Milk Fat Globule Membrane Components—A Proteomic Approach

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Abstract The milk fat globule membrane (MFGM) is the membrane surrounding lipid droplets during their secretion in the alveolar lumen of the lactating mammary gland. 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. Despite their low classical nutritional value, MFGM proteins have been reported to play an important role in various cellular processes and defense mechanisms in the newborn.

Using a proteomic approach, such as high-resolution, two-dimensional electrophoresis followed by direct protein identification by mass spectrometry, it has been possible to comprehensively characterize the subcellular organization of MFGM.

This chapter covers the description of MFGM proteomics from the first studies about 10 years ago through the most recent papers. Most of the investigations deal with MFGMs from human and cow milk.

Milk Fat Globule Membrane

The principal lipids of milk are triacylglycerols secreted in the alveolar lumina in the form of droplets, coated with a cellular membrane, called the milk fat globule membrane (MFGM) (Mather & Keenan, 1998). MFGM is a tripartite structure, consisting of the typical bilayer membrane as the outer coat, with an electron-dense material on the inner membrane face, and finally, the monolayer of proteins and polar lipid that covers the triacylglycerol droplet core.

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Lactating mammary cells assemble and release lipid droplets by a unique mechanism; microlipid droplets (<0.5-μm diameter) originate in or on the surfaces of rough endoplasmic reticulum membranes. These droplets are released from the endoplasmic reticulum into the cytosol with a surface coat of proteins and polar lipids. Microlipid droplets grow by fusion with each other and form larger cytoplasmic lipid droplets (>1-μm diameter). These droplets migrate unidirectionally from their sites of origin, mostly in basal and lateral cell regions, to the apical region, probably with the involvement of the cytoskeletal elements. The materials on the surface of the lipid droplets appear to remain associated with the droplets when they are secreted as milk fat globules (Cavaletto et al., 2004; Heid & Keenan, 2005).

Cytoplasmic lipid droplets approach the apical surface, are gradually coated with plasma membrane, and then are released into the alveolar lumen completely surrounded by plasma membrane, as first described by Bargmann and Knoop (1959) and reviewed by Mather and Keenan (1998). In some cases, a cytoplasm inclusion is entrapped into the secreted globules and appears as "crescent" material between the outer membrane layer and the lipid globule.

An alternative mechanism of lipid globule secretion has been described by Wooding (1971), who proposed the progressive fusion of secretory vesicles on the surface of the lipid droplet, leading to the formation of an intracytoplasmic vacuole released by exocytosis; in this case the outer membrane of the lipid globule would be entirely derived from the secretory vesicle membrane. Such a mechanism may be common during the periparturient period or when milk secretion is inhibited (Mather & Keenan, 1998). See Fig. 1 for a schematic representation of the two proposed mechanisms for milk fat globule secretion.

Until now no definitive conclusion has been made on the contribution of the apical plasma membrane or the secretory vesicle membrane to the MFGM, and a combination of the two mechanisms of secretion may be possible.

New proteomic studies on the MFGM characterization will help in defining the molecular basis of the biological processes, involved in the origin and secretion of milk fat by mammary epithelial cells.

Proteomic Analysis

The proteome, or the protein complement of genome, is the full set of proteins expressed by a genome under a particular set of environmental conditions (Pandey & Mann, 2000). Proteomics is a relatively new field and one of the fastest-growing areas of biological research, thanks to its potential to unravel biological mechanisms not accessible by other technologies. Since proteins do not work in isolation, but function in large arrays that form

