***Effects of Milk Processing on the Milk Fat Globule Membrane
Constituents***

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“We don’t receive wisdom; we must discover it for ourselves after a journey that no one
can take for us or spare us”

-Marcel Proust

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ABSTRACT

Effects of Milk Processing on the Milk Fat Globule Membrane Constituents

Xiomara E. Elías-Argote

The milk fat globule membrane (MFGM) is avidly studied by many groups of scientists around the world due to its unprecedented nutritional and functional properties; however, limited research has been performed on the effects of milk processing on the chemical changes of the MFGM. Thus, this study highlights the changes that lipids and proteins undergo from the time milk leaves cow's udders. Cooling (4 °C) was included along with subsequent pasteurization by different traditional thermal processes and cold pasteurization by pulse light ultra violet treatment. Cooling milk to 4 °C had a measureable effect on the MFGM composition, resulting in protein alterations, particularly to butyrophilin and adipophilin. Thermal treatments disturbed the native structures of molecules and increased the adsorption of milk and whey proteins on the globules, especially α -lactalbumin and β -lactoglobulin. As the heat intensity increased, the aggregation of fat globules became more pronounced due to protein interactions. Intrinsic MFGM proteins also varied in relative abundance during the processing steps. The concentrations of polar lipids did not change during processing, with the exception of phosphatidylserine, which decreased during the cooling and thermal treatments. Cold pasteurization (UV treatment) had a minimal effect on fat globules and MFGM proteins. Since the MFGM promises to deliver nutritional effects and more when included in food products, currently HTST pasteurization was shown to be the best method to process milk and obtain MFGM isolates for further supplementation.

Keywords: milk fat globule membrane (MFGM), proteomics, thermal treatment, milk processing, UV-light treatment.

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I. INTRODUCTION

With consumers becoming more health-conscious, the demand for food products that deliver nutritional and functional benefits has increased. In the dairy industry, the interest in the milk fat globule membrane (MFGM) is no exception. This milk component was initially described in the 1970's and 1980's as the membrane that surrounds fat globules in milk, preventing coalescence and rancidity of lipids (Keenan *et al.*, 1971; Mather and Keenan, 1975; Jarasch *et al.*, 1977; Mather and Keenan, 1998; Patton, 2004; Singh, 2006; Dewettinck *et al.*, 2008). In the last two decades, the nutraceutical properties of the MFGM have been explored (Argov *et al.*, 2008a; Spitsberg, 2005). In fact, anticancer and antihypercholesterolemic activities, antimicrobial and antiviral properties such as inhibition of the ulcer-forming bacterium *Helicobacter pylori* and rotavirus, and suppression of diseases such as multiple sclerosis have been ascribed to the MFGM. In addition, in clinical studies, complementing infant food with MFGM and micronutrients leads to more copper and vitamin B12 uptake by infants (Lonnerdal *et al.*, 2006). Also, MFGM complementation has reduced the incidence and prevalence of diarrhea in infants (Zavaleta *et al.*, 2006). Contemporary research and innovation in the MFGM has been in the pharmaceutical field where liposome formation for the delivery of drugs, vitamins, and nutrients is being designed and tested (Bezelgues *et al.*, 2009; Thompson and Singh, 2006).

The manufacture of butter and cheese generate by-products that have not found a place in the market or that are underutilized commercially although they have high nutritional and functional potential. In this category are buttermilk and butter serum, which have unprecedented nutritional and functional properties that could be capitalized upon when incorporated in innovative food products. The presence of the MFGM in these

food or dairy products promises to deliver more than nutritional benefits by including nutraceuticals for health and well-being; however, there is still a lot to know, test, prove and develop about this component of milk.

Buttermilk originates during cream churning where the fat globules are destabilized with subsequent agglomeration and coalescence, displacing the MFGM and water-soluble components from cream into the aqueous phase. This by-product, known as sweet buttermilk, is the main commercial source of MFGM, which has a low cost but currently very limited market (Jiménez-Flores and Brisson, 2008; Jiménez-Flores and Higuera-Ciapara, 2009; Lopez *et al.*, 2010b). A second source is butter serum, which is popular in countries where butter is made from very high fat content cream (plastic cream), and there is very little traditional buttermilk production. Currently, in the US industry, sweet buttermilk is mainly used in food products such as ice cream and bakery goods, with very limited use as surface-active ingredient (Dewettinck *et al.*, 2008; Jinjark *et al.*, 2006; Sodini *et al.*, 2006). In fact, in 2007 the use of buttermilk in the United States was mainly for baking to improve flavor and texture, representing 79.9% of the 49.8 million pounds of dry buttermilk sold. The dairy industry also utilized buttermilk (13.9%) in products such as ice cream, recombined milk, and cheese, and about 3.4% was used for prepared dry mixes (American Dairy Products Institute, 2007). In 2008, 1,644 billion pounds of butter were produced in the United States, generating approximately the same amount of buttermilk (United States Department of Agriculture). We could be using this product to complement food and obtain the health benefits ascribed to MFGM constituents such as proteins and phospholipids (PL), which are present in concentrations of 32.9 and 2.03 grams per 100 grams of dry buttermilk, respectively (Jiménez-Flores and Brisson, 2008).

The genesis of PL characterization in milk started in the 1960's (Smith and Jack, 1959; Sprecher *et al.*, 1965; Webb *et al.*, 1974). More recently, with the use of novel technologies, their physicochemical properties have been exploited, highlighting the emulsification and anti-foaming properties of MFGM. These lipids have been associated with enhanced health, especially those phospholipids present in specific animal tissues such as sphingomyelin and phosphatidylcholine, cerebrosides and gangliosides (considered important glycolipids). In fact, PL and their metabolites have important biological roles such as cell and membrane communication, myelination in the central nervous system, and as membrane receptors. Sources with high concentrations of PL such as the MFGM should be considered of high value by food technologists and efforts need to be made to include them in food products that not only to deliver essential nutrients but functional compounds.

Despite all the health benefits offered by the milk fat globule membrane, little effort has been given by the technological community to incorporate MFGM constituents into food products, and the MFGM still remains the least understood component of nature's most nutritious food: milk (Argov *et al.*, 2008a). With the latest techniques in biology, chemistry and physics, proteomics, genomics, glycomics (Tao *et al.*, 2008) and other reliably objective physical and chemical tools the complex biological structure and interaction of fat globules and MFGM can be characterized *in situ* or with very little destructive manipulation and, thereby, provide knowledge of the native components that benefit mammalian neonates.

This thesis gives a current view on the relevant discoveries that place the MFGM in a unique position for food and dairy products applications, and presents findings on the chemical, biochemical, and physical changes of the MFGM as a result of milk processing

throughout collection, storage, and pasteurization, as depicted in Figure 1. In addition, a revolutionary technology in cold pasteurization, pulsed UV-light, is introduced and compared with conventional heat pasteurization. Since heat pasteurization has high capital and operational costs, light treatment may become an alternate pasteurization technique for the dairy industry due to its efficiency and fast results in deactivating microorganisms, especially foodborne pathogens. However, little information is available about its effects on the conformational changes in proteins or the production of by-products that could be carcinogenic or affect consumer acceptance.

Figure 1. Milk Processing in the dairy industry. Upon collection, milk is transferred to a storage unit at 4 °C where it awaits pasteurization for further processing (Adapted from Dairy Processing Handbook).

II. LITERATURE REVIEW

A. *Brief Outline*

This review highlights the chemical, biochemical, and nutritional characteristics of the milk fat globule membrane as well as its dairy sources. It attempts to give an overview of the origin, composition, structure, health benefits, and technical aspects of the MFGM that have been identified for this novel ingredient as a potential nutraceutical. In addition, the effect of milk processing such as temperature, pressure, and irradiation on the structure and composition of the MFGM will be discussed as well as current methodologies for further noninvasive investigation. These facts are offered as a basic platform from which readers can find ideas and inspiration for furthering the knowledge and application of this milk component and resource.

B. *Origin and Function of the Milk Fat Globule Membrane*

Milk lipids are mainly composed of triacylglycerols (TAG) that accumulate within the bilayer of the rough endoplasmic reticulum (rER) of epithelial cells of the mammary glands where they start their journey towards the apical membrane (Nielsen *et al.*, 1999; Bionaz and Loor, 2008). However, freeze-fracture electron microscopy further pinpointed that lipid droplets developed alongside the rER membrane. Regardless of the lipid droplet biogenesis, a membrane known as the milk fat globule membrane (MFGM) surrounds the lipids. This trilayer membrane acts as an emulsifier and prevents coalescence of fat droplets and lipase activity (Dewettinck *et al.*, 2008).

Newly-formed triacylglycerols are first assembled into microdroplets (Bionaz and Loor, 2008), which range in diameter from less than 0.5 μm to more than 4 μm , at the basal membrane of the secretory mammary cells (Keenan *et al.*, 1971). A monolayer,

consisting of polar phospholipids and proteins, surrounds the assembled triacylglycerols, which do not form part of this monolayer (Wooding and Kemp, 1975). As the microdroplets migrate through the cytoplasm, they coalesce to form bigger droplets called cytoplasmic lipid droplets (CLD). This formation is promoted by calcium and by ganglioside mediation (Mather and Keenan, 1998; Keenan and Mather, 2006; Jiménez-Flores and Brisson, 2008). CLD accumulation may be promoted by decreased lipid storage in mammary adipocyte tissue (Russell *et al.*, 2007). The CLDs then approach the apical surface, probably assisted by GTP-binding proteins (Spitsberg and Gorewit, 1997), are gradually enveloped in the membrane, and bud out of the secretory cells (Keenan and Mather, 2006; Mather, 2000; Dewettinck *et al.*, 2008). It has been hypothesized that migration is assisted by microtubules; however, current research is inconclusive (Keenan and Mather, 2006). The apical membrane that surrounds MFG may represent a line of defense against pathogens at the mucosal surface of the mammary glands, so proteins associated with host defense may be associated with the MFGM (Smolenski *et al.*, 2006).

During the enveloping process, a distance of 10 to 20nm is kept between the outer surface of the droplet and the cytoplasmic face of the plasma membrane, which appears to be filled with electron-dense material when observed in micrographs. The origin of such material is still unknown (Keenan and Mather, 2006; Dewettinck *et al.*, 2008). The formation of secretory vesicles that pick up microdroplets has also been suggested for milk fat globule (MFG) excretion, but cytochemical evidence favors the plasma membrane envelopment mechanism (Mather and Keenan, 1998; Keenan and Mather, 2006). In fact, butyrophilin, an integral MFG protein, plays a role in globule secretion because it is highly expressed on the apical membrane and concentrated at budding locations (Robenek *et al.*, 2006a; Keenan and Mather, 2006; Mather and Keenan, 1998;

Dewettinck *et al.*, 2008). Molecular data suggest that butyrophilin, xanthine oxidase (XO), and the lipid droplet surface protein, adipophilin (ADPH) play a tripartite role in milk fat secretion according to their relative mRNA abundance (Bionaz and Loor, 2008). However, evidence from freeze-fracture replica immunolabelling (FRIL) has revealed the location of these proteins and contradicts the former hypothesis suggesting that milk lipid secretion is solely mediated by butyrophilin (McManaman *et al.*, 2007). Despite this controversy, Bionaz *et al.* (2008) consolidated recent and detailed data on milk fat synthesis during lactation and introduced a pathway based on genomic data that depicts the role of proteins involved in fatty acid synthesis as well as milk droplet excretion (Figure 2).

In the secreted milk, lipid globule diameter ranges from 0.15 μm to more than 15 μm with slight variation in their fatty acid composition between small (1 to 3 μm) and large (6 μm) globules (Michalski and Januel, 2006a; Fauquant *et al.*, 2005). According to Fauquant *et al.* (2007), small MFG, ranging between 2.3 μm to 8.0 μm , have no significant difference in their phospholipid and sterol content. Later studies indicate that small milk fat globules (1.6 μm) contain almost three times more polar lipid, lower amounts of phosphatidylcholine and sphingomyelin, and larger curvature than large fat globules (6.6 μm). The latter ones are especially enriched in phosphatidylcholine. In addition, the fatty acid composition in small globules was enriched in C12:0, C14:0, C16:0, C18:1 trans, and C18:2 (Lopez *et al.*, 2010a). Avalli and Contarini (2005) reported higher polar lipid content in cream obtained from natural creaming as supposed to cream from centrifugation, highlighting that such observations were due to the presence of small fat globules. Lopez *et al.* (2010a) attributed the variation in PL in different globule size to the mechanism of fat globule secretion or to the rearrangement in

the MFGM post-secretion from the mammary secretory cell and hypothesized that the greater curvature fat globules may exclude choline-containing polar lipids.

The fat globule size depends on many factors such as cow breed and lactation stage. In fact, fat globules from Jersey cows are larger than those from Friesian cows, with average diameters of 4.5 μm and 3.5 μm , respectively (Singh, 2006). Argov *et al.* (2008) speculated that the distribution in diameter might imply a different role than just fat delivery to neonates (Kemsley, 2008). In fact, membrane vesicles consisting of phospholipids and proteins, with 30-100nm and 100-1000nm diameters, respectively known as exosomes and microvesicles, have been observed in milk without association of triglycerides and cholesterol (Silanikove *et al.*, 2006; Argov *et al.*, 2008b). Their genesis and functions are unknown, but they have been associated with immunoregulation and antitumoral activities (Silanikove *et al.*, 2006). Admyre *et al.* (2007) identified these vesicles as exosomes that have the capacity to influence immune responses in human breast milk. Argov *et al.* (2008) have designated them as lactosomes in human milk whereas Silanikove and Shapiro (2007) called them whey membrane particles (WMP) in bovine milk.

Figure 2. Pathway of milk fat synthesis regulation in bovine mammary glands. This model incorporates the most recent information in milk droplet secretion and enzymes involved in milk fat synthesis (Taken from Bionaz *et al.*, 2008)

In approximately 1% of bovine fat globules, some cytoplasm will be entrapped between the bilayer membrane and the fat droplet surface, which forms a crescent or “signet” (Wooding *et al.*, 1970). Keenan and Mather (2006) support the original suggestion by Patton (2004) that crescents might be a pathway for the introduction of hormones or other bioactive molecules in milk. Knowledge about their molecular formation may help us decipher the mechanism that controls milk fat secretion, which is still an area of speculation. Also, there is a lot of emphasis on post-secretion modification of the MFGM, which may be the origin of membrane fragments seen in skim-milk; however, the extent of this event is uncertain (Mather and Keenan, 1998; Mather, 2000; Keenan and Mather, 2006; Singh, 2006). In fact, mechanical treatments like heating, homogenization, aeration, and agitation may enhance MFGM release into the serum phase (Rombaut *et al.*, 2006a; Evers, 2004a).

C. Composition and Structure of the MFGM

The MFGM is a complex structure. It is 10-50 nm thick and contains phospholipids, sphingolipids, and specific membrane proteins (Figure 3). Phospholipids and proteins account for over 90% of the membrane's dry weight (Singh, 2006). Some of the proteins are an integral part of the membrane such as xanthine oxidase and adipophilin, and others are peripheral or loosely attached within the trilayer membrane including butyrophilin and glycosylated proteins respectively (Dewettinck *et al.*, 2008; Lopez *et al.*, 2008). Recent studies with trypsin and pronase E shows that butyrophilin may be located on the exterior of the MFGM as well as adipophilin, and xanthine oxidase may have a dual position within the membrane (Vanderghem *et al.*, 2011).

The first membrane, derived from the rER, is a monolayer containing phospholipids and proteins that is in direct contact with the intracellular lipid core (Vanderghem *et al.*,

2011). The second membrane is a bilayer containing glycosylated and non-glycosylated proteins, glycerophospholipids and sphingolipids, enzymes, cholesterol, and other minor components. Between the inner membrane and the bilayer, there is electron-dense coat rich in proteins (Lopez *et al.*, 2008; Rombaut *et al.*, 2006a). A glycocalyx forms a fourth layer, which acts as a source of specific bacterial and viral ligands and varies during lactation (Spitsberg and Gorewit, 1997; Wilson *et al.*, 2008; Evers, 2004a). Interactions among milk constituents and other molecules occur mainly on this outer surface (Vanderghem *et al.*, 2011). The structural composition of the oligosaccharides in glycoproteins is designed as an innate protection to clear out unwanted pathogens from the gastrointestinal tract of the newborn offspring, especially those of human milk (Wilson *et al.*, 2008). Studies have shown that the MFGM is not a continuous membrane (Kobylka and Carraway, 1973), and even some membrane material is destroyed during secretion leaving only the inner layer on parts of the fat globules (Robenek *et al.*, 2006b; Vanderghem *et al.*, 2011).

Figure 3. Schematic representation of the structure of the milk fat globule membrane
(Taken from Lopez *et al.* 2011).

The gross composition of the MFGM, reported in literature, differs as a result of isolation, purification, and techniques used in analysis (Keenan and Mather, 2006; Dewettinck *et al.*, 2008; Evers, 2004a). Furthermore, the composition can be altered by physiological, chemical/enzymatic, and physical/mechanical factors (Evers, 2004b). The latter includes cooling, drying, separation, agitation, heating, and homogenization (Dewettinck *et al.*, 2008; Lopez *et al.*, 2008; Jiménez-Flores and Brisson, 2008; Michalski and Januel, 2006a). With respect to physiological factors, Lopez *et al.* (2008) observed large differences in the fatty acid (FA) composition of the phospholipids in the milk from cows fed a regular diet and a diet rich in polyunsaturated FA.

Table 1. Factors that affect the composition and structure of the MFGM (Adapted from Ward *et al.* 2006, and Evers, 2004a).

Factor	Effect	Reference
Chemical/Enzymatic		
Processing origin	Loss of membrane components, adsorption of milk plasma components	
Bacterial origin	Production of lipases, phospholipases, proteinases, or glycosidic hydrolyases may change MFGM	(McPherson and Kitchen, 1983; Erwin and Randolph, 1975; Evers, 2004a)
Physiological		
Diet, breed, stage of lactation	Affect amount of membrane material, fat size distribution, and relative distribution of membrane components	(Mondy and Keenan, 1993; Ye <i>et al.</i> , 2002; Murgiano <i>et al.</i> , 2009; Reinhardt and Lippolis, 2008; Lopez <i>et al.</i> , 2008)
Physical/Mechanical (handling & processing)		
Air bubbles	Loss of membrane material	(Walstra and Jenness, 1984)
Ageing	Effects depend on temperature	Little is known
Cooling	Loss of copper, loss of phospholipids, absorption of proteins	(Anderson <i>et al.</i> , 1972; Dickow <i>et al.</i> , 2011; Patton <i>et al.</i> , 1980; Baumrucker and Keenan, 1973)
Pumping, stirring, agitation	Depends on the degree of air incorporated. High shear forces are required to change the MFGM in the absence of air	(Mulder and Walstra, 1974; Stannard, 1975)
Homogenization	Adsorption of serum protein, rupture of membrane	(Anderson <i>et al.</i> , 1972; Cano-Ruiz and Richter, 1997; Mondy and Keenan, 1993; Lee and Sherbon, 2002; Ye <i>et al.</i> , 2004a)
	Formation of PL or membrane vesicles	(Michalski <i>et al.</i> , 2002)
	Redistribution of protein and lipids in globules	(Keenan <i>et al.</i> , 1983)
Heating	Adsorption of copper, whey proteins & caseins Aggregation of BTN and XO, loss of PAS 6/7 and phospholipids	(Koops and Tarassuk, 1959; Greenbank and Pallansch, 1961; Corredig and Dalgleish, 1996; Sharma and Dalgleish, 1993; Kim and Jiménez-Flores, 1995; Houlihan <i>et al.</i> , 1992; Sharma and Dalgleish, 1994; Lee and Sherbon, 2002; Ye <i>et al.</i> , 2002; Ye <i>et al.</i> , 2004b; Garcia-Amezquita <i>et al.</i> , 2009)
Spray-Drying	Reduce globule size and increase surface area for adsorption of serum proteins	(Landstrom <i>et al.</i> , 2000; Ye <i>et al.</i> , 2007)

A diet rich in polyunsaturated FA resulted in a significant decrease in saturated fatty acid content in milk, which enhances milk's nutritional quality, especially for people with coronary heart disease risks (Lopez *et al.*, 2008; Jensen, 2002). Enzymatic changes are due to microbial growth from both the cow's udder and the external environment (Evers,

2004a), and according to Erwin and Randolph (1975), mastitis milk has lower amounts of phospholipid, higher levels of cholesterol, and changes in enzyme activity such as xanthine oxidase. Table 1 summarizes the factors that affect the structure and composition of MFGM, effects, and respective references. El-Loly (2011) has recently published the latest review on the MFGM, which includes composition, properties, and factors that affect MFGM constituents.

1. Lipids of the Milk Fat Globule Membrane

Polar lipids, such as glycerophospholipids and sphingolipids, constitute less than 1% of the MFG total lipids; nevertheless, they function as intracellular signaling molecules, provide a framework structure, and have nutritional and functional properties such as emulsification (Jiménez-Flores and Brisson, 2008; Lopez *et al.*, 2008). Research has also found a relationship between polar lipid consumption and enhanced health (Spitsberg, 2005). Polar lipids are amphiphilic molecules comprised of a hydrophobic tail and a hydrophilic head. Their morphology is influenced by the presence of water (Fedotova and Lencki, 2008). On the MFGM, they are distributed heterogeneously and present in different percentages (Lopez *et al.*, 2008; Dewettinck *et al.*, 2008; Jiménez-Flores and Brisson, 2008).

These phospholipids contain high levels of unsaturated long-chain fatty acids (FA) such as palmitic, stearic, and tricosanoic acids (Sánchez-Juanes *et al.*, 2009; Singh, 2006; Carrasco-Pancorbo *et al.*, 2009). No difference has been observed in the FA composition of MFGM phospholipids regardless of season (Fauquant *et al.*, 2005); however, changes have been observed during lactation, especially an increase in medium-chain fatty acids and a decrease in polyunsaturated fatty acids (18, 20, and 22 carbons) as lactation progressed (Bitman and Wood, 1990). During milk processing, polar lipids migrate to

aqueous phases such as skimmed milk, buttermilk, and butterserum, making these products good sources for further purification, especially butterserum, which was enriched in sphingomyelin (Rombaut *et al.*, 2006c).

Figure 4. Schematic representation of the different types of lipids seen in the milk fat globule membrane (Nelson and Cox, 2000).

Other lipids such as triglycerides (TG), diglycerides, monoglycerides, sterols, and sterol esters are mainly present in the milk fat globule core and function as energy storage (Table 2). Among these, triglycerides represent about 60% of the neutral lipids in the milk fat globules, and cholesterol accounts for 90% of the total sterols in milk fat globules (Mather, 2000; Keenan and Mather, 2006; Dewettinck *et al.*, 2008; Rombaut *et al.*, 2006c). Figure 4 classifies the different types of lipids found in the MFGM. Recently, bioactive sterols such as lanosterol, lathosterol, demosterol, stigmasterol, and β -sitosterol were identified (Fauquant *et al.*, 2007). Other constituents are hexoses, hexosamines, and sialic acids, which are associated exclusively with proteins and glycosphingolipids (Keenan and Mather, 2006). Small globules contain more medium-chain fatty acids and less stearic acid within the triglyceride core (Fauquant *et al.*, 2005).

Table 2. Composition of milk lipids and their distribution among the milk fat globule (MFG) core, the milk fat globule membrane (MFGM), and the skim milk (adapted from Jiménez-Flores and Brisson, 2008; Michalski *et al.*, 2006; Jensen, 2002)

Lipid Class	Content in total Fat (g/kg)	Fraction in (%)		
		Globule core	MFGM	Skim phase
Neutral glycerides				
Triacylglycerol	958–983	100		
Diacylglycerol	2.8–22.5	• 90	• 10	?
Mono-acylglycerol	0.3–3.8	Traces	Traces	Traces
Free fatty acids	1.0–4.4	60	• 10	30
Phospholipids [†]	2.0–11.1	–	65	35
Small globule (3 µm) ^a			28.4 [‡]	
Large globule (6 µm)			36.9 [‡]	
Cerebrosides	1.0	–	70	30
Gangliosides	0.1	–	• 70	• 30
Sterols		80	10	10
Cholesterol	3.0–4.6			
Cholesteryl ester	• 0.2			
Carotenoids + Vit. A	0.02	• 95	• 5	Traces

[†]PL in the MFGM pellet

^a Small globules tend to contain more sphingomyelin

[‡]PL% in total lipids of the MFGM from microfiltrated globules

a) Phospholipids

Glycerophospholipids have a glycerol backbone with two fatty acids attached on positions *sn-1* and *sn-2*. The third hydroxyl group has a phosphate group esterified to an organic compound such as choline or serine (Rombaut *et al.*, 2006c) as observed in Figure 5. Phosphatidylcholine (PC) represents up to 35% of the total phospholipids and is a major constituent of cell membranes, which provides structure and maintains the permeability barrier (Dewettinck *et al.*, 2008; Jiménez-Flores and Higuera-Ciapara, 2009). Depending on the water concentration and temperature, PC can take on up to four morphologies including lamellar crystalline (L_c), laminar β' ($L_{\beta'}$), laminar α (L_α), and oblique β' ($P_{\beta'}$) (Fedotova and Lencki, 2008).

Name of glycerophospholipid	Name of X	Formula of X	Net charge (at pH 7)
Phosphatidic acid	—	— H	—1
Phosphatidylethanolamine	Ethanolamine	— CH ₂ -CH ₂ -NH ₃ ⁺	0
Phosphatidylcholine	Choline	— CH ₂ -CH ₂ -N ⁺ (CH ₃) ₃	0
Phosphatidylserine	Serine	— CH ₂ -CH(NH ₃ ⁺)COO ⁻	—1
Phosphatidylglycerol	Glycerol	— CH ₂ -CH(OH)-CH ₂ -OH	—1
Phosphatidylinositol 4,5-bisphosphate	<i>myo</i> -Inositol 4,5-bisphosphate		—4
Cardiolipin	Phosphatidyl-glycerol		—2

Figure 5. Glycerophospholipid structure showing the glycerol backbone with two fatty acids (top). Different alcohol substituents that form polar lipid derivatives at the second alcohol via a phosphoester linkage (bottom table) (Nelson and Cox, 2000).

