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Composition, Applications, Fractionation, Technological and Nutritional Significance of Milk Fat Globule Membrane Material

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6.1. Introduction

Among the components of mammalian milks, the milk fat globule membrane (MFGM) is the least close to being fully understood. As a result of the synthesis and secretion process, the fat globules in milk are composed of a non-polar lipid core, surrounded by a layer of phospholipids and proteins. This structure is then bounded by a membrane bilayer that is, in turn, derived from the apical surface of the mammary epithelial cell. Substantial biochemical investigations have been conducted to elucidate the details involved in the synthesis, transport and secretion of milk fat globules, yet there has been little discussion on the uniqueness of this system, and the evolutionary forces that have led to its development. On one hand, the complex structure of the globules may have arisen as a result of the physiological constraints on the secretion process, and would therefore not be expected to contribute a significant benefit to the health of the offspring. It should be recognized, however, that the simple process of lipoprotein secretion, which does not require the addition of extra cell bilayer surface, had evolved well before the appearance of mammals and would presumably have been a simple and easy alternative for fat secretion. Thus, it is also possible that there is a nutritional or physiological benefit that may be conferred to

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the consumer of milk by the structure of the fat globules, which would have led to the Darwinian selection of this process during mammalian evolution.

According to evolutionary theory, individuals of a species tend to be selected over time as a function of survival and reproductive success, and hence pass on their genetic material. Parturition is a tenuous time in the mammalian life cycle, as the constant threat of microbial pathogens is compounded by developmental delays in mucosal and systemic immunity (Goldman, 2002). However, the biological role of milk is to provide a support system that promotes metabolic regulation, assists in rapid physical development and complements the functionally immature immune system. Consequently, infants who consume breast milk experience phenotypic benefits, which we cannot explain with our current understanding of nutrition, food composition and structure. Although alternate formulae match the macronutrient, vitamin and mineral composition of breast milk, it must be the composite, non-essential structures in milk, not present in formulae, which are responsible for this extent of differential benefit. The milk fat globule membrane is composed of an interesting mixture of proteins, lipids and carbohydrates. With its similarity to epithelial cell surfaces, it may well have functions and activities above the simple delivery of the nutrients it contains.

In food and nutrition research, there is growing awareness of dietary components, which, while not essential, nonetheless provide tangible benefits to health when consumed. Many of the components of the MFGM have been associated positively with health, whether or not the mechanism of action is understood. Importantly, the identification and subsequent characterization of the constituents responsible for these effects will not be as straightforward as was the scientific research on the essential nutrients. If a nutrient is essential, removing it from the diet will always result in specific, reproducible insults to health. Yet, food science and nutrition research has not yet been as successful in developing research protocols to probe the importance and function of conditionally essential nutrients. As milk contributes to the survival and fitness of mammalian young, it is not simply a participant in evolution, but rather a driver, with simultaneous selection for efficacy and efficiency. Therefore, it is hard to imagine that there is anything in milk, including the MFGM, which is not interesting from a nutritional perspective.

6.2. Nutritional and Physiological Significance of the Milk Fat Globule Membrane

Most of the literature on milk fat globules has been generated using bovine, goat or human milk, and thus it is possible that alternate lipids may be present in other species. However, to date this has not been reported. The

lipids in milk are present as colloidal particles, due to their thermodynamic incompatibility with the aqueous serum, and the assembly of these insoluble components into stable multi-molecular units is the result of a unique secretory process. Milk fat globules are structurally complex, as a result of their synthesis and secretion, and it is possible to see many levels of nutritional value in the various stages of their architecture. The fat globule is primarily a core of triacylglycerides synthesized within the endoplasmic reticulum of the mammary epithelial cell. This core of triglycerides is bounded by a monolayer of polar lipids, such as phospholipids, derived from the endoplasmic reticulum membrane of the cell. This layer is also characterized by a dense proteinaceous coat located on the outside of the triglyceride core. The entire globule, however, is surrounded by a bilayer plasma membrane, which enrobes the globule as it exits the epithelial cell (Keenan and Mather, 2002). The MFGM is composed of the lipids and proteins of the epithelial cell plasma membrane, including significant quantities of cholesterol, phosphatidylcholine and sphingomyelin. Additional components unique to the external surface of the native fat globule include glycolipids, gangliosides and significant quantities of membrane glycoproteins and mucins. The membrane helps to stabilize the fat globules as an emulsion in the aqueous environment of milk. The average diameter of the milk fat globules ranges from less than 1 μm to 10 μm (Jensen, 2002), with three main size populations (Keenan and Patton, 1995). The small globule distribution is centered at less than 1 μm in diameter, the intermediate globule size distribution at roughly 3–5 μm, and large globule distribution at about 8–10 μm. Figure 6.1 is a scanning electron microscope image of milk fat globules from bovine milk. Most of the globules visible in the micrograph fall in the intermediate size range.

The colloidal properties of fat globules give rise to interesting compositional features. Numerically, most globules in milk (70–90%) are less than 1 μm in diameter and yet account for less than 5% of the total milk lipid, whereas those of intermediate size account for 10–30% of the globules, yet 90% of the total lipid. The proportion of polar lipid (phospholipids) surrounding the core of neutral lipids (triglycerides, cholesterol esters) increases as the globule size decreases but in a globule of 1 μm diameter, the MFGM accounts for only 3% of the volume. In a 0.5 μm diameter globule, the membrane makes up 6% of the globule volume. Although the membrane makes up between 1 and 5% of the lipid fraction, the surface area of the fat globules in 1 ml of mature human milk is estimated to be 500 cm^2 (Ruegg and Blanc, 1981). In colostrum, in which the fat occurs almost exclusively as very small globules, the proportion of membrane lipids is even higher.

To date, little attention has been given to the native structural properties of milk lipids. In particular, little is known about how these structural

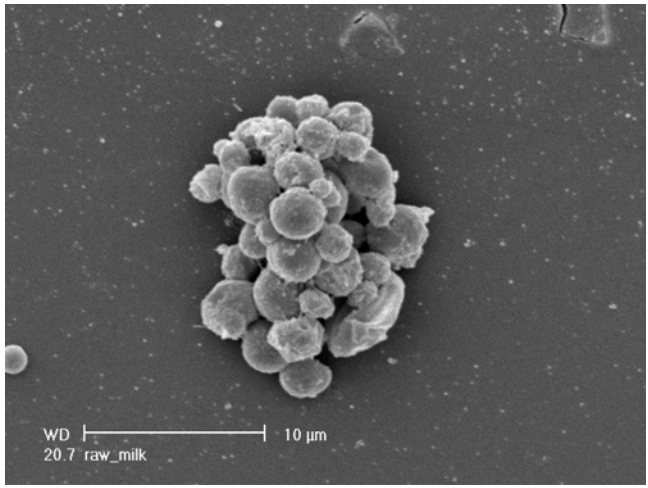

Figure 6.1. Scanning electron micrograph of a cluster of fat globules from fresh, unhomogenized whole milk. Sample was fixed with glutaraldehyde and post-fixed with OsO_4.

features relate to the nutritional and functional properties of particular components and the overall properties of milk as an intact delivery system. The fact that these structures vary widely, but consistently, among all mammalian species and at different periods of lactation implies that structure is of functional value. However, the techniques necessary to describe the structures of lipids are not fully developed. As these techniques become available, research must address how variations in structure influence biological and nutritional properties.