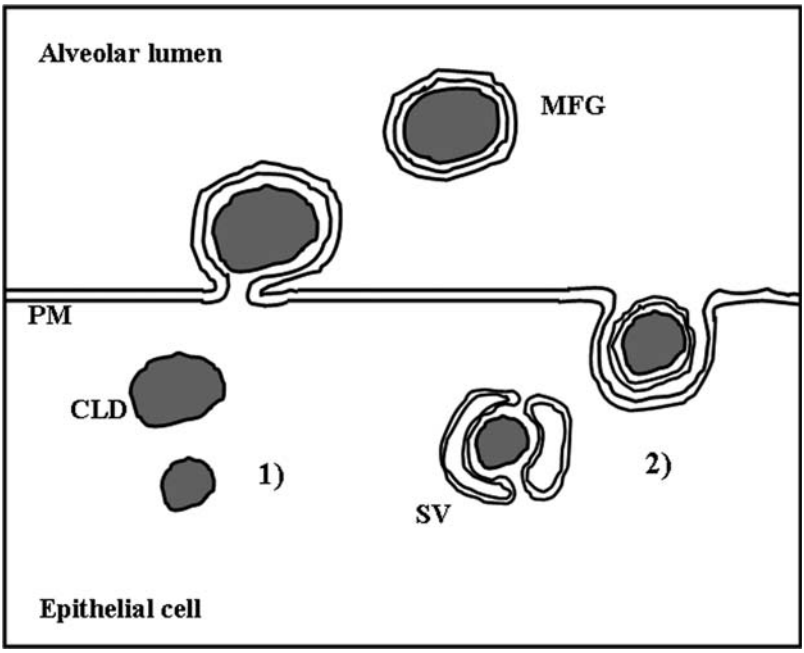

Fig. 1 Schematic representation of the two secretion mechanisms of the lipid globules in the apical region of the mammary epithelial cell. (1) Secretion by apical membrane envelopment of CLD. (2) Secretion by fusion of secretory vesicles on the surface of CLD, followed by release by exocytosis. The tripartite structure of MFGM is shown, with an intervening space between the lipid droplet surface and the surrounding outer bilayer. Size distributions between lipid droplets volume and plasma membrane bilayer are not to scale. (CLD = cytoplasmic lipid droplet; MFG = milk fat globule; PM = plasma membrane; SV = secretory vesicle.)

protein machines, proteomics is exciting because it allows one to dissect and analyze this complex machine into its component parts and to understand how it is assembled, how the proteins interact with one another, and what goes wrong in disease.

The combination of isoelectric focusing (IEF) and sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE), commonly known as two-dimensional electrophoresis (2-DE), was developed in the early 1970s. It is still the method of choice for high-resolution profiling of proteins in biological samples (O'Farrel, 1975; Görg et al., 2004). With 2-DE, several thousands of proteins can be resolved on a single-slab gel, also named a bidimensional map.

Following electrophoresis, 2-DE maps may be compared between samples obtained under different physiological and/or experimental conditions; then, using image analysis software, it is possible to specifically detect up- and downregulated proteins (comparative proteomics). Recently, a number of

sensitive and specific fluorescent stains have been developed that allow multiplex staining of different groups of proteins on the same gel, thus enhancing differential analysis (Patton & Beechem, 2001).

Protein identification after 2-DE separation is typically accomplished using trypsin in-gel digestion of corresponding protein spots, followed by peptide mass fingerprinting (PMF) via mass spectrometry (MS), or peptide sequencing via tandem MS (MS/MS). Proteomic MS employs soft, nondestructive ionization methods such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). The most common analyzer platforms range from the quadrupole (Q), the ion trap (IT), to the time of flight (TOF). Several software algorithms compare the observed peptide masses and the fragmentation masses against those predicted from theoretical peptides within the sequence database (McDonald & Yates, 2000).

Although proteomic technology is advancing, some limitations become evident, such as lack of automation and insufficient dynamic range. Biological samples are characterized by large differences in the concentrations of the most and least abundant cellular proteins (approximately 5-log difference). Many proteins involved in signal transduction are present in low abundance and thus are not readily detectable in crude extracts.

Other limitations include detection of proteins with extremes in pI and molecular weight and membrane-associated proteins.

As an alternative to gel-based proteomic investigations, multidimensional liquid chromatographic methods have been combined with MS (LC MS/MS) to enable the profiling of complex protein mixtures (MudPIT technology). In general, this strategy includes a strong cation exchange in line with a reverse-phase column and allows one to directly analyze the digests of protein mixtures, yielding good results for the identification of hydrophobic proteins (Link et al., 1999).

In order to detect low-abundance proteins, a powerful strategy is prefractionation of the sample, leading to the subcellular proteome characterization. The identification of subsets of proteins at the subcellular level is therefore an initial step toward the understanding of protein translocation and cellular function (Dreger, 2003). With the fractionation of organelles and subcellular compartments, minor proteins, such as regulatory proteins or integral membrane proteins, are enriched and more easily characterized.