Phosphatidylethanolamine (PE) makes up to 30% of the total phospholipids in MFGM and is mainly found in nerve tissue and brain, accounting for approximately half

of the phospholipid content in the latter. In fact, PE is implicated in optimal brain development and boosting memory (Zeisel, 2006). PE derived from MFGM is highly unsaturated (68%) with high levels of C18:1 and C18:2 and less C14:0 and C16:0 compared to PE from whole milk (Sánchez-Juanes *et al.*, 2009). Phosphatidylinositol (PI) and phosphatidylserine (PS) are anionic phospholipid present in lower amounts in the MFGM. PI acts as a substrate for several enzymes involved in cell signaling and can be phosphorylated to form phosphatidylinositol phosphate (PIP). PS is a negatively charged component of cell and blood platelets that assist in blood coagulation. In addition, PS may reduce the risk of dementia and cognitive dysfunction in the elderly, as presented in Table 3 (Dewettinck *et al.*, 2008). The distribution of these phospholipids is asymmetrical throughout the MFGM (Deeth, 1997). PE, PS, and PI are concentrated in the inner surface whereas PC, sphingomyelin (SM), and glycosphingolipids such as cerebrosides and gangliosides are observed in the bilayer membrane (Deeth, 1997; Lopez *et al.*, 2008).

In the market, sour and sweet buttermilk and quark-skimmed cheese are the highest polar lipid-containing products (Jiménez-Flores and Brisson, 2008). With all the health benefits and biological roles that phospholipids and sphingolipids have, it will be beneficial to fortify food with these low-price products; however, it is important for the food technologist to know the structure of these molecules and understand their chemistry. Phospholipids are good emulsifiers but differ in properties. PC and PE tend to stabilize water-in-oil emulsions due to their low hydrophilic-lipophilic balance (HLB) value while SM, PI, and PS tend to form oil-in-water emulsions (Fedotova and Lencki, 2008; Fedotova and Lencki, 2010). As Jiménez-Flores and Brisson (2008) stated, it is essential to understand the biological mechanism of nutritional delivery to incorporate

MFGM constituents into foods, so food scientists can formulate products that preserve the optimal structure of these lipids and deliver the proper nutrition and functionality.

Table 3. Nutritional aspects of polar lipids of the MFGM (adapted from Dewettinck *et al.*, 2008; Jiménez-Flores and Brisson, 2008; Rombaut *et al.*, 2006).

Component	PL %*	Nutritional Aspects
Sphingolipids and metabolites	18.0-34.1 ^a	Reduction of the number of aberrant crypt foci and adenocarcinomas
		Shift in tumor type (malignant $\Rightarrow$ benign)
		Anticholesterolemic, especially from dairy origin
		Protection of the liver from fat- and cholesterol induced steatosis
		Suppression of gastrointestinal pathogens
		Neonatal gut maturation
		Myelination of the developing central nervous system
		Endogenous modulators of vascular function
Sphingosine 1-phosphate		Associated with age-related diseases and the development of Alzheimer
Phosphatidylcholine (PC)	19.2-37.3	Mitogenic
		Support liver recovery from toxic chemical attack or viral damage
		Protects the human GI mucosa against toxic attack
Lysophosphatidylcholine (lysoPC)	2	Reduction of necrotizing enterocolitis
		Alleviates orotic acid-induced fatty liver
Phosphatidylethanolamine (PE)	19.8-42.0	Bacteriostatic and bactericidal capacity
Phosphatidylinositol (PI)	5-11%	Strong gastroprotective role in the duodenal mucosa
		Maintains hemostasis
Phosphatidylserine (PS)	1.9-10.5	Brain development & boost memory
		Substrate in cell signaling
		Promotes plasma cholesterol transport and metabolism
		Restore normal memory on a variety of tasks
		Positive effects on Alzheimer patients
		Improve exercise capacity of exercising humans

* Relative phospholipid content (g per 100 g of polar lipid including sphingomyelin)

^a Sphingomyelin content

b) Sphingolipids

Sphingolipids are highly bioactive molecules characterized by a sphingoid base, which is a long-chain aliphatic amine (12-22 carbon atoms) (Rombaut *et al.*, 2006c). Attachment of a fatty acid via a hydroxyl group forms a ceramide that is the basic unit for the formation of a sphingophospholipid such as sphingomyelin and lactosylceramide (Figure 6). The difference in headgroups as well as the length and saturation of the fatty

acid and sphingoid chains have contributed to the identification of more than 400 sphingolipids (Duan, 2011).

Figure 6. Basic structure of sphingolipids, which are composed of a sphingosine base link to a fatty acid on its amide group and a headgroup (R) on its hydroxyl group (Top). Most common phosphosphingolipids and glycosphingolipid headgroups (bottom table) (Nelson and Cox, 2000).

Sphingomyelin (SM) is the most abundant sphingolipid (Duan, 2011) and constitutes up to one third of the MFGM polar lipid fraction (Dewettinck *et al.*, 2008). It is composed of a phosphorylcholine headgroup and a fatty acid linked to the amide nitrogen

of the sphingoid long chain base. The high degree of saturation of the acyl chains facilitates complex formation with cholesterol (Figure 3) (Avalli and Contarini, 2005; Duan). This liquid-ordered state, also known as a lipid raft, affects membrane structure and function (Vesper *et al.*, 1999; Duan, 2011; Lopez *et al.*, 2010b). Lipid rafts are rigid domains implicated in cellular processes like signal transduction, endocytosis, and cholesterol trafficking (Lopez *et al.*, 2008; Lopez *et al.*, 2010b; Dewettinck *et al.*, 2008). Other sphingolipids present in lower percentages in the MFGM are glucosylceramide, lactosylceramide, and gangliosides. Galactosylceramide, ganglioside, and SM have only been identified in mammalian tissue but not in plant tissue (Duan, 2011).

The metabolism of dietary and membrane sphingolipids, which mainly takes place in the middle region of the small intestine, generates ceramide, sphingosine, sphingosine-1-phosphate, and ceramide-1-phosphate (Duan and Nilsson, 2009; Duan, 2011). Among these intermediate products, ceramide is the most important because other sphingolipids can be derived from it (Duan, 2011). These molecules act as bioactive lipid messengers that influence cellular functions such as growth, differentiation, apoptosis, response of mucosal cells to pathogens, and have anticancer activities, especially in colorectal, liver, and gastric cancer (Figure 7) (Duan, 2011). Foods high in sphingolipids are eggs and soybeans with over 2,200 $\mu\text{mol/kg}$ and dairy products such as cream and cheese with over 1,300 $\mu\text{mol/kg}$ (Yeh, 1984). For infants the only source of sphingolipids is milk, which has approximately 160 $\mu\text{mol/kg}$ (Yeh, 1984; Duan, 2011).

Figure 7. Functions of sphingomyelin and metabolites as well as factors that can modulate sphingolipid metabolism and cell regulation (Adapted from Vesper *et al.* 1999).

Sphingomyelin is primarily located in the outer membrane of the MFGM and is present in higher percentages in milk derived from cows fed with unsaturated fatty acids due to the presence of smaller globules and consequently more surface area (Lopez *et al.*, 2008). Some studies indicate that starter cultures may contribute to the amount of PL, especially sphingomyelin, present in dairy products and by-products (Umemoto and Sato, 1973; Morin *et al.*, 2006); however, another research group found similar sphingolipid concentrations in the raw material and the final fermented product, indicating that starter cultures do not contribute to SM in dairy foods (Ahn and Schroeder, 2002). In the contrary, they degrade some phospholipids, and thereby, explain the presence of phosphatidic acid in fermented foods.

SM has been shown to reduce aberrant crypt foci and the appearance of colon adenocarcinoma (Schmelz *et al.*, 1996; Snow *et al.*, 2010), to influence neonatal gut maturation (Duan, 2011; Duan and Nilsson, 2009), and to contribute to myelination of the developing rat central nervous system (Yeh, 1984; Dewettinck *et al.*, 2008). Rats fed with milk sphingolipids had a significant reduction in plasma cholesterol levels and liver triacylglycerols (Nyberg *et al.*, 2000; Watanabe *et al.*, 2010). Sphingomyelin derived from dairy seems to be more efficient in reducing cholesterol levels than egg SM (Noh and Koo, 2004). The ceramide fraction of milk polar lipids had similar results in diabetes/obese model mice; it decreased the level of cholesterol and TAG in the liver and total cholesterol in the plasma (Watanabe *et al.*, 2010).

2. *Milk Fat Globule Membrane Proteins*

MFGM proteins constitute 1-2% of the total bovine milk protein, and depending on the milk source and how it is processed, 25-70% of the MFGM may be polypeptides, ranging in molecular weight (MW) from 15,000 to 240,000 Da (Dewettinck *et al.*, 2008; Jiménez-Flores and Higuera-Ciapara, 2009; Riccio, 2004; Ye *et al.*, 2002). Indeed, Murgiano *et al.* (2009) detected differences between Chianina and Holstein cattle in the amount of proteins associated with mammary gland development, lipid droplet formation, and host defense mechanisms. A detailed description and suggested nomenclature of the known and major MFGM proteins are presented by Mather (2000). Using proteomic techniques, Reinhardt and Lippolis (2006) identified 120 proteins, which were grouped into seven categories as seen in Table 4; however, studies by Fong and Norris have demonstrated eight predominant proteins in the MFGM: butyrophilin, adipophilin (ADPH), xanthine oxidase, cluster of differentiation 36, fatty acid-binding protein (FABP), lactadherin, mucin 1, and mucin 15 (Fong and Norris, 2009).

Table 4. MFGM Protein classification using proteomics techniques (adapted from Reinhardt and Lippolis, 2006).

Protein Functionality	%
Membrane/protein trafficking	23
Cell signaling	23
Fat transport and/or metabolism	11
Transport	9
Protein synthesis, binding &/or folding	7
Immune functions	4
Milk proteins: α -and β -casein	2
Unknown function	21

Early studies demonstrated in-vitro intrinsic phosphorylation of butyrophilin and fatty acid binding protein (FABP) (Spitsberg and Gorewit, 1997). In fact, there have been detected about 28 enzymes in the MFGM including some protein kinases, oxidases, 5'-nucleotidase, adenosine triphosphatase, and phosphodiesterase (Reinhardt and Lippolis, 2006; Keenan and Mather, 2006). Half of these enzymes belong to the hydrolase class, and the most abundant enzymes are alkaline phosphatase and xanthine oxidase (Jarasch *et al.*, 1977; Singh, 2006). The purpose of some enzymes is still unclear. It has been hypothesized that they might originate from the cytoplasmic crescents, making them transient MFGM proteins (Keenan and Mather, 2006; Mather and Keenan, 1998). The biological role of the MFGM-associated enzymes has been focused on their damaging effects, which makes their presence undesirable in dairy products because they might cause off-flavors or affect processing properties (Keenan and Mather, 2006). One fourth of the identified proteins still have unknown functions. Table 5 presents the health benefits attributed to MFGM proteins and components.

Table 5. Health benefits of MFGM components (adapted from Dewettinck *et al.*, 2008; Fong *et al.*, 2007; Jiménez-Flores and Brisson, 2008; Michalski and Caroline, 2006 ; Riccio, 2004; Pan *et al.*, 2006; Spitsberg, 2005).

Proteins	MW (Kg/mol)	Reported Health Benefits/Functions
Mucin 1 (MUC1) [†]	160	Antiviral action/Anti Rotavirus especially in neonates

Mucin 15 (MUC15 or PAS III)†	94-100	Antiviral action
Butyrophilin (BTN)†	66	Suppression of multiple sclerosis
Xanthine oxidase (XO)	150-155	Bactericidal agent
Cluster of differentiation (CD36 or PAS IV)†	78	Glycoproteins that act as receptors due to high sugar content
Fatty acid binding protein (FABP)	15	Cell growth inhibitor Anti-cancer factor (FABP as selenium carrier)
BRCA1 and BRCA2	210	Inhibition of breast cancer
Lactadherin (PAS 6/7)†	43-59	Role of epithelialization, cell polarization, cell movement and rearrangement, neurite outgrowth, synaptic activity in the central nervous system, protection against viral infection in the gut. Phospholipid binding
Adipophilin (ADPH)	52	Milk synthesis
Other components*		
β-Glucuronidase inhibitor		Inhibition of colon cancer
Helicobacter pylori inhibitor		Prevention of gastric diseases
Cholesterolemia-lowering factor		Anti-cholesterol activity
Vitamin E and carotenoids		Anti-oxidants
Phosphoproteins		Source of organic phosphorus and Ca-phosphate

† Glycosylated protein

* Phospholipids described in Table 4

As mentioned before, the protein content also varies throughout the cow's lactation phase (Ye *et al.*, 2002). Two of the major proteins in the MFGM, xanthine oxidoreductase (XOR) and the mammary gland specific butyrophilin (BTN), fluctuate during the 300-day lactation period. They are high during the early and late stages compared with the mid-lactation. Despite the change in protein concentration, the fat globule size was not influenced, and the molar ratio (4:1, BTN:XOR) between these proteins remained the same throughout, suggesting a close interaction (Ye *et al.*, 2002; Keenan and Mather, 2006; Singh, 2006). Further studies by Reinhardt and Lippolis (2008) characterized changes in the bovine MFGM proteome during transition from colostrum to mature milk. From day one (colostrum) to day seven (milk), 26 proteins were upregulated and 19 proteins were downregulated. Some of the most important MFGM proteins, including those regulated during the transition period, are discussed below. Among the upregulated proteins, mucin-1 and mucin-15 had the highest expression change with a 7-fold increase in mature milk (Reinhardt and Lippolis, 2008).

a) Butyrophilin

Butyrophilin (BTN) is a glycosylated protein that is mainly found in the apical side of epithelial cells, in budding fat droplets, and in the inner coat of the MFGM (Franke *et al.*, 1981). Recent data also localizes BTN on the cytoplasmic side of the lipid monolayer (McManaman *et al.*, 2007). Mather and Jack (1993) were the first ones to hypothesize that BTN has multiple origins, from the apical membrane and from intracellular sources. It is the most abundant protein of the MFGM, constituting about 40% by weight of the total MFGM proteins in milk from Holstein cows and approximately 20% from Jersey milk (Singh, 2006; Jiménez-Flores and Higuera-Ciapara, 2009). Its apparent molecular weight is 62-67 kDa with a 5% carbohydrate content (Mather and Jacks, 1993; Singh, 2006). Fatty acids are also covalently bound to butyrophilin, which merits its name from the Greek *butyros* and *philos*, signifying “affinity for butter” (Keenan and Mather, 2006); palmitic, stearic, and oleic acid are the predominant protein-bound fatty acids in both BTN and XOR (Singh, 2006). Butyrophilin is part of the immunoglobulin (Ig) superfamily of adhesive and receptor proteins. Indeed, its N-terminus has two Ig-like folds, and the C-terminus comprises a B30.2 domain, which is predicted to have two Ig folds that function as a protein-binding domain (Keenan and Mather, 2006; Mather, 2000; Mather and Jacks, 1993).

Butyrophilin is highly expressed during lactation and forms complexes with XO to envelop the nascent fat globule via adipophilin (Keenan and Mather, 2006). Freeze-fracture localization studies have shown that milk fat secretion is controlled by interactions between butyrophilin present in plasma membrane and in the secretory granule phospholipid monolayer (Robenek *et al.*, 2006a). Jeong *et al.* (2009) provides evidence that the cytoplasmic domain of butyrophilin binds to xanthine reductase as it

was originally hypothesized. In addition, it has been suggested that BTN transfers phosphorus groups to tyrosine residues in proteins, activating them and playing a critical role in controlling the physiological activity of proteins in the secretory cells and within the MFGM. The gene controlling the expression of the BTN gene (BTN1A1) is described in detail in Bionaz and Loor (2008).

To date, the consumption of BTN continues to be a controversial topic due to its association with multiple sclerosis (MS), an autoimmune-mediated inflammatory disease that affects the central nervous system (Dewettinck *et al.*, 2008; Rutter, 2006; Mather, 1999). Some research suggests that dairy products exacerbate symptoms or even produce the disease because BTN resembles a polypeptide named myelin oligodendrocyte glycoprotein (MOG), a putative auto-antigen that induces experimental autoimmune encephalomyelitis (EAE). This disease has clinical symptoms similar to human MS (Dewettinck *et al.*, 2008; Riccio, 2004); however, in other experiments, mice treated with BTN, either before or after MOG injections, did not show clinical manifestations of EAE (Dewettinck *et al.*, 2008). Mather and Linington (1999) have modified BTN to suppress an autoimmune response, which can also be used to screen for MS susceptibility. BTN has also been associated with autism due its resemblance with neurospecific antibodies; however, its etiopathogenesis is unknown (Riccio, 2004). Until the role of BTN with respect to MS and autism is elucidated, dairy products should be included in our daily diet to receive its health benefit, as the etiology of these diseases is a combination of genetic susceptibility and environmental factors (Rutter, 2006).

b) Xanthine Oxidase

Xanthine oxidoreductase (XOR) is a member of the molybdopterin hydroxylase family, which is widely expressed in tissues but has higher activity in bovine milk as compared

to goat, sheep, and human milk (Silanikove and Shapiro, 2007). Moreover, XOR is highly expressed during pregnancy and parturition (Keenan and Mather, 2006). It can be present as xanthine dehydrogenase (XD) and converted to xanthine oxidase (XO) by proteolysis or formation of disulphide bonds (Mather, 2000; Silanikove and Shapiro, 2007; Spitsberg and Gorewit, 1998). It has been shown that plasmin activity is responsible for this interconversion, and the low-oxygen and low-enzyme environment of the inner-membrane decreases the conversion to XO, resulting in a mixed population of enzymes.

XD is distributed in the CLD monolayer, with a non-enzymatic role in fat globule secretion (McManaman *et al.*, 2007; Silanikove and Shapiro, 2007). Vanderghem *et al.* (2011) recently demonstrated that XO may be located both peripherally and within the MFGM. XD is not very stable in solution and reverts slowly to XO at pH 7.5 (Spitsberg and Gorewit, 1998). The active molecule is a homodimer with monomeric molecular masses of 150 kDa, which accounts for up to 20% of the coat proteins where XOR binds with high affinity to the carboxyl end of BTN. Each subunit contains one molybdenum, one flavin adenine dinucleotide, and two nonidentical iron-sulfur redox centers (Martin *et al.*, 2004; Keenan and Mather, 2006; Mather, 2000).

During MFGM isolation, XOR disperses in the supernatant but remains tightly bound to the membrane, and can be isolated using high salt and non-ionic detergents (Keenan and Mather, 2006; Mather, 2000). Keenan and Mather (2006) have proposed that the XDH/BTN complex associates with other proteins to mediate interactions between the lipid core and the membrane. Besides purine catabolism, XOR generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite that act as antimicrobial agents in the neonatal gut. Silanikove and Shapiro

(2007) concluded that XD is a source of freely available extra-membrane protein that plays an essential role in the development of innate immunity (Martin *et al.*, 2004; Jiménez-Flores and Higuera-Ciapara, 2009). Specifically, 33% of the free XD is found in the outer surface of the MFGM, 46.7% in solution, and 20.5% in the outer surface of exosomes. This form may also affect the physicochemical oxidative properties of milk, especially during storage (Silanikove and Shapiro, 2007).

c) Adipophilin

Adipophilin, also known as adipocyte differentiation-related protein (ADRP), is ubiquitously expressed in the presence of long chain free fatty acids, especially in the mammary glands and in differentiating pre-adipocytes (Chang and Chan, 2007). It is one of the five members of the perilipin family (Brasaemle, 2007), and ADPH is the human homolog. These proteins localize on the monolayer and bilayer that surround MFG (McManaman *et al.*, 2007). Clusters of ADRP have been observed in the cytoplasmic leaflet of the rough endoplasmic reticulum where lipid droplet biogenesis might take place. According to Robenek *et al.* (2006b), adipophilin is involved in the growth of lipid droplets, which are enclosed within endoplasmic reticulum (ER) cups. Immunofluorescence evidence by Russell *et al.* (2007) suggests that ADPR mediates lipid droplet accumulation in differentiating epithelial cells. In fact, its expression may be one of the factors that initiate lipid droplet accumulation (Russell *et al.*, 2007).

During preadipocyte differentiation, adipophilin is highly expressed, and the protein embeds itself in the periphery of the intracellular lipid droplets. As adipocytes mature, ADRP is replaced by proteins known as perilipins; however, this mechanism is absent in the epithelial cells of the mammary glands (Nielsen *et al.*, 1999; Dewettinck *et al.*, 2008). Perilipins are proteins localized in the periphery of intracellular lipid droplets and are

essential for droplet formation as well as lipolysis, but recent molecular evidence suggests that its role in mammary lipid droplet formation is not significant (Bionaz and Loor, 2008).

In the fat globules, adipophilin associates with the XOR/BTN complex and facilitates the envelopment of the globules with plasma membrane during milk fat droplet secretion (Nielsen *et al.*, 1999). Proteomic data shows that these three proteins are upregulated individually during the transition period from colostrum to mature milk, indicating an early developmental shift in milk fat transport (Reinhardt and Lippolis, 2008). When the membrane is disrupted, adipophilin remains attached in the MFGM-associated coat material (Keenan and Mather, 2006). The apparent molecular weight of adipophilin is 52 kDa, but calculations from the cDNA result in a lower molecular mass, suggesting post-translational modification, especially acylation (Nielsen *et al.*, 1999). Also, differences in mass and pI may be due to close associations with fatty acids, especially myristate, palmitate, stearate, and oleate (Mather, 2000; Vanderghem *et al.*, 2008).

d) Glycoprotein PAS6 and PAS7

Early studies by Welsch *et al.* (1990) demonstrated the presence of glycoproteins in true ruminants. It was postulated that such glycosylation plays a role in the guts of the newborns (Welsch *et al.*, 1990). In 1992, Kim *et al.* characterized two main glycoproteins in the MFGM, PAS6 and PAS7. The name is derived from the staining technique, periodic acid Schiff glycoprotein (PAS), which is used to identify protein with sugars. Their respective molecular masses are 50 kDa and 47 kDa, and their polypeptide core are identical, suggesting that the difference in molecular weight is due to the N-linked carbohydrate moieties. Three glycosylation sites have been detected in PAS6 while PAS7 only includes two sites (Kim *et al.*, 1992; Hvarregaard *et al.*, 1996). These molecules are

loosely attached to the MFGM and can be removed with salt treatment such as MgCl_2 (Singh, 2006; Mather, 2000).

The human homologue, known as lactadherin, has been shown to bind to rotavirus and protect the gastrointestinal tract of newborn infants from infection (Keenan and Mather, 2006). Studies have demonstrated that this protein plays a crucial role in vascular endothelial growth factor (VEGF)-dependent neovascularization, inducing the growth of new blood vessels in-vitro and in-vivo (Silvestre *et al.*, 2005). Later studies showed that alterations in lactadherin production/function might contribute to the initiation or progression of Alzheimer's disease (Boddaert *et al.*, 2007). The mouse homologue, milk fat globule factor-E8 (MFG-E8), has been involved in regulation of apoptotic neurons, and disruption in such mechanism may lead to neurodegenerative diseases (Fuller and Van Eldik, 2008; Elliott and Ravichandran, 2010). A mutation in this protein has also been implicated in atherosclerosis (Ait-Oufella *et al.*, 2007). The bovine PAS-6/7 has been associated with lipid binding, specifically to phosphatidylserine, -inositol, -glycerol and phosphatidic acid. Such binding had a higher affinity towards MFGM polar lipids as supposed to a pure polar lipid fraction (Andersen *et al.*, 1997).

e) Mucins

Mucins are highly glycosylated proteins that might work as receptors on the surface of cells, especially epithelial cells, where they may inhibit adhesion of intestinal bacteria to host epithelial cell receptors by acting as competitive inhibitors (Wilson *et al.*, 2008; Parker *et al.*, 2010). The predominant mucin in bovine MFGM is related to the human mucin (MUC1), and because of allelic polymorphism, MUC 1 appears as a diffuse band from pooled milk with molecular weight ranging from 160 to 200 kDa. Wilson *et al.*

(2008) demonstrated that bovine and human MUC1 differs in their glycosylation, which implies a different protection against pathogens.

MUC 1 is incorporated onto the MFGM during the budding and release of lipid droplets at the cell surface (Mather, 2000), and milk processing such as cooling and stirring may contribute to its release from fat globules (Peterson *et al.*, 1998). In vitro studies indicate that these glycosylated proteins prevent binding of *H. pylori* to mucosal cells (Peterson *et al.*, 1998). In addition, Parker *et al.*, (2010) determined that MUC inhibits bacterial binding to human intestinal cells, especially Gram (-) enteric bacteria such as *E. coli* and *B. subtilis* compared to Gram (+) bacteria. Another glycosylated protein, MUC15 has also been characterized and shown to be present in the bovine MFGM as well as in nonfat containing fractions such as skim milk and whey. Its molecular weight is about 130 kDa, from which 65% represent the glycans (Pallesen *et al.*, 2007). Both MUC can be used as prophylactic molecules to prevent illness caused by enteric pathogens by inhibiting bacteria's ability to adhere to intestinal epithelial cells or displacing bacteria from cells after microbial binding has occurred (Parker *et al.*, 2010); however, care must be taken during MFGM isolation as these proteins, especially MUC 1, are readily dissociated from the fat globules into skim milk when milk is cooled and agitated (Singh, 2006).

f) *Lactoferrin*

Lactoferrin is an iron-binding protein with varying degrees of glycosylation depending on the state of lactation and exhibiting a high degree of homology with transferrin. The actual concentration in milk differs in response to inflammation or infection (Farrell Jr *et al.*, 2004; Baumrucker *et al.*, 2006), but a decline has been observed during lactation (Reinhardt and Lippolis, 2008). When fully saturated with Fe^{3+} ,

lactoferrin is known as hololactoferrin whereas the iron deficient molecule is called apo-lactoferrin. The iron-lactoferrin is resistant to proteolysis (Baumrucker *et al.*, 2006), and iron dissociates only below pH 3.5 (Farrell Jr *et al.*, 2004). Its first recognized biological function was binding to the outer membrane of gram (-) bacteria and causing the release of polysaccharides (Farrell Jr *et al.*, 2004). In breast milk, a major portion of iron is bound to lactoferrin, which has a specific receptor on the membrane of the small intestine of infants (Suzuki *et al.*, 2005).