6.2.1. Biological Significance of Native Globules

The structure and size distribution of milk fat globules affects the presentation of the membrane surface, and the rate of lipolysis. In addition, there are discrete compartments within the globule, which may have functional consequences. It has long been supposed that the presence of a vast MFGM surface area in milk can serve as a decoy for intestinal pathogens that seek to adhere to epithelial cells. Certain constituents have been associated with binding of pathogens, such as the mucins, lactadherin and gangliosides. Binding to host epithelial tissues is a prerequisite for infection by some pathogens, and for many, the cognate ligands for their bacterial adhesion are complex glycans. Indeed, as the surface of milk fat globules is derived from the apical membrane of epithelial cells, it presents a glycan-rich glycocalyx in milk, similar to the host epithelial cells. In colostrum, the

majority of the globules are small, and this increases the surface area to lipid ratio. Not surprisingly, colostrum is also the richest source of other substances involved in protection against pathogens, such as the immunoglobulins and lysozyme. Although the non-covalent interactions between proteins and carbohydrates are of low affinity and thus have equilibrium constants in the millimolar range, multiple interactions of receptors and ligands increase binding avidity, and make the attraction stronger.

A second functional consequence of the structure of milk fat globules is its effect on lipolysis. Of the three lipases (gastric, pancreatic, and bile salt-stimulated) that act on triglycerides in human infants, only gastric lipase is able to penetrate the lipid bilayer of the MFGM to initiate digestion (Bernback *et al.*, 1989). Without the prior action of this enzyme in an *in vitro* assay, the ability of the two other lipases to release fatty acids from milk fat globules is greatly reduced. Furthermore, as evidenced by studies on rats, this enzyme is more active on triglycerides containing short-chain fatty acids, which are antimicrobial, and which are absorbed directly in the stomach (Jensen, 1989). It has not been reported whether or not the human gastric lipase is more active on triglycerides containing short-chain fatty acids. Putting these data together, it is tempting to speculate that the native structure of the milk fat globule along with the specificity of gastric lipase leads to selective hydrolysis of short-chain fatty acids in the stomach where they provide protection against pathogens, and also provide a ready source of energy. The result of this enzymatic activity would then leave the fat globules more susceptible to hydrolysis by pancreatic and bile-salt stimulated lipases in the small intestine.

In some milk fat globules, small aqueous compartments are located beneath the membrane bilayer, which have been termed cytoplasmic crescents (Huston and Patton, 1990). Whether or not this cytoplasmic inclusion provides some benefit is unknown. Yet, as this aqueous compartment is protected from the bulk serum phase by the MFGM, constituents located therein are presumably afforded some protection, at least initially, from gastric hydrolysis. Huston and Patton (1990) found crescents in all samples of milk they examined, and they were more prevalent in human (7.3% of globules), than in bovine (1% of globules) milk. Furthermore, there was considerable individual and diurnal variation. The structure of a cytoplasmic inclusion, surrounded by an intact plasma membrane on one side and a fat globule surface on the other, may allow certain labile constituents to be protected until they reach their proper site of bioactivity. At this point it is not known whether the crescents have a purpose or are simply the result of inefficiencies in the secretion process. As it is possible to isolate milk preparations enriched in cytoplasmic crescents, there is an opportunity to determine the nature of the materials found within. This unusual biocompartment may prove to be a model of food structure for biodelivery.

6.2.2. MFGM Consumption Studies: Physiological and Nutritional Effects

Considering the large quantity of bovine MFGM that is produced industrially each year as a byproduct in butter churning, and is available as a food ingredient with a unique polar lipid and membrane protein profile, few studies have been conducted to assess the physiological and nutritional effects of its consumption. Its presence in milk suggests beneficial bioactivities, and this perspective is reinforced by analysis of the effects on health of its individual components. However, feeding studies are still necessary to provide scientific evidence in support of synergisms, or perhaps antagonisms, and resolve whether putative benefits can be detected on the system under investigation.

Not surprisingly, most of the studies performed on the health aspects of consuming the MFGM during the past three decades have investigated solely its effect on serum cholesterol. Several studies have indicated that the consumption of MFGM lowers serum cholesterol, but other studies failed to reproduce this effect. After noting low serum cholesterol in the East African Masai, a group with a large daily milk intake, Mann (1977) reported that the consumption of four liters of whole milk per day lowered the level of serum cholesterol, and reduced the incorporation of radiolabelled acetate into cholesterol. Howard and Marks (1977) fed individuals various milk fractions in an attempt to identify the causal factor. After a two-week study, they noticed that butterfat (80 g/day) significantly raised serum cholesterol, whereas an equal amount of fat as cream did not. Furthermore, spray-dried buttermilk significantly reduced the level of serum cholesterol. The authors concluded that the effect on serum cholesterol may involve the MFGM. To test this hypothesis explicitly, Antila *et al.* (1980) fed volunteers either cultured buttermilk or cultured skim milk, and found that while both lowered serum cholesterol, the former was more effective. The cultured buttermilk in this study was derived from the aqueous byproduct of butter production, is rich in MFGM, and should not be confused with the commercially available cultured buttermilk, which is produced from skim milk.

The diets used in these initial studies on the effect of consuming buttermilk on serum cholesterol were not standardized, and involved a small number of individuals. Hussi *et al.* (1981) fed a large group of healthy volunteers with either 2.7 L/day of skim milk or 2 L/day of buttermilk or a control diet for three weeks. All diets were standardized for macronutrient and energy level, and all volunteers consumed the control diet for 3 weeks prior to the study. No significant differences were found in the serum lipid or lipoprotein profiles between the control and test groups.

The ability of MFGM to inhibit intestinal β-glucuronidase activity was measured by Ito *et al.* (1993). This enzyme is a product of colonic

enterobacteria, and when present in the colon has the ability to activate carcinogenic precursors to carcinogens (Simon and Gorbach, 1984). Since this activity is inhibited by sialoglycoproteins from porcine salivary glands (Sakamoto, 1974), the possibility that sialoglycoproteins in bovine milk might provide this benefit was investigated by Ito *et al.* (1993) in *in vitro* enzymatic assays and, in *in vivo* feeding studies with mice. In the enzyme assay, 0.2%, w/v, purified MFGM inhibited β-glucuronidase activity on phenolphthalein-β-D-glucuronide by 90%, while κ-casein at the same concentration inhibited the reaction by 35%. To determine whether this activity would survive gastrointestinal transit, the authors fed mice diets supplemented with 5, 10 or 20% MFGM for five days, and measured β-glucuronidase activity in faeces. The diets containing 5 or 10% MFGM caused 15–20% inhibition of faecal β-glucuronidase activity, whereas when 20% MFGM was added to the standard diet, the level of inhibition was 50%.

6.3. Composition and Bioactivity of Individual Components

In addition to being an essential structural component (emulsifies) of milk fat globules, the MFGM is composed of many molecules that have been associated both individually and collectively with beneficial nutritional bioactivities. Although very few of the constituents of MFGM preparations are essential in our diet, many components are increasingly being documented as providing specific nutritional benefits. A discussion of the current nutritional understanding of the major constituents of the MFGM follows.

6.3.1. Phospholipids

The MFGM is a rich source of phospholipids. Dietary phospholipids have antioxidative activity (Saito and Ishihara, 1977), as well as antimicrobial and antiviral properties (van Hooijdonk *et al.*, 2000). Additionally, there is evidence that the consumption of phospholipids can protect against gastric ulceration (Kivinen *et al.*, 1992). In a rat model for the ulcerative action of HCl administered to the stomach lumen, raw rat or bovine milk, and also pasteurized-homogenized bovine milk provided protection. However, this effect was not found when phospholipids were removed from the milk (Dial and Lichtenberger, 1984). In the duodenum, dietary phospholipids are converted to their lyso-forms by phospholipases and, to a lesser extent, by pancreatic lipase. Lysophospholipids are strong surfactants, and can cause lysis of Gram-positive bacteria. Sprong *et al.* (1999) tested the effects of phosphatidylcholine, phosphatidylethanolamine and their lyso-forms on *Listeria monocyogenes* in cultured cells *in vitro* and in rats fed diets based on lactase-treated sweet buttermilk powder. *In vitro*, lysophosphatidylcholine

inhibited the growth of *L. monocytogenes*, and in rats orally infected, the number of luminal and mucosal bacteria was significantly reduced by a buttermilk diet compared with a skim milk diet. These investigators concluded that buttermilk phospholipids might improve the resistance of the host to infection by *L. monocytogenes* by enhancing the gastrointestinal killing of this pathogen.