In this context, milk proteins can be fractionated by centrifugation into three major subsets: soluble whey proteins, the pellet of casein micelles, and the floating proteins associated with the MFGM (Cavaletto et al., 2004). The proteome of the MFGM succeeds in profiling this class of milk membrane proteins, which represent only 1–4% of total milk proteins and usually are lacking in the proteome of the whole milk, masked by the most abundant caseins.

Figure 2 summarizes the principal approaches to the proteomic analysis of the MFGM.

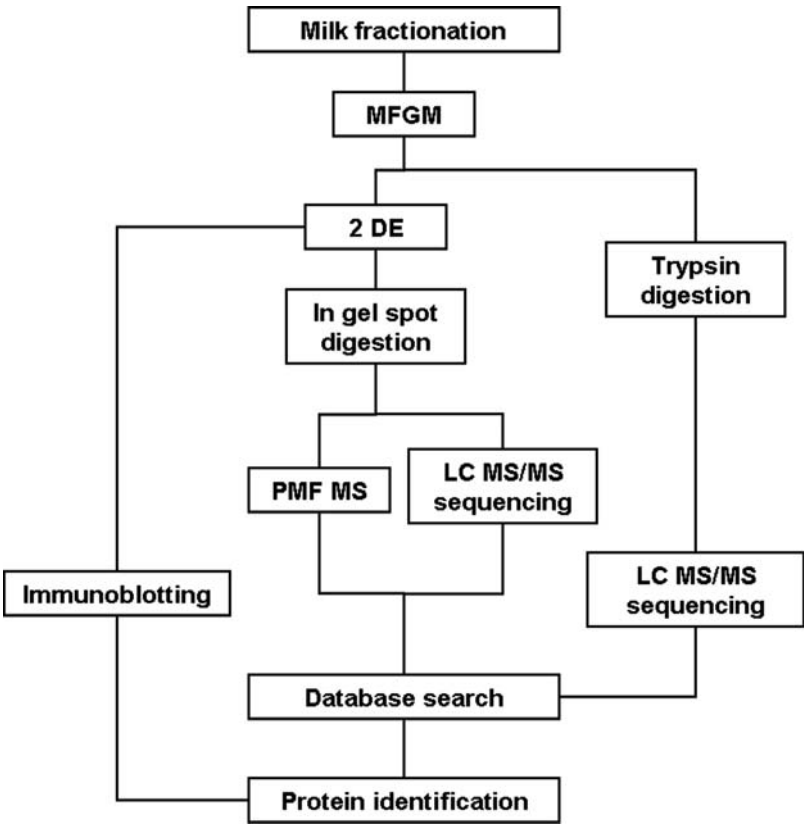

Fig. 2 Strategies applied to the proteomic analysis of the MFGM

Proteomic Approach to MFGM Characterization

Bovine MFGM

The first 2-DE separation of bovine MFGM protein was reported in the review of Mather (2000). The review describes the major proteins associated with the bovine MFGM; these corresponded to seven major bands when separated by SDS-PAGE, while in 2-DE each band was resolved into a series of related isoelectric variants. Major proteins included mucin 1, xanthine oxidase, CD36, butyrophilin, adipophilin, PAS6/7 (lactadherin), and fatty acid binding protein. Identification of the MFGM components was based largely on comparison of electrophoretic mobilities, staining characteristics, and reaction with specific antibodies. The review reported the protein characterization by means of molecular cloning, sequencing, and comparative analysis with MFGM proteins from other species.

In 2002, the effect of heat treatment on bovine MFGM proteins from early, mid-, and late season was characterized using one- and two-dimensional SDS-PAGE under reducing and nonreducing conditions (Ye et al., 2002). It was found that xanthine oxidase and butyrophilin formed aggregates via intermolecular disulfide bonds after heating.

Two papers have recently described the proteome of bovine MFGM (Fong et al., 2007; Reinhardt & Lippolis, 2006). Fong et al. (2007) used the classic proteomic approach to profile the protein and lipid composition of bovine MFGM. Protein identification was carried out using PMF and MS/MS analysis, while lipid composition was determined with a combination of capillary gas chromatography and LC-MS. The composition of MFGM resulted in 69–73% lipid and 22–24% protein; polymeric immunoglobulin receptor, apolipoprotein A and E, 71-kDa heat shock cognate protein, clusterin, lactoperoxidase, and peptidylprolyl isomerase have been identified among minor proteins.