Currently, lactoferrin is associated with antimicrobial activities due its iron-binding capacity, inhibition/modulation of cytokine activity by neutrophils, promotion of growth of epithelial cells, anti-inflammatory responses, and prevention of apoptosis (Baumrucker *et al.*, 2006; Pan *et al.*, 2006). Due to these biological activities, lactoferrin is purified in commercial scale and even included in infant formula (Arla, 2011) since milk processing has little effect on lactoferrin structure (Sánchez *et al.*, 1992), and research has shown that MFGM fractions incorporated in food or supplements exert health-promoting properties (Lonnerdal *et al.*, 2006; Zavaleta *et al.*, 2006; Arla, 2011).

g) Cluster of Differentiation (CD36)

CD36 has been identified in the MFGM, playing a role in milk fat excretion. It is a glycoprotein with an apparent molecular weight of 77 kDa containing about 24% carbohydrate (Singh, 2006). Genomic data indicates that CD36 also plays a role in fatty acid (FA) synthesis by importing FA from blood into milk fat globules. Indeed, its expression level increases during lactation, and CD36 may be the most important regulator in endothelial long-chain fatty acid transport (Bionaz and Loor, 2008).

h) Fatty acid-binding protein (FABP)

FAB proteins are well known for their role in fatty acid transport and lipid metabolism; however, recent studies have linked FAB proteins with cellular proliferation and differentiation, and cell growth inhibition including breast cancer cells (Spitsberg and Gorewit, 2002; Jiménez-Flores and Higuera-Ciapara, 2009). Studies by Spitsberg *et al.*, (1995) indicate that FABP associate with CD36, a protein involved in fatty acid import (Bionaz and Loor, 2008), and are under hormonal regulation as shown by a correlation between level of FABP phosphorylation within the MFGM and the level of milk production (Spitsberg *et al.*, 1995). Further research indicated that FABP associated with the cytoplasmic domain of CD36 (Spitsberg and Gorewit, 2002).

D. Health Benefits of the Milk Fat Globule Membrane

Progress in the knowledge of the composition and role of milk fat globule membrane components has led to the realization that some MFGM constituents possess biological properties beyond their nutritional significance. Spitsberg (2005), in his review, refers to the MFGM as a nutraceutical with nutritional and pharmaceutical potential. In fact, the MFGM has antimicrobial proteins, illness suppressors, micronutrient-binding proteins that bind compounds such as iron, zinc, copper, folate, and vitamin B1, and other components with anti-carcinogenic properties as seen in Table 5. Indeed, Wat *et al.* (2009) supplemented bovine PL to mice fed a high-fat diet, resulting in a reduction in liver weight, total liver lipid, and serum lipid levels, which might be of therapeutic benefit in humans with non-alcoholic fatty liver disease, especially obese and diabetic patients (Wat *et al.*, 2009).

1. *MFGM in Infant Development*

In a clinical study, Lonnerdal *et al.* (2006) complemented infant food with bovine MFGM and micronutrients to evaluate its effect on children's growth, nutrition, and morbidity. In a double-blind study with 6-11 month-old-infants (n=550), fortification of food was beneficial for the copper and vitamin B12 status among the subjects, which enhance growth and normal function of the brain and nervous system, respectively (Lonnerdal *et al.*, 2006). This fortifying complementary food was introduced in the market by Arla as LACPRODAN MFGM-10 (Arla Foods Ingredients, Denmark). It is rich in lactoferrin and phospholipids resembling human milk. The latter has more sphingomyelin and lactoferrin (95-130 mg/L and 1.7-7 g/L, respectively) as compared to cow's milk (25 mg/L and 20-200 mg/L, respectively) (Arla, 2011).

In 2008, another clinical study was launched by the Umeå University in Sweden to determine the effects of MFGM-enriched formula with reduced energy and protein content on the growth and development of newborns (NCT00624689). Breast-fed and formula-fed infants usually differ in their growth pattern, risk for obesity and cardiovascular disease, neurological development and morbidity in infections (Timby, 2011). The study will conclude in 2016, but scientists expect to see minimal differences between breast-fed and formula-fed infants due to the bioactive compounds in the MFGM.

2. *Anticancer Properties of the MFGM*

Many scientists have proposed the use of MFGM as a food supplement due to its effects on cancer cell growth (Spitsberg, 2005; Jiménez-Flores and Higuera-Ciapara, 2009; Jiménez-Flores and Brisson, 2008). In fact, the fatty acid binding protein (FABP)

has been found to inhibit the growth of some breast cancer cell lines *in vitro* at very low concentrations (Spitsberg *et al.*, 1995; Spitsberg and Gorewit, 2002). The breast-ovarian cancer susceptibility (BRCA1 and BRCA2) proteins, which regulate lipid synthesis via an interaction with acetyl-CoA carboxylase (Moreau *et al.*, 2006b), have also been identified in the MFGM (Spitsberg and Gorewit, 1999; Vissac *et al.*, 2002). These proteins inhibit the growth of various cell types and influence cellular proliferation and differentiation. Mutations on these genes may be responsible for a significant number of breast and ovarian cancers, and it is speculated that BRCA1 exerts its tumor suppressor function through a lipogenic mechanism (Moreau *et al.*, 2006b). According to Spitsberg (2005), a MFGM-supplement will work by the consumption of MFGM material and subsequent release of peptides that can be absorbed in the digestive tract. The absorbed peptides can then exert their inhibitory action on cells undergoing carcinogenic transformation.

In addition, several lipids found in the MFGM can act as intracellular signaling molecules. Sphingomyelin has been shown to possess anti-cancer properties (Table 3). Its metabolites, sphingosine and ceramide, mediate numerous imperative cell functions including regulating cell growth, development and differentiation. Studies have shown sphingomyelin to be effective in suppressing colon tumors and increasing the efficacy of chemotherapy (Lemonnier *et al.*, 2003; Modrak *et al.*, 2002). Recently, it was shown that an ingredient rich in bovine PL from the MFGM prevented intestinal cancer in rats (Snow *et al.*, 2010).

3. *Antimicrobial and Antiviral Properties of the MFGM*

Milk and colostrum contain several antimicrobial factors, especially low-abundance proteins in colostrum, which are transferred from the mother to the neonate and

contribute to the protection against infectious diseases (Yamada *et al.*, 2002; Affolter *et al.*, 2010). For many species, the milk-derived antimicrobial system is crucial for survival of the neonate in early lactation. The components of the MFGM are involved with anti-infection or anti-adhesion processes, and hence with protection of the neonate from various viral and bacterial infections (Lopez *et al.*, 2008). Currently there is evidence that the protective effect of milk on viral infections is a complex mechanism involving MFGM components, which include glycosylated proteins (Wilson *et al.*, 2008). Zavaleta *et al.* (2006) tested the antimicrobial and antiviral properties of bovine MFGM on the incidence and duration of diarrhea in infants. In a double-blind study with 6-11 month old infants, MFGM fortification reduced diarrhea incidence and prevalence.

The antimicrobial and antiviral activities of proteins and lipids are currently receiving increasing interest, especially in the context of the preparation of infant formula and medical food. A review by Pan *et al.* (2006) summarized the current knowledge of the protein lactoferrin, most comprehensively studied for its beneficial immunological, antimicrobial and anticarcinogenic properties. This review examined the potential mechanism of lactoferrin as a powerful inhibitor of viral infection. Bovine lactoferrin was found to be more effective against viral infections than human lactoferrin. Isaacs *et al.* (1995) conducted an *in vitro* study in which fatty acids and monoglycerides were added to infant formula. They found that fatty acids and monoglycerides with chain lengths varying from 8 to 12 carbons were more antiviral and antibacterial than long chain monoglycerides (Isaacs *et al.*, 1995). On the other hand, Sánchez-Juanes *et al.* (2009) failed to observe binding between individual PL and *E. coli* in in-vitro studies; therefore, they discard the idea that PLs may block the adhesion of pathogen to the gastrointestinal tract.

E. Biochemical Properties of the MFGM in Food Products

1. Emulsifying and stabilizing properties of MFGM

MFGM is the native emulsifier in milk. The food industry recognizes the amphiphilic nature of the components in MFGM, phospholipids and glycoproteins, all of which are found in buttermilk powder and are excellent emulsifying agents (Kanno *et al.*, 1991; Corredig and Dalgleish, 1997a; Roesch *et al.*, 2004). The water distribution properties of the MFGM (either purified or in dairy ingredients) aid in thickening formulations and foaming of processed foods. However, the liability is lipid oxidation, causing the powder to go rancid in approximately six months. The major advantages of using buttermilk solids in functional food systems pertain to their significant antioxidant activity and buffering capacity (Wong and Kitts, 2003) and to their high phospholipid content, and their suppressive effect on certain pathogens (Jiménez-Flores and Higuera-Ciapara, 2009; Evers, 2004a).

MFGM components have also been used to stabilize emulsions and enhance the absorption of fat-soluble nutrients such as vitamin D₃ and vitamin A. It has been speculated that MFGM-stabilized microdispersions interact with bile salts and enzymes. Indeed, Bezelgues *et al.* (2009) utilized micelles (monolayers) stabilized with MFGM components to evaluate the capacity of liposoluble compounds to be incorporated into mixed micelles during digestion. Emulsions stabilized with MFGM had a 2-fold accumulation of tocopherol and lycopene when compared to emulsions stabilized with milk proteins, suggesting a nutritional application as absorption promoters of liposoluble nutrients in low-fat food products. Many researchers are starting to see that the value of buttermilk extends beyond emulsification to include the benefits of the MFGM components.

2. *Potential delivery systems derived from MFGM*

The rich content of phospholipids (PL) in the MFGM, and their unique chemical composition, facilitates the use of its constituents in the formation of liposomes, which are used in the pharmaceutical and cosmetic industries (Singh, 2006; Thompson and Singh, 2006). These are vesicles formed through the self-assembly of amphiphilic molecules such as PL, creating bilayers with an aqueous core. During their formation, hydrophobic molecules can be incorporated into the bilayer while hydrophilic molecules comprise the core (Singh, 2006). In the food industry, liposomes can be utilized to protect sensitive ingredients or to increase the efficacy of additives (Thompson and Singh, 2006); however, the popular methodologies to make liposomes are not feasible on a large scale due to kinetics and the use of detergents and solvents. Furthermore, the current PL sources, soy and egg, have high purification costs and a different composition to those PL from milk. Thus utilization of low-cost sources such as buttermilk or butter serum could alleviate the restrictions of liposome production. Thompson and Singh (2006) have successfully used microfluidization, which uses high pressure to force fluid into microchannels to overcome the limitations of other techniques (Thompson and Singh, 2006). This method can generate a large volume of liposomes in a reproducible manner without promoting oxidation, and the inclusion of MFGM phospholipids increases liposome dispersion and stability (Thompson and Singh, 2006).

3. *Application of MFGM Components in Food Systems*

MFGM components have been incorporated into food systems mainly as buttermilk (Vanderghem *et al.*, 2010). The use of condensed sweet cream buttermilk at low levels of supplementation improved the yield of pizza cheese without affecting compositional,

rheological or sensory properties (Govindasamy-Lucey *et al.*, 2006). Buttermilk, originating from raw or pasteurized cream was also integrated into casein matrixes to simulate cheese, resulting in increased yield due to the water-holding capacity of phospholipids (Morin *et al.*, 2008). Fedotova *et al.* (2008) fortified butter with MFGM phospholipids, which stabilized crystal morphology; however, at elevated levels their addition caused a coalescence of the aqueous phase, creating water pockets that may affect sensory properties. A subsequent study by the same group showed that addition of PL increases butter hardness and reduces the tendency to oil-off at elevated temperatures. The optimal butter product contained a blend of 60-80% globule fat (Fedotova and Lencki, 2010). Recently, Vanderghem *et al.* (2010) has summarized the effect of the addition of buttermilk on the physico-chemical and technofunctional properties of food products.

Another dairy product of interest is the milk produced during the first week of lactation, known as colostrum, which is free of caseins and rich in immunoglobulins and MFGM proteins (Affolter *et al.*, 2010). One of the main proteins present is lactoferrin, which is correlated with beneficial immunological, antimicrobial, and anticarcinogenic properties. Some current nutraceutical formulations utilize lactoferrin for the treatment of various ailments, including cardiovascular disease (Mao *et al.*, 2007). Methods have been developed for the processing of colostrum to diminish the microbial load and obtain a stable and available product, which may be added to a variety of foods (Scammell, 2001). In addition, milk oligosaccharides from human milk are linked to intestinal protection from pathogens (Wilson *et al.*, 2008), and their potential use in infant formula is being considered, especially for premature infants who are susceptible to necrotizing enterocolitis (Kemsley, 2008).

F. Sources of the Milk Fat Globule Membrane: a Novel Dairy Product

The industrial and potential health benefit properties of the MFGM components have led to its isolation and concentration from dairy products. The commercial source is sweet buttermilk, which is the aqueous phase resulting from butter churning that contains proteins, lactose, MFGM, and other minor constituents (Sodini *et al.*, 2006; Rombaut *et al.*, 2006a). Compared to skim milk and whole milk, buttermilk has seven times more phospholipids on a dry basis (0.28 g per 100g versus 2.03 g per 100 g, respectively) (Sodini *et al.*, 2006). Despite its high content of MFGM components and low cost, buttermilk has very limited commercial value (Jiménez-Flores and Brisson, 2008; Jiménez-Flores and Higuera-Ciapara, 2009; Singh, 2006). There are also other sources of buttermilk such as cultured (sour) and whey buttermilk, derived from churning of cultured cream and whey cream; however, they do not have high demand in the industry. Sodini *et al.* (2006) analyzed buttermilk's functional properties including protein solubility, viscosity, emulsification, and foaming and compared them to skim milk and whey. The fat content was higher in buttermilk, but the presence of MFGM components imparted greater emulsification and lower foaming capacity to sweet, sour, and especially whey buttermilk as compared to skim milk and whey. The functional properties of the buttermilk were pH-dependent with the exception of whey buttermilk, whose functionality makes it ideal for low pH foods (Sodini *et al.*, 2006).

Table 6. Phospholipid and protein composition in dairy products (adapted from Dewettinck *et al.*, 2008; Fong *et al.*, 2007; Jiménez-Flores, 2009; Morin *et al.*, 2006).

Product	Phospholipids (g/100g)		Protein Percentage [‡] in	
	Wet basis	Dry basis	Product	Dry
Raw milk	0.035	0.28	3.3	-
Skim milk	0.02	0.28	3.41	7.55
Cream	0.19	0.40	3.16	-
Isolated MFGM	0.65	6.5*	1.8	60*

Butter	0.19	0.23	0.85	-
Sweet Buttermilk	0.16	2.03	3.31	32.95*
Butterserum	1.25	11.54	-	-
Fresh acid buttermilk ^a	0.31	1.86	3.31	-
Acid buttermilk whey	0.10	1.84	0.99	84.7*
Whey buttermilk powder (WBP) *	-	14.81*	-	47.39*
SPE-WBP* ^b		60.59*		72.69*
Cheddar cheese	0.15	0.25	-	24.89
Cheddar cheese whey	0.02	0.26	3.49	35.2

[‡] Average from several sources and laboratory data.

*Numbers from corresponding author's laboratory data

^a Sour buttermilk

^b Solid phase extracted (SPE) whey buttermilk powder (WBP)

Besides sweet buttermilk, other sources such as sour and whey buttermilk have been characterized as denoted in Table 6. Among them, butter serum offers the highest concentration of phospholipids on a dry basis and has promise to become a potential source of MFGM (Rombaut *et al.*, 2006a). However, in any of these sources, other milk proteins 'dilute' the concentration of MFGM components. Thus, much research has been undertaken to adapt filtration techniques such as microfiltration and ultrafiltration to concentrate or purify MFGM without the use of chemical solvents. At the same time, the effects of homogenization and heat treatment on the native structure of the MFGM and the potential implications these may impart on product functionality and nutrition need to be evaluated.

Another source of MFGM is butter serum, a by-product of anhydrous milk fat production. It can be made by either separation of butter upon melting and centrifugation, or homogenization of 75% cream until phase inversion is induced, creating butter serum (Rombaut *et al.*, 2006b). The phospholipid content in this fraction is five times higher than that of buttermilk (11.54g per 100g on dry basis, see Table 1). Two more MFGM-enriched milk fractions are whey protein concentrate (WPC) and buttermilk protein concentrate (BMP), which have been characterized in terms of their protein composition (Affolter *et al.*, 2010). Affolter *et al.* (2009) found 244 proteins in

WPC and 133 in BMP, from which 28 in these two fractions can be accounted for in the research performed by Reinhardt *et al.* (2006). Nevertheless, the main problem with MFGM purification is the presence of caseins and whey proteins, which has triggered the quest for novel purification techniques involving membranes.

G. Isolation and Purification of the MFGM

1. Laboratory Production of MFGM

The yield and composition of the MFGM, as mentioned previously, depends on the technique utilized for its isolation from cream, buttermilk, or whole milk. Loosely bound proteins and especially the amount of skim milk proteins, varies in the final MFGM fraction (Singh, 2006; Keenan and Mather, 2006). Also, the concentration of triacylglycerols differs due to cross-contamination from the globule core (Keenan and Mather, 2006; Mather, 2000). On micro- and macro-scales, MFGM proteins are purified using a common four-step procedure that involves cream separation, cream wash, globule disruption, and membrane collection (Mather, 2000; Dewettinck *et al.*, 2008).

The fat globules are first isolated out of the milk by centrifugation with a physiological buffer layer that also washes the fat globules, eliminating the second step and increasing yield of MFGM material (Patton and Huston, 1986). Alternatively after centrifugation, the globules can be washed two or three times with buffers (Anderson and Brooker, 1974; Basch *et al.*, 1985; Keenan and Mather, 2006); nevertheless, some membrane disruption and loss of at least 16% of the membrane proteins occurs (Anderson and Brooker, 1974). In addition, cream washing affects the densities of the lipoprotein complexes (Houlihan *et al.*, 1992). To minimize MFGM loss, a milk salt buffer or sucrose is usually added to increase the density of serum (Singh, 2006). Then,

the globules are disrupted by either churning (agitation), cycles of freezing and thawing, treatment with detergents, or by suspension in polar and aprotic solvents (Keenan and Mather, 2006). This step may lead to loss of proteins such as XOR or BTN due to their dispersion in the salt/detergent phase. Once the membrane is disrupted, the proteins can be collected by ultra-centrifugation, protein precipitation at low pH, or induction of aggregation with ammonium sulfate followed by centrifugation (Singh, 2006; Keenan and Mather, 2006). The pathways used to isolate MFGM in a laboratory setting or in a pilot plant are detailed and compared in Figure 8.

Figure 8. Milk fat globule isolation in a laboratory and pilot plant setting. MFGM proteins can be purified using a four-step procedure: Step 1. cream (fat globule) isolation; Step 2. washing of globules; step 3. Disruption of globules, and step 4. Membrane collection (Dewettinck *et al.*, 2008).

2. *Commercial Production of MFGM*

On a commercial-scale MFGM isolation is more suitable from buttermilk, whey serum, or acid serum. Acid and whey serums have 0.5 to 1.03% (in dry-matter) residual fat that interferes with whey protein concentrate production (Glass and Hedrick, 1977); therefore, side purification techniques have been developed to obtain MFGM material and facilitate concentration of whey proteins (Rombaut and Dewettinck, 2007). Currently, microfiltration and ultrafiltration are the most widely used techniques to purify or concentrate milk fat.

a) Microfiltration

Microfiltration features a pressure-driven membrane filtration, with pressure less than one bar and membrane pore size between 10^{-1} - 10^1 μm , which is commonly used for the removal of bacteria and separation of macro molecules (Corredig *et al.*, 2003; Morin *et al.*, 2006). In the dairy industry, this is employed to remove lipids from whey for production of whey protein concentrate (WPC). With regard to MFGM purification, casein makes microfiltration difficult even after the use of casein micelle dissociating agents such as citrate. Corredig and Dalgleish (2003) used sodium citrate to dissociate casein micelles resulting in a MFGM concentrate of approximately 60% (w/w) protein and 35% (w/w) lipid. However, citrate dissociation requires the use of diafiltration to wash out minor constituents, and results in high permeate losses (Rombaut *et al.*, 2006b). Morin *et al.*, (2004) demonstrated that microfiltration alone cannot achieve optimal separation of lipids and protein for the production of novel ingredients. The pore size of the membrane and temperature also influenced the final product as well as the starting material (Morin *et al.*, 2004).

Despite permeate loss, citrate treatment and microfiltration resulted in a MFGM isolate that showed better creaming stability than whole buttermilk concentrate. Such effects were attributed to the compositional difference between these two ingredients (Roesch *et al.*, 2004). Morin *et al.* (2006) fractionated buttermilk and whey buttermilk using microfiltration, resulting in two-fold and 50% increases in phospholipid concentration, respectively. Another study by the same group used washed cream to remove some of the caseins and whey proteins that disturb milk fractionation. A two-fold increase in the permeation flux was observed as well as a 66% increase in the phospholipid content compared to regular cream buttermilk (Morin *et al.*, 2007a). Rombaut *et al.* (2006b) suggested coagulation of casein by acid or rennet addition, followed by MFGM purification using ultrafiltration or microfiltration techniques. Michalski *et al.* (2006) patented a microfiltration method to isolate fat globules with different diameters. This technique results in large globules (6 μm) that can be churned easily for butter making (Fauquant *et al.*, 2007). Finding effective ways to utilize the by-products of microfiltration, such as the citrate-containing permeate and the precipitated caseins, should be an active area of research to find relevant applications in foods (Singh, 2006).

b) Ultrafiltration

Ultrafiltration is utilized to concentrate large and macromolecules such as the proteins in milk and whey. It uses pressure of 1-10 bar and membrane pore sizes between 10^{-2} - 10^{-1} μm . Despite its functionality, this technique has not been fully used to purify MFGM components. Early attempts in the utilization of ultrafiltration resulted in the production of buttermilk concentrate with a high protein content (12.87%) and excellent rheological as well as emulsifying properties (Real del Sol and Corrales, 1991). Our group is

currently exploring ultrafiltration to obtain whey buttermilk powder in conjunction with supercritical fluid extraction (SFE). The use of SFE as a solvent to extract non-polar lipids has revolutionized the solvent-extraction methods because it does not leave solvent residues in the final product. This technique concentrates the phospholipids but does not separate out proteins from them (Singh, 2006). Spence *et al.* (2009) utilized microfiltration and SFE to remove triglycerides and concentrate the phospholipid content in buttermilk powder, generating powders with a 70% decrease in total lipids (Spence *et al.*, 2009). Rezende-Costa *et al.* (2010) produced a whey buttermilk powder enriched in phospholipids utilizing ultrafiltration/diafiltration and SFE. The total lipid content was reduced by 57%, and the protein was increased up to 53% (Rezende-Costa *et al.*, 2010). Such techniques can concentrate the phospholipids and MFGM protein content from cheese or butter-by-products, and the resulting fractions can later be incorporated into food matrixes or pharmaceuticals.

c) Purification through Aggregation

MFGM fragments can also be purified through precipitation from cheese whey. Attebery (1968) patented the use calcium in conjunction with pH adjustment and moderate heat treatment to clarify whey (Attebery, 1968); such method was later applied to all types of whey (Fauquant *et al.*, 1985; Maubois *et al.*, 1987). Rombaut *et al.* (2007) optimized these settings to purify MFGM fragments from acid buttermilk cheese whey. Their findings indicate that MFGM recovery is highly dependent on pH and less dependent on temperature and calcium addition. An optimal treatment at 55 °C, pH 7.7, and 0.205 g of calcium/L of whey resulted in a 91% recovery of whey polar lipids and minimum whey protein association in the aggregates (Rombaut and Dewettinck, 2007).

With similar techniques, MFGM material can be isolated from whey protein concentrate production and be used in food products.

H. Physical and Chemical Effects of Processing on Fat Globules and MFGM

Drinking milk undergoes a series of processing steps that physically and chemically affect the composition and characteristics of the MFGM. Other milk-derived products such as buttermilk and butterserum also endure similar conditions that result in MFGM damage; therefore, a lot of research has been done to characterize such changes (Table 4), but without a general definition of the MFGM, measuring these alterations in a constant manner may be difficult. In his review, Evers (2004) was the first one to clearly define the MFGM in order to provide methodologies to measure the damage to fat globules and the membrane (Evers, 2004b). What follows is a summary of the main milk handling practices that affect this membrane and their effects.

1. Temperature Effect

a) Cool Temperatures

Temperature treatments are aimed at preventing spoilage, preserving foods, and increasing the quality of products by either inhibiting microbial growth, killing microorganisms, or inactivating enzymes (Walstra *et al.*, 2006; Huppertz and Kelly, 2006). The intensity of the treatment depends on the combination of temperature and duration to establish specific product or ingredient properties. In the dairy industry, milk goes through cycles of cooling and heating depending on the final product, and these conditions have an effect on the fat globules, especially the proteins and lipids of the MFGM.

At low temperatures, milk globules undergo mainly physical changes. A cream layer tends to form, which is minimized by regular stirring and by aeration. The latter, in conjunction with temperature fluctuations, contributes to cycles of fat crystallization and melting, which leads to fat globule disruption and clumping. During cold storage, milk fat globules tend to rise in large clusters at a higher rate than individual globules due to the presence of immunoglobulin IgM (Huppertz and Kelly, 2006). Such phenomenon is known as cold agglutination and requires a heat-labile component, IgM, which functions as a cold-agglutinin, and a homogenization-labile skim milk membrane (SMM), which consists of lipoprotein particles present in the aqueous phase of milk. Fat globules agglutinate by interacting with IgM alone, but extensive clustering is seen in the presence of SMM, which acts a cross-linking agent and interacts with IgM only (Huppertz and Kelly, 2006).

