results, as they possibly depend on various factors, such as the dairy sources for MFGM isolation, the intensity and frequency of heat treatment (different processing for milk, cream and butter) as well as the preparation conditions of the emulsions. The denaturation of MFGM proteins, the complexation between MFGM proteins and lipid

fractions, and the association of whey proteins to the MFGM, caused by heat treatment, may decrease the solubility of the MFGM isolate [Corredig & Dalgleish, 1998b]. Hence, it may be necessary to completely hydrate the MFGM isolate before using it in emulsion preparations. The temperatures during the preparation of the emulsions may also affect the functional properties [Innocente *et al.*, 1997].

Several MFGM isolate applications, which are mainly based on their emulsifying properties, have been reported. The polar lipids are amphiphilic molecules with a hydrophobic tail and a hydrophilic head group, which largely contribute to the emulsifying capacity of the membrane. They are applied as a baking improver to ameliorate fat dispersion and antistaling, as additives to chocolate to reduce viscosity and prevent crystallization, as wetting enhancer to improve the wetting stabilization of instant products, and as stabilizer of margarine to prevent spattering and browning [Szuhaaj, 1983; Vanhoutte *et al.*, 2004; Vannieuwenhuyzen, 1976, 1981]. The application of polar lipids in other industrial fields includes uses as a drug delivery carrier and as fat liquoring for leather tanning, *etc.* [Guo *et al.*, 2005; Kisel *et al.*, 2001; Vannieuwenhuyzen, 1981]. In contrast with the polar lipid fraction of plants, dairy products contain a substantial part of sphingolipids, which can be used as raw material for the production of ceramides, applicable in the cosmetic industry [Becart *et al.*, 1990]. Due to its major role in maintaining the water-retaining properties of the epidermis, ceramide is of great commercial potential in cosmetic and pharmaceutical industries, such as in hair and skin care products [Zhang *et al.*, 2006].

## NUTRITIONAL ASPECTS OF MFGM COMPONENTS

A general overview of the nutritional aspects of the lipid and protein fraction of the MFGM material is given in Table 6, 7. Several health-promoting effects have been attributed to the MFGM material, but some researchers also reported a link between the MFGM fraction or individual components and disease.

### Lipid fraction of the MFGM

#### *Bioactivity of sphingolipids and metabolites*

The polar lipid fraction of the MFGM consists of glycerophospholipids and sphingolipids, which may represent the most structurally diverse category of lipids in nature. Sphingolipids are functional ingredients, due to their regulatory properties, in addition to their structural functionality, and their effectiveness at low concentrations [Schmelz, 2000]. SM is the major sphingolipid membrane component, which is highly bioactive through its metabolites ceramide, sphingosine and sphingosine 1-phosphate (S1P) [Rombaut *et al.*, 2006].

SM and cerebroside undergo little cleavage in the stomach, but are hydrolyzed in all subsequent regions of the small intestine and colon of rats and mice [Vesper *et al.*, 1999]. The sequential hydrolysis of dietary SM by intestinal alkaline sphingomyelinase and neutral ceramidase results in ceramide and sphingosine by removal of the head groups and the FA [Duan *et al.*, 1995; Nilsson, 1968, 1969]. The metabolites are rapidly absorbed by intestinal cells and reincorporated into complex sphingolipids that remain associated primarily with

TABLE 6. Nutritional aspects of polar lipids of the MFGM and other MFGM components.