Milk fat contains small amounts of ether lipids, which include alkyldiglycerols and alkylglycerophospholipids (reviewed by Parodi, 1996). In these molecules, the *sn*-1 position of glycerol has an ether-linked acyl chain, compared to an ester-linked chain in phospholipids. While the non-polar lipids of milk contain 0.01% by weight of these molecules, the phospholipid fractions contains up to 0.16% by weight (Hallgren *et al.*, 1974), and presumably they partition with the MFGM in the churning of butter. Human milk contains 10 times more ether lipids than bovine milk (Hallgren and Larsson, 1962). In the intestinal lumen of rodents and humans, dietary ether lipids are converted to *sn*-1-monoalkyglycerols and absorbed, with the ether linkage intact. They are then transported to the liver and used to synthesize membrane alkylglycerolipids and plasmalogens (Blank *et al.*, 1991; Das *et al.*, 1992). The biological effect of ether lipids is believed to be a result of their influence on the properties of membranes. They have been shown to have anticancer effects, by preventing growth and metastasis, and preventing induction of differentiation of tumors (Berdel, 1991; Diomede *et al.*, 1993).

6.3.2. Ceramide Sphingolipids and Glycosphingolipids

Sphingolipids are not essential nutrients but are increasingly being recognized as important in nutrition, as was reviewed by Vesper *et al.* (1999). In mammalian tissues and milk, the sphingolipids include ceramides, sphingomyelins, cerebrosides, gangliosides and sulfatides.

Both sphingolipids and gangliosides are present in human and bovine milk and are enriched in products such as cream and cheese. The dominant phospholipid in milk is sphingomyelin, and it is reported to represent about one-third of total bovine milk phospholipids (Pfeuffer and Schrezenmeir, 2001), and 38% of total human milk phospholipids (Motouri *et al.*, 2003). Unlike phospholipids, which are built on a phosphoglycerol backbone, sphingolipids are based on sphingosine, an amino alcohol with a long unsaturated hydrocarbon chain. Whereas in phospholipids both acyl chains are linked to the phosphoglycerol backbone by ester bonds, sphingolipids have one acyl chain linked *via* an amide bond to sphingosine. This core of sphingolipids and gangliosides, *N*-acylsphingosine, is also known as a ceramide. Sphingolipids also contain a polar group, such as phosphocholine; gangliosides are further derivatized on the polar head groups with addition

of neutral and acidic sugars. Sphingolipids are among the most structurally diverse categories of lipids in nature.

The profile and concentration of gangliosides in milk vary across mammalian species. GD3 and GM3 are the predominant gangliosides in bovine milk, whereas in human colostrum, GD3 predominates and in mature human milk, GM3 predominates (Rueda *et al*., 1998). GM1 has been shown to prevent diarrhea caused by *Escherichia coli* and *Vibrio cholerae* enterotoxins. Furthermore, a ganglioside-supplemented infant formula modifies the intestinal ecology of pre-term infants, increasing the numbers of *Bifidobacteria* and lowering that of *E. coli*. The proposed mechanism of action is that soluble membranes, like that on milk fat globules, serve as false intestinal receptors for some strains of pathogenic bacteria. GD3 and other gangliosides are involved in mechanisms of lymphocyte activation and differentiation, and thus milk gangliosides might function in the development of intestinal immunity. The levels of neutral glycolipids and other glycosphingolipids in bovine milk have been tabulated, with references, by Jensen (2002).

The *per caput* sphingolipid consumption in the U.S. is estimated to be 150–180 mmol (~115–140 g) per year, or 0.3–0.4 g/day (Vesper *et al*., 1999). Though there is no nutritional requirement for sphingolipids (Vesper *et al*., 1999; Berra *et al*., 2002), studies in a rat model indicate significant benefits at particular life stages, for example in promoting gut maturation in the suckling infant. Suckling rats fed 0.5% sphingomyelin had a significantly lower level of intestinal lactase, vacuolated cells in intestinal villi were restricted to the tip of villi, and the Auerbach nerve plexus area of the ileum was significantly greater than in the control group. These results suggest that sphingomyelin plays an important role in neonatal gut maturation during the suckling period. In additional studies (Oshida *et al*, 2003), suckling rat pups were injected daily with an inhibitor of sphingolipid biosynthesis from 8 days after birth (2 days before the onset of myelination) to 17 days after birth. The experimental group was then fed supplemental sphingomyelin until 28 days of life. Lipid analysis and morphometric analyses of the optic nerve showed that dietary spingomyelin contributed to myelination of the developing rat central nervous system.

Reports indicate that the digestion and delivery of exogenous sphingomyelin to the intestinal cells and the interaction of these dietary components with endogenous sphingomyelin in the intestinal mucosa are relevant to optimal cell regulation and preventing such defects as colon cancer (Duan, 1998). There is experimental evidence that the consumption of sphingolipids inhibits the early stages of colon carcinogenesis, as determined by the appearance of aberrant crypt foci in mice (Dillehay *et al*., 1994; Schmelz *et al*., 1996, 1997, 2000). Dietary sphingolipids also reduce serum LDL-cholesterol

and elevate HDL (Imaizumi *et al.*, 1992; Kobayashi *et al.*, 1997). These findings have been interpreted to indicate that sphingoliplids are ''functional'' components of foods.

The digestion products of sphingolipids (ceramides and sphingoid bases) are highly bioactive compounds that regulate cell growth, differentiation and apoptosis (programmed cell death), all of which are processes that are lost in cancer (Merrill *et al.*, 2001). Sphingolipids are involved in functions that range from structural protection to signal transduction and protein sorting, and participate in lipid raft assembly (Slimane and Hoekstra, 2002). Cholesterol-sphingolipid microdomains (lipid rafts) are part of the machinery that ensures the correct intracellular trafficking of proteins and lipids (Ikonen, 2001).

Smith and Merrill (2002) presented a mini-review prologue for a series covering the current understanding of sphingolipid biosynthesis, intracellular transport and turnover. In this mini-series, Merrill (2002) discussed the fact that *in vivo* biosynthesis of sphingolipids is probably required although sphingolipids are present in most foods, including the MFGM. The *de novo* pathway must be controlled because so many of the intermediates are highly bioactive, especially ceramides, which are the immediate precursors of sphingomyelins and glycosphingolipids and are one of the important mediators in signalling cascades of apoptosis, proliferation and stress responses (Hannun and Obeid, 2002; Spiegel and Milstien, 2002). In the same mini-series, van Meer and Lisman (2002) reviewed the role of sphingolipids in the structure of membrane rafts, sphingolipid biosynthesis, and translocators important in directing sphingolipid distribution in cells.

Recently, milk sphingomyelins were reported to interact significantly with the physical state of cholesterol, which correlated positively with reduced uptake and esterification of cholesterol by Caco-2 cells; they also significantly reduced cholesterol absorption in mice, even at 0.1% of the diet (Eckhardt *et al.*, 2002). An earlier study showed the regulation of cholesterol absorption by the content of sphingomyelin in intestinal cell membranes (Chen *et al.*, 1992).