Reinhardt and Lippolis (2006) fractionated MFGM by monodimensional electrophoresis, digested gel slices, and performed protein identification via the LC-MS/MS approach. Among the 120 identified MFGM proteins, 71% were membrane-associated, while 29% were cytoplasmic or secreted proteins; functional immune proteins such as CD14 and Toll-like receptors 2 and 4 have also been detected in the MFGM.

In another recent study, the proteome of bovine MFGM has been compared in three different conditions: from peak lactation, during the colostrum period, and during mastitis (Smolenski et al., 2007). The work is the most comprehensive characterization to date of minor proteins in bovine milk (fractionated in skim milk, whey, and MFGM); 95 distinct gene products were identified, comprising 53 proteins identified through direct LC-MS/MS and 57 through 2-DE followed by MS. The authors demonstrated that a significant fraction of minor proteins are involved in protection against infection.

Human MFGM

The first separation of human MFGM by 2-DE was described in Goldfarb (1997), in which 17 proteins were identified by immunoblotting with specific immunoprobes. The high resolution of 2-DE brought to the detection multiple spots of different pIs due to the presence of multiple isoforms. Besides the typical MFGM proteins, such as xanthine oxidase, butyrophilin, and fatty acid binding protein, other proteins were mapped, including the IgM μ chain, the IgA α chain, the HLA class I heavy chain, immunoglobulin light chains, secretory piece, J chain, actin, α acid glycoprotein, albumin, and casein, with particular attention to the pattern of apolipoproteins E, A-I, A-II, and H.

In 2001, the map of human colostral MFGM was published (Quaranta et al., 2001). This was the first report of MFGM proteome in which proteins were directly identified by PMF MS and/or N-terminal sequencing. Using a new

MFGM double-extraction method with SDS followed by urea/thiourea/CHAPS, 23 protein spots were identified. The main spots corresponded to lactadherin, adipophilin, butyrophilin, and carbonic anhydrase; the latter had not previously been detected in association with the MFGM. Proteomic analysis revealed the presence of other minor identified MFGM components, including α-lactalbumin, casein, disulphide isomerase, and clusterin (or apolipoprotein J), the latter two as newly identified proteins in human MFGM.

Human butyrophilin expression was evaluated in a comparative proteomic approach (Cavaletto et al., 2002) between colostral and mature milk. While searching the protein complement of seven human butyrophilin transcripts, known only at the mRNA level and mapping on chromosome 6, the authors found 14 multiple forms of butyrophilin; among them, a butyrophilin at pI 6.5 was shown in mature MFGM, whereas the putative butyrophilin, named BTN2A1, was detected for the first time at a protein level.

In 2002, proteomics was applied to the characterization of N-glycosylation (glycomics) of MFGM proteins (Charlwood et al., 2002). The composition of N-linked sugars was analyzed in a hybrid mass spectrometer (MALDI-Q-TOF), after in-gel enzymatic release and subsequent derivation of glycans. Four proteins, clusterin, lactoferrin, polymeric Ig receptor, and lactadherin, were found to possess a wide range of different sugar motifs. In particular, multiple fucosylation products, probably linked to infant protection against bacterial and viral infections, were highlighted.

The first annotated database of human colostral MFGM proteins separated by 2-DE was published in 2003 (Fortunato et al., 2003) and is available in the WORLD-2DPAGE List database at http://www.expasy.org/ch2d/2d-index.html as a partially federated map (Appel et al., 1996). With PMF by MALDI-TOF MS and sequencing by nanoESI-IT MS/MS, 107 protein spots were identified, many of which were present as multiple spots due to posttranslational modifications. On the whole, they derived from 39 genes or gene families. About 60% of the identified proteins were typical MFGM or mammary gland–secreted proteins, and 10% were linked to protein folding and destination, among them cyclophilin, a peptidylprolyl isomerase involved in the response to inflammatory stimuli. Proteins involved in intracellular trafficking and/or receptorial activities were detected in 9%. The cargo selection protein or TIP47, which could interact with the lipid droplet surface, adipophilin and butyrophilin in the process of budding and secretion of the MFGM, has been identified in this group. The remaining minor proteins are correlated with signal transduction, complement complex, and glutathione metabolism.

Mouse MFGM

Wu et al. (2000) described a comparative proteomic analysis between the mouse MFGM and the cytoplasmic lipid droplets (CLDs) of the mouse liver and the

mammary gland. The authors tried to dissect the complexity of the lipid secretion and provided evidence that mammary CLDs were intimately associated with membrane-like structures originating from the endoplasmic reticulum.