Component	Nutritional aspects	References
<b>Polar lipids</b>		
Sphingolipids and metabolites	Reduction of the number of aberrant crypt foci and adenocarcinomas	Dillehay <i>et al.</i> [1994]
	Shift in tumor type (malignant → benign)	Schmelz <i>et al.</i> [1996, 2000]; Symolon <i>et al.</i> [2004]; Spitsberg [2005]
	Anticholesterolemic	Noh & Koo [2003, 2004]; Nyberg <i>et al.</i> [2000]; Eckhardt <i>et al.</i> [2002]
	Protection of the liver from fat- and cholesterol-induced steatosis	Duivenvoorden <i>et al.</i> 2006
	Suppression of gastrointestinal pathogens	Sprong <i>et al.</i> [2002]; Vesper <i>et al.</i> [1999]; Pfeuffer & Schrezenmeir [2001]
	Neonatal gut maturation	Oshida <i>et al.</i> [2003]
	Myelination of the developing central nervous system	Oshida <i>et al.</i> [2003]
	Endogenous modulators of vascular function Associated with age-related diseases and the development of Alzheimer	Michel <i>et al.</i> [2007] Parodi [2001]; Spitsberg [2005]
Sphingosine 1-phosphate	Mitogenic	Zhang <i>et al.</i> [1990, 1991]
Phosphatidyl-serine (PS)	Restore normal memory on a variety of tasks	McDaniel <i>et al.</i> [2003]
	Positive effects on alzheimer patients	Crook <i>et al.</i> [1992]; Heiss <i>et al.</i> [1994]; Pepeu <i>et al.</i> [1996]; Gindin <i>et al.</i> [1998]; Hashioka <i>et al.</i> [2004]
	Improve exercise capacity of exercising humans	Kingsley [2006]
Phosphatidylcholine (PC)	Support liver recovery from toxic chemical attack or viral damage	Kidd [2002]
	Protects the human GI mucosa against toxic attack	Anand <i>et al.</i> [1999]
	Reduction of necrotising enterocolitis	Carlson <i>et al.</i> [1998]
Lysophosphat-idylcholine (lyso PC)	Bacteriostatic and bactericidal capacity	van Rensburg <i>et al.</i> [1992]
	Strong gastroprotective role in the duodenal mucosa	Kivinen <i>et al.</i> [1992]; Kivinen <i>et al.</i> [1992]; Kivinen <i>et al.</i> [1995]
<b>Other components</b>		
Vitamin E and carotenoids	Antioxidants	Spitsberg [2005]

the intestine, or are further degraded to FA *via* fatty aldehydes [Schmelz *et al.*, 1994]. However, not all of the ingested sphingolipids are absorbed and a part of the dietary sphingolipids is excreted *via* the faeces [Nilsson, 1969]. Significant, dose-dependent amounts of SM and its metabolites were found in the intestinal contents, colon and excreted faeces [Nyberg *et al.*, 1997]. SM digestion is slow and is affected by luminal factors such as bile salt, cholesterol, and other lipids. SM and its metabolites may influence triglyceride hydrolysis, cholesterol absorption, lipoprotein formation, and mucosal growth in the gut [Nilsson & Duan, 2006].

#### *Effects of sphingolipids on cancer and bowel-related diseases*

Normal intestinal cells undergo rapid turnover, except in cancer in which normal growth arrest and apoptosis is delayed [Duan, 2005]. SM might exert an effect on colon cancer cells, mainly through its metabolites, ceramide and sphingosine, which induce growth arrest, differentiation and/or apoptosis [Merrill *et al.*, 2001]. Sphingolipids were found to inhibit both the early and the late stages of colon carcinogenesis, in tests on mice in which tumorigenesis was caused by an inherited genetic defect or chemically induced by a chemical agent. Sphingolipid supplementation reduced the number of aberrant crypt foci and, with longer feeding, also the number of

adenocarcinomas [Dillehay *et al.*, 1994]. Moreover, a significant shift in tumor type from the malignant adenocarcinomas to the more benign adenomas was observed [Schmelz *et al.*, 1996, 2000; Spitsberg, 2005; Symolon *et al.*, 2004].

Sphingolipids were found to be chemopreventive as well as chemotherapeutic, *i.e.*, tumor reduction was observed when mice were fed SM before and after tumor initiation [Lemonnier *et al.*, 2003]. It is important to note that the concentrations of sphingolipids that had a detectable effect (0.025–0.5% of the diet of mice) were close to the estimated human consumption (0.01–0.02% of the diet) in the United States [Vesper *et al.*, 1999]. Furthermore, different studies suggest that the enzyme sphingomyelinase, which catalyzes the conversion from SM to ceramide, might have antiproliferative effects on colon cancer cells [Duan *et al.*, 2003].