Sphingolipids have also been implicated as mediators of bone health. As a testament to their bioactive potential, two recent patents (Takada *et al.*, 2001a,b) describe their use as drugs. One drug containing ceramides, sphingomyelins, sphingoglycolipids, and gangliosides is for the treatment of periodontal diseases. Application of a solution containing gangliosides prepared from milk to the teeth of hamsters with experimentally-induced periodontal disease significantly suppressed the decrease in alveolar bone.

The second patent defines drugs for the prevention and treatment of osteoporosis, fracture, lumbago, and rheumatic arthritis. These drugs contain compounds such as ceramides, sphingomyelins, sphingoglycolipids and

gangliosides, and optionally calcium, vitamin D and/or vitamin E. In ovariectomized osteoporotic rats fed diets containing gangliosides prepared from milk, milk calcium and vitamin D, the decrease in the amount of bone minerals was suppressed.

In a commercial response to the potential value of sphingolipids as functional food components, a patented process for preparing milk products enriched in both phospholipids and sphingolipids was developed (Dewettinck and Boone, 2002). These products are obtained by ultrafiltration of byproducts from the direct processing of milk or from the further processing of directly acquired byproducts. The ultrafiltration membrane used had a cut-off value ranging from 5,000 to 20,000 Da.

6.3.3. Proteins

Although the proteins in the MFGM represent only about 1% of the total milk protein, they are unique in their functionality. Identification of the component proteins is based largely on electrophoretic mobility, more specifically on sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The composition, structure and function of the proteins and glycoproteins in bovine MFGM has been reviewed extensively (McPherson and Kitchen, 1983; Kanno, 1990; Danthine *et al.*, 2000; Mather, 2000; see Chapter 4). Mather (2000) referred to the complex banding pattern of MFGM proteins separated on two dimensional (2D) gels as evidence of the complexity of the membrane proteome. Many of the more quantitatively abundant proteins have been characterized, and Mather (2000) provided detailed information on eight proteins, from the primary sequence to post-translational modifications. The major proteins in bovine MFGM are: mucin MUC1, xanthine dehydrogenase/oxidase, PAS III, CD36, butyrophylin, a group of glycosylated proteins called PAS 6/7, and adipophilin (see also Chapter 4).

In addition to the major proteins, many bioactive constituents work at low concentrations, and thus it is of interest to catalogue and functionally annotate all of the proteins present. To that end, a few proteomic studies have been conducted on human milk fat globules using 2D-PAGE gels coupled with subsequent identification using mass spectrometry (Quaranta *et al.*, 2001; Charlwood *et al.*, 2002; Fortunato *et al.*, 2003). Global proteomic investigations are also helping to unravel the assembly process of milk fat globules. Wu *et al.* (2000) separated murine proteins from cytoplasmic lipid droplets on 2D-PAGE gels, digested them with trypsin, and then identified the resulting peptide fragments using tandem mass spectrometry. By comparing the gel pattern of mammary cytoplasmic lipid droplets to that of the milk fat globules and liver cytoplasmic lipid droplets, they were able to identify proteins that are likely mediators of the cross-cell lipid traffic.

6.3.4. Butyrophilin

Butyrophilin, the most abundant protein in bovine MFGM, has a molecular weight of 66 000 Da. Although the function of butyrophilin is still subject of active debate, the protein is a member of a large family of immunoglobulins related to activity of the immune system. Most butyrophilin is found associated with the membrane and seems to be an integral protein. Sequence homology studies have indicated that domains of butyrophilin are highly conserved among species and may have a universal function in protein-protein interactions. It was hypothesized that butyrophilin and Xanthine oxidoreductase play an important role in the formation of a supermolecular complex (also with adipophilin), which may be an essential step in the assembly of the MFGM and the secretion of the fat globules (Mather and Kennan, 1998; see Chapter 4). Disulfide bonds play a role in stabilizing its association with the membrane. The levels of butyrophilin and Xanthine oxidoreductase are highest in early lactation and then decrease as lactation progresses to its midpoint. The molar ratio is reported to be between 4:1 and 3:1 (Mondy and Keenan, 1993; Ye *et al.*, 2002). Direct evidence of a thiol-dependent complex between Xanthine oxidoreductase, butyrophilin and adipophilin has been shown in support of the hypothesis that membrane association of the most abundant MFGM proteins is crucial for the secretion process of the mammary epithelial cells (McManaman *et al.*, 2002).

Although the function of butyrophilin is under debate, epidemiological associations have been interpreted to suggest that it may be involved in the etiology of the autoimmune disorders, Multiple Sclerosis (MS) and autism. MS is an inflammatory autoimmune disease of the central nervous system that results in demyelination of neurons due to a disruption of immunological self-tolerance (Mana *et al.*, 2004). The mechanistic cause of MS remains completely unknown and a variety of hypotheses have been proposed and tested. One hypothesis is that immunogenic determinants of important (self) proteins can be mimicked by food-based molecules, and this is the proposed mechanism for the involvement of butyrophilin. Indeed, there seem to be roles for both genetic predisposition as well as environmental factors in triggering the onset of the disease, making it difficult to discover a single casual agent or mechanism behind the disease. Epidemiological studies seem to suggest that the consumption of dairy products may be one of many environmental factors associated with higher rates of MS in susceptible individuals (Lauer, 1997). Whereas a biological effect does seem plausible according to the data from some of the studies, many confounding factors are present. Further studies are needed using randomized trials to substantiate the relationship between MFGM and the disease (Lauer, 1997).

Butyrophylin contains an Ig-like domain that is common to an extended family of B7-like proteins. Another member of this family is the myelin oligodendrocyte glycoprotein (MOG), which is a central nervous system protein thought to be an important target of the autoimmune response in MS. The implication of butyrophilin in the etiology of MS comes as a result of its greater than 50% peptide sequence identity to MOG. Furthermore, in an animal model for the disease, experimental autoimmune encephalomyelitis (EAE), MOG is a major target of the autoimmune response (Stefferl *et al.*, 2000). In Dark Agouti rats, which are genetically susceptible to EAE, the authors have shown that the $CD4^+$ T cell response to intravenously injected MOG is mutually cross reactive with the IgV-like extracellular domain of native butyrophilin, and *vice versa*. Such cross reactivity of endogenous and exogenous epitopes is known as "molecular mimicry," and has been suggested to be the cause of other autoimmune disorders. Stefferl *et al.* (2000) also found that transmucosal exposure (*via* intranasal administration) to both the MOG and the butyrophilin antigens modulated the severity of MOG-induced EAE. This finding indicates that dietary butyrophilin may actually promote oral tolerance to the antigen, and through cross-reactivity may suppress autoimmune MOG-reactive T-cells. Speculating about a plausible role for butyrophilin in inducing MS, the authors point out that oral tolerance is poorly developed at birth, and exposure to dietary antigens at this time could activate the immune system, rather than provide tolerance.

Mana *et al.* (2004) have shown that the link between molecular mimicry, environmental antigens and autoimmunity does not necessitate pathogenesis, building on the findings that transmucosal exposure to antigens can modulate the immune response. Working with C57BL/6 mice, a strain genetically susceptible to EAE, they found that treatment with butyrophilin before immunization with MOG can prevent pathogenesis, and treatment after MOG immunization can suppress the disease symptoms. The effect seemed to lie in the reduction of immune cell proliferation, and the reduction of Th1 related cytokines, IFN-γ, IL-2, IL-12 and GM-CSF, with an up-regulation of IL-10. One important finding of this study is that the existence of self-reactive antibodies does not lead to autoimmunity.