In regards to globule size, batch pasteurization or indirect ultra-high temperature (UHT) heating of milk at 90-150 °C does not influence the volume surface-weighted mean diameter d_{vs} . In contrast, direct UHT decreases d_{vs} as the temperature increases (Huppertz and Kelly, 2006). Coalescence of fat globules is prevented by the presence of the polar head of phospholipids in the MFGM. Removal of such entities results in oiling-off (Huppertz and Kelly, 2006). Indirect UHT treatment can cause aggregation of fat globules.

Furthermore, when fresh milk is cooled, the association of proteins to the MFGM changes (Hohe *et al.*, 1985; Steffensen *et al.*, 2004). The casein proteins, especially β -casein, dissolve from the casein micelles and go into the serum phase and may be incorporated onto the fat globules (Walstra, 1969). Cooling milk also induces the release of MFGM phospholipids into the serum phase (Baumrucker and Keenan, 1973) as well as

the liberation of xanthine oxidase from the MFGM and the migration of copper from the fat globules to the milk plasma (Huppertz and Kelly, 2006). Cool temperatures induces the binding to lipoprotein lipase (LPL) and increases the activity of this one, especially after three to five hours after milking when heparin is present (Sundheim and Bengtsson-Olivecrona, 1985). No further association of LPL with the MFGM is observed beyond this point (Dickow *et al.*, 2011). The activity of xanthine oxidase also increases in cooled milk (Steffensen *et al.*, 2004).

b) Thermal Temperatures

Milk pasteurization lowers the rate of deterioration caused by microbial and enzymatic action. During heating, the loss of oxygen decreases the rate of oxidation reactions, and the amount of inorganic phosphate increases by hydrolysis of phospholipid esters, especially those of casein micelles, and phospholipids (Walstra *et al.*, 2006; Huppertz and Kelly, 2006). Acylglycerols are also hydrolyzed and interesterified, and lactones and methyl ketones are formed from fat (Walstra *et al.*, 2006).

With respect to proteins, free sulfhydryl groups are formed as shown in Table 7, reactions 2 and 4. Lactose and proteins also undergo Maillard reactions, as denoted by reaction 8, and provide a brownish color to milk. Most of the serum proteins are denatured and rendered insoluble, especially β -lactoglobulin, which becomes covalently bound to kappa casein and to some proteins of the fat globule membrane (Walstra and Jenness, 1984).

Table 7. Biochemical reactions of side chain groups of amino acid residues linked in the peptide chain (I) of proteins at high temperature (Adapted from Walstra *et al.* 2006).

Rxn	Reactant	Direction	Product
1	$\text{I-CH}_2\text{CONH}_2 + \text{H}_2\text{O}$ Asparagine ⁺	→	$\text{I-CH}_2\text{—COO}^- + \text{NH}_4^+$ Aspartic acid
2	$\text{I-CH}_2\text{—SH} + \text{OH}^-$ Cysteine	↔	$\text{I-CH}_2\text{—S}^- + \text{H}_2\text{O}$ Charged sulhydryl group
3	$\text{I-CH}_2\text{—S}^- + \text{S-CH}_2\text{—I}$ Cysteine	Oxidation-> <-Reduction	$\text{I-CH}_2\text{—S—S-CH}_2\text{—I} + 2e$ Cystine
4	A $\text{I-CH}_2\text{—S—S-(CH}_2)_n\text{—I}$ B $\text{I-CH}_2\text{—S—}$ Sulphydryl exchange	↔	A $\text{I-CH}_2\text{—S—}$ B $\text{I-CH}_2\text{—S—S-CH}_2\text{—I}$
5	$\text{I-CH}_2\text{—S}^-$ Cysteine	→	$\text{I=CH}_2 + \text{HS-}$ Dehydroalanine
6	$\text{I=CH}_2 + \text{HS-CH}_2\text{—I}$ Dehydroalanine Cysteine	→	$\text{I-CH}_2\text{—S—CH}_2\text{—I}$ Lanthionine
7	$\text{I-(CH}_2)_4\text{—NH}_3^+ + \text{H}_2\text{C=I} + \text{OH}^-$ Lysoalanine ⁺⁺	→	$\text{I-(CH}_2)_4\text{—NH—CH}_2\text{—I} + \text{H}_2\text{O}$ Lysoalanine
8	$\text{I-CH}_2\text{—COOH} + \text{H}_2\text{N-(CH}_2)_4\text{—I}$ Aspartic Acid Lysine	→	$\text{I-CH}_2\text{—CO—NH-(CH}_2)_4\text{—I} + \text{H}_2\text{O}$ Isopeptide
8	$\text{I-(CH}_2)_4\text{—NH}_2 + \text{C}_6\text{H}_{12}\text{O}_6$ Lysine Glucose	→	$\text{I-(CH}_2)_4\text{—NH—C}_6\text{H}_{11}\text{O}_5 + \text{H}_2\text{O}$ Amadori product

+ Rxn occurs with glutamine residues
++ Rxn occurs with histidine residues

The composition of the MFGM is further altered by thermal treatment and homogenization due to protein denaturation and interactions with serum proteins (Lee and Sherbon, 2002). Upon heat treatment, a layer of whey protein, especially β -lactoglobulin and α -lactalbumin, deposits on the MFGM, possibly through intermolecular disulfide bonding (Kim and Jiménez-Flores, 1995; Michalski and Januel, 2006a; Singh, 2006). Bermudez-Aguirre *et al.* studied the surface of fat globules microscopically in raw and thermosonificated milk. According to their findings, heat and ultrasonic waves are responsible for disintegrating MFGM and the release of triacylglycerols. Fat globules with a rougher surface (Figure 9) are observed in heat-treated samples due to MFGM disintegration and interaction with casein micelles. Also, smaller fat globules are seen after thermosonification (Bermúdez-Aguirre *et al.*, 2008).

Figure 9. Fat globule in raw milk (left, 7000x) with no change on surface and thermally treated globule (right; 63 °C, 30 min, 6000x) exhibiting deposits of casein proteins. Images captured with scanning electron microscopy (Adapted from Bermudez-Aguirre, D. *et al.* 2008)

If milk is heated after homogenization, then β - and α -lactoglobulins absorb to the casein micelles already incorporated onto the membrane (Ye *et al.*, 2004b), making the outside membrane more resistant to coalescence (Bermúdez-Aguirre *et al.*, 2008). The exact mechanism of protein interaction has not been elucidated; however, it has been speculated that it could be via sulfhydryl-disulfide interchange (reaction 4 in Table 7) or by displacement of the original MFGM material (Michalski and Januel, 2006a; Singh, 2006). The MFGM's association with β -lactoglobulin starts at 60-65 °C and increases with heating time until a plateau level of about 1.0 mg/g of fat is reached (Corredig and Dalgleish, 1996; Ye *et al.*, 2004b; Ye *et al.*, 2004c); this accounts for 1% of the β -lactoglobulin found in milk. There is no evidence on the specific MFGM proteins that associate with β -lactoglobulin (Singh, 2006).

Association of α -lactalbumin displays a similar mechanism but less protein is absorbed onto the membrane. Also, small amounts of κ -casein seem to interact with the

MFGM when milk is heated above 80 °C; such interaction may be bridged by the already absorbed β -lactoglobulin. According to Kim *et al.* (1995), higher temperature treatments (140 °C for 16 s) cause less casein adsorption, but studies in direct and indirect UHT determined adsorption of caseins and whey proteins, especially κ -casein and β -lactoglobulin in the MFGM (Hillbrick *et al.*, 1999). On the other hand, Dalglish and Bank believed that presence of casein on the surface of fat globules may originate from partial homogenization, and their data did not support the association of κ -casein onto the MFGM (Dalglish and Banks, 1991). Later studies by Ye *et al.* indicated that κ -casein facilitates the adsorption of casein micelles to fat globules through disulfide bonding between κ -casein and MFGM components in heated milk (65-95 °C, 10 min) (Ye *et al.*, 2004c). Other proteins affected during heating are PAS 6 and 7, which migrate into the serum phase and begin to aggregate at temperature above 80 °C (Houlihan *et al.*, 1992; Kim and Jiménez-Flores, 1995; Lee and Sherbon, 2002; Ye *et al.*, 2004c); however, in the absence of whey protein, PAS 7 is stable (Ye *et al.*, 2002). Ye *et al.* (2002) hypothesized that PAS 7 forms a complex with β -lactoglobulin to later migrate to serum phase.

Heating also decreases the triacylglycerol content of the MFGM and promotes migration of copper from plasma to fat globules. With regard to polar lipids, there is inconclusive evidence for a correlation between the concentration of PLs and heat treatment (Huppertz and Kelly, 2006). Morin *et al.* (2006) found that production of buttermilk from pasteurized cream results in buttermilk with higher lipid content. In addition, the total MFGM protein that can be isolated from cream depends on the heat treatment (Morin *et al.*, 2007a; Ye *et al.*, 2002). In fact, cream processed at 50 °C for 10

min results in approximately 50% MFGM protein loss and formation of aggregates via intermolecular disulfide bonds in the absence of serum proteins (Ye *et al.*, 2002). Gassi *et al.* (2008) also observed formation of fat globules aggregates in cream that underwent high heat treatment (70 °C, 2 hr followed by 30 s at 88 °C, and 94 °C for 80 s). Modifications in the MFGM proteins involve alterations in interfacial properties resulting in fat globule aggregation. Figure 10 represents a schematic representation of these changes in the MFGM and consequently in the fat globules upon heat treatment.

Figure 10. Changes in the MFGM and fat globules as a function of thermal treatment in creams. A) Schematic representation of native fat globules or at low heat, and B) after high heat treatment, which adsorbs aqueous phase proteins, especially whey proteins (Taken from Gassi *et al.*, 2008).

2. Homogenization

In the dairy industry, homogenization is performed to counteract creaming and provide stability toward partial coalescence during the product's shelf life. Coalescence is prevented by reducing the diameter of the fat globules due to the application of high

pressures (8-20 MPa) through a valve needle (Michalski *et al.*, 2002; Walstra and Jenness, 1984). Droplets of fat globules are deformed and possibly disrupted by shear stress, inertial forces, and cavitation, which cause particles to coalesce and have a bigger surface areas where adsorption of proteins occurs. Temperatures between 42 to 72 °C are applied during homogenization to increase efficiency; nevertheless, most processes are performed at 60°C (Michalski *et al.*, 2002). The type of homogenizer, the homogenizing pressure, and temperature are some of the main factors that affect globule size (Michalski *et al.*, 2002; Walstra *et al.*, 2006).

The main effect of homogenization is the disruption of casein micelles, displacement of MFGM components to the skim milk, and casein adsorption onto the newly formed interface of the fat globules as denoted on Figure 11 (Michalski and Januel, 2006a). The amount of adsorbed proteins increases with homogenization pressure; however, the protein composition of the MFGM is not affected by homogenization alone but also significantly by the heat treatment applied before homogenization (Cano-Ruiz and Richter, 1997). Furthermore, the phospholipid content appears to decrease in the new membrane (McPherson and Kitchen, 1983). Keenan *et al.* (1983) reported a loss in total lipids, polar lipids, and cholesterol decreased in homogenized milk samples compared to unprocessed samples, but the phospholipid distribution remained the same (Keenan *et al.*, 1983). Homogenization also seems to improve the digestibility of milk; however, premature babies have better digestion of human milk fat globules than the homogenized lipid droplets of infant formula (Favé *et al.*, 2004).

Homogenized milk appears to be responsible for allergic reactions. Michalski *et al.* (2006) explains that allergenic milk proteins absorb onto the new increased surface area, or perhaps homogenization exposes proteins that may trigger putative allergenic effects.

Xanthine oxidase (XO) is one of these proteins, and it has also been associated with atherosclerosis. Controversial findings indicate that XO liberated during homogenization favors the development of coronary diseases, but Spitsberg (2005) and Michalski (2006) argue that populations with high intake of buttermilk or cheese rich in MFGM are not associated with coronary mortality. Other authors claim that MFGM proteins provoke autoimmune and neurological diseases such as diabetes I and multiple sclerosis (Michalski and Januel, 2006a). Despite the controversy, there is a plethora of research highlighting the health effects of MFGM proteins.

→ Organization of an homogenized lipid droplet

→ General organization of homogenized milk

Figure 11. Effects of homogenization on milk fat globules. Native fat globules lack adsorption of casein micelles and whey protein. Upon homogenization, serum proteins and casein attached to the interphase of fat droplets (top) and MFGM fragments are desorbed (bottom) (Adapted from Michalski and Januel, 2006).

3. *Influence of Spray-drying*

Products are usually dried to increase their storage stability, reduce packaging requirements, and reduce shipping weights (Landstrom *et al.*, 2000). In the dairy industry, spray drying is the most common method used to concentrate milk solids, due to milk's seasonal variations, and to produce milk-derived powders, especially whole milk powder (WMP) and skim milk powder (SMP) as fresh milk substitutes (Schuck, 2002; Ye *et al.*, 2007; Singh and Ye, 2010). Milk must undergo heat treatments, homogenization, evaporation, and spray drying to produce powders. First, the liquid product is atomized at the top of the drying chamber and mixed with dry heated air (180-220 °C), which evaporates the moisture in the droplets. Atomization provides a large surface area but damages fat globules by shear, causing changes in the fat globule surface protein layer. Since thermal evaporation damages the MFGM, milk to be evaporated is usually homogenized to deposit casein on the fat globules and make the membrane stronger for spray-drying (Morin *et al.*, 2007b).

Rennie *et al.* (1999) correlated small particle size with stickiness and powder deposition in dairy powders. WMP had twice the powder cohesion than SMP as temperature increased from 30 to 65 °C due to the presence of fat. Ye *et al.* (2007) investigated the behavior of milk fat globules during spray drying of whole milk, and concluded that upon spray-drying, the average fat globule size (d_{32}) of powders was smaller than the initial products and formation of large globules (4-80 μm) occurred. In regard to phospholipids, spray-drying decreases the PL content in buttermilk powders, probably due to complex formations between PL and MFGM proteins from the heat treatment, rapid evaporation, and increased ionic strength created by spray drying (Morin *et al.*, 2007b). In conclusion, milk powder undergoes a lot of processing that causes

physical and chemical interactions among milk components, and such interactions determine the functional properties for powder reconstitution in water and consumers' acceptability (Schuck, 2002; Ye *et al.*, 2007; Singh and Ye, 2010).

I. Cold Pasteurization-UV Pulsed Light Treatment

As an effort to reduce the risk of foodborne illnesses associated with pathogens, especially *E. coli* O157:H7, *Salmonella*, and *Campylobacter*, the U.S. Department of Agriculture (USDA) approved the use of irradiation in food products in 1963. Currently, in the United States irradiation is applied to meat and poultry and includes the use of gamma rays, electron beams, and x-rays in government-approved irradiation facilities (USDA). More than 40 food products are irradiated in 37 countries including wheat and wheat powder, spices, fresh shell eggs, fresh produce, and recently apple juice in countries such as Canada (Health Canada).

For half a century, irradiation has been used to treat food products because no significant changes have been observed. In fact, the American Medical Association and the United Nations' World Health Organization endorsed the use of this technique due to its efficient antimicrobial effects (USDA). Data collected on apple juice concluded that the only degradation products that would occur from UV treatment of juice/cider are by-products that occur naturally from sunlight (Health Canada). With such findings, irradiation must find its way into the dairy industry to substitute for heat pasteurization of milk, of which in 2010, 193 billion pounds were produced in the United States totaling \$31.4 billion in cash receipts from marketing (Milk Production, Disposition, and Income 2010 Summary, USDA). It is expected that UV light treatment will minimize the number of spores in USA milk powder and increase its commercialization in other countries that currently reject products that do not meet their standards.

1. Bactericidal Effects of UV-light Treatment

UV-light has a broad range of antimicrobial effects including vegetative cells, especially with spore-formers and pathogenic bacteria, fungi, and yeast (Krishnamurthy *et al.*, 2004; Elmnasser *et al.*, 2007; Smith *et al.*, 2002; Anderson *et al.*, 2000). In addition, microorganisms do not develop resistance to UV treatments (Elmnasser *et al.*, 2007). Chang *et al.* (1985) investigated the effect of continuous UV-light sterilization on *E. coli*, *S. typhi*, *S. sonnei*, *S. faecalis*, and *S. aureus* and concluded that these microorganisms have different resistances to UV-light. All of them were inactivated with 7 mW-sec/cm² energy to get a 3-log₁₀ reduction with the exception of *S. faecalis* that required more energy (Chang *et al.*, 1985).

Smith *et al.* (2002) used pulsed UV treatment to inactivate *S. aureus* and concluded that a minimum dose of 12.6 J/cm² is needed to achieve a 2-log₁₀ reduction. In addition, they observed a decrease in spore-forming bacteria colonies. Krishnamurthy *et al.* (2004) also conducted inactivation of *S. aureus* with a pulsed UV-chamber, finding similar results but emphasizing that the volume and the distance between the UV source and the sample as well as energy intensity and time are important in opaque products such as milk. Differences on the microbial lethality between gram-negative and gram-positive bacteria were also investigated and attributed to the composition in bacterial cell wall and their protective and repair mechanisms. Gram (-) microorganisms such as *S. enteritidis* and *E. coli* are more susceptible to pulsed-UV light than gram (+) *B. cereus* (Anderson *et al.*, 2000).

2. Chemical Effects of UV-light Treatment

Extensive work has been done on the bactericidal effects of UV-light, but there is still little information available on the effects of light treatment on the conformational changes in milk proteins or the production of by-products that could be carcinogenic or affect consumer acceptance. Elmnasser *et al.* (2008) concluded that pulsed-light treatment does not contribute to conformational changes in milk proteins besides the aggregation of β -lactoglobulin, which results in the formation of dimers with molecular weight of 36 kDa due to disulfide bonding. Light treatment may change the polarity of microenvironments, making some amino acids such as tryptophan more exposed to hydrophilic areas and changing protein folding and aggregation as seen in fluorescence spectrophotometry of intrinsic tryptophan. Such alteration can be measured by fluorescence by exciting a sample at 290 nm and scanning between 305-450nm. In addition, a decrease in riboflavin concentration, a marker for oxidation that involves radical formation and consequent protein and lipid degradation, resulted in a decrease of the maximum emission wavelength; nevertheless, the authors concluded that there was an absence of oxidation reactions of proteins by lipids (Elmnasser *et al.*, 2008).

On the other hand, goat milk treated with an UV fluid processor resulted in lipid oxidation, as denoted by an increase in thiobarbituric acid (TBA) and acid degrees values (ADV); however, these changes were not enough to detect organoleptic differences in treated samples. Volatiles such as pentanal, hexanal, and heptanal were detectable in treated samples after 1.3 mJ/cm² energy (Matak *et al.*, 2007). Furthermore, temperature increments have been observed during prolonged UV treatment, and without the proper cooling system, the quality and nutritional profile of foods may be compromised (Elmnasser *et al.*, 2007).

J. Methodology to Monitor the Biological Activity of the MFGM

With the advances in the scientific and technological fields, there are new tools to monitor the biological activity of MFGM in foods, which can increase our knowledge of the membrane structure, especially before processing. Such findings will allow us to select methods to preserve the native structure of the MFGM and set standards for generating a product similar to what the neonate receives. Among the latest technologies, atomic force microscopy, confocal microscopy, and laser tweezers have captivated the attention of scientists due to their ease of use, non-invasive methodology, and plethora of results.

Atomic force microscopy (AFM) has similar resolution to that of electron microscopy (Evers, 2004b). It allows the visualization of monolayer domain formation, binding events, as well as membrane-membrane interactions, among other features (Jiménez-Flores and Brisson, 2008). It has been used to characterize the structure and properties of the MFGM. In fact, Gallier *et al.* (2010) monitored the physical behavior of phospholipid monolayers from various dairy products using atomic force microscopy and found that milk processing alters the phospholipid and fatty acid composition in the MFGM and consequently the physical behavior of PL monolayers.

Confocal laser scanning microscopy, lipophilic probes, and fluorescent lectin wheat germ agglutinin (WGA) were first utilized to investigate the organization of the MFGM in milks from animal species (Evers *et al.*, 2008). With the same confocal technique, Lopez *et al.* (2010b) revealed the heterogeneous organization of the MFGM, especially the lateral structure of polar lipids, glycolipids, and glycoproteins. The MFGM surface, especially the phospholipid monolayer, has been characterized by microscopic techniques at our facility (Gallier *et al.*, 2010a).

The use of laser tweezers, also known as optical tweezers, has been applied in the MFGM field. This technique arises from the principle that small particles/objects can be imprisoned in the center of a strongly focused laser beam, experiencing a restoring force if they try to leave the high intensity volume. The optical tweezer system has been used for direct manipulation of a variety of micrometer-sized objects and for force measurement in the pico-Newton (pN) region (Jiménez-Flores and Brisson, 2008). In fact, Argov *et al.* (2008) utilized laser tweezers Raman spectroscopy to characterize submicron-sized particles in human milk. They observed a significant difference in the triacylglycerol composition of human fat globules, depending on their diameter, especially in nanoparticles (less than 1 μm), implying that these components have other functions besides fat delivery. Such findings open the door to nanoscience, which can help us reveal the association between food structure and functionality. Furthermore, our research group has used laser tweezers to objectively measure binding force between lactic acid bacteria and the MFGM (Brisson *et al.*, 2010).

K. Principles of Analysis Methods Utilized

1. Cold Pasteurization

Cold pasteurization is a nonthermal method that utilizes broadspectrum electromagnetic energy to inactivate microorganisms. Its efficiency depends on the intensity ($\text{J} \cdot \text{cm}^{-2}$) and the number of pulses delivered, which inhibits bacterial replication by damaging DNA as pyrimidine dimers are formed (Krishnamurthy *et al.*, 2004; Oms-Oliu *et al.*, 2008). Microbial cells can also be disintegrated by the UV photothermal effects, which might cause instantaneous overheating of the cellular constituents and rupture of the cell membrane (Oms-Oliu *et al.*, 2008).

UV-light sterilization can currently be accomplished using two models: a continuous UV-light or pulsed UV-light treatment. According to research, pulsed UV-light seems to be more efficient in deactivating microorganisms due to the intensity of the energy delivered in short-duration pulses. Continuous UV-light has poor penetration depth and low emission power as compared to pulsed UV-light. The emission power achieved by the continuous system ranges from 100 to 1,000 W whereas the pulsed system may have a peak of 35 MW (Oms-Oliu *et al.*, 2008). The Food and Drug Administration (FDA) approved the use of the latter technology in 1996, and since then, different systems have come to the market. The SteriPulse-XL system generates UV/Visible light with a xenon flashlamp that delivers up to 505 Joules per pulse (1.27 Joules/cm^2) at a rate of 3 pulses/s, providing short pulses (360 μs width) that prevent excessive heat buildup.

2. *Milk Analysis*

a) *Analysis of Milk Lipids*

The analytical procedure to determine lipid compounds involves lipid extraction from a protein matrix, analytical separation, identification, and quantification (Carrasco-Pancorbo *et al.*, 2009). In the dairy industry, fat extraction and quantification is mainly performed by the standard Mojonnier method (Association of Official Analytical Chemists, AOAC, 989.05). However, this procedure has its limitations due to the use of ammonium hydroxide to denature proteins in conjunction with heat to evaporate solvents, which can lead to oxidation and hydrolysis of phospholipids (Rombaut *et al.*, 2006c). In addition, the Mojonnier method may not be efficient in extracting all the phospholipids (Morin *et al.*, 2007b). Cold methods such as the Folch method are preferred to purify fat from tissue, utilizing chloroform/methanol to extract lipids in low-fat products and saline

aqueous solution to wash out non-lipid components (Folch *et al.*, 1957). Its efficiency in extracting lipids is higher, but its reproducibility is lower than the Mojonnier method (Avalli and Contarini, 2005). In addition, the solvents used in the Folch method are highly toxic, and to extract the equivalent of fat in the Mojonnier method, larger volumes of these solvents will be needed using Folch's protocol (Min and Ellefson, 2010).

Phospholipids are further concentrated for analysis through differences in their polarities via solid phase extraction (SPE), which fractionates analytes according to their affinity between a solid and a mobile phase (Descalzo *et al.*, 2003). Bitman *et al.* (1984) utilized SPE columns to separate neutral lipids from polar lipids using hexane/ether and chloroform/methanol/water, respectively (Bitman *et al.*, 1984). Vaghela and Kilara (1995) presented a protocol to purify phospholipids using methanol preceded by the use of chloroform/isopropanol and acetic acid in ether to eliminate more neutral lipids and free fatty acids, but aminopropyl SPE columns were utilized instead (Vaghela and Kilara, 1995). In 2005, Avalli and Contarini introduced a protocol similar to Bitman, but the ratio of hexane/ether differed to extract more neutral lipids from the final product (Avalli and Contarini, 2005). In a comparison study, the previous three methods were used to extract PLs from buttermilk. Bitman samples were enriched in phosphatidylcholine, sphingomyelin, and saturated lipids while Avalli and Vaghela samples eluted more phosphatidylethanolamine and phosphatidylinositol than Bitman's samples. Such difference in the phospholipid profile played a role in the type of lipid monolayer films observed under fluorescence microscopy (Nelson and Rios, 2007).

Phospholipid analyses have traditionally been performed in thin layer chromatography (TLC) or gas chromatography (GC) (Sprecher *et al.*, 1965; Descalzo *et al.*, 2003); however, the former is tedious and laborious (Fagan and Wijesundera, 2004),

and the latter requires extensive derivatization. Thus, high performance liquid chromatography (HPLC) has become the method of choice for separation and quantitative determination of the different classes of phospholipids (Rombaut *et al.*, 2005; Rombaut *et al.*, 2006c; Vaghela and Kilara, 1995). This technique has been employed in direct mode or with on-line pre-concentration using a cation-anion exchange or silica guard column to separate the major PL (Fagan and Wijesundera, 2004; Carrasco-Pancorbo *et al.*, 2009).