#### *Sphingolipids and age-related diseases*

Sphingolipids are associated with age-related diseases and the development of Alzheimer's disease [Parodi, 2001; Spitsberg, 2005], as sphingolipid signalling may play a role in the progressive loss of cell function during the aging process. In many tissues, aging results in changes in the SM content [Eisenberg *et al.*, 1969; Jenkins & Kramer, 1988; Levi *et al.*, 1989; Yechiel & Barenholz, 1986]. Furthermore, ceramide has been implicated

TABLE 7. The major proteins and other components of the milk fat globule membrane with their abbreviation, molecular mass (M), iso-electric point (IEP), function and health aspects.

Component	Abbr.	M (kDa)	IEP	Function	Health aspects	References
Fatty acid binding protein	FABP	13	5–5.5	Transport of fatty acids	Cell growth inhibitor	Spitsberg <i>et al.</i> [1995]; Peterson <i>et al.</i> [1998a]
				Regulation of lipid metabolism	Anticancer factor (FABP as selenium carrier)	Kromminga <i>et al.</i> [1990]; Bansal & Medina [1993]; Whanger [2004]; Spitsberg & Gorewit [2002]; Riccio [2004]
				Increase of lipid droplets in cytoplasm	Similar to P2 myelin protein involved in EAN	
Butyrophilin	BTN	66–67	5.32	MFG secretion	Suppression of MS	Mana <i>et al.</i> [2004]; Guggenmos <i>et al.</i> [2004]
				Belongs to Igs	Induces or modulates EAE	Johns & Bernard [1999]; Stefferl <i>et al.</i> , [2000a]; Stefferl <i>et al.</i> [2000b]
					Influences pathogenesis of autistic behavior	Vojdani <i>et al.</i> [2002]
Xanthine oxidase	XDH /XO	146 (300)	7.8	Structural, lipid secretion	Bactericidal agent	Martin <i>et al.</i> [2004]; Hancock <i>et al.</i> [2002]
				Role in purine metabolism	Redox reaction / anti-inflammatory	Spitsberg [2005]; Fong <i>et al.</i> [2007]
Mucin 1	MUC1	160–200	< 4.5	Protection from physical damage	Protective effect against rotavirus infection	Kvistgaard <i>et al.</i> [2004]
Breast cancer type 1 susceptibility protein	BRCA1			Cancer suppressor	Inhibition of breast cancer	Spitsberg & Gorewit [1997, 1998]
Breast cancer type 2 susceptibility protein	BRCA2			Cancer suppressor Direct regulator of cytokinesis	Inhibition of breast cancer	Vissak <i>et al.</i> [2002] Daniels <i>et al.</i> [2004]
Lactadherin	PAS 6/7	47	6–6.6	Member of cadherins	Protection from viral infections in the gut	Kvistgaard <i>et al.</i> [2004]
				Ca-dependent adhesive properties	Role in epithelialization, cell polarization, cell movement and rearrangement, neuriteout growth	Riccio [2004]
				Phospholipid binding	Synaptic activity in the central nervous system	
Proteose peptone 3	PP3	18–30/14	6.3	Membrane associated Expressed in lactating mammary gland		Fong <i>et al.</i> [2007]
Adipophilin	ADPH	52	7.5–7.8	Uptake and Transport of FA/TAG		Riccio [2004]
Periodic acid Schiff 3	PAS 3	95–100	< 4.5	Marker of the secretory and ductal epithelium		Riccio [2004]
Cluster of differentiation	CD 36	76–78	< 7	Macrophages marker Phagocytosis by Neutrophils		Riccio [2004]
Other components						
$\beta$ -glucuronidase inhibitor					Inhibition of colon cancer	Ito <i>et al.</i> [1993]
<i>Helicobacter pylori</i> inhibitor					Prevention of gastric diseases	Wang <i>et al.</i> [2001]
					Organic phosphorus /Ca-phosphate source	Spitsberg & Gorewit [1997]
MFGM antigens					Coronary Atherogenic effects	Moss & Freed [2003]

EAE: Experimental autoimmune encephalomyelitis, EAN: Experimental allergic neuritis.

as a mediator of senescence in a cell culture model for aging [Lee & Obeid, 1997; Venable *et al.*, 1995; Vesper *et al.*, 1999].