The cross-reactivity of an autoimmune antibody to butyrophilin has also been described in autism, a developmental disorder whose etiology includes genetic, environmental, neurological and immunological components. Using an enzyme-linked immunosorbent assay, Vojdani *et al.* (2002) demonstrated higher levels of antibodies against nine different neuron-specific antigens, two microbial antigens and butyrophilin in the serum of autistic children versus non-autistic controls. While this study confirms the observation in the MS studies that antibodies raised to MOG will cross-react

with butyrophilin, these findings do not indicate that it was butyrophilin, and not MOG, to which the immune response was initially raised.

In both the MS and autism cases, more information is needed on the digestion and survival of milk protein-derived peptides in the gut, their interaction with the gut-associated lymphoid tissue, the microflora, and their role in the disease etiology. Most critically, the basic mechanistic understanding of immune development remains poor. The suggestion by some scientists that dietary butyrophilin may play a role in promoting MS and autism, and the inability of other scientists to support or refute this suggestion is a vivid demonstration of our poor understanding of the basic mechanisms of immunological development and the role of diet in its regulation. Ironically, the ability of milk to support the appropriate immune development of infant mammals argues that a scientific model with which to study the effect of diet on appropriate immune development is milk itself.

6.3.5. Mucins

Mucins are high molecular weight, highly glycosylated glycoproteins and unlike the secreted mucins of goblet cells, those in the MFGM are present as integral membrane components (Patton *et al.*, 1995). Milk fat globule mucins have been detected in many species, and are thought to be orthologues to human MUC1. Carbohydrates may constitute as much as 50% of the mass of the mucin molecule, and sialic acid in the terminal position of the oligosaccharide chains gives them a negative charge. Human MUC1 contains more galactose and *N*-acetylglucosamine, and less *N*-acetylgalactosamine and sialic acid than bovine MUC1. Carbohydrate epitopes on milk mucins have been probed using peanut, wheat germ and jack bean lectins, which detect the T-antigen (β-D-galactosyl (1-3)-*N*-acetyl-D-galactosamine), sialic acid and mannose, respectively. Both bovine and human milk mucins show T-antigen activity and sialic acid, whereas only bovine mucin seems to contain mannose, which is consistent with detection of *N*-glycosylation on this protein. The negative charge, which results from sialic acid, is thought to have functional implications in the prevention of globule coalescence, or in the regulation of the fat globule size (Patton *et al.*, 1995). Unlike human MUC1, which consists of a single polypeptide chain, bovine mucin is a heterodimer that is synthesized as a monomer, and cleaved during post-translational processing into a transmembrane domain and an extracellular glycosylated domain. Although the two segments remain bound noncovalently, bovine mucin is unstable to cooling and washing, and a portion of the extracellular glycosylated domain can be recovered in the serum phase when milk is cooled (Patton, 1999). The reason for this modification is not known.

As MUC1 extends from the epithelial and milk fat globule membranes, one function seems to be in providing protection for cells by acting as a physical barrier. Schroten *et al*. (1992) found that components of the human MFGM bind to S-fimbriated *Escherichia coli*, and that mucin showed the highest activity. Furthermore, mucins with a molecular weight greater than 200 kDa, which were isolated from the feces of breast-fed but not from feces of formula-fed infants, prevented the attachment of the bacteria to the buccal epithelium. MFGM mucins also bind rotavirus and perhaps respiratory syncitial virus, and prevent replication (Yolken *et al*., 1992). Deglycosylation of the mucin resulted in the loss of this activity.

To investigate the effect of gastrointestinal transit on MUC1, Patton (1994) analyzed fecal extracts of seven breast-fed and seven bottle-fed infants aged 20 days to 6 months using monoclonal antibodies directed against a tandem repeat of this mucin. Fragments of 200 kDa were detected in three of the seven breast-fed infants, but in none of those fed formula, which would be expected, as MUC1 is not present in infant formula. In a related study, Midtvedt *et al*. (1994) monitored mucin degradation activity in a group of 30 healthy Swedish children for 2 years. For those infants fed breast milk exclusively for at least 4 months, mucin degradation was initiated significantly later than in those who had received at least some formula. After one year, 21 children degraded mucin completely, while all children did so after 2 years of age.

The degradation of mucins requires the concerted action of proteases, esterases, sulfatases and glycosidases (Hoskins, 1992). These depolymerizing enzymes are not produced by the host, but rather by certain microbial residents at the entrance to the colon, representing about 1% of the total culturable fecal bacteria. They are Gram-positive, non-sporulating, obligately anaerobic, and non-pathogenic bacteria, and have been identified as *Ruminococcus torques, Ruminococcus gnavus* and *Bifidobacterium spp*. (Hoskins, 1992). With hog gastric mucin, which contains both the blood group A and H determinants (carbohydrate structures), as a substrate, ruminococci were able to degrade roughly 90% of mucin carbohydrate, while the bifidobacteria could degrade between 60 and 80%. The difference was attributed to the ability of ruminocci to cleave the terminal *N*-acetylgalactosamine A antigen. Further characterization of these isolates indicated that the glycosidases produced by the ruminococci were constitutively produced, released extracellularly, and were resistant to proteolysis, whereas, the glycosidases of the bifidobacteria are cell-bound. Co-culturing of both organisms led to increased overall oligosaccharide degradation and bacterial growth, due to the cooperative contribution of glycosidases, suggesting that this may be a characteristic feature of the gut microflora. Analysis of the spent medium indicated the presence of L-fucose, D-galactose,

N-acetylglucosamine, *N*-acetlygalactosamine, and the disaccharides lactosamine and galactose ($\beta 1 \rightarrow 3$) *N*-acetlygalactosamine. Thus, the presence of certain strains of bacteria enables the cooperative degradation of glycoconjugates, which in turn increases the nutrient availability to other members of the gut microflora.

In the MFGM of human milk, mucin is associated with two other glycoproteins, butyrophilin and lactadherin. Yolken *et al.* (1992) found that a non-immunological fraction of human milk inhibited the replication of rotaviruses in tissue culture, and prevented the development of gastroenteritis in an animal model. Further characterization indicated that virus bound to mucin and to the associated 46 kD protein (identified as lactadherin). The biological functions of lactadherin may be related to its ability to interact physically with a wide variety of molecules.

6.3.6. Xanthine Oxidoreductase

The protein product of a highly conserved housekeeping gene, Xanthine oxidoreductase (XOR), catalyses the oxidative degradation of the purine, xanthine, to uric acid and plays a role in the secretion of the milk fat globule (McManaman *et al.*, 2002). A hemizygous murine knockout of XOR is able to initiate, but not sustain, fat globule secretion (Vorbach *et al.*, 2002). Although the role of this protein in milk is not completely understood, it is thought to act outside of its characterized enzymatic role. XOR may have a functional role against pathogens, playing a part in the overall immune activity provided by milk (Vorbach *et al.*, 2003). The antimicrobial activity of XOR is a result of generating reactive oxygen and nitrogen species (ROS and RNS, respectively), and has been recognized for decades (Green and Pauli, 1943; Lipmann and Owen, 1943). Early studies used high enzyme concentrations, high oxygen tensions, and the effect was monitored by plate counts. The antimicrobial effects were attributed to the generation of the ROS, hydrogen peroxide. Using a strain of *Escherichia coli* that expressed a constitutive luminescent reporter, Hancock *et al.* (2002) showed that both bovine and human milk had bacteriocidal activity, and that it was reduced by boiling the milk, or by the addition of an XOR inhibitor, oxypurinol. The assay was conducted under hypoxic conditions, and was dependent on nitrite, presumably for the generation of RNS. When a little oxygen is present, nitric oxide (NO) and superoxide are formed, and can react to form the powerful bacteriocidal agent, peroxynitrate. As the K_M of XOR for nitrite is in the millimolar range and as some bacteria produce this concentration as a result of nitrate reductase, Hancock *et al.* (2002) suggested that the metabolism of the bacteria may result in their own undoing.