HPLC is most commonly coupled with UV or evaporative light scattering detector (ELSD) for PL detection (Rombaut *et al.*, 2005). While UV is limited to compounds that possess a chromophore, ELSD is more efficient for detecting non-volatile compounds such as phospholipids because it is a universal mass detector. With the aid of a nebulizing gas, the solvent evaporates, and the analyte atomize, scattering a laser beam at the end of a heating tube to a sensitive photomultiplier (Carrasco-Pancorbo *et al.*, 2009). A new detector, charged aerosol detector (CAD), offers the same sensitivity as ELSD without the trouble of instrument parameter optimization such as temperature adjustments (Bailey *et al.*; Moreau, 2006a). It has a high-voltage corona needle to charge molecules, so the response is independent of the analyte's structure (Bailey *et al.*; Crafts *et al.*). In addition, CAD has a nearly linear mass-to-peak area relationship for many types of lipids (Moreau, 2006a). A comprehensive review on analytical techniques for lipid is presented by Carrasco-Pancorbo *et al.* (2009).

b) Fourier Transform Infrared (FTIR) Milk Analysis

Conventional dairy analyses generate high waste volumes, have expensive labor costs, and require a lot of time to obtain results for laboratories or production environments. In order to increase the efficiency of these facilities, infrared milk

analyzers (IRMA) emerged to reduce cost (Biggs, 1967; Van de Voort *et al.*, 1987). Later developments introduced fourier transform infra-red (FTIR) as an analytical technique in industrialized countries due to its speed, accuracy, quick sample preparation, and relatively low cost (Rodriguez-Otero *et al.*, 1997). This analytical method is based on infrared spectroscopy, which measures the absorption of different frequencies of infrared radiation by solids, liquids, and gases (Wehling, 2010; Barbano and Clark, 1989). When molecules absorb IR energy at a particular wavelength, chemical bonds vibrate at different frequencies, which can be used to identify characteristic functional groups in a sample (Wehling, 2010).

Mid-IR spectroscopy (4000 cm^{-1} to 400 cm^{-2}) revolutionized the dairy industry with FTIR spectroscopy because it performs qualitative and quantitative analysis of ingredients and finished foods (Rodriguez-Otero *et al.*, 1997; Barbano and Clark, 1989). It is currently an AOAC and an International Dairy Foundation (IDF) accepted method (Rodriguez-Otero *et al.*, 1997). Currently, the Foss Milkoscan FT2 is widely used in the dairy industry for simultaneous milk composition analysis of lactose, protein, and fat (Rodriguez-Otero *et al.*, 1997).

3. *Particle Size Analysis*

Laser diffraction techniques are utilized to determine the particle sizes distribution in wet or dry samples (Xu, 2001). They are non-destructive and non-intrusive techniques capable of measuring particles in the size range 0.02 to 2000 microns (Kippax). Modern laser diffraction instruments, such as the Coulter LS-230, target a laser beam through a sample, which scatters the incident light onto a Fourier lens (Kippax). The benefits of this technique are the wide dynamic measuring range, flexibility of samples, rapid data acquisition, and high repeatability.

4. Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis (SDS-PAGE)

A protein mixture can be separated and visualized by SDS-PAGE according to the protein's electrophoretic mobility. This technique was first used in the 1950's to separate proteins, and later the use of reducing agents and detergents such as β -mercaptoethanol and sodium dodecyl sulfate (SDS), respectively, were included to isolate protein subunits. Since then, SDS-PAGE has evolved to separate proteins with simple and quick staining protocols and high protein recovery. In 1975, O'Farrell introduced high-resolution two-dimensional gel electrophoresis (2DE). This technique separates proteins first by isoelectric focusing and secondly by molecular weight in SDS-PAGE (O'Farrell, 1975). 2DE has the advantages of high-resolution separation, estimation of molecular weight, quantification of individual proteins, and visualization of the sample's proteome (O'Donnell *et al.*, 2004; Hamdan and Righetti, 2005). Protein expression requires comparison of gels after staining and statistical analysis (Lindmark-Mansson *et al.*, 2005). Some disadvantages include the modest detection limit, detection of only highly abundant proteins, and poor results in extreme pH as well as high and low molecular weights (Hamdan and Righetti, 2005).

The staining techniques have also been important in protein visualization. According to Candiano *et al.* (2004), staining procedures can be divided into four categories: organic dyes, silver stains, negative stains, and fluorescent stains. The latter group includes SYPRO Ruby, with a sensitivity of up to 0.5-1.0 ng protein /mm² but requires UV excitation to visualize the proteins. Among the organic dyes, there exist Coomassie Blue R-250 and Coomassie Blue G-250, which bind one mg of protein per 1.2 and 1.4 mg of dye, respectively (Candiano *et al.*, 2004). The most sensitive protocol is silver staining that relies on the reduction of ionic to metallic silver onto the protein surface. However,

irreproducibility and dependence on other parameters makes this stain rather erratic. The most common dye used is Coomassie blue, but it is limited at detecting glycosylated proteins, which require a periodic acid-Schiff (*PAS*) reagent method to detect oxidizable carbohydrate groups. In proteomics, Coomassie Blue is preferred due to its high sensitivity, simple use, and fewer preparation steps, but protocols have been adapted to have a more sensitive colloidal stain for proteome analyses (Candiano *et al.*, 2004; Westermeier, 2006).

5. *Liquid Chromatography and Proteomics*

In 1995, M. Wilkins introduced the term proteomics from the word “proteome,” which indicates “the entire protein complement expressed by a genome, or by a cell or tissue type” (Takahashi and Isobe, 2007). This field of science developed from the need to identify, quantify, and characterize the structure, biochemical activity, and functionality of all the proteins in an organism or specimen and from the necessity to establish how these properties vary in time or physiological state (Gagnaire *et al.*, 2009; Hamdan and Righetti, 2005). This technique became even more popular as protein sequence databases increased, along with arising revolutionary equipments in spectrometry (Gagnaire *et al.*, 2009). Nowadays, proteomics is mostly performed to identify and quantify proteins or modifications such as glycosylation that change in time (Reinhardt and Lippolis, 2008; Beddek *et al.*, 2008). In the dairy field, the first proteomic studies were performed to characterize milk and whey proteins (Lindmark-Mansson *et al.*, 2005; Manso *et al.*, 2005). Current research focuses on low-abundance and/or bioactive proteins such as those in the MFGM (Yamada *et al.*, 2002; Smolenski *et al.*, 2006; Gagnaire *et al.*, 2009; Reinhardt and Lippolis, 2008).

There are two approaches for protein identification, the top-down and bottom-up approach. In the first approach, complex protein mixtures are digested into peptides from which fragmentation data is obtained and match to a predicted spectra from peptide amino acid sequence (Takahashi and Isobe, 2007). The top down approach is more challenging because the intact mass of a protein is measured, followed by protein fragmentation, and identification from the fragmentation pattern.

Proteins are usually separated in 1D or 2D electrophoresis gels, cation/anion exchange, or isoelectric focusing to decrease the complexity of the dynamic range of proteins (Takahashi and Isobe, 2007). Upon separation, isolated protein or spots are digested with trypsin, and peptides are separated and identified with mass spectrometry (MS). In MS, the samples are introduced, ionized, separated, and detected. Nonvolatile, high-molecular-weight compounds such as peptides and proteins can be ionized by matrix-assisted laser-desorption/ionization (MALDI) or electrospray ionization (ESI) (Hamdan and Righetti, 2005; Takahashi and Isobe, 2007). MALDI is a solid-phase technique utilized on silicon chips that detects singly-charged ions and is usually coupled with a high mass range analyzer such as a time of flight (TOF) mass analyzer. MALDI-TOF requires fairly pure samples, so it is commonly used in conjunction with protein fractionation with 1DE or 2DE (Hamdan and Righetti, 2005). Identification of proteins is performed by peptide mass fingerprinting (PMF), which compares a list of experimental peptide masses with a theoretical list of peptide masses in protein databases. On the contrary, ESI generates gas-phase ions from liquid samples, and it is usually coupled with on-line chromatographic techniques, such as reversed phase HPLC and anion exchange, to identify ions via quadrupoles and quadrupole ion traps. These instruments are more readily configured as tandem mass spectrometers for mass-selecting and fragmenting

single components of a mixture (Hamdan and Righetti, 2005). Despite its sensitivity, ESI has limited application in the analysis of complex mixtures unless high-resolution chromatographic equipments are utilized (Hamdan and Righetti, 2005; Takahashi and Isobe, 2007).

Current research attempts to identify minor milk proteins using both direct liquid chromatography-tandem mass spectrometry and 2DE followed by MALDI-TOF (Smolenski *et al.*, 2006). Molecules involved in host defense have been of special interest in the dairy field, and the need to quantify changes during milk lactation has driven many scientists to come up with more sensitive techniques to achieve such goals. O'Donnell *et al.* (2004) provides a good review of proteomics techniques and the challenges faced in milk proteomics.

L. Summary of Literature

Fat lipid formation has been reviewed along with the origins of the crescent milk fat globule membrane, which is constituted of proteins and polar lipids. These components are associated with health and well-being benefits and even nutraceutical properties, which emphasizes the importance of studying the MFGM to its fullest. However, further research to optimize the extraction of MFGM from the richest sources, buttermilk and butter serum, is necessary to maximize the yield. Therefore, the latest procedures in filtration were presented to recover intact MFGM constituents. Also, the technical aspects and methodologies that reveal the MFGM composition with minimal invasion were summarized to provide alternate techniques to study the effects of milk processing, such as cooling, thermal treatment, and homogenization.

M. Justification for Present Research

The milk fat globule membrane is part of the most nutritional product that nature has provided: milk; however, its health-promoting and nutraceutical properties are still unknown to most consumers. A lot of research has been done in characterizing the milk fat globule membrane. Yamada *et al.* (2002) first attempted to identify low-abundance protein in colostrum and mature milk, identifying specific proteins to each set of milk. Later, developmental changes in the transition from colostrum to milk were published (Reinhardt and Lippolis, 2008) as well as milk glycan using proteomics techniques (Wilson *et al.*, 2008). The latest work profiled the metabolic proteomic of bovine mammary tissue (Beddek *et al.*, 2008), but even with the plethora of studies about the health benefits of MFGM proteins and phospholipids, no work has been performed in studying the damage that MFGM constituents undergo during milk processing in the same line of milk.

With this study, an in depth characterization of the effects of milk processing on the milk's constituents, especially proteins and polar lipids, was performed utilizing established methodologies and sensitive proteomic techniques such as LC/MS and 2-DE gels coupled with MALDI-TOF. Furthermore, protocols for the study of MFGM were established for future research, to determine the best source of MFGM for commercial production, and for incorporation into the food products.

The knowledge obtained from the effect of milk processing on the MFGM will further expand our understanding of the fundamentals that will allow the design of an optimal processes to maintain the function of the MFGM throughout the line of production and absorption of nutrients in the human intestine and receive benefits similar to the ones neonates acquire.

III. MATERIALS AND METHODS

A. Milk Collection & Processing

Milk was collected from Cal Poly's milking parlor before it reached the cooling tank and kept at room temperature until analysis were done; this, sample was denoted "Fresh." Then, the milk was left in the cooling unit for 72 hrs, and processing was performed as shown in Figure 12. Three independent replicates were performed in different weeks

Figure 12. Milk Collection and Processing.

1. Heat Pasteurization

Milk processing was conducted at Cal Poly's pilot plant. Batch pasteurization was done in a double walled vessel (Groen DN/RA-60) where steam was applied until the unit reached 63 °C and maintained for 30 min. Temperature was monitored with a thermometer. The milk was immediately placed at 4 °C after processing. High temperature short time (HTST) pasteurization was performed with a heat plate exchanger

(APV, SR II, Cropoco, Inc) controlled with Hardy instrument software (HI2151/20 WC). The conditions were 72 °C for 16 seconds followed by immediate cooling to 4 °C. Ultra high temperature (UHT) pasteurization was performed in an autoclave (2540E, Tuttnauer Brinkmann). Milk was placed in 500ml pyrex glass bottles and heated to 121 °C for 3 minutes.

2. *Cold Pasteurization- Pulsed UV Light*

Cold pasteurization was performed according to Elmnasser *et al.* (2008) and Krishnamurthy *et al.* (2004)'s descriptions, using a SteriPulse-XL system (RS-3000C, Xenon Corp., USA) equipped with a lamp housing with 16 in quartz flashlamp type C (XL-1957), a sterilization chamber (LH840 LMPHSG), a liteMark-XL 1000 light monitor, and a controller (RC-847). The system was located under a hood to ventilate ozone being produced. The milk sample, five to ten mL, was pipetted into a glass bottom petri dish (9 cm diameter, Pyrex, USA) and placed at the midpoint of the flashlamp (7.9cm depth x 20.3 cm wide). To receive the maximum energy, the petridish was located 4.5 cm (1.76 in) from the face of the sterilization chamber's window, which has an intensity of approximately 1.0 Joules/cm²/pulse.

a) *Microbial Reduction in Milk*

To determine the best settings to reduce the microbial population in a non-transparent liquid such as milk, microbial studies were performed on UV treated milk and compared to raw milk. Two parameters were analyzed, the volume of the fluid placed in the petri dish since this determines the depth penetration of the lamp and the exposure time. Volumes in the range from five to ten mililiters were tested in combination with treatment time of two up to nine seconds. Microbial reduction in aerobic and

E.coli/coliform counts were enumerated using 3M™ Petrifilm™ Aerobic Count Plates and 3M™ Petrifilm™ E. coli Count Plates (EC), respectively, after 24 and 48 hrs of incubation. Dilutions were made with butterfield phosphate buffer (D599, Hardy Diagnostics, USA), and the samples were plated in duplicates and incubated at 25 °C. One-way ANOVA tests were performed using MiniTab (v.16.1.0, Minitab Inc., State College, PN) to find difference ($p < 0.05$) among samples. Paired comparisons between means were carried out using the Tukey's test when a significant difference was observed.

b) Milk Processing

Milk that was kept in the cooling unit for 72 hrs was utilized for this experiment and maintained cold throughout the pulsed UV light treatment. Seven mL of milk were placed in a petridish and given five pulses of UV light for a total of 5,970 Joules (15 Joules/cm²) of energy. Three petridishes were located on the tray along the midpoint of the flashlamp, and their product consolidated. A final volume of 800 mL of UV-treated milk was collected and kept at 4 °C until needed.

B. Physical Properties of the Milk Fat Globule Membrane

1. Milk Fractionation

To separate cream, 35 g of processed milk was measured in ultracentrifuge tubes (357002, Beckman, CA) and transferred to a pre-chilled Beckman L7-35 Ultracentrifuge at 7,000 rpm for 30 min at 4 °C (Basch *et al.*, 1985). Upon centrifugation, the cream was scooped out with a spatula and placed on a Whatman #1 filter paper (No. 100150) for 5 min and later placed into a 15 mL falcon tube. The skim milk was decanted into clean

falcon tube containing a net (hair net) to prevent cream chunks from entering the final fluid. Cream and skim milk were kept at room temperature previous to analysis.

2. Milk Fat Globule Size Distribution

Particle size diameter distribution of whole milk, skim milk, and 40% cream were determined according to the method described by Menard *et al.* (2010) using a laser diffraction particle size analyzer in the Polarization Intensity Differential Scattering (PIDS) optical mode (Beckman Coulter, model LS 230, CA). The analysis chamber was filled manually with one liter of deionized water containing 16 mL of 35 mM EDTA/NaOH pH 7 buffer (>99% disodium salt dehydrate, Sigma) for a final concentration of 0.1 mL buffer per liter of water. The instrument was allowed to adjust for electrical offsets and align the laser prior to measuring background, which was measured for 60 sec. Sample loading was conducted for 90 seconds. The obscuration rate was held 10% for milk during the runtime. For whole and skim milks, one mL of 35mM EDTA buffer was added to 10 mL of milk five min before measurements (Mènard *et al.*, 2010). For cream, 35 mM EDTA buffer was use to make the final cream concentration 40% (w/v). The pump speed for all the samples was 50%; laser power, 80%; run length, 60 s, and temperature, 23 to 25°C.

Standard parameters were calculated by the software (Coulter LS Software v.3.29 August 2003), and measurements were conducted in triplicate. Five parameters were calculated to describe the milk fat globule size distribution in milk, cream, and skim milk. The volume-weighted average diameter ($d_{4,3}$), which is defined by Equation 1 as:

$$d_{4,3} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \quad (\text{Eq. 1})$$

Where n_i is the number of fat globules of diameter (d_i). The second parameter is the volume–surface average diameter ($d_{3,2}$), described by Equation 2.

$$d_{3,2} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \quad (\text{Eq. 2})$$

The modal diameter (mode) was measured to represent the most important population of fat globules in volume. The fourth parameter is the specific surface area (SSA), which is shown via Equation 3:

$$SSA = \frac{6\phi}{d_{3,2}} \quad (\text{Eq. 3})$$

Where ϕ is the volume fraction of milk fat, and the size distribution width was calculated using Equation 4 as indicated below. For this equation, $d_{v,0.9}$, $d_{v,0.1}$, and $d_{v,0.5}$ represent the diameter below which lies 90%, 50%, and 10%, respectively, of the globule volume.

$$\text{Span} = \frac{d_{v,0.9} - d_{v,0.1}}{d_{v,0.5}} \quad (\text{Eq. 4})$$

C. Chemical Composition of Processed Milk

1. Evaluation of Milk Composition

The protein, casein, lactose, fat, and total solids content of processed milks were determined with a Milkoscan FT2 (Foss, Eden Prairie, MN) utilizing Foss Integrator software package version 1.5.0 (Foss). Prior to the analysis, the instrument was calibrated with DQCI Servies: Dairy Quality Control California Milk Standards (DQCI, Mounds View, MN). The samples were warmed to 40 °C for five minutes before measurements, and each sample was conducted in triplicates.

2. *Fat Extraction-Mojonnier*

Milk fat was extracted using the Mojonnier method (AOAC Method 989.05) (Min and Ellefson) with slight modifications to prevent phospholipid degradation. All the reagents were analytical grade and purchased from Fisher Scientific. Prior to extraction, each sample was warmed to 38 °C for five min to assure a complete homogenization of sample. Five mL of milk (25 °C) were weighed in a Mojonnier extraction flask (EW-34590-65, Cole-Parmer, IL) followed by addition of warmed water (38 °C) and mixing for 30 sec. The sample was shaken vigorously for 45 sec after each chemical addition with the exception of ammonium hydroxide, which was done for 30 sec. To neutralize acids and denature proteins, 1.5 mL of NH_4OH (A669-212) were added. Gel formation was prevented by adding 10 mL of 95% ethanol (A407-4), and the lipids were dissolved by the addition of 25 mL of ethyl ether (E199-4). A couple drops of a 1% phenolphthalein solution (SP62) were added to distinguish between the organic and inorganic phases. More lipids were extracted with 25 mL of petroleum ether (E120-4), which also removed moisture from the organic phase. The sample was spun down in a Garver electrifuge (308M, Garver Manufacturing, IN) for 45 sec. The organic phase was decanted in a pre-weighed Mojonnier fat tin, which was previously dehydrated at 100 °C for 30 min and placed in a dessicator. A second extraction was performed without the addition of ammonium but with 5 mL of ethyl alcohol, 15 mL of ethyl ether, and 15 mL of petroleum ether. The solvents were then evaporated on a hot plate at approximately 60 °C and transferred to a pre-warmed isotemp vacuum oven (281A, Fisher Scientific, CA) for 30 minutes at 60 °C with at least 20 in Hg vacuum. The tins were transferred to the dessicator and allowed to cool down before weighing them. The milk fat was transferred to a 4-ml amber vial with the addition of chloroform/methanol (2:1, v/v) to a final

concentration of 100 mg/mL. Nitrogen gas was introduced into the vial for 5 sec to reduce fat oxidation, and the samples were kept at -15 °C until needed.

3. *Determination of Phospholipids in the MFGM*

a) *Phospholipid Solid Phase Extraction (SPE)*

Milk phospholipids were obtained from the extracted fat according to the method described by Bitman *et al.* (1984). The reagents were of analytical grade and purchased from Fisher Scientific. The amber vials containing fat were allowed to thaw and placed in a 50 mL round bottom flask to evaporate the solvents in a rotatory evaporator with a diagonal condenser (Labconco Corp.). The water bath was kept at 45 °C to prevent lipid degradation, especially with sphingomyelin. Approximately 0.1 g of fat was utilized to load 2g/12 ml Strata Si-1 Silica (55um, 7A) solid phase extraction tubes (8B-S012-KDG, Phenomenex). The cartridges were placed in a SPE 12-position vacuum manifold (AH0-6023, Phenomenex), and 9-mL glass tubes were placed under the columns, which were first cleaned by adding 20 ml of chloroform and conditioned by adding 10 ml of hexane. The fat was then transferred onto the columns with two mL of hexane, and an extra two mL volume of hexane was added to rinse any residue and added to the column. The lipids were allowed to be absorbed onto the columns for approximately five minutes, and the hexane was allowed to drip by gravity. Neutral lipids (fraction 1) were eluted with 40 ml hexane/ethyl ether (1:1 v/v) by gravity or by applying vacuum at approximately 5 in Hg. This fraction was discarded, and the glass tubes were replaced with clean ones to elute the phospholipid fraction (fraction 2) with 20 ml methanol, which removes 80% of the PL. The eluent was transferred into pre-weighed round bottom flasks, and the remaining PL was eluted with 20 ml of chloroform/methanol/water (3:5:2) (fraction 3). The second

and third fractions were consolidated and evaporated under vacuum with a rotatory evaporator to evaporate organic solvent with the previous settings. The round bottom flasks were then immersed in liquid nitrogen to freeze the water containing PL and placed in a freeze dryer holder (75408, Labconco Corp.) to sublime the solvent using a freeze dry system (Freezon 4.5, Labconco Corp.) for three hours or until a white residue was observed. The PL powder was dissolved in one ml of chloroform/methanol (2:1, v/v), transferred to amber vials, and stored at -20 °C.

b) Phospholipid Separation & Quantification

For identification purposes, pure polar lipids such as L- α phosphatidylcholine (PC), L- α phosphatidylethanolamine (PE), L- α phosphatidylinositol (PI), L- α phosphatidyl-L serine (PS), and sphingomyelin (SM) from bovine brain were purchased from Sigma Aldrich. Chloroform, methanol, and formic acid were of HPLC grade from Fisher Scientific. Samples were collected and analyzed as indicated in Figure 13.

Figure 13. Analysis of milk phospholipids involves fat extraction from milk, isolation of PL from other simple lipids via solid phase extraction (SPE), and separation and quantification of the different phospholipid classes via HPLC.

Phospholipid separation was performed by high performance liquid chromatography (HPLC) according to the methodology described by Rombaut *et al.* (2005). A Shimadzu prominence ultra flow liquid chromatography system (LC-20AB, Shimadzu, USA Inc.) consisted of a communication bus module (CBM-20A, Shimadzu), an autosampler (Sil-20A HT, Shimadzu), a degasser (DGU-20A₃, Shimadzu), and a column oven (CTO-20A, Shimadzu) connected to a charge aerosol detection (CAD) detector (Corona Plus, Dionex, USA). Nitrogen was used as a carrier gas at a backpressure of 2.4 bar. A 150mm x 3.0 mm Prevail silica column with a 3 μ m particle diameter (Part N. 99341, Grace Division, USA) and a 7.5mm x 3.0 mm guard silica column with a 5.0 μ m particle diameter (Prevail™ All-Guard™ Part N. 99354, USA) were used. The column and guard column were equilibrated at 40 °C. Samples were kept at room temperature, and 10 μ L were injected. The injection loop was rinsed with chloroform/methanol (2:1, v/v) in between injections. The phospholipid elution was performed with a linear gradient with 87.5: 12: 0.5 (v/v/v) chloroform; methanol; triethylamine (TEA) buffer (pH 3, 1 M formic acid) at t = 0 min with a flow at 0.5 mL/min. The TEA buffer was prepared by making a formic acid buffer of 1 M, which was neutralized with triethylamine to a final pH of 3 (see Appendix A.2). At t=16 min, the mobile phase was 100% eluent B (28: 60: 12, v/v/v). The mobile phase was brought back to 100% eluent A at t=17 min, and the column was equilibrated until the next injection at 28 min (Rombaut *et al.*, 2005).

The data was collected and analyzed using LCSolution (Shimadzu). Identification of phospholipids was carried out by comparison with the retention time of pure standards. For each PL, calibration curves were created from the area values obtained by injecting 10 μ L of serially diluted solutions of PE (25-300 μ g), PI (2.5-30 μ g), PS (1.67-20 μ g), PC (21-250 μ g), and SM (25-300 μ g). A solution of chloroform/methanol (2:1, v/v) was

utilized for the solutions. All the samples and standards were run in triplicates, and a linear regression equation ($y = a + bx$) was applied to each phospholipid standard.