Since liver CLDs differed from mammary CLDs in protein composition, it was elucidated that different lipid secretion mechanisms occurred in the mammary epithelial cells and in the hepatocytes. Finally, a subset of the MFGM proteins were found also to be present in mammary CDLs, thus suggesting that the membranes and the adherent proteins associated with CDLs were involved in the secretory process.

MFGM Proteins: From Classic to Newly Identified by Proteomics

While MFGM proteins have very low classical nutritional value, they play important roles in various cell processes and in the defense mechanism for the newborn. In addition, the molecular pathways underlying the secretion of milk fat globules have not yet been elucidated, mostly due to the lack of established cell lines that secrete lipid globules.

The proteomic approach to the study of MFGM complex organization will help in defining the roles of MFGM at the level of both the mammary gland and the newborn gastrointestinal tract.

The proteomic investigations dealing with MFGM have directly confirmed, by MS identification, the presence of the classic major proteins associated to MFGM, and in some cases posttranslational modifications have been highlighted.

Thanks to its high-resolution power and high sensitivity, proteomics has resulted in the identification of numerous minor proteins that were not known to be associated with the MFGM and whose function in secreted milk still has to be elucidated. Table 1 lists the minor MFGM proteins, identified by proteomics.

Table 1 List of the Minor MFGM Proteins, Identified by Proteomic Tools

Minor Protein	Function
Actin	Cell motility
Albumin	Binding and transport
Aldehyde dehydrogenase	Metabolic enzyme
α1-Acid glycoprotein	Structural protein
Annexin 1, A2	Structural protein
Apolipoprotein A-1	Transport and lipoprotein metabolism
Apolipoprotein A-2	Transport and lipoprotein metabolism
Apolipoprotein A-4	Transport and lipoprotein metabolism
Apolipoprotein C1	Transport and lipoprotein metabolism

Table 1 (continued)

Minor Protein	Function
Apolipoprotein E	Transport and lipoprotein metabolism
Apolipoprotein H	Transport and lipoprotein metabolism
ATP synthase	Metabolic enzyme
Breast cancer suppressor 1	Mediator of metastasis suppression
α-Casein	Transport of calcium phosphate
β-Casein	Micelle stability
Cathelicidin	Protection
CD14	Immune system
CD36	Receptor and adhesion
CD59	Inhibitor
Cholesterol esterase	Triglyceride hydrolysis
Clusterin	Apoptosis
Complement C4 γ-chain	Complement activation
CRABP II	Cell differentiation
Disulfide isomerase	Protein destination
Dynein intermediate chain	Microtubule motor
Endoplasmin	Protein destination
Enolase 1	Metabolic enzyme
ERcarboxylesterase	Triglyceride synthesis
ERP29	Secretion
ERP99	Secretion
Fatty acid binding protein	Lipid transport
Fatty acid synthase	Lipid synthesis
Fibrinogen	Platelet aggregation
Gelsolin	Cytoskeletal structure
Gephyrin	Cytoskeletal interaction
Glucose regulated protein 58 kDa	Chaperone
Glutamate receptor	Signal transduction
γ-Glutamyl transferase	Glutathione metabolism
GAPDH	Metabolic enzyme
Glycerol-3-P DH	Metabolic enzyme
GTPbinding protein	Signal transduction
GTPbinding protein SAR1b	Transport
GRP 78	Protein destination
GSHH	Protection
Heat shock 27 kDa	Chaperone
Heat shock 70 kDa	Chaperone
Heme binding protein	Transport
Histone H2, H3	DNA binding
HLA class I	Immune system
Immunoglobulin A	Secretory immunity
Immunoglobulins D, G, M	Immune system
Isocitrate DH	Metabolic enzyme
J chain	Immune system
Keratin type II	Cytoskeletal structure
KIAA1586 protein	DNA binding

Table 1 (continued)