#### Anticholesterolemic effects of sphingolipids

Sphingolipids are also involved in the intestinal uptake of cholesterol. SM was found to dose-dependently lower the in-

testinal absorption of cholesterol and fats in rats [Eckhardt *et al.*, 2002; Noh & Koo, 2003; Nyberg *et al.*, 2000].

Duivenvoorden *et al.* [2006] reported that dietary sphingolipids play an important role in lowering plasma cholesterol and triacylglycerol and protecting the liver from fat- and cholesterol-induced steatosis. They suggested that

dietary sphingolipids should be considered as compounds that not only treat or ameliorate the lipid component of cardiovascular disease, but also the inflammatory processes involved in atherosclerosis and insulin resistance. They concluded that dietary sphingolipids hold great potential to treat multiple aspects of the metabolic syndrome, such as dyslipidemia, insulin resistance and cardiovascular diseases.

#### *Bactericidal effect of sphingolipids*

Dietary sphingolipids could have protective capacities against bacterial toxins and infection by bacteria or viruses. It is plausible that food sphingolipids can compete for and act as cellular binding sites [Bibel *et al.*, 1992; Fantini *et al.*, 1997], since many bacteria, as well as bacterial toxins and viruses, use glycosphingolipids to bind to cells [Karlsson, 1989]. As the adherence of the pathogens to the intestinal mucosa is often the first step in infection, the competition leads to an elimination of pathogens from the intestine, which causes a shift in the bacterial population of the colon [Pfeuffer & Schrezenmeir 2001; Vesper *et al.*, 1999]. When infants are given a formula supplemented with gangliosides, significantly fewer *Escherichia coli* and more bifidobacteria were observed in their faeces than those of the control group [Rueda *et al.*, 1998].

Sprong *et al.* [2001, 2002] even reported direct *in vitro* bactericidal activities of digestion products of sphingolipids. Ceramide was not bactericidal at the tested concentration of 100 mmol/L, but lysophingomyelin appeared highly bactericidal against *Campylobacter jejuni*, *Listeria monocytogenes* and *Clostridium perfringens*, and showed moderately lowered viable counts of *E. coli* and *Salmonella enteritidis*. Sphingosine decreased viable counts of all pathogens tested. These authors also observed a decreased colonization of *L. monocytogenes* in rats fed diets based on sweet buttermilk powder compared with rats fed skim milk diets, which leads to the suggestion that bovine milk sphingolipids may also protect against gastrointestinal infections.

#### *Phospholipids*

The major types of polar lipids (PL) present in the membrane are phosphatidylcholine (PC), 35%; phosphatidylethanolamine (PE), 30%; sphingomyelin, (SM), 25%; phosphatidylinositol (PI), 5%; phosphatidylserine (PS), 3%. Glucosylceramide (GluCer), lactosylceramide (LacCer) and gangliosides (Gang) are present in trace amounts [Danthine *et al.*, 2000; Deeth, 1997].

Many neuronal effects of ageing in animals are attenuated by PS, and it was also shown to restore normal memory on a variety of tasks. Preliminary findings with humans, though, are limited [McDaniel *et al.*, 2003]. At elevated doses of 200 mg per day, clinical trials with patients suffering from Alzheimer's disease showed positive effects [Crook *et al.*, 1992; Gindin *et al.*, 1998; Hashioka *et al.*, 2004; Heiss *et al.*, 1994; Pepeu *et al.*, 1996]. PS supplementation on exercising humans showed that PS might alter neuroendocrine function and positively influenced perceived muscular soreness and well-being. Furthermore, oral supplementation with soybean PS has recently been demonstrated to improve exercise capacity during high-intensity cycling [Kingsley, 2006].

Generally, the contribution of dairy products will be of limited significance, since PS is only present in small amounts [Rombaut & Dewettinck, 2006].