4. Measurements of S-S and SH group levels

The S-S and SH group levels in the MFGM were determined according to the method of Beveridge *et al.* (1974) with slight modifications by Lee and Sherbon (2002). To determine free SH groups, 0.5 ml of MFGM suspension was mixed with 2.5 ml of 5 g SDS/ per liter of Tris-gly-8M urea (Appendix A.6.h) (Cui *et al.*, 2009) in a polycarbonate ultracentrifuge bottle (357002, Beckman, CA). Then, 20 μ l of Ellman's reagent at 4mg/ml (5,5'-dithiobis-2, 2'-nitrobenzoic acid (DTNB) in Tris-glycine buffer) were added, and the mixture was allowed to stand for 1 hour at 25 °C. There was no need to clarify solution with the addition of chloroform, but the mixture was centrifuged at 5000g for 5 min at 25 °C to avoid solids. The absorbance of the supernatant was measured at 412 nm with a spectrophotometer, and the SH groups were quantified using Equation 5.

$$\frac{\text{Free SH } \mu\text{M}}{\text{g MFGM proteins}} = \frac{73.53 \times \text{Abs}_{412} \times (6.04)}{\text{mg/ml of protein}} \quad (\text{Eq. 5})$$

The term A_{412} is the absorbance at 412 nm, 6.04 is the dilution factor of the sample, and 73.53 is derived from the conversion from molar to $\mu\text{M/mL}$ and from mg solids to g solids (10^6) over the molar absorptivity of the Ellman's reagent (1.36×10^4) (Beveridge *et al.*, 1974). The total SH was quantified by mixing 0.2 ml of MFGM homogenate with one of 5 g SDS/ per liter of Tris-gly-8M urea 8M and 20 μ l of β -mercaptoethanol. After incubation for 1hr at 25 °C, 10 ml of a solution of 12% (w/v) trichloroacetic acid (TCA) was added and incubated for 1 hr at 25 °C. The solution was then centrifuged at 5000 g for 15 min, and the precipitation was resuspended in six ml of TCA, and centrifuged at

5000 g for 15 min to remove β -mercaptoethanol. A second wash was repeated with 6 ml of TCA followed by centrifugation. The precipitate was dissolved in 3 ml of 8 M urea and 5g SDS/l in tris –glycine buffer, and 30 μ l of Ellman's reagent was added followed by incubation for 1 hour at room temperature. There was no need to add chloroform, but centrifugation was performed for 5 min at 5000xg. The total SH (SH+ reduced SS) were quantified using Equation 6.

$$\frac{\text{Total SH } \mu\text{mol}}{\text{g MFGM proteins}} = \frac{73.53 \times \text{Abs}_{412} \times 15}{\text{mg/ml of protein}} \quad (\text{Eq. 6})$$

The term 15 is the dilution of the sample in the final volume. The S-S groups were calculated from the difference in free SH from total SH as indicated by equation 7.

$$\frac{\text{SS group } \mu\text{mol}}{\text{g MFGM proteins}} = \frac{\text{Total SH} - \text{Free SH}}{2} \quad (\text{Eq. 7})$$

D. Milk Fat Globule Membrane Extraction

Milk was brought to room temperature before MFGM extraction, which was performed according to the method of Patton and Huston (1986) with some modifications. The density of serum was increased by adding sucrose to milk at 5 g per 100 ml, and the solution was allowed to dissolve completely for 10 m. Fifteen mL of phosphate buffer saline (PBS) (1X) (see Appendix A.1) were placed in a 50 mL conical centrifuge tube, and 35 mL of the dense milk was carefully deposited underneath the buffer layer using a 25 mL disposable pipette (Patton and Huston, 1986). The conical tubes were carefully placed in a centrifuge (Eppendorf 5810R) and spun down at 4,000 rpm (3200 x g) for 20 min at 21 °C to collect the cream (Basch *et al.*, 1985). The cream layer was removed with a spatula and placed in a 50 mL tube. The cream was

resuspended with cold (4 °C) 1X PBS to a final 40% (w/v) solution, and Tween 80 (Fisher, T164) was added to a final 0.2% (w/v) concentration. The mixture was blended for 45 sec using a commercial blender (Waring Blender 51BL31) in high setting and a pre-chilled (4 °C) steel cup (Eberbach Corporation). The churned cream was placed inside an isotemp bath (Isotemp205, Fisher Scientific, CA) at 42 °C for 30 min and spun down in a pre-warmed centrifuge (Eppendorf 5810R) at 4,000 rpm for 20 min at 40 °C. Centrifugation separated the cream into buttermilk, which contains the MFGM proteins, and oil layers are depicted in Figure 14.

Figure 14. Milk fat separation upon churning and centrifugation. Left. Actual conical tube containing cream layers. Right. Schematic representation. The buttermilk, which contains MFGM proteins, was isolated for the next step in MFGM purification.

The unsaturated and saturated fat layers were discarded with a Pasteur pipette, and the aqueous portion of the churned cream was isolated using a 25 mL plastic pipette and transferred into a clean 50 mL conical tube. Then, sodium citrate was added to disintegrate casein micelles at 2% (w/v) and gently agitated to dissolve the citrate

completely. The buttermilk was incubated overnight at 4 °C to break emulsions. Upon incubation, the buttermilk solution was transferred to a polycarbonate ultracentrifuge bottle (357002, Beckman) and spun down for two hours in a pre-chilled (4 °C) Beckman L7-35 Ultracentrifuge at 25,000 rpm (50,000x g). The aqueous phase was decanted without disturbing the pellet, and any remaining saturated fat was removed manually. The pellet was washed with 25 mL of cold 1X PBS to remove any remaining detergent, and the tube was placed back in the centrifuge with the same settings for 45 min. Upon centrifugation, the aqueous phase was discarded, and the dry pellet was transferred to a 1.5 mL microcentrifuge vial and stored at -4 °C.

E. MFGM Protein Profile from One Dimensional Analysis

1. Protein Homogenization

The MFGM protein pellet was put into solution by adding cold 1X PBS into siliconized microcentrifuge at a final concentration of 0.1 mg/uL. Then, the solution was sonicated using a Sonic Dismembrator (500, Fisher Scientific) with a CE Branson microtip (102C, Fisher Scientific) using three cycles of 12 seconds at 10% amplitude. The vial containing the homogenized pellet was kept in ice during sonication to avoid local heating, and the tip was washed with acetone and deionized water between samples. The homogenized solution was stored at -15 °C.

2. Protein Quantification

Protein concentration was determined using a BCA[™] protein assay kit (23227, Pierce, USA), which is based on bicinchoninic acid (BCA) for colorimetric detection and quantitation of total protein. Briefly, a standard curve was constructed from bovine serum albumin (BSA). An aliquot of 25µL of sample or standard was pipetted into a microplate

well, and 200 μL of working reagent was added followed by mixing on a shaker for 30 sec. The plate was transferred to an incubator at 37 °C for 30 min and cooled before reading absorbance at 562 nm using a spectrophotometer. Samples and standards were prepared in duplicates.

3. *Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis*

The stock MFGM protein solution was diluted to 1.6 $\mu\text{g}/\mu\text{L}$ using filtered 1X PBS buffer before SDS-PAGE analysis. Protein samples were prepared for reduced gel electrophoresis using NuPAGE® Novex® reagents (Invitrogen, CA). Five μL of sample was mixed with 2.5 μL NuPAGE LDS sample buffer (4X) (NP008), one μL NuPAGE reducing agent (10X) (NP0004), and 5 μL nanopure water to a final concentration of 0.8 $\mu\text{g}/\mu\text{L}$. The samples were heated at 70 °C for 10 minutes and immediately cooled down to room temperature with top water. An aliquot of 12 μL of the reduced sample was loaded into 1.0 mm x 12 well NuPAGE 4-12% Bis-Tris gel (NP0322), which was mounted onto XCell Surelock™ Mini-Cell chamber (EI0001). Novex Sharp pre-stained protein standards (LC5800S), 10 μL , were included in each gel. The cell was filled with 1X NuPAGE MOPS SDS running buffer (NP0001) containing 500 μL of NuPAGE antioxidant (NP0005) at a concentration of 5 $\mu\text{L}/\text{mL}$. The cell unit was connected to a BioRad power unit (PAC300, BioRad) and run at 200 V for approximately 50 minutes. The gel was then transferred to a clean container and washed with nanopure water three times for 5 min. SimplyBlue safe staining solution (LC6060) was added and incubated overnight on a rotatory incubator (Max 2000 E-class, Barnstead, IA) at room temperature. Upon staining, the gels were washed with nanopure water three times for 5

minutes, and images were captured with an Epson Scanner (Epson Expression 1680) in conjunction with Epson Scan Software in professional mode.

4. *Protein Densitometry & Statistical Analysis*

Quantiy One 1-D Analysis Software (v.4.6.3, BioRad) was used to quantify protein bands. Trace quantity (peak density x area) was utilized to compare the band intensities in each sample per treatment batch.

5. *LC-MS/MS Analysis*

LC-MS/MS was performed on 1DE gels at UC Davis Genome Center Proteomics Core Facility. First, four sections were excised from a 1DE gel using a razor over a gel sheet (165-7018, BioRad) to avoid contamination: higher than 150 kDa, between 150 and 55 kDa, between 55 and 35 kDa, and less than 35 kDa. These samples were sent to UC Davis for protein identification. Briefly, the gel sections were digested overnight with trypsin, and the peptides were purified for further analysis as described by the Proteome Center (Tran). A Thermo Finnigan LTQ ion trap mass spectrometer (Thermo Fisher) coupled with a paradigm HPLC (Michrom Bioresources, Auburn, CA, USA), and a Michrom captive spray ionization source were utilized for peptide identification. The digested peptides were loaded onto a trap column (Zorbax300SB-C18, 5 μ m, 5 $\times$ 0.3 mm, Agilent Technologies, Santa Clara, CA, USA) and desalted online followed by elution from the trap column using an increasing gradient of organic solvent (B) (A=0.1% Formic Acid, B= 100% Acetonitrile) onto a reverse-phase Michrom Magic C18AQ (200 μ m $\times$ 150 mm) column at a flow rate of 2 μ L per min. Peptide elution started with 2%B-35%B for 30 min, 35%B to 80%B for two min, and held at 80%B for one min. Eluent B decreased from 80%B to 2%B over 1.5 min, and the column was re-equilibrated at 2%B

for 26.5 min. The complete run was 60 min, and the following parameters were selected: spray voltage 1.8kV, heated transfer capillary 180°C, full scan mass range of each sample was 350-1400 mass to charge (m/z) ratio, and the MS/MS data was collected using 35% normalized collision energy for collision-induced dissociation and an isolation width of 1.0.

6. *Database Searching and Criteria for Protein Identification*

For database searching, tandem mass spectra were extracted by Bioworks version 3.2. All MS/MS samples were analyzed using X! Tandem (The GPM, thegpm.org; version TORNADO (2010.01.01.4)). The program was set up to search Uniprot Human complete proteome database (December 2010) and the cRAP common laboratory artifacts database along an equal number of reverse decoy sequences assuming the digestion enzyme trypsin. Tandem was searched with a fragment ion mass tolerance of 0.40 D and a parent ion tolerance of 1.8 Da. The iodoacetamide derivative of Cys (carbamidomethyl) was specified in X! Tandem as a fixed modification. For variable modifications, deamination of asparagine and glutamine, oxidation of methionine and tryptophan, sulfone of methionine, tryptophan oxidation to formylkynurenin of tryptophan and acetylation of the N-terminus were specified.

For protein identification, Scaffold (version Scaffold_3_00_05, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. This software verifies peptide identifications assigned by SEQUEST and Mascot using the X!Tandem database searching program. Scaffold then probabilistically validates these peptide identifications using Peptide Prophet algorithm (Keller *et al.*, 2002) and derives corresponding protein probabilities using Protein Prophet algorithm (Nesvizhskii *et al.*, 2003). Peptide identifications were accepted if they could be

established at greater than 70.0% probability as specified by the Peptide Prophet, and protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Using the above criteria the false discovery rate (FDR, Decoy; Target) was calculated as 0.1% on the peptide level and 3.4 % on the protein level using a target-decoy (reverse) search strategy (Tabb, 2007). Protein function was determined using www.uniprot.org and <http://www.ncbi.nlm.nih.gov>.

F. Statistical Analysis

Statistical analyses were conducted for milk composition (FTIR), MFGM extraction, particle size distribution, phospholipids, and 1DE SDS-PAGE band intensity, using the General Linear Model (GLM) command in Minitab. Week was a random effect, and treatment was a fixed effect. Differences between the treatment means were compared at the 5% level of significance using Tukey's method. In addition, a histogram of residuals, normal plot of residuals, residuals versus fits, and residuals versus order were used to test model adequacy and normality.

G. MFGM Protein Profile from Two-Dimensional Analysis

1. Protein Homogenization

The reagents for 2D analysis were of electrophoresis purity grade from BioRad unless otherwise stated. The working homogenization buffer was prepared fresh by adding 50μL of immobilized pH gradient (IPG) buffer (0.5% ampholyte) and 62mg dithiothreitol (DTT) (40mM) per 10 ml of homogenization buffer (7M urea, 2M thiourea, 1% ASB-14, 40mM Tris base, 0.001% bromophenol blue, 0.5% ampholyte, and 40mM DTT, see

Appendix A.3.a). An aliquot of 1.2 ml of homogenization buffer was added per 100 to 150 mg of MFGM protein pellet. The membrane was homogenized gently using pellet pestles (749515, Kontes, NJ) into a 1.5 ml centrifuge tube until no fragments of tissue were visible. The samples were kept at room temperature. Homogenized membrane was transferred into 2mL siliconized microcentrifuge (02-681-321, Fisher Scientific) tubes and allowed to sit on the bench at room temperature for one hour to solubilize the proteins. Upon incubation, samples were centrifuged at room temperature for 30 min at 13,500 rpm. Solutions were decanted into fresh siliconized tubes, and the solids were left behind and discarded. 400 μ L aliquots were prepared in 2ml siliconized microcentrifuge tubes to proceed with a precipitation step.

2. *Protein Precipitation*

To precipitate proteins, 10% trichloroacetic acid (TCA)/acetone solution (kept at -20 °C) was added at 4x times the volume of protein solution. Thus, 1.6 ml of cold 10% TCA/acetone (see Appendix A.3.g) was added to each 400 μ l aliquot of protein solution. Microtubes were vortexed briefly and stored overnight at -20 °C. Upon incubation, microcentrifuge tubes were spun down at 4°C in a pre-cooled centrifuge (Microfuge 22R, Beckman Coulter) for 15 minutes at 13,500 rpm. The acetone mixture was decanted into a waste container, and 1.6mL of cold 100% acetone (kept at -20 °C) was added to each tube to wash out TCA and any contaminants out of the protein pellet. The pellets were broken down by vortexing twice for 10 seconds. The centrifuge tubes were immediately transferred to the pre-chilled centrifuge and spun at 4 °C for 15 minutes at 13,500 rpm. The acetone solution was immediately decanted into a waste container, and the tubes were air-dry for a few seconds. To rehydrate the precipitated proteins, 0.4 mL of fresh

working IPG rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 2% NP-40, 0.002% bromophenol blue, 0.5% ampholyte and 100mM DTE, see Appendix A.3.b) was added to each tube and vortexed for about 10 sec. Working IPG rehydration buffer was prepared by adding 6.25 μ L of IPG buffer and 20 mg DTE per 1.25 mL of IPG rehydration buffer. Samples were allowed to sit at room temperature for one hour and vortexed every 10 min for 10 sec to bring the proteins into solution. The tubes were then centrifuged at room temperature for 10 min at 13,500 rpm. The protein solutions were pooled into a clean siliconized microcentrifuge, and any gelatinous pellet was discarded. Solutions were stored at -80°C until needed.

3. *Protein Quantification*

Protein quantification was performed by using a 2D Quant kit (80-6483-56, GE Healthcare, NJ, USA) according to the manufacturer's instructions. Briefly, the precipitated sample first was diluted 1:10 by adding 4.5 μ L of protein solution into 40.5 μ L of nanopure water. A standard curve was prepared using bovine serum albumin (BSA). Samples and standards were prepared in duplicates in siliconized microcentrifuge tubes. To precipitate the proteins out of solution, 500 μ L of precipitant was added to each sample and standard followed by a brief vortex. The microtubes were then incubated for 3 min at room temperature, and upon incubation, 500 μ L of coprecipitant was added. The tubes were mixed by inversion and spun down at 10,000xg for 5 min to sediment the protein. Following centrifugation, the supernatant was decanted without disturbing the protein pellet. To dissolve the precipitated protein, 100 μ L of copper solution and 400 μ L of nanopure water were added to each microtube followed by a quick vortex. The solutions were then incubated at room temperature for 15-20 min, and their absorbances were read at 480 nm by transferring 200 μ L of solution into a 96-well plate in triplicate.

4. *Isoelectric Focusing (IEF)-First Dimension*

Protein solutions were diluted at a final concentration of 0.5 µg/µL using fresh IPG rehydration buffer, and 200 µL of this solution were loaded into a lane in a BioRad focusing tray (165-4030) without introducing bubbles. Immobilized pH gradient strips (pH 3-10, 11 cm; 163-2014, BioRad) were placed with the gel side down in a IEF tray lane making sure the + end of the strip touched the left side of the tray. 1.5mL of mineral oil (163-2129) were immediately added to each IEF strip, and the IEF tray was placed in a BioRad Protean IEF cell to separate proteins according to their isoelectric point. Isoelectric focusing started with a passive rehydration step (5 hr), followed by 12 hr of active rehydration (50 V). The protocol continued with a gradual voltage increase, 500 V for 1 hr, 1000 V for 1 hr, and 8000 V for 2.5 hrs (all voltage changes occurred in rapid mode). Upon separation, the strips were removed from the IEF tray and placed between western blot filter papers to remove excess mineral oil. The strips were then transferred gel side up into a rehydration/equilibration (165-4035) tray and kept at -80 °C overnight or until ready to proceed with the second dimension.

5. *SDS-PAGE-Second Dimension*

Frozen strips were thawed and equilibrated first with 1.6 mL of DTT/equilibration buffer (10mg DTT/mL, 375 mM tris pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, Appendix A.3.c) followed by 1.6 ml of iodoacetamide/equilibration buffer (25mg/mL. Equilibration was done at room temperature for 15 min each on a bidirectional Max/rotator (4361, Barnstead Int., IA, USA), and the equilibration buffer was removed from the strips by decantation. 12.5% polyacrylamide gels were prepared according to Appendix A.4, using BioRad Criterion cassettes, IPG +1 well 1.0 mm (345-

9906). The strips were loaded onto the polymerized gels after a quick immersion in 1X running buffer (see Appendix A.3.d), making sure the (-) side was placed on right side of the gel. An IEF electrode wick (165-4071) containing low range molecular weight standard (161-0304, see Appendix A.3.e) was placed in the far left side of the gel, which was covered with melted 0.8% agarose containing Laemmli SDS electrophoresis buffer (25 mmol/L tris-base, 192 mmol/L glycine, 0.1% SDS, Appendix A.3.f). Once the agarose was solidified, the cassettes were placed inside a BioRad Criterion Dodeca cell and filled with 1x buffer. The dodeca box was connected to a cooling system (HAAKE K10, Thermo Scientific, IA, USA) set at 10 °C, and slow stirring was initiated. The unit was connected into a BioRad Power Pac HC and set at 200V constant current for approximately 55 min, or until the blue loading dye reached the bottom of the gel.

6. *Sensitive Stepwise Colloidal Staining*

An adapted protocol described by Westermeier (2006) was utilized for visualization (Westermeier, 2006). The gels were washed three times in nanopure water for five min to remove any remaining detergent followed by overnight fixing in a 50% ethanol/3% phosphoric acid solution. Upon incubation, the gels were washed three times for 20 minutes with nanopure water and pre-incubated for hour in staining solution (34% (v/v) CH₃OH /3% (v/v), H₃PO₄/17% (w/v) (NH₄)₂SO₄, see Appendix A.5). Then, Coomassie brilliant blue G-250 powder (BP-100, Fisher Biotech) was added at a final concentration of 350 mg per liter of staining solution. The gels were stained for 5 days on a rotator shaker at room temperature. Upon staining, the gels were washed three times with nanopure water for 20 min. Images were captured with an Epson scanner (Epson Expression 1680) in conjunction with Epson Scan Software in professional mode.

7. *Fluorescent Staining-Sypro-Ruby*

The gels were washed three times in nanopure water for five min to remove any remaining detergent followed by overnight fixing in 10% methanol/7% acetic acid, v/v, (100mL per gel) in a clean plastic staining tray. Incubation was performed over a rotational shaker at room temperature, making sure the gels were protected from light during the staining protocol. Upon incubation, the fixing solution was replaced with diluted SYPRO Ruby (170-3127, Biorad, 1:5 with nanopure water) solution. After overnight incubation at room temperature, the gels were transferred into a new clean container to minimize the deposition of stain speckles and washed three times with 10% methanol/ 7% acetic acid, v/v, for 30 min each at room temperature and rotation at 40 rpm. After washing, the gels were transferred into a large volume of nanopure water to remove methanol and rotated for 10 min.

The gels were scanned with Typhoon Trio⁺ variable mode imager (98115, Amersham Biosciences). The Typhoon was allowed to warm up for 20 minutes, and the following scanning parameters were selected: fluorescence acquisition mode; 610 BP30 deep-purple, SYPRO Ruby, EtBr emission filter; 575 PMT; blue 532 nm laser; normal sensitivity; 200 microns pixels; platen focal plane, and ImageQuant analysis. The glass surface was washed with 70% ethanol followed with dH₂O, and the gels were placed face down, removing bubbles and blotting puddles around edges with Kimwipe. Once scanning was complete, the file was saved and reopened in ImageQuant 5.2 (Amersham Biosciences) to save them as “tif” files.

8. 2-DE Gel Image & Statistical Analysis

Digitized two-dimensional gel images were analyzed using Delta 2D (version 4.0.8 final 06, Decodon, Greifswald, Germany). The images were imported and connected through match vectors using *Group Warping Strategy*. Then, all the images were fused to a composite image (proteome map, which represents average volume for each spot) by using *Union* as fusion type, which adjusts the common regions and eliminates background. After background subtraction, protein spot volumes were normalized against total spot volume of all proteins in a gel image.

Normalized spot volumes were analyzed using an analysis of variance (two-factor ANOVA) for all the treatment. A second statistical analysis was done with all the treatments except UHT because this heat process caused many changes in protein levels, making difficult to overlap gel images. In addition, a two-way ANOVA was performed between Fresh milk and each treatment for a total of seven statistical analyses. For all the analyses, a null distribution was generated using 1000 permutations, and a p-value of < 0.01 was used to limit the number of false positives. To construct a hierarchical clustering, Pearson correlation, and average linking within the statistical tool suite within Delta2D was used.

9. Protein Excision & Digestion from SDS-PAGE

The reagents were of LC/MS grade and kept at room temperature unless otherwise indicated. Eppendorf Safe-Lock microcentrifuge tubes and tips were used to avoid plastic contamination, and glassware was washed with appropriate solvents before use. Proteins with different expression levels were excised from SDS-PAGE gels using a manual tissue arranger puncher (MP15-1.5 mm diameter, Beecher Instruments, Prairie, WI, USA). The

gels were placed over a gel sheet (165-7018, BioRad) during excision. The gel plugs were stored at -20 °C.

The gel plugs were thawed, and 200 μL of destaining buffer (25mM NH_4HCO_3 /50% acetonitrile, see Appendix A.6.a) were added to each tube. Vials were incubated on shaker at room temperature for 30 min. Upon incubation, the liquid was pipetted out and discarded carefully without discarding the gel plug. If Coomassie stain was still noticeable, an additional destaining step was performed. To dehydrate the plugs, 200 μL of 100% acetonitrile was added to each vial and incubated at room temperature for 10 min. This step was repeated if the gel was not dehydrated, as seen by semi-clear gel plugs. The acetonitrile was discarded, and the samples were allowed to dry completely under the hood for a few minutes with the tube open. To digest the proteins, 15 μL of prepared trypsin solution in a 40mM NH_4HCO_3 /10% acetonitrile was added to the bottom of vials at a final concentration of 11 $\mu\text{g}/\mu\text{L}$ (see Appendix A.6.c); vials were incubated at 37°C overnight. Upon incubation, the liquid containing the digested protein was transferred into a fresh Eppendorf tube (0.6ml), and 25 μL of analyte solution (0.1% TFA/ acetonitrile 2:1 v/v, see Appendix A.6.b) was added to each sample to elute the remaining peptides out of the gel. Samples were incubated for 30 min at room temperature, and peptide elution was repeated after removing the liquid and consolidating into the collection tube. The consolidated sample (~65 μL) was concentrated in a SpeedVac (Eppendorf Vacufuge TM AG 22331, USA) at 45°C for 50-60 min. Once the samples were dry, six and 12 μL of freshly prepared matrix solution (HCCA, - hydroxycyanocinnamic acid, see Appendix A.6.e) were added to each sample and peptide calibration standard (see Appendix A.6.d), respectively. On an AnchorchipTM Target (MTP Anchorchip 600/384 targus, Bruker DaltonicsPlate), one μL of this matrix/analyte

sample was plated in duplicates onto the 600 μm anchors. Samples were allowed to dry under the hood. One replicate of standards and samples was rinsed gently with 10 μL of wash buffer (1.15 $\text{NH}_4\text{H}_2\text{PO}_4$ mg/mL 0.1% TFA, see Appendix A.6.f) by pipetting up and down twice without touching the sample on the target. After drying, the matrix was recrystallized by adding one μL of recrystallization buffer (see Appendix A.6.g) directly to each spot on the target. Spots were allowed to dry before placing in the M/S analysis.

10. Mass Spectrometry- Protein Identification with MALDI-TOF

Peptide mass fingerprints (PMGs) were obtained by using a matrix-assisted laser desorption ionization (MALDI) mass spectrometer (Ultraflex II, Bruker Daltonics Inc., Billerica, MA, USA). Twelve peptides were chosen to conduct tandem mass spectrometry (time of flight, TOF) in order to obtain formation about the b- and y-ions of the peptide sequence. Peptide spectra were analyzed with FlexAnalysis (version 3.0, Bruker Daltonics, Inc.) and applied the TopHat algorithm for baseline subtraction, the Savitzky-Golay analysis (with 0.2 m/z; number of cycles =1) for smoothing and the SNAP algorithm to detect peaks (signal-to-noise ratio: 6 for MS and 1.5 for MS/MS). The charge state of the peptides was assumed to be +1, and porcine trypsin was used as an internal standard.

Proteins were identified using Mascot (version 3.1, Matrix Science inc., MA, USA) and combined PMFs and tandem mass spectra in a search against NCBI database for *Bos taurus*. Oxidation of methionine and carbamidomethylation of cysteine were selected as variable modifications, and one missed cleavage during trypsin digestion was allowed. The precursor-ion mass tolerance was set to 0.6 Da for tandem mass spectrometry, and two matched peptides was the lower limit for a significant hit that indicates a positive identification. Most identifications were to bovine sequences although some matches to

other mammals were accepted if there was not a corresponding bovine sequence in the database. Theoretical pI and molecular weight were predicted using Compute pI/Mw in monoisotopic mode at www.expasy.ch.

IV. RESULTS

A. Microbial Reduction in Milk

The efficiency of UV pulsed light to kill aerobic microorganisms was compared to batch, HTST, and UHT pasteurization as seen in Figure 15. In non-transparent fluids, such as milk, the depth of the sample is critical in determining the penetration of irradiation, thus, milk was treated with different pulse times (seconds, s) at different volumes. While conventional methods were able to achieve a log reduction, UV light treatment only reduced 44% of the initial microbial population present in raw milk. Nevertheless, a decrease in microbial growth is seen as the volume of the sample is reduced.

Figure 15. Aerobic bacterial counts in pasteurized and UV pulsed light treated milk. The volume is shown in ml, and the irradiation time is in seconds (s). Each second delivers three Joules/cm². Counts after 48 hrs of incubation at 37 °C.