6.4. Fractionation and Technological Significance of Milk Fat Globule Membrane Material

MFGM is found in significant quantities in dairy products such as cheese, cheese whey, butter and buttermilk. Milk fat globules are concentrated in cream and during the manufacture of butter the fat globules are disrupted mechanically. This process destabilizes the oil-in-water emulsion and results in two phases, fat granules and an aqueous phase rich in MFGM. The latter phase is traditionally called buttermilk, and is different in composition from the cultured product available commercially (Corredig and Dalgleish, 1998a). This byproduct of the industrial production of butter is enriched in MFGM. Figure 6.2 shows the presence in buttermilk of MFGM fragments and casein micelles (shown as elongated and round structures, respectively).

Increased recognition of the nutritional significance of components of the MFGM has led to a number of studies dedicated to the extraction and production of MFGM from buttermilk. Specific processes have been designed to obtain MFGM isolates free from other milk constituents, with the explicit objective of their use as bioactive and functional food ingredients. The differences in composition between isolates produced from different

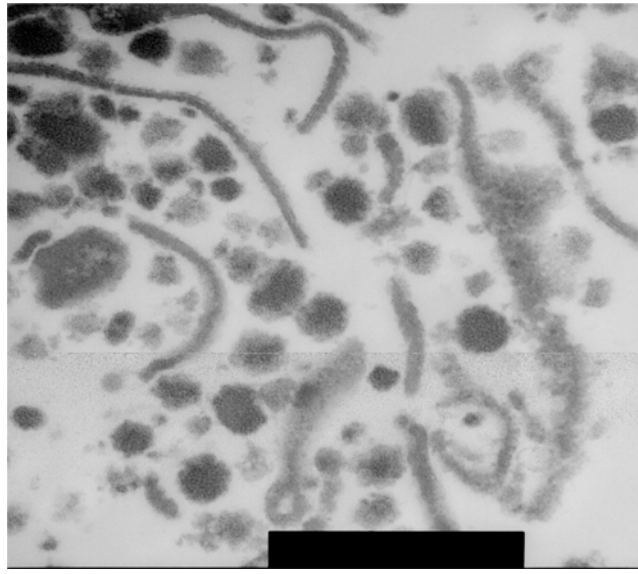

Figure 6.2. Electron microscopy image of buttermilk sedimented by centrifugation. The sample was fixed with glutaraldehyde and post fixed with OsO_4. Bar = 0.70 μm. Fragments of the MFGM are clearly visible.

sources and using alternative processes are not yet understood. However, understanding how different methodologies used to produce MFGM affect its composition will allow for fractions to be produced with unique functionalities, and could increase the value of buttermilk as a food ingredient.

6.4.1. Effect of Processing on the Composition and Functionality of the MFGM

Agitation, cooling and ageing are just a few of the processes that cause changes to the composition of the MFGM post secretion (Evers, 2004). Table 6.1 summarizes some of the work reported in this area. It is important to note that the results of the analysis of the composition of MFGM may be affected by differences in the isolation method used. Despite careful handling of the fat globules in raw milk, supermolecular complexes between the MFGM proteins, Xanthine oxidoreductase, butyrophilin and adipophilin, are found in native milk fat globules. These large protein complexes fundamentally affect not only the biological functionality of the MFGM components, but also their stability and their changes with processing. While in fresh whole milk, the membrane consists mainly of phospholipids and MFGM proteins, on heating, the fat globule surface becomes coated with a layer of denatured proteins derived from the serum phase of milk (mainly whey proteins) (Dalgleish and Banks, 1991), and this effect is even more pronounced in cream (McPherson *et al.*, 1984). Very little is known about the

Table 6.1. Factors that affect the structure and composition of the MFGM

Chemical/enzymatic	
Loss of membrane components, ions, adsorption of milk plasma components, enzymatic activity	Anderson *et al.* (1972); Walstra (1983); McPherson and Kitchen (1983).
Physiological	
Diet, breed, stage of lactation	Anderson and Cawston (1975); Mondy and Keenan (1993).
Handling and processing	
Pumping, stirring, agitation	McPherson and Kitchen (1983).
Air	Van Boekel and Walstra (1989).
Cooling and ageing	Anderson *et al.* (1972).
Homogenization	van Boekel and Walstra (1989); Sharma and Dalgleish (1993); Lee and Sherbon (2002).
Heating	Dalgleish and Banks (1991); McPherson *et al.* (1984); van Boekel and Walstra (1995); Corredig and Dalgleish (1996); Ye *et al.* (2002).

changes that occur in the phospholipid fraction of MFGM with thermal processing.

When fat globules are heated in the absence of serum proteins, high molecular weight complexes form between butyrophilin and Xanthine oxidoreductase in less than 10 min at a temperature as low as 60°C (Ye *et al.*, 2002). In the presence of whey proteins, large amounts of β-lactoglobulin and α-lactalbumin associate with the MFGM (Corredig and Dalgleish, 1996; Ye *et al.*, 2004). Direct evidence of heat-induced covalent disulfide interactions between whey proteins and MFGM proteins can be obtained by electrophoresis and isoelectric focusing of the heated MFGM (Kim and Jiménez Flores, 1995; Corredig and Dalgleish, 1996; Ye *et al.*, 2002). The heat-induced formation of protein complexes on the surface of the MFGM may include denaturation of the individual proteins with the formation of aggregates containing MFGM proteins alone (butyrophylin, Xanthine oxidoreductase, PAS6/7) or MFGM proteins with whey proteins. The details of these reactions have been described by Ye *et al.* (2004).

Mechanical treatments such as agitation, pumping and high shear can cause changes in the composition of the MFGM, as well as changes in the size of the fat globules (McPherson and Kitchen, 1983). Homogenization is often employed to reduce the size of the fat globules, improve stability and delay creaming. During homogenization, the interfacial area increases significantly. Rearrangement of the original MFGM material occurs and considerably more protein is necessary to cover the newly formed interface. For this reason, casein micelles are adsorbed on the milk fat globules. This effect explains the observation that the fat globules in homogenized milk have a much higher protein load than untreated fat globules (Sharma and Dalgleish, 1993). Homogenization and heating are unit operations that are usually combined during milk processing. Differences in homogenization as well as the conditions of thermal treatment result in differences in the protein load on the MFGM surface and alter the ratio of whey proteins to caseins (Sharma and Dalgleish, 1993, 1994).

The formation of complexes between skim milk-derived proteins and MFGM proteins is of significance in milk processing. For example, the association of α-lactalbumin and β-lactoglobulin with the MFGM, which occurs with heat treatment, strongly affects the functional properties of MFGM isolates when used as ingredients in foods. Furthermore, the stability of oil-in-water emulsions prepared with MFGM isolates depends on the heat treatment of the original cream. The functional properties of the MFGM extracted from thermally-treated creams decrease as a result of heat treatment, even at a mild temperature (65°C) (Corredig and Dalgleish, 1998b).

6.4.2. Isolation of MFGM

Various bench-top extraction protocols have been developed to isolate MFGM for compositional analysis, and to facilitate an understanding of the secretion process of the fat globules. These investigations have provided strategies for the isolation of this material on a large scale. To achieve optimal isolation of the MFGM components (proteins and phospholipids), extractions should be carried out on freshly collected milk that has not been cooled (Mather, 2000). The unit operations used during the storage and processing of milk cause major changes to the MFGM, as has been demonstrated by the compositional analysis of the material extracted from various sources (for example, raw milk, heat-treated milk or cream).