Minor Protein	Function
α-Lactalbumin	Lactose synthesis
Lactoferrin	Iron transport
Lactoperoxidase	Metabolic enzyme
Lysozyme	Protection
Macrophage protein 65 kDa	Protection
Macrophage scavenger receptor	Protection
β2-Microglobulin	Protection
Migration inhibitor factor MIF	Cytokine
Oxoprolinase	Metabolic enzyme
Peptidoglycan recognition protein	Protection
Peroxiredoxin IV	Protection
Peroxisome coactivator 1	DNA binding
Poly Ig receptor	Ig superfamily
Prohibitin	Signal transduction
14-3-3 Protein	Signal transduction
Proteose peptone 3	Structural protein
Pyruvate carboxylase	Lipogenesis
Rotamase (cyclophilin)	Folding
S100 Ca binding protein	Transport
SCY1-like2	Signal transduction
Secretory piece	Immune system
Selenium binding protein	Transport
TER ATPase	Membrane fusion
TIF32/RPG1	Cytoskeletal structure
Toll-like receptor 2, 4	Immune system
Transforming protein RhoA	Signal transduction
Tubulin	Structural protein
Villin 2	Structural protein
Vimentin	Structural protein
Voltage-dependent anion channel	Signal transduction
WNT-2B protein	Cellular development

Major MFGM Proteins

Butyrophilin, the most abundant protein in MFGM, is a type 1 membrane glycoprotein. It consists of two extracellular immunoglobulin-like domains and a large intracellular domain homologous to ret finger protein. Butyrophilin may have some receptorial function; it has been exploited to modulate the encephalitogenic T-cell response, supporting its possible involvement in autoimmune diseases (Cavaletto et al., 2002).

Mucin 1 is a highly glycosylated transmembrane protein, a fragment of which co-migrates with butyrophilin (Fong et al., 2007). Mucin is resistant to degradation in the stomach due to its high degree of glycosylation.

Additionally, O-linked sugar chains confer a protective role against the attachment of fimbriated microorganisms (Hamosh et al., 1999).

Lactadherin, the human glycoprotein homologue of bovine PAS 6/7 and mouse MFG-E8, consists of an epidermal growth factor-like domain and two C1 and C2 domains similar to those found in coagulation factors V and VIII. It does not contain the transmembrane domain but might bind to the membrane bilayer by acylation or hydrophobic interaction. Lactadherin promotes cell adhesion via integrins and inhibits rotavirus binding and infectivity (Quaranta et al., 2001).

Adipophilin and TIP47 (Fong et al., 2007; Fortunato et al., 2003; Sztalryd et al., 2006) are typical proteins associated with the surface of the cytoplasmic lipid droplets, suggesting an important structural role for lipid droplet packaging and storage. TIP47 was first identified as a cargo protein involved in the trafficking of the mannose-6-phosphate receptor. Adipophilin and TIP47 are both widely distributed among nonadipogenic tissues.

Carbonic anhydrase is a glycosylated enzyme present in many biological fluids and mostly in saliva. It has been shown to be an essential factor in the normal growth and development of the gastrointestinal tract of the newborn (Karhumaa et al., 2001; Quaranta et al., 2001).

Lactoferrin inhibits the classical pathway of complement activation and can have a bacteriostatic action by competing with bacteria for iron. Also, lactoferrin may function as an antibiotic agent for its structural properties (Cavaletto et al., 2004; Charlwood et al., 2002; Smolenski et al., 2007).

Xanthine oxidase is a cytosolic enzyme concentrated along the inner face of the MFGM. Protein–protein interactions are responsible for the formation of a supramolecular complex among xanthine oxidase, butyrophilin, and adipophilin, probably involved in lipid globule secretion (Mather, 2000). Due to its enzymatic activity, it can act as a defense protein. As reported in Aoki (2006), analysis using knockout mice has revealed that xanthine oxidase and butyrophilin are indispensable for milk fat secretion. With the accumulation of milk fat within mammary epithelial cells, larger lipid droplets with disrupted MFGMs have been described in mice where xanthine oxidase or butyrophilin expression was damaged or abolished.

Conclusions

The proteomic approach applied to the study of the MFGM components has resulted in the identification and characterization of a large set of proteins associated with this particular subcellular compartment. For the major proteins, it is now possible to depict their functional involvement in milk secretion, but for those newly identified by proteomics and for minor ones, their function is still an open and unknown field.

As a source of bioactive components, MFGMs can contribute greatly to the nutraceutical value of milk, in healthy and in pathological conditions; and milk research could explore their utility as functional food ingredients.

In the near future, the completion of the MFGM proteome and the exploitation of the progress in proteomics will elucidate the complex network of interactions between the MFGM and both the mammary gland (site of origin) and the newborn gastrointestinal environment (final destination).

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