The microbial reduction of coliforms was also determined with UV irradiation (Figure 16). No coliforms were seen in milk after treatment with the conventional methods. On the other hand, a different pattern was observed in the microbial reduction

with UV pulsed light. The highest reduction was seen in the 7mL/9sec milk sample with a 22% decrease in microbial population.

Figure 16. Coliform counts in pasteurized and UV pulsed light treated milk. The volume is shown in ml, and the irradiation time is in seconds (s). Each second delivers three Joules/cm². A 22% reduction is observed in the 7ml/9sec UV pulsed treated sample. Counts after 24 hrs of incubation at 37 °C.

To determine the best parameters that allow processing a high volume of samples and decreasing the microbial population, a second study was performed with different sample volumes and irradiation times. As seen in Figure 17, a 7mL-sample with UV energy of 15 Joules/cm² resulted in the highest microbial reduction among the treated samples. This treatment was significantly different from the raw material ($p < 0.0001$), with a 70% decrease in microbial population.

Figure 17. Microbial reduction in aerobic bacteria in UV pulsed treated milk. The volume is shown in ml, and the irradiation time is in seconds (s). Each second delivers three Joules/cm². Counts after 48 hrs of incubation at 37 °C. Statistically significant difference in samples is denoted by (p < 0.0001)

With regard to coliforms, the highest reduction, 53%, is seen in a 5mL sample with 15 Joules/cm² of energy followed by the 7mL/5sec sample with a 44% decrease in microbial population as depicted in Figure 18. If the volume is held constant, higher irradiation times are more efficient in killing coliforms and aerobic bacteria; however, higher energy intensities results in more bacterial counts. In fact, in both aerobic and coliforms, 21 Joules/cm² (7s) of energy is the turning point to observe more CFU. Experiments in our laboratory have demonstrated that higher energy intensities result in spore germination (data not shown). This may explain the higher CFU counts observed in samples that received more energy. There is not a linear relationship that explains this observation, but it can be expected that higher energy intensities at a constant volume may result in similar outcomes.

Figure 18. Microbial reduction in coliforms in UV pulsed treated milk. The volume is shown in ml, and the irradiation time is in seconds (s). Each second delivers three Joules/cm². Counts after 24 hrs of incubation at 37 °C.

B. Fat Globule Size Distribution in Milk Processing

The dispersion of MFG size in processed whole milk, cream, and skim milk was analyzed with five parameters: the volume-weighted average diameter ($d_{4,3}$), the volume–surface average diameter ($d_{3,2}$), the mode, the specific surface area (SSA), and the size distribution width (Span). To eliminate an apparent increase in the average globule diameter, a chelating agent, EDTA, was used to sequester Ca^{+2} and dissolve casein micelles, which range between 130 and 206 μm (Gassi *et al.*, 2008). Disintegration of casein micelles diminishes fat globule aggregation formation through casein interaction. Figure 19 illustrates the percent volume of whole milk fat globules (MFG) as a function of particle diameter (μm) in milk for the six treatments. The plot resembles a Gaussian distribution, which is in accordance with previous studies with milk (Mènard *et al.*, 2010; Garcia-Amezquita *et al.*, 2009). The fat globules range from 0.4 μm to 10 μm , and similar distributions were observed among the trials.

Figure 19. Milk fat globule (MFG) size distribution (volume %) in milk as a function of particle diameter (μm). Data for Trial II. Similar results were observed with the other trials (I, III, and IV). Values are average of three measurements.

A visual analysis does not indicate a vast change, but statistical analysis of the five parameters determined a significant difference in the volume–surface average diameter, $d_{3,2}$, which is sensitive to smaller particles ($p < 0.001$), and consequently in the specific surface area (SSA) ($p < 0.001$) of milk (Table 8). The Fresh sample has the highest $d_{3,2}$ and smallest specific surface area indicating a difference in the active surface of the fat globules.

Table 8. Parameters that describe the fat globule size distribution in milk.

Treatment	D(3,2) μm	Mode μm	SSA cm^2/mL	D(4,3) μm	SPAN
Fresh	3.02 ^a	4.05	19890 ^b	3.89	1.33
4C	2.90 ^b	3.93	20700 ^a	3.83	1.39
Batch	2.91 ^b	3.93	20590 ^a	3.83	1.38
HTST	2.91 ^b	3.93	20600 ^a	3.89	1.40
UHT	2.92 ^b	3.93	20543 ^a	3.86	1.41
UV	2.88 ^b	3.93	20838 ^a	3.85	1.41
s•N	0.01	0.05	103	0.03	0.02

s•N, standard error of mean among treatments. N, sample size, three trials

($d_{4,3}$), volume-weighted average diameter; ($d_{3,2}$), volume–surface average;

SSA, specific surface area; SPAN, size distribution width.

Means with different subscripts within a column are statistically different ($p < 0.05$).

Despite the variation observed in whole milk, no significant difference ($p < 0.05$) was described in the particle size distribution of isolated creams, which ranges from $0.4\ \mu\text{m}$ to $200\ \mu\text{m}$ depending on the treatment (Figure 20). Three peaks are visible in the fat globules distribution that center at $4\ \mu\text{m}$, $35\ \mu\text{m}$, and at about $70\ \mu\text{m}$. The first peak belongs to the regular fat globules diameters (Lopez *et al.*, 2010b) while the other two peaks are expected to be medium and large fat globule aggregates. The third peak distribution is very prominent in milk that underwent heat pasteurization, especially in HTST and batch milks with a center at about $90\ \mu\text{m}$ and $100\ \mu\text{m}$, respectively. UHT milk follows a similar pattern, but the large fat aggregates peak at $60\ \mu\text{m}$. The samples that did not undergo heat treatment have a higher percent volume in the normal size fat globule range. In fact, the Fresh and UV samples depict fewer fat aggregates at the $35\ \mu\text{m}$ and $70\ \mu\text{m}$ peaks whereas the 4°C sample shows a higher percent volume in the $100\ \mu\text{m}$ fat globule diameter region. Unfortunately, no trials were performed with 1% SDS to measure individual fat globule diameter. SDS dissociates fat globule aggregates by providing a net negative charge on the surface of globules that inhibits agglomeration. In addition, the samples that underwent heat treatment present smaller fat globules ($< 0.5\ \mu\text{m}$), which may correspond to vesicles formed by MFGM fragments composed of phospholipids and proteins.

Figure 20. Fat globule (FG) size distribution of 40% cream as a function of particle diameter (μm). Data are shown for Trial IV. Similar results observed for other trials.

As previously observed with whole milk, similar fat globule size distribution patterns were obtained with cream among the trials (Appendix E). The Fresh sample has smallest volume-weighted average diameter ($d_{4,3}$), volume–surface average diameter ($d_{3,2}$), mode, and size distribution width (SPAN), but these results are not significantly different from the other treatments (Table 9).

Table 9. Parameters that describe the fat globule size distribution in 40% cream.

Treatment	D(3,2) μm	Mode μm	SSA cm^2/mL	D(4,3) μm	SPAN
Fresh	3.23	3.63	18657	6.19	3.32
4C	4.17	21.89	15429	18.80	5.52
Batch	4.16	28.88	15308	19.26	3.57
HTST	4.13	50.07	15383	21.59	4.34
UHT	4.27	39.19	15150	23.44	3.35
UV	4.21	27.85	15264	16.72	4.69
s/•N	0.34	13.94	1058	3.86	0.94

s/•N, standard error of mean among treatments. N, sample size.

($d_{4,3}$), volume-weighted average diameter; ($d_{3,2}$), volume–surface average;

SSA, specific surface area; SPAN, size distribution width.

A change in the simulated Gaussian plot distribution of fat globules indicates a change in globule size (Evers, 2004b). Statistically, there is not significant difference,

probably due to the way the data is analyzed; the measured parameters only indicate a central tendency that does not take into account different regions. Figure 20 clearly shows a defined pattern in cream, so with a novel analysis by Garcia-Amezquita *et al.* (2009) a difference may be seen. They analyzed MFG size distribution by dividing the plot into four areas: smallest globules (0.1-1 μm), medium size globules (2-4 μm), large globules (5-11 μm), and out of range globules (20-70 μm). For this data set, four distinct areas are seen: small globules (0.4 -1.0 μm), mid-large globules (1.5-10 μm), out of range (20-60 μm), and aggregated globules (70-200 μm).

The centrifugation of milk, which was performed at 7,000g to concentrate fat globules, left residual fat globules with normal particle diameter (0.4 to 10 μm) and globules with bigger diameter (40 to 250 μm , fat globules aggregates) in the skim milk as seen in Figure 21.

Figure 21. Fat globule (FG) size distribution in skim milk showing residual fat globules aggregates and normal size fat globules.

Table 10 presents the calculated parameters that describe the size distribution in skim milk. Even though the data is not statistically different ($p > 0.05$), the modal diameter is

lower in UHT milk compared to other treatments. In regard to surface-volume, smaller values are observed for cooling temperature as well as UHT treatment.

Table 10. Parameters calculated from the size distributions of fat globules in skim milk.

Treatment	D(3,2) μm	Mode μm	SSA cm ² /mL	D(4,3) μm	SPAN
Fresh	50.83	146.95	2297	122.47	0.86
4C	18.77	140.93	3629	115.38	0.88
Batch	66.32	143.29	1412	130.33	0.70
HTST	62.12	144.81	2013	127.12	0.83
UHT	24.23	95.21	2556	79.03	1.55
UV	48.61	149.53	2771	124.76	0.92
s/•N	25.48	2.77	1148	18.01	0.32

s/•N, standard error of mean among treatments. N, sample size.

($d_{4,3}$), volume-weighted average diameter; ($d_{3,2}$), volume–surface average; SSA, specific surface area; SPAN, size distribution width.

C. Chemical Composition of Milk during Milk Processing

1. Milk Composition by FTIR Analysis

The fat, lactose, total solid, and protein content of milk were measured using a FTIR instrument. No significant difference was seen in the above parameters among the samples, as indicated in Table 11. On the other hand, FTIR artifact of β -lactoglobulin and casein complexes results in a lower casein content for the ultra high temperature (UHT) milk ($p = 0.05$). Citric acid content also shows a marginal difference ($p = 0.05$), especially in UHT milk, but such observations are attributed to detection limits of FTIR, as it has been shown that citric acid does not change with heating (Sommer and Hart, 1918). In addition, the urea content is high in UHT milk ($p < 0.0001$) probably due to induced protein catabolism at 121 °C and 15 psi, as urea naturally decomposes into ammonia and carbon dioxide (Metwalli *et al.*, 1996; Metwalli and van Boekel, 1998). With regard to free fatty acids (FFA), a significant lower content is observed in Fresh and UHT

compared to the other treatments ($p < 0.0001$). Similar findings were described in previous studies (Burton, 1988; Kintner and Day, 1965).

Table 11. Fat, lactose, total solid, and protein content of milk during milk processing.

Sample	g/100 mL milk							Acidity °TH	Urea mg/dL	Density SG	FPD mC	FFA mEkv/l
	Fat	Prot	Cas	Citric	Lac	TS	SNF					
Fresh	3.82	3.34	2.46 ^{a,b}	0.17 ^{a,b}	4.78	12.94	8.88	16.29	365 ^b	1028.41	515.94	0.294 ^b
4-C	3.91	3.34	2.43 ^{a,b}	0.17 ^{a,b}	4.76	13.04	8.99	15.86	320 ^c	1028.41	513.56	0.450 ^a
Batch	3.95	3.40	2.54 ^{a,b}	0.18 ^{a,b}	4.86	13.22	9.13	15.63	347 ^{b,c}	1029.26	522.44	0.517 ^a
HTST	3.88	3.25	2.35 ^b	0.17 ^b	4.64	12.78	8.76	15.68	327 ^{b,c}	1027.51	495.56	0.438 ^a
UHT	3.94	3.39	2.65 ^a	0.19 ^a	4.75	13.10	9.00	15.83	437 ^a	1028.85	521.06	0.324 ^b
UV	3.90	3.36	2.48 ^{a,b}	0.17 ^{a,b}	4.78	13.04	9.01	15.71	332 ^{b,c}	1028.72	513.00	0.535 ^a
S/•(N)	0.17	0.04	0.05	0.00	0.05	0.22	0.09	0.18	7.19	0.35	6.75	0.02

Prot, protein; Cas, casein; Citric, citric acid; Lac, lactose; TS, total solid; SNF, solid nonfat; FPD, freezing point depression; FFA, free fatty acid;
 °TH, Thorner degrees; SG, specific gravity; mEkv/l, milliequivalents/l
 S/•(N), standard deviation of the mean, which was calculated from three independent trials
 Subscripts within a column indicate statistically significant values ($p < 0.05$)

2. Isolation of Milk Fat Globule Membrane

The isolated MFGM material was not in accordance with previous reports mainly due to the extraction procedure, which did not include washes (Kitchen, 1977; Sánchez-Juanes *et al.*, 2009); however, the MFGM isolates in this project differed depending on the sample's temperature, as observed by Kim and Jiménez (1995). They described that higher temperatures resulted in less membrane and more insoluble product. In fact, there was a clear reduction in the isolated MFGM pellet as the pasteurization temperature increased. Figure 22 illustrates and compares the extracted MFGM where the UHT isolate is significantly different from the other treatments ($p < 0.0001$). As pasteurization temperatures increased, the final MFGM material decreased. Samples without heat treatment, such as Fresh, 4 °C, and cold pasteurization UV, had higher membrane material than those where heat was applied.

Figure 22. MFGM isolates of samples collected during milk processing. Values represent the mean weight of dry membrane material of four independent experiments, expressed as mg of MFGM (pellet upon ultracentrifugation) per liter of milk. Samples with different subscript are statistically significant different ($p < 0.0001$).

A discoloration was also observed in samples that underwent heat treatment, especially UHT (Figure 23). Corredig and Dalgleish (1998) also reported similar results in heated samples. According to their findings, the color of the MFGM material changed from brown-red in unheated isolates to white-yellow in samples derived from high heat treatments. They attributed the discoloration to the loss of iron content in the MFGM and hypothesized that heat induces the release of iron from xanthine oxidase, a metalloflavo protein, which has four residues holding iron molecules. Structural changes due to XO denaturation liberate iron as a function of heating temperatures since a decrease in iron is seen in temperatures as low as 62 °C. It also exposes the sulfhydryl bonds for chemical interchange and further denaturation. Unfortunately in this project, the iron content was not measured to correlate such observations. Discoloration was more intense in UHT followed by HTST samples.

Figure 23. Final MFGM material isolated from milk following a modified protocol by Patton *et al.* (1986). From left to right: Fresh, UHT, and UV MFGM pellets.

3. *Changes in the Amount of Protein in the Milk Fat Globule Membrane*

The extracted MFGM material was collected as pellet, and the amount of protein was measured using a colorimetric assay. Figure 24 illustrates the amount of protein present in each isolate where HTST had the highest amount of protein per 100 mg of MFGM pellet ($p < 0.0001$) and Fresh the lowest amount.

Figure 24. Effect of milk processing on the amount of protein in the milk fat globule membrane. Values represent the mean of three independent experiments, expressed as mg of protein per 100 mg of MFGM pellet. Samples with different subscript are statistically significant different ($p < 0.0001$; $n = 3$ experiments;; $n = 3$ measurement).

4. *Changes in the Phospholipid of the Milk Fat Globule Membrane*

The major five phospholipids in the MFGM were separated and quantified by HPLC in accordance to a protocol described by Rombaut *et al.* (2005). Figure 25 depicts a

typical chromatogram of the phospholipid standards where distinct peaks are seen for each PL. Sphingomyelin from bovine brain had three peaks, which are caused by the absence or presence of an extra hydroxyl group (Rombaut *et al.*, 2006c). These peaks are present in higher intensities compared to the first peak as reported in previous studies (Rombaut *et al.*, 2006c; Fong *et al.*, 2007). Sphingomyelin derived from eggs has only two peaks, peak 2 and 3 (Data not shown).

Figure 25. Normal-phase high performance liquid chromatography (HPLC) coupled with charged aerosol detector (CAD) chromatogram of phospholipid standards to quantify the PL from the MFGM, phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC) and sphingomyelin (SM) with three peaks.

Calibration curves were constructed from the standards to determine PL concentration in each milk sample. The area under each PL peak was calculated with ShimadzuLC software and correlated to individual PL concentration. Since the CAD response is approximately linear over a narrow range, a linear equation was applied to the standard curves, and the resulting formulas are presented in Table 12. All the R^2 values are higher or equal to 0.99, indicating a linear relationship between PL area and PL concentration.

Table 12. Linear equations of phospholipid standards and respective retention time (T_R).

PLs	T_R (min)	Equation*	R^2
PE	7.35	$PE_{con} = (3.8722 \times 10^{-5}) \times \text{Area} - 20.3624$	0.99
PI	8.42	$PI_{con} = (1.9307 \times 10^{-5}) \times \text{Area} - 1.0866$	0.99
PS	8.99	$PS_{con} = (2.1852 \times 10^{-5}) \times \text{Area} + 0.0872$	1.00
PC	9.91	$PC_{con} = (2.5123 \times 10^{-5}) \times \text{Area} - 13.6201$	1.00
SM	11.28	$SM_{con} = (3.3228 \times 10^{-5}) \times \text{Area} - 5.1631$	0.99

* Concentration was graphed as a function of PL area,

The actual sample chromatograms were a little different from the standards as seen in Figure 26. Even after SPE concentration and purification, the samples still had some neutral lipids (NL) and other unidentified peaks, which some of them have previously been identified as lactosylceramide, phosphatidic acid, or as oxidative products of PL. In addition, each PL class is composed of a complex mixture of fatty acids with different acyl chain length (Lopez *et al.*, 2008; Sánchez-Juanes *et al.*, 2009). In the chromatograms of samples, the PC peak is composed of four individual peaks as reported by Mènard *et al.* (2010).

Figure 26. Normal-phase liquid chromatography (LC) coupled with charged aerosol detector (CAD) chromatograms of (Black) PL standards and (Blue) 4 °C sample. PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin; NL, neutral lipids; PA, phosphatidic acid.

The reported effects of heating on the phospholipids of the milk fat globules are contradictory (Evers, 2004a). It is well established that milk lipids differ with lactation

(Bitman and Wood, 1990), diet (Lopez *et al.*, 2008) as well as with cow's breed among other factors; therefore, in this study, the same line of milk was used during the processing to discard variations from these factors. Statistical differences ($p < 0.05$) were observed among each trial (II, III, and IV), which can be attributed to physiological factors such as stage of lactation, time of the year, and sample preparation. The latter encourages lipid oxidation and promotes changes in total phospholipid content. In fact, higher PL concentrations are seen in trial IV compared to trial II (Appendix F).

After accounting for these variations by using blocks and trials as random effects in Minitab, no statistical difference was observed among the six samples (Figure 27). Phospholipids present in lower concentrations such as PI and PS are seen in Figure 28. The concentration of polar lipids that constitute part of the outer bilayer membrane, PC and SM, was very constant throughout milk processing as well as in the innermembrane PI and PS phospholipids. PE, which is located inside the membrane, illustrates a pattern that indicates a change in PE concentration as a function of thermal treatment, but more trials are needed to confirm such observations. These results add to the pool of uncertainty about the fluctuation of phospholipids during processing.

Figure 27. Polar lipid concentration (mg of PL per g of total lipids) in the milk fat globules. The PL means of two independent experiments are presented with the standard error of mean (s/•N).

Figure 28. Concentration of PI and PS (mg of PL per g of total lipid) in the milk fat globules. These PLs are found in lower amounts in the MFGM. The means of two independent experiments are presented with the standard error of mean (s/•N).

The reported polar lipid content in the MFGM is less than 1%, and in this study, the PL content is in the range of the data in literature (Rombaut *et al.*, 2006c; Sánchez-Juanes *et al.*, 2009; Jensen, 2002). In their review, Rombaut and Dewettinck (2006c) reported 0.35 to 0.53 g of polar lipids per 100 g of total lipid in isolated creams whereas Ménard *et*

al. (2010) reported 3.64 mg of polar lipid per g fat in milk. The amount of polar lipids in cream was 0.57 g of polar lipid per 100 of total lipids while the PLs in milk ranged from 7.7 to 20.5 mg of polar lipid per 100 g of milk.

The content of each polar lipid was analyzed as a percentage of the total five polar lipids present in the fat globules as mainly reported in most literature (Table 13). The last trial (IV) was included because the percentage of PLs were within the same range as the previous trials.

Table 13. Phospholipid content in fat globules

PL	Phospholipid g per 100 g polar lipid				
	PE	PI	PS	PC	SM
Fresh	18.70	3.96	1.66	31.20	44.49
4C	12.34	3.18	0.30	37.83	46.35
Batch	14.64	3.58	0.25	36.79	44.74
HTST	17.07	2.39	0.44	35.29	44.81
UHT	18.42	2.78	0.23	36.88	41.69
UV	11.50	4.23	0.77	36.99	46.52

Mean percentages of three independent studies

No statistical difference was observed in the analysis of PL percentages; however, the individual PL percentages are in accordance with previous reports (Astaire *et al.*, 2003; Rombaut *et al.*, 2006a). Among the PLs, SM was present in the highest concentration in all the samples (44.5-46.5%) followed by PC (31.2-37.0%) and PE (11.5-18.7%). PI and PS were present in lower concentrations, (2.4-4.2%) and (0.2-1.7%), respectively. Figure 29 shows a graphical representation of the mean of three trials (II, III, IV).

Figure 29. Phospholipid content of milk fat globules presented as percentage of the total phospholipids. The PL means of three independent experiments presented with standard error of mean (s/•N).

The experimental design did not allow for an evaluation of the phospholipid variation during processing, but a pattern may be observed in the mg/g fat data set. In fact, Figure 30 shows a global representation of the PL content in fat globules and illustrates that with a larger sample size a difference in polar lipid may be observed. The exact quantification of phospholipid is complicated by the different fatty acid moieties present on the acyl chains, which depend on different factors such as ruminants' diet.

Figure 30. Global representation of phospholipids in treated samples for three independent trials (II, III, and IV). PL content is presented as mean of three HPLC measurements (mg PL per g of fat).

5. *Changes in the Sulfhydryl (SH) & Disulfide SS Groups*

Thermal treatment induced SH/S-S exchange reactions and led to an increase in S-S groups. Figure 31 denotes a reduced in SH group level and an increased in SS group level as a function of thermal treatment. Disulfide bonds stabilize the folded conformation of proteins and decreases the conformational entropy leading to more SS group levels as heat treatment was increased. In fact, UHT milk had the lowest free SH groups and the highest SS groups.

Figure 31. Effect of milk processing on the free and total sulfhydryl (SH) and disulfide (SS) groups of milk fat globule membrane protein

D. Protein Profile from One Dimensional Gels Coupled with LC/MS

1. Milk Fat Globule Membrane Protein Profile

When milk is heated above 70 °C, whey protein denatures and deposits on the surface of the milk fat globules interacting with MFGM proteins. These protein interactions are important because they determine the physicochemical and functional properties of MFGM isolates. Figure 32 depicts a representative pattern of MFGM proteins resolved in 4-12% bis-tris NuPAGE gel after Coomassie staining. The protein profile displays several major bands identified in previous studies by molecular weight (Mather and Keenan, 1975; Evers, 2004a). Butyrophilin ran at apparent molecular weight of 60 kDa when its literature value is 67kDa, probably due to post-translational modifications such as phosphorylation that may affect its electrophoretic mobility. Some glycosylated proteins of the MFGM are not seen because a more sensitive staining technique is needed to identify their location by molecular weight.

Figure 32. Sodium dodecyl sulfate-PAGE pattern (4-12% gradient Bis-Tris NuPAGE gel) of milk fat globule membrane material isolated from different fractions of milk processing. 1&8) Novex Sharp Standards, 2) Fresh, 3) 4 °C, 4) Batch, 5) HTST, 6) UHT, and 7) UV.

Heat treatment resulted in high incorporation of β -lactoglobulin (β -LG, 18 kDa) in the MFGM isolates (lanes 4, 5, 6) as denoted by the staining intensity in the gel. The UHT treatment, in particular was enriched in caseins and β -LG. Even with a reducing gel, some high-molecular bands are observed in this sample (lane 6), indicating formation of protein complex as a function of time. According to Ye 2004, xanthine oxidase (XO) and butyrophilin (BTN) form complexes above 60 °C, which may be present in these bands. In fact, the bands that identify these two proteins are less intense in the UHT sample compared to the other treatments, confirming XO-BTN complex formation. In addition, fluctuations in other MFGM proteins are observed by changes in band intensity; however, these proteins were identified by comparing molecular weights were performed.

2. MFGM Protein Quantification by SDS-PAGE Densitometry

Table 14. Relative abundance of MFGM proteins during milk processing.