In general, the common steps for the isolation of the MFGM include concentration of the fat globules (cream) using gravitational separation (often centrifugation), a series of washing steps to remove contaminants which adsorb loosely to the globules, and finally a step to destabilize the emulsion and separate the lipid and aqueous phases. These steps are general and common to bench-top and processing practices. The main difference between MFGM extracted in the laboratory versus that produced as a byproduct of industrial butter production is that the former is handled more carefully, and is washed and extracted using detergents, high speed centrifugation or salting out (McPherson *et al.*, 1984). A physical method such as strong agitation, churning or freeze-thawing is usually used to destabilize and rupture the MFGM.

Unlike the preparation obtained using laboratory isolation procedures, which normally include various steps to wash the fat globules to remove loosely associated contaminants, industrially processed buttermilk contains a large amount of serum proteins. Butter is prepared from cream containing of about 40% fat, and large amounts of caseins and whey proteins are still present in the aqueous phase. Buttermilk has physico-chemical properties similar to those of skim milk: it contains casein micelles with similar size and zeta-potential to those in skim milk, and a similar amount of total protein and protein soluble at a pH of 4.6 (O'Connell and Fox, 2000). In spite of the similarities in protein composition with skim milk, buttermilk is very distinct from any other dairy product and is recognized as a valuable source of phospholipids (Malmsten *et al.*, 1994; Sachedva and Buchheim, 1997). Sphingomyelin, phosphatidylcholine and phosphatidylethanolamine are present in an approximate ratio of 1:1:1 and represent most of the phospholipids in the MFGM (Parodi, 1997). This ratio makes the phospholipid extracts from the MFGM unique compared to the other sources of lecithin (egg and soy, for example). In spite of the availability of large amounts of these high-value components, buttermilk is still viewed as a

by-product and has very few applications as a value-added functional ingredient, mainly because to its low stability to oxidation.

A byproduct of cheese manufacture, whey cream, is the fat fraction separated from whey after removal of the curd and is also a good source of MFGM. Whey cream contains less skim milk-derived proteins, and MFGM can be recovered from the aqueous phase, which results from the destabilization of the fat globules. Whey cream, buttermilk as a by-product of the manufacture of whey butter, butter serum from the manufacture of anhydrous milk fat and buttermilk are less valuable than skim milk, because of the polyunsaturated fatty acids present, which are labile to oxidation. When compared to skim milk, buttermilk has high batch-to-batch variability, a characteristic that limits applications in food processing. All the processing steps involved in the production of buttermilk affect the interactions of the serum proteins with the MFGM. For this reason, a better understanding of the factors underlying these interactions, coupled with careful control of the processing parameters will be necessary to obtain products of consistent quality. Currently, this is often not the case for buttermilk and whey cream, as their processing history is not fully controlled. This presents an opportunity for research to resolve the inconsistencies found in these byproducts, by understanding how changes in the functionality of MFGM relate to the variations in manufacturing conditions.

Improvement of membrane separation technology has resulted in the isolation of MFGM-enriched material from commercially available products. A phospholipid-rich fraction can be extracted from whey (Boyd *et al.*, 1999) and buttermilk (Sachedva and Buchheim, 1997) with a reported yield of 0.25 g of phospholipids/g of protein in buttermilk (Sachdeva and Buchheim, 1997). Microfiltration of whey derived from the Cheddar cheese process, using 0.2 μm ceramic filters results in a fraction containing two major phospholipids, phosphatidylcholine and phosphatidylethanolamine, and lesser amounts of phosphatidylinositol, phosphatidylserine, sphingomyelin and cerebrosides (Boyd *et al.*, 1999). The phospholipid fraction separated from the total lipids contains a larger proportion of mono- and polyunsaturated fatty acids (mainly oleic, $C_{18:1}$ and linoleic, $C_{18:2}$) compared to the total lipid and the neutral lipid fraction (Boyd *et al.*, 1999).

While in the laboratory it is possible to extract a MFGM fraction free from contaminants, although with some losses of membrane material during the washing steps, the isolation of MFGM from commercial dairy products is more challenging. In addition to the MFGM polypeptides, buttermilk and whey may contain significant amounts of components derived from skim milk (whey proteins, caseins). Only a few reports describe the separation of MFGM from casein micelles (Sachdeva and Buchheim, 1997; Corredig and Dalgleish, 1998c; Corredig *et al.*, 2003), which are comparable in size to the

MFGM fragments, and cannot be isolated by filtration only. The primary challenges are not in the optimization of MFGM fractionation with little protein contamination, but in scaling up of the technology. MFGM material can be separated from the other proteins and lactose in commercial buttermilk using centrifugation, by adding agents that disrupt the casein micelles and increase casein solubility (Corredig and Dalgleish, 1998c); however, this technique is not applicable to industrial-scale separation of MFGM. Different buttermilk fractions can be prepared by adsorbing the MFGM material on different types of biosilicates. These materials selectively extract the high molecular weight fraction of the MFGM from buttermilk, and have a higher binding affinity for phospholipids than neutral lipids (Fryksdale and Jiménez-Flores, 2001).

The caseins can be precipitated by treating buttermilk with rennet or citric acid. The phospholipid-rich serum can be filtered using 0.2 μm membranes with good recovery of the MFGM fraction. The yield varies depending on coagulation conditions, especially pH (Sachdeva and Buchheim, 1997).

Microfiltration has shown the best potential to extract MFGM-rich fractions from buttermilk. Microfiltration with a small nominal pore-size membrane (0.1 μm) is often used to produce micellar casein, native phosphocaseinate, or to modify the ratio of caseins in milk (Mistry and Maubois, 1993; Pouliot *et al*., 1994). Phospholipids can be concentrated using a 0.1 μm membrane (Morin *et al*., 2004). The use of reconstituted buttermilk, rather than fresh buttermilk, results in compositional differences in the final product (Morin *et al*., 2004). However, microfiltration alone cannot achieve complete separation of MFGM lipids and proteins from caseins and whey proteins. Because of the comparable size of the components, retention of caseins during microfiltration of buttermilk can be reduced by increasing the amount of soluble casein (Corredig *et al*., 2003). The separation of MFGM from skim milk-derived caseins can be achieved by filtering through 0.1 μm pore size membrane after addition of sodium citrate to buttermilk. This optimizes the retention of fat and the high permeation of the caseins, and results in an enriched fraction from buttermilk containing about 80% of MFGM material (Corredig *et al*., 2003). MFGM-enriched fractions obtained from buttermilk, although containing a significantly reduced amount of caseins, still contain a large amount of whey proteins (Corredig *et al*., 2003). The lack of permeation of whey proteins, especially β-lactoglobulin and to a lesser extent α-lactalbumin, shows the strong binding of whey proteins with MFGM components. The protein aggregates containing whey proteins are large enough to be retained during microfiltration and these complexes may be heat-induced polymers of whey proteins or, more likely, complexes with skim milk proteins (e.g., κ-casein) or MFGM proteins.

A combination of microfiltration (with 0.8 μm pore-size membranes) and supercritical fluid extraction has also been used to obtain MFGM isolates rich in phospholipids (Astaire *et al.*, 2003). Supercritical fluid extraction can be used to extract lipid and lipid-soluble materials from complex matrices. Analysis of the extracts obtained by first concentrating buttermilk by cross-flow microfiltration and then extracting the concentrate using supercritical fluid to remove the neutral lipids showed that the MFGM isolates contained a significantly reduced concentration of non-polar lipids and an increased concentration of polar lipids derived from the MFGM (Astaire *et al.*, 2003). Although it is possible to obtain a phospholipid-rich extract using this technology, it would be quite expensive and skim milk-derived material would still be present in significant quantities as contaminants.