PC is believed to clinically support liver recovery from toxic chemical attack (pharmaceuticals, alcohol, mushroom poisoning) or acute or chronic viral (hepatitis) damage [Kidd, 2002]. PC also protects the human gastrointestinal mucosa against toxic attack [Anand *et al.*, 1999] and reduces life-threatening necrotizing enterocolitis in hospitalized preterm infants [Carlson *et al.*, 1998].

As in the case of SM, PC is a source of choline, which is an essential nutrient for humans. It is believed to promote synthesis and transmission of neurotransmitters important to memory, and might also be involved in brain development [Blusztajn, 1998; Szuhaj & Nieuwenhuyzen, 2003]. Furthermore, some phospholipids are digested in the gastrointestinal tract to compounds that might possess antimicrobial activity [van Hooijdonk *et al.*, 2000].

### **Protein fraction of the MFGM**

#### *Anticancer effects*

Spitsberg & Gorewit [1997] studied the effect of some bovine mammary gland proteins and some MFGM proteins on cancer cell growth. One of the isolated proteins of bovine MFGM, namely fatty acid binding protein (FABP), has been found to inhibit the growth of some breast cancer cell lines *in vitro* at extremely low concentrations [Kromminga *et al.*, 1990; Peterson *et al.*, 1998b; Spitsberg & Gorewit, 2002; Spitsberg *et al.*, 1995]. Furthermore, they demonstrated the presence of the onco-suppressor BRCA1 protein in bovine and human MFGM [Spitsberg & Gorewit, 1998]. Vissak *et al.* [2002] detected BRCA1 and BRCA2 in extracts obtained from human and bovine MFGM using affinity chromatography. Both the BRCA2 and BRCA1 proteins are involved in DNA repair processes, although BRCA2 has an additional function as one of the direct regulators of cytokinesis [Daniels *et al.*, 2004].

#### *Butyrophilin and multiple sclerosis*

Multiple sclerosis (MS) is a progressive autoimmune condition that affects the CNS. It is a chronic neurodegenerative disease of the CNS, in which relentless attacks of autoimmune-mediated inflammation result in demyelination, loss of oligodendrocytes and axonal degeneration. Although the etiology of MS remains unknown, both genetic and environmental factors are believed to be involved. It has been suggested that milk and dairy products may cause MS, or exacerbate symptoms or progression in MS patients [Butcher, 1976, 1986; Lauer, 1997; Malosse & Perron, 1993; Malosse *et al.*, 1992]. BTN can both trigger the development of experimental autoimmune encephalomyelitis (EAE), or suppress the disease [Berer *et al.*, 2005; Stefferl *et al.*, 2000b].

#### *Butyrophilin and autism*

Autism is a chronic neurodevelopmental disorder characterized by social and language impairments and stereotyped, repetitive patterns of behaviour [Bailey *et al.*, 1996; Kolevzon *et al.*, 2007]. As in the case of MS, its etiology is still unknown and it may have a variety of causes including genetic, environmental, immunological and neurological factors [Riccio, 2004].



Immunological research has suggested that the immune system plays an important role in the development of autism [Ashwood & Van de Water, 2004; Pardo *et al.*, 2006; Stigler, 2006]. Moreover, Vojdani *et al.* [2002] detected antibodies against nine different neuronspecific antigens in the sera of children with autism. The antibodies could bind with different encephalitogenic molecules, which have sequence homologies with the milk protein BTN. These results suggest a role for antibodies against brain cross-reactive food antigens and infectious agents in the pathogenesis of autistic behaviour.

#### Direct antibacterial effects of MFGM proteins

The role of XDH/XO as an antimicrobial agent in the gastrointestinal tract has been reviewed recently Harrison [2006] and Martin *et al.* [2004]. XDH/XO is expressed in different cells of the gastrointestinal lining, and its antimicrobial function is related to production of reactive oxygen species, superoxide and hydrogen peroxide in the gut. It may also catalyze the reduction of inorganic nitrite to nitric oxide, and in the presence of oxygen to peroxyxynitrite, which both show bactericidal properties. Pathogenic bacteria interact with epithelial membranes of the digestive tract, but can also bind to similar receptors on the MFGM. The latter can act as a decoy to avoid that bacteria interact with their primary target site. At the same time, these bacteria are subjected to the antimicrobial effects of XDH/XO, since XDH/XO is a major component of the MFGM. Chromatographically purified XDH/XO was shown to inhibit the growth of *Staphylococcus aureus*, *E. coli* and *Sal. enteritidis*, through the effect of hydrogen peroxide formation or the stimulation of the lactoperoxidase system in milk [Harrison, 2004, 2006; Martin *et al.*, 2004].

Lactophorin, a 23-residues cationic peptide derived from bovine milk component-3 of proteose peptone (PP3), and presumably part of the MFGM, displays growth-inhibitory activity against some Gram-positive (*Streptococcus thermophilus*) and Gram-negative (*Salmonella*) bacteria, but no activity was observed against *E. coli*. The interaction of lactophorin with natural lipid membranes possibly causes the inhibitory effect [Campagna *et al.*, 2004].

#### Antiadhesive effects of the MFGM

Some forms of stomach diseases, such as chronic type B gastritis, peptic ulcer disease and stomach cancer, can etiologically be attributed to the colonization of stomach mucosa with *Helicobacter pylori* [Atherton, 2006; Cover & Blaser, 1992; Fox & Wang, 2007], which can cause *in vitro* hemagglutination. Mucins separated from human gastric juices inhibit sialic acid-specific hemagglutination of *H. pylori*, an activity that is significantly reduced after removal of sialic acids from the mucins. Delipidated bovine MFGM material shows inhibitory potencies similar to that of these gastric mucins, while low molecular mass components from milk like glycomacropptide (GMP) and sialyl ( $\alpha$ -2,3)-lactose show much less inhibitory activity. These results suggest that the high molecular mass mucin-like components from bovine MFGM are most important for the inhibitory potency of the MFGM [Hirmo *et al.*, 1998].

#### MFGM proteins and coronary heart disease

Based on epidemiological analysis, Moss & Freed [2003] reported the presence of antibodies against the MFGM proteins. CHD death is also associated with circulating antibodies against MFGM proteins, raising the possibility that MFGM antigens have both biochemical and immunological coronary atherogenic effects [Riccio, 2004].

### FUTURE PERSPECTIVES

Further work is needed on the quantification of MFGM components in various dairy products, and on the optimization of food-grade downstream production processes for MFGM components that can be applied in the food industry. Higher quantities of well-characterized MFGM-components are also needed to further evaluate the technological, nutritional and bioactive properties of these valuable components.

Further research should increasingly focus on studying the milkfat globule in its native environment (*i.e.* milk plasma) and in real time. Such studies will contribute significantly to the understanding of the MFGM and how it changes post secretion by the mammary secretory cell. This knowledge will lead to improved milk-handling practices, more efficient manufacturing processes and better dairy products.

Further experiments would require a controlled feeding regime, as factors other than stage of lactation, such as feed (the quality of which can be season dependent), may have confounded the results obtained in previous studies.

Furthermore, the results of the study on buffalo milk suggest that mammary gland development plays a significant role [Singh & Ganguli, 1976], particularly in the days immediately post partum. On the basis of the fact that large compositional changes occur in the first few days post partum [Anderson & Cheeseman, 1975], it is postulated that significant changes in fat globule stability take place during this period.

Further research is required, as in most disease cases the evidence for the presumed beneficial or detrimental effects of MFGM components is quite weak or even absent.

The future challenge of MFGM analysis will depend on the development of new strategies in the field of protein-protein interaction technologies, protein chips, and mass spectrometry. All these technological advances will contribute to clarify the multifunctional roles of MFGM proteins in milk.

From the literature, by means of the MFGM material, new cheeses or yoghurts with different functional and nutritional properties can be produced. This perspective could also bring economical profits by increasing the product yield or using low value by-products from the dairy industry, such as buttermilk. However, the use of processed MFGM material may result in different effects compared with native MFGM. This has to be taken into account when developing products with desired and controlled characteristics.

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