6.4.3. Application and Utilization of MFGM as a Functional Ingredient in Foods

Although some components of buttermilk, butter serum or whey cream could, potentially, be isolated and marketed as dietary supplements, these products are also valuable when added to foods because of their functional properties as ingredients. Buttermilk has traditionally been considered to have superior functionality compared to skim milk in bakery and ice cream manufacture, because it contains MFGM material. For this reason, it is often used as an ingredient in food products because of its emulsifying properties and in low-fat products to improve flavor and texture. For example, the incorporation of ultrafiltered buttermilk into reduced-fat cheese improves the mouth-feel, body and meltability of the cheese. The addition of buttermilk to cheese milk increases the yield of low-fat Cheddar cheese by increasing the moisture content (Mistry *et al.*, 1996; Turcot *et al.,* 2001). The presence of MFGM has also been reported to improve the stability of processed foods (e.g., the addition of buttermilk enhances the heat stability of reconstituted evaporated milk) (Singh and Tokley, 1990).

Another suggested use for buttermilk solids as a value-added ingredient is to stabilize certain food matrices against lipid peroxidation (Wong and Kitts, 2003). Buttermilk solids are ineffective in delaying the onset of lipid oxidation, but reduce the severity of lipid oxidation during propagation. The concentrations tested were 0.1–0.2% in emulsion models, and at the same concentrations, whey proteins were less effective (Wong and Kitts, 2003). The antioxidant activity of buttermilk cannot be attributed solely to the total sulphydryl content. In fact, heating reduces the total sulphydryl content of buttermilk to levels lower than those reported for milk and whey (Taylor and Richardson, 1980).

MFGM fractions isolated from native milk fat globules have shown high surface activity. The measured interfacial tension on surfaces covered by native MFGM is similar to that coved by caseins (Chazelas *et al*., 1995). These results confirmed other research reports on the emulsifying properties of untreated MFGM fractions. MFGM can be used as an emulsifier in reconstituted milk fat emulsions; MFGM can stabilize 25 × its mass of milk fat, forming droplets comparable in size to those in homogenized milk (Kanno, 1989; Kanno *et al*., 1991). Model soy oil-in-water emulsions are also stabilized at neutral pH by MFGM prepared from untreated cream, but they are unstable at low pH (Corredig and Dalgleish, 1998c). The newly-formed oil droplets, covered by MFGM material, behave differently from emulsions stabilized by other milk proteins: no displacement occurs on the addition of small molecular-weight surfactants, and the addition of β-lactoglobulin or caseins after emulsification does not seem to affect the composition of the interface. In all these studies, the MFGM material was derived from cream, which had not been treated and no other protein was present in solution during emulsification.

The behavior of buttermilk as an ingredient in foods may be attributed in part to the presence of the MFGM, but it is predominantly determined by the presence of the skim milk-derived material, especially caseins (Corredig and Dalgleish, 1998a). Whey proteins and caseins constitute a large percentage of the total functional protein in commercial buttermilk. For this reason the functional properties of buttermilk result from the contribution of both skim milk-derived proteins and MFGM material. When buttermilk powder is used to prepare oil-in-water emulsions, caseins make up about 50% of the total protein adsorbed at the interface (Corredig and Dalgleish, 1998a). In these emulsions, the MFGM material does not show preferential adsorption over skim milk proteins and large aggregates are adsorbed at the interface. The behavior of oil-in-water emulsions prepared with buttermilk is different from that of skim milk, as in skim milk-stabilised emulsions less protein is needed to stabilize the oil droplet surfaces than in buttermilk-stabilised emulsions. In addition, preferential adsorption of the caseins over the whey proteins takes place in homogenized skim milk but not in buttermilk (Walstra and Oortwijin, 1982).

The functional properties of the MFGM fragments present in buttermilk cannot be related directly to those of MFGM extracted from untreated cream, because the processing history of MFGM strongly influences its functional properties. Treatment at a temperature as low as 60°C affects the emulsifying properties and solubility of MFGM isolates. MFGM fractions prepared from various heat-treated creams contain considerable amounts of associated whey proteins, and their emulsifying properties are poor compared to those of MFGM material extracted from unpasteurized

cream (Corredig and Dalgleish, 1998b). The decrease in emulsifying properties and solubility are related directly to the amount of whey protein associated with the MFGM and the temperature applied (Corredig and Dalgleish, 1998b). Such changes in functionality of the MFGM with heat treatment and processing need to be understood to explain the variability of the sources of buttermilk available commercially. The functionality of MFGM prepared from heat-treated cream can be modified by proteolysis with trypsin or chymotrypsin. When adsorbed onto oil droplets, the MFGM isolated from treated buttermilk (commercially available) is more accessible to enzymatic treatment than MFGM prepared from untreated cream (Corredig and Dalgleish, 1997). In addition, the emulsifying properties of the MFGM preparations can be improved by treatment with trypsin or chymotrypsin.

6.4.4. Conclusions and Future Research Directions

Living mammals are the progeny of those for whom lactation was successful. The genetic legacy of the relentless selective pressure on milk as a nourishing food is encoded in the genes responsible for milk production (i.e., the milk genome). From the compositional information, which exists on milk from different species, at different stages of lactation and in response to various external inputs, it is clear that this biomaterial is highly dynamic. To be successful in competitive habitats, lactation must be effective in providing nourishment and protection for the neonate while not excessively taxing the mother's resources. As is true for much of molecular evolution, many of the components of milk have been co-opted from other functions in biology, and in most cases it is not clear why. Yet, it can be argued that components that provided no benefit, or which were no longer needed, would tend to be lost over time. Against this background of evolutionary selection, comparative milk genomics becomes a relevant scientific endeavour for beneficial nutritional bioactivities. The challenge to the nutritional and food sciences is to understand the molecular basis of these benefits. Understanding how milk functions will ultimately allow similar benefits to be extended to other foods and other consumers.

The arrival of the genomic age offers a range of new tools for scientists to approach these complex biological questions. The assembly of vast nucleotide and protein sequence databases, together with the concomitant development of the bioinformatic tools to analyze them, gives researchers a unique window into the mammalian genome information space. As the first pass sequencing of the human genome is now largely completed, work has begun to annotate all the genes for their endogenous function. The inherent power of this approach is that the databases are additive, and integrative. As proteins are

identified as constituents of the MFGM, the following information should contribute to an understanding of their function, such as the primary sequence, domain structure, and function in other cell types. Identification of the genetic basis for milk components specific to particular functional motifs, coupled with the physiological knowledge of their mechanisms of action will give insight as to how these components are functioning.

The MFGM contains a large amount of components that contribute to the compositional diversity in milk. In addition, numerous MFGM constituents have recognized bioactivity. While research continues to reveal evidence of the relationship between dietary consumption of MFGM and enhanced health, more research is needed to understand the functionality of the various components present in this material. The process engineering of dairy streams could be modified to take advantage of the MFGM if such information was available. For example, at present, high heat is applied to cream for butter processing; if a less, severe heat treatment was used, highly functional MFGM material would be available, which could be marketed as a value-added ingredient for its nutritional functionality as well as its processing functionality (e.g., as an ingredient that improves texture and mouth-feel).

Only a few reports are available on the preparation of MFGM from commercially available sources and the opportunities to exploit fully the utilization of MFGM as a functional material are so far limited by the lack of available products and commercially feasible preparation methods. The development of methods for the extraction of MFGM from buttermilk through microfiltration may increase the opportunity to produce this ingredient on a commercial scale. On the other hand, before the economics of such processes can be appreciated, the unique functionality of MFGM isolates needs to be understood better.

The conditions to which fat globules are subjected during processing compromises the native structure and composition of the MFGM and are likely to have dramatic consequences on its functionality. Hence, greater exploitation of the value of MFGM may require redesigning of some of the dairy processes to which the globules are subjected.

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