MFGM Proteins	MW (kDa)	Obs. MW	Relative Protein Abundance					
			F	4 °C	B	H	U	UV
Mucin 1 (MUC1)*	160-500	252	1.9	1.0	1.4	1.8		
BRCA1 and BRCA2	210	217	1.9	1.1	1.1	1.0	6.4	3.8
		181	1.0	1.2	1.1	1.3	1.6	1.0
		174	1.0	1.2				1.9
Xanthine oxidase (XO)+	150-155	151	4.6 ^a	4.7 ^a	4.2 ^a	4.3 ^a	1.0 ^b	4.3 ^a
		140	1.7	1.7	1.0	1.3	1.2	1.3
		131	2.9	1.6	1.0	1.5		1.9
		124					1.0	
		120	1.4	1.3	1.0	1.5		1.2
		110			1.1			1.0
		106			1.0			
Mucin 15 (MUC 15 or PAS III)*	94-100	101	1.2	1.0	1.3	1.0		1.5
Cluster of Differentiation (CD36 or PAS IV)	88	89-87	6.1	2.2	10.5	5.3	3.3	1.0
		80	1.1	1.0				1.3
Lactoferrin	78	76-77		2.2	1.7	1.0	1.5	1.5
Serum Albumin	69	70	8.9 ^a	11.5 ^a	11.7 ^a	9.7 ^a	1.0 ^b	10.4 ^a
		63	1.0	1.3	1.4	1.3		1.3
Butyrophilin (BTN)	59	59	2.1 ^a	2.3 ^a	2.0 ^a	2.2 ^a	1.0 ^b	2.0 ^a
		55-56	5.5	4.4	3.8	1.8	1.0	2.4
		53	1.3	1.0	1.8	2.8	3.5	1.0
Adipophilin (ADFP)	52	51-52	5.1 ^a	6.7 ^a	5.8 ^a	6.5 ^a	1.0 ^b	5.8 ^a
Lactadherin (PAS 6/7)++	43-59	49-50	6.7 ^a	9.1 ^a	7.0 ^a	8.9 ^a	1.0 ^b	8.0 ^a
MFG-E8								
Monocyte differentiation antigen (CD14)	40	41-43	1.0	2.9	2.0	1.7		4.6
Annexin	39	39	3.1 ^a	3.4 ^a	1.0 ^b	1.1 ^b	3.1 ^a	1.9 ^{a,b}
Mucin 15	36	38	3.6 ^a	2.9 ^b	1.0 ^c	1.1 ^c		2.3 ^b
		33-34	1.3	1.2	1.0	1.1	1.5	1.1
		31	1.0	1.2	2.0	1.2		1.0
		29			1.3	1.0		
α-(s2)- casein	24	25-26	1.0 ^c	1.6 ^{b,c}	2.8 ^b	1.4 ^c	6.3 ^a	1.3 ^c
α-(s1)- casein	23	21-22	5.6 ^a	4.8 ^{a,b}	2.1 ^{b,c}	2.5 ^{a,b,c}	1.0 ^c	4.7 ^{a,b}
κ-casein	19	20	1.4	1.0	1.2	1.1		1.1
β-lactoglobulin	18	18	1.8 ^c	1.2 ^c	10.3 ^b	10.2 ^b	20.5 ^a	1.0 ^c
α-lactalbumin	14.4	13	1.6 ^{b,c}	1.7 ^{b,c}	2.4 ^b	2.0 ^{b,c}	3.8 ^a	1.0 ^c

* Usually identified by PASS staining (Riccio, 2004)

++ Also known as MFG-E8

In order to assess the change in MFGM proteins upon milk processing, protein bands were identified and quantified by densitometry using the band trace tool (intensity peak $\times$ mm) in BioRad Quantity One software. The program was automated with the same settings for NuPAGE gels, and the relative abundance of proteins was calculated (Raw data, Appendix G. Table 14 shows the relative abundance of MFGM proteins among samples. Proteins were identified according to their molecular weight in literature, and those bands without a molecular weight match are expected to be unidentified proteins, polypeptides, or complexes of proteins. High-molecular weight proteins were more intense and detectable in Fresh sample (308 kDa and 284 kDa) as well as in the 4 °C sample (284 kDa). They may correspond to fatty acid synthase (275 kDa) and acetyl-CoA carboxylase (265 kDa). A 104 kDa band is also seen in UHT.

3. *MFGM Protein Identification through LC/MS*

SDS-PAGE is an excellent technique to visualize proteins; however, identification of proteins is limited by protein resolution; therefore, to assess the effect of milk processing into more depth, liquid chromatography connected with tandem mass spectrometry (LC-MS/MS) was used to detect more proteins from 1DE gel. Results were in accordance to previous studies where more than 120 proteins were identified in bovine MFGM (Reinhardt and Lippolis, 2006). In this study, 117 proteins were identified, which fluctuated depending on the milk processing treatment, especially when heat was applied. Among these proteins, 15 of them are still uncharacterized bovine proteins. Table 15 presents the probabilities of proteins that were identified according to the threshold parameters designated in Scaffold. The false discovery rate (FDR) for peptide identification was determined by Scaffold as 0.1%; thus, yielding high confidence in the identified proteins.

Table 15. MFGM proteins identified by LC-MS/MS upon milk processing.

Accession Number	MW (kDa)	Identified Proteins ⁺	Protein Probability (Identification)					
			Fresh	4°C	Batch	HTST	UHT	UV
<i>Lipid transport/metabolism</i>								
BT1A1_BOVIN	59	Butyrophilin subfamily 1 member A1	100	100	100	100	100	100
PLIN2_BOVIN	49	Perilipin-2 (ADFP)	100	100	100	100	100	100
XDH_BOVIN	147	Xanthine dehydrogenase/oxidase	100	100	100	100	100	100
FABPH_BOVIN	15	Fatty acid-binding protein, heart	100	100	100	100	100	100
CD36_BOVIN	53	Platelet glycoprotein 4	100	100	100	100	100	100
Q0VCZ8_BOVIN	78	Acyl-CoA synthetase long-chain family member 1	100	100	100	100	100	100
ACACA_BOVIN	265	Acetyl-CoA carboxylase 1	100	100	100	100	100	100
FAS_BOVIN	275	Fatty acid synthase	100	100	100	100	98	98
A4FUX1_BOVIN	25	CIDEA protein	100	100	100	100	16	100
Q3SX32_BOVIN	48	Mannose 6 phosphate receptor binding protein 1	100	100	83	100		100
Q9TSF1_BOVIN	14	Butyrophilin (Fragment)	42	98	97	100		
Q3ZC65_BOVIN	46	Ancient ubiquitous protein 1	30			99		
ERG7_BOVIN	83	Lanosterol synthase	58		74	100	100	
A9XTK4_BOVIN	38	17-beta hydroxysteroid dehydrogenase	100	99		100		41
NSDHL_BOVIN	40	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	73			100	58	100
A6H7G0_BOVIN	34	CYB5R3 protein		83	83	100	100	74
A6H7D4_BOVIN	52	Lysophosphatidic acid acyltransferase, zeta			83	100	40	71
A7YWR0_BOVIN	36	Apolipoprotein E			83	100		53
LIPL_BOVIN	53	Lipoprotein lipase				100	21	
FABP7_BOVIN	15	Fatty acid-binding protein, brain			100			
<i>Immunity & defense</i>								
Q3T0K7_BOVIN	48	MFGE8 protein	100	100	100	100	100	100
PIGR_BOVIN	82	Polymeric immunoglobulin receptor	100	100	100	100	100	100
Q3T101_BOVIN	25	IGL@ protein	100	100	100	100	100	100
A7E340_BOVIN	36	Mucin 15, cell surface associated	58	71	79	99	98	51
CTHL1_BOVIN	18	Cathelicidin-1	100	100	100	100		100
MUC1_BOVIN	58	Mucin-1	100	100	71	50		100
Q3SYR8_BOVIN	18	Immunoglobulin J chain	100	100	100	71		74
ANXA1_BOVIN	39	Annexin A1	73			50		100
B0JYP6_BOVIN	26	IGK protein	100	100	100			100
GLCM1_BOVIN	17	Glycosylation-dependent cell adhesion molecule 1		100	100	100	80	
A6QNL0_BOVIN	40	CD14		63	100	100	98	
Q1RMN8_BOVIN	25	Immunoglobulin light chain, λ gene cluster		100	100		75	

Accession Number	MW (kDa)	Identified Proteins [†]	Protein Probability (Identification)					
			Fresh	4°C	Batch	HTST	UHT	UV
PERL_BOVIN	81	Lactoperoxidase		63		50	99	
B5T263_9CETA	90	Toll-like receptor 2				97		
<i>Membrane/protein trafficking</i>								
RAB18_BOVIN	23	Ras-related protein Rab-18	100	100	100	100	100	100
RB11A_BOVIN	24	Ras-related protein Rab-11A	100	99	100	100	70	100
Q2T9M8_BOVIN	23	Synaptosomal-associated protein 23 isoform SNAP23A	100	100	99	100		100
SAR1A_BOVIN	22	GTP-binding protein SAR1a	100	100	98	100		100
RAB7A_BOVIN	24	Ras-related protein Rab-7a	100	100	100	100		74
YKT6_BOVIN	22	Synaptobrevin homolog YKT6	35	100		71		100
ARF1_BOVIN	21	ADP-ribosylation factor 1	100	73				74
A6QLH3_BOVIN	33	STX3 protein	100	51		50		71
RAB13_BOVIN	23	Ras-related protein Rab-13	73			100		
Q2TBH2_BOVIN	24	Related RAS viral (R-ras) oncogene homolog	100	99				100
RAB5C_BOVIN	23	Ras-related protein Rab-5C	73	100				
A5D7E0_BOVIN	23	RAB35 protein		100		71	39	74
RAB8A_BOVIN	24	Ras-related protein Rab-8A	99					
<i>G-protein mediated cell signaling</i>								
A1L528_BOVIN	23	RAB1A, member RAS oncogene family	100	100	100	100	100	100
GBB2_BOVIN	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	100	100	100	100	100	100
GBB1_BOVIN	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	100	83	83	100	100	99
GNAS2_BOVIN	46	Guanine nucleotide-binding protein G(s) subunit alpha isoforms short	100	73	83	100	32	
RAC1_BOVIN	21	Ras-related C3 botulinum toxin substrate 1	100	73	73	100		99
RAP1A_BOVIN	21	Ras-related protein Rap-1A	100	100	73	99		98
ARL15_BOVIN	23	ADP-ribosylation factor-like protein 15	98	97		100		74
B6VAP7_BOVIN	21	CDC42 protein	100	100		99		
A5D7R9_BOVIN	21	NRAS protein			97			100
RHOC_BOVIN	22	Rho-related GTP-binding protein RhoC	73			100		
GNAI1_BOVIN	40	Guanine nucleotide-binding protein G(i) subunit alpha-1	99				31	
<i>Protein synthesis, binding and/or folding</i>								
Q861V9_BOVIN	16	Similar to peptidylprolyl isomerase A (Cyclophilin A) (Fragment)	100	100	100	100	43	100
SEP15_BOVIN	18	15 kDa selenoprotein	73	73	99	100		74
B0JYL8_BOVIN	19	Cofilin 1	73	100	73	71		100
HSP7C_BOVIN	71	Heat shock cognate 71 kDa protein	100			50		

Accession Number	MW (kDa)	Identified Proteins [†]	Protein Probability (Identification)					
			Fresh	4°C	Batch	HTST	UHT	UV
Q99BK3_9ALPH	88	Ribonucleotide reductase large subunit	61	100	79			
A5D7N2_BOVIN	14	HIST2H2BE protein		99				98
A2VDV1_BOVIN	22	Ankyrin repeat domain 22		73		99		74
EF1A1_BOVIN	50	Elongation factor 1-alpha 1	100					28
A5D792_BOVIN	18	DCK protein						100
<i>Homeostatic regulator</i>								
D5HSX1_BOVIN	87	cGMP-dependant type II protein kinase	100	64		100		66
Q6QRN7_BOVIN	34	PP1201 protein	73	100		71		100
Q2KIS4_BOVIN	34	Dehydrogenase/reductase (SDR family) member 1	97			100		
B3VTM3_BOVIN	78	Lactoferrin			100	100	100	
ANXA5_BOVIN	36	Annexin A5			38	100		
ALBU_BOVIN	69	Serum albumin				100	100	
ACOC_BOVIN	98	Cytoplasmic aconitate hydratase	100					
<i>General Transporter</i>								
ABCG2_BOVIN	73	ATP-binding cassette sub-family G member 2	100	100	100	100	100	100
A8E4P3_BOVIN	31	STOM protein	100	100	100	100	50	100
NPT2B_BOVIN	76	Sodium-dependent phosphate transport protein 2B	100	100	100	100	100	
Q8MKB7_BOVIN	73	Na ⁺ /glucose cotransporter	73	83			100	51
5NTD_BOVIN	63	5'-nucleotidase	19	71		100	100	
CHP1_BOVIN	22	Calcium-binding protein p22	73	100				100
<i>Cell Structure</i>								
A6QNZ7_BOVIN	55	Keratin 10	100	100	71	100	100	98
A4FV94_BOVIN	61	KRT6A protein	73	100	79	100	100	74
A5D7M6_BOVIN	63	KRT5 protein	99	100		99	100	20
ACTB_BOVIN	42	Actin, cytoplasmic 1	100	100	100	100	58	100
Q17QL7_BOVIN	49	KRT15 protein	73	100			50	
GIPC2_BOVIN	35	PDZ domain-containing protein GIPC2	100			100		
<i>Cell surface receptor mediated signal transduction</i>								
A5PKG9_BOVIN	59	FGR protein	73			100		
ANXA2_BOVIN	39	Annexin A2	73	83		99	45	
D4QGC1_BOVIN	59	Tyrosine-protein kinase Lyn				100		
<i>Milk proteins</i>								
CASA1_BOVIN	25	Alpha-S1-casein	100	100	100	100	100	100

Accession Number	MW (kDa)	Identified Proteins [†]	Protein Probability (Identification)					
			Fresh	4°C	Batch	HTST	UHT	UV
CASK_BOVIN	21	Kappa-casein	98	100	100	100	100	100
CASA2_BOVIN	26	Alpha-S2-casein	73	100	100	100	100	100
Whey proteins								
B5B0D4_BOVIN	20	Major allergen beta-lactoglobulin	73	100	100	100	100	100
LACB_BOVIN	20	β-lactoglobulin			73	100	100	
Q28049_BOVIN	14	α-lactalbumin (Fragment)				100	100	
Q9TRB9_BOVIN	2	Enterotoxin-binding glycoprotein PP20K (Fragment)			28	100	100	
Unknown function								
ENPP3_BOVIN	100	Ectonucleotide pyrophosphatase/phosphodiesterase family member3	100	100	100	100	100	100
Q0IIA4_BOVIN	59	Glycoprotein 2	100	100	100	100		100
PPBT_BOVIN	57	Alkaline phosphatase				99		
A4FUH8_BOVIN	21	Chromosome 1 open reading frame 93 ortholog				99		

HTST, high temperature short time; UHT, ultra high temperature; UV, UV pulsed light treatment

It is well known that heat induces protein interactions and protein displacement as well as protein adsorption on the milk fat globule membrane (Singh, 2006; Evers, 2004a). LCMS identified these proteins and permitted the graphical representation of the number of proteins identified in each treatment (Figure 33). HTST milk had the highest protein identification, 83%, from the 102 total identified proteins. Tyrosine-protein kinase Lyn, alkaline phosphatase, chromosome 1 open reading frame 93 ortholog, and most importantly toll-like receptor 2 were four proteins exclusively found in HTST, indicating that heating may have contributed to fix these proteins to the MFGM. The Fresh and 4 °C treatments had almost the same number of proteins identified, 76 and 71, respectively, and UHT had the least number of identified proteins.

Figure 33. Percentage of identified proteins in each milk treatment from the 102 total characterized proteins identified by LC-MS .

The deoxycytidine kinase (DCK) enzyme was exclusively present in the UV pulsed treatment, and the ras-related protein Rab-8A and the cytoplasmic aconitase hydratase proteins were only present in the Fresh treatment. The elongation factor 1-alpha-1 was also present only in Fresh and with a low probability in the UV treatment. Appendix J shows the protein probability, identified peptide, and peptide coverage for each protein according to every treatment.

For MFGM, one of the greatest obstacles is identifying low abundance proteins due to the high abundance of some proteins such as butyrophilin, which results in fewer proteins being identified with high confidence; therefore, in order to minimize the high dynamic range in protein abundance that complicates proteomic analysis, 1DE gels were cut into molecular weight regions (I, II, III, and IV) for protein identification. Tables 16 through 19 depict the proteins present at specific intervals in the 1DE gels and possible interactions among MFGM proteins.

Table 16. Proteins identified in region I (higher than 150 kDa) of MFGM isolates through LC/MS.

Accession Number	MW (kDa)	Identified Proteins in >150 kDa Region (I)*	Protein Probability					
			F	4°C	B	H	U	UV
BT1A1_BOVIN	59	Butyrophilin subfamily 1 member A1	100	100	100	100	100	100
PLIN2_BOVIN	49	Isoform 2 of Perilipin-2	100	100	100	100	100	100
XDH_BOVIN	147	Xanthine dehydrogenase/oxidase	100	100	100	100	100	100
ACACA_BOVIN	265	Acetyl-CoA carboxylase 1	100	100	100	100	100	100
ABCG2_BOVIN	73	ATP-binding cassette sub-family G member 2	100	100	100	100	100	100
ENPP3_BOVIN	100	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	100	100	100	100	100	100
FAS_BOVIN	275	Fatty acid synthase	100	100	100	100	99	99
CD36_BOVIN	53	Platelet glycoprotein 4	100	100	100	100	100	100
Q3T0K7_BOVIN	48	MFGE8 protein	100	100	68	100	100	100
A6QNZ7_BOVIN	55	Keratin 10	100	100	68	100	100	98
K2C7_BOVIN	52	Keratin, type II cytoskeletal 7	100	100	68	99	50	58
Q0VCZ8_BOVIN	78	Acyl-CoA synthetase long-chain family member 1	100	100		100	100	100
A4FV94_BOVIN	61	KRT6A protein	100	100	68		100	99
MUC1_BOVIN	58	Mucin-1	100	100	68	50		100
NPT2B_BOVIN	76	Sodium-dependent phosphate transport protein 2B	100	100		100	100	
		Guanine nucleotide-binding protein G(I)/G(S)/G(T)						
GBB2_BOVIN	37	subunit beta-2	53	55			100	30
K2C71_BOVIN	57	Keratin, type II cytoskeletal 71	97	55			29	98
A7E340_BOVIN	36	Mucin 15, cell surface associated	30					17
RAB18_BOVIN	23	Ras-related protein Rab-18	99			49	50	58
Q17QL7_BOVIN	49	KRT15 protein	53				50	
B5B0D4_BOVIN	20	Major allergen beta-lactoglobulin			68	100	100	100
		Isoform Short of Polymeric						
PIGR_BOVIN	59	immunoglobulin receptor			100	100	100	
FABPH_BOVIN	15	Fatty acid-binding protein, heart				100	50	
ALBU_BOVIN	69	Serum albumin				100	100	
		Enterotoxin-binding glycoprotein						
Q9TRB9_BOVIN	2	PP20K (Fragment)				50	22	
PERL_BOVIN	81	Lactoperoxidase				98	100	
CASB_BOVIN	25	Beta-casein					50	58
		Pentatricopeptide repeat-containing protein 2					96	
PTCD2_BOVIN	44							
A4FUX1_BOVIN	25	CIDEA protein		98			22	100
		cGMP-dependant type II protein						
D5HSX1_BOVIN	87	kinase		99				58
		Glycosylation-dependent cell adhesion molecule 1		22		50	50	
GLCM1_BOVIN	17							
A6QQR3_BOVIN	36	APOL3 protein	98					
CASA1_BOVIN	25	Alpha-S1-casein	30					
HSP7C_BOVIN	71	Heat shock cognate 71 kDa protein	53					
Q2T9M8_BOVIN	23	Synaptosomal-associated protein		26				
		RAB1A, member RAS oncogene family		55				
A1L528_BOVIN	23							

Accession Number	MW (kDa)	Identified Proteins in >150 kDa Region (I)*	Protein Probability					
			F	4°C	B	H	U	UV
ANXA2_BOVIN	39	Annexin A2			68			
Q8MKB7_BOVIN	73	Na ⁺ /glucose cotransporter					100	
5NTD_BOVIN	63	5'-nucleotidase					50	
A8E4P3_BOVIN	31	STOM protein					50	
GBB1_BOVIN	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1					45	
A6QLW3_BOVIN	37	RPRD1B protein					99	
B3VTM3_BOVIN	78	Lactoferrin					100	
LACB_BOVIN	20	Beta-lactoglobulin					100	
Q28049_BOVIN	14	Alpha lactalbumin (Fragment)					100	
CASK_BOVIN	21	Kappa-casein					100	
ANXA1_BOVIN	39	Annexin A2						58

F, Fresh; B, Batch; H, HTST; U, UHT; UV

A total of 48 proteins were identified.

Table 17. Proteins identified in region II (higher than 55 and less than 150 kDa) of MFGM isolates through LC/MS.

Accession Number	MW (kDa)	Identified Proteins in >55<150 kDa Region (II)*	Protein Probability					
			F	4 °C	B	H	U	UV
BT1A1_BOVIN	59	Butyrophilin subfamily 1 member A1	100	100	100	100	100	100
PLIN2_BOVIN	49	Isoform 2 of Perilipin-2	100	100	100	100	100	100
XDH_BOVIN	147	Xanthine dehydrogenase/oxidase	100	100	100	100	100	100
Q3T0K7_BOVIN	48	MFGE8 protein	100	100	100	100	100	100
ABCG2_BOVIN	73	ATP-binding cassette sub-family G member 2	100	100	100	100	100	100
PIGR_BOVIN	59	Isoform Short of Polymeric immunoglobulin receptor	100	100	100	100	100	100
CD36_BOVIN	53	Platelet glycoprotein 4	100	100	100	100	100	100
Q0VCZ8_BOVIN	78	Acyl-CoA synthetase long-chain family member 1	100	100	100	100	100	100
A7E340_BOVIN	36	Mucin 15, cell surface associated	98	98	73	100	98	64
Q0IIA4_BOVIN	59	Glycoprotein 2 (Zymogen granule membrane)	100	100	100	100		100
NPT2B_BOVIN	76	Sodium-dependent phosphate transport protein 2B	100	100	100	100	99	
A4FV94_BOVIN	61	KRT6A protein	100	100	73	100	100	
K2C7_BOVIN	52	Keratin, type II cytoskeletal 7	50	100	73	100	100	
K2C71_BOVIN	57	Keratin, type II cytoskeletal 71		99		50	99	
A6QNZ7_BOVIN	55	Keratin 10	50	100		100	100	
GBB2_BOVIN	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	98				100	
D5HSX1_BOVIN	87	cGMP-dependant type II protein kinase	100			100		
ERG7_BOVIN	83	Lanosterol synthase	50		59	99	100	
ACHE_BOVIN	55	Acetylcholine receptor subunit epsilon	19			50	96	25

Accession Number	MW (kDa)	Identified Proteins in >55<150 kDa Region (II)*	Protein Probability					
			F	4 °C	B	H	U	UV
5NTD_BOVIN	63	5'-nucleotidase	25	99		100	100	
Q8MKB7_BOVIN	73	Na ⁺ /glucose cotransporter	50	54				64
Q9TSF1_BOVIN	14	Butyrophilin (Fragment)		19	98	16		62
CASA1_BOVIN	25	Alpha-S1-casein		100		50	99	64
GLCM1_BOVIN	17	Glycosylation-dependent cell adhesion molecule 1		100		50		
B3VTM3_BOVIN	78	Lactoferrin			100	100	100	
B5B0D4_BOVIN	20	Major allergen beta-lactoglobulin				100	100	
ALBU_BOVIN	69	Serum albumin				100	100	
Q9TRB9_BOVIN	2	Enterotoxin-binding glycoprotein PP20K (Fragment)				13	47	
PERL_BOVIN	81	Lactoperoxidase				12	50	
HSP7C_BOVIN	71	Heat shock cognate 71 kDa protein	100			50		
GBB1_BOVIN	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	12					
A6QQR3_BOVIN	36	APOL3 protein	100		98			
RAB18_BOVIN	23	Ras-related protein Rab-18	100					
ACOC_BOVIN	98	Cytoplasmic aconitate hydratase	100					
A4FUX1_BOVIN	25	CIDEA protein	37					
FABPH_BOVIN	15	Fatty acid-binding protein, heart	14					
Q08E14_BOVIN	138	Collagen, type III, alpha 1		100				
Q08DZ4_BOVIN	98	Smoothelin		98				
PPBT_BOVIN	57	Alkaline phosphatase				100		
LACB_BOVIN	20	Beta-lactoglobulin					100	
Q28049_BOVIN	14	Alpha lactalbumin (Fragment)					100	
CASK_BOVIN	21	Kappa-casein					100	
A6QNL0_BOVIN	40	CD14 protein					50	
Q17QL7_BOVIN	49	KRT15 protein					50	
A1L528_BOVIN	23	RAB1A, member RAS oncogene family					17	

F, Fresh; B, Batch; H, HTST; U, UHT; UV

A total of 46 proteins were identified

Table 18. Proteins identified in region III (higher than 35 and less than 55 kDa) of MFGM isolates through LC/MS.

Accession Number	MW (kDa)	Identified Proteins in >35<55 kDa Region (III)*	Protein Probability					
			F	4 °C	B	H	U	UV
BT1A1_BOVIN	59	Butyrophilin subfamily 1 member A1	100	100	100	100	100	100
PLIN2_BOVIN	49	Isoform 2 of Perilipin-2 (ADFP)	100	100	100	100	100	100
XDH_BOVIN	147	Xanthine dehydrogenase/oxidase	100	100	100	100	100	100
Q3T0K7_BOVIN	48	MFGE8 protein	100	100	100	100	100	100
ABCG2_BOVIN	73	ATP-binding cassette sub-family G Member 2	100	100	100	99	100	100
GBB2_BOVIN	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	100	100	100	100	100	100

Accession Number	MW (kDa)	Identified Proteins in >35<55 kDa Region (III)*	Protein Probability					
			F	4°C	B	H	U	UV
K2C7_BOVIN	52	Keratin, type II cytoskeletal 7	100	100	75	51	50	73
ACTB_BOVIN	42	Actin, cytoplasmic 1	100	100	100	100	50	100
GBB1_BOVIN	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	100	80	98	100	100	100
CASA1_BOVIN	25	Alpha-S1-casein	99	68	75	100	100	100
A4FV94_BOVIN	61	KRT6A protein	100	100		100	100	73
A9XTK4_BOVIN	38	17-beta hydroxysteroid dehydrogenase	100	80		100		64
A6QNZ7_BOVIN	55	Keratin 10	100	100		99	50	
GNA11_BOVIN	42	Guanine nucleotide-binding protein subunit alpha-11	100		36	100	99	73
GNAS2_BOVIN	44	Isoform Alpha-S1 of Guanine nucleotide-binding protein G(s) subunit alpha isoform	100		98	100	45	
Q3SX32_BOVIN	48	Mannose 6 phosphate receptor binding protein 1	100		75	100		100
NSDHL_BOVIN	40	Sterol-4-alpha-carboxylate 3-dehydrogenase	99			100	98	100
CD36_BOVIN	53	Platelet glycoprotein 4	99		75		100	73
A8E4P3_BOVIN	31	STOM protein	99			100		73
K2C71_BOVIN	57	Keratin, type II cytoskeletal 71	63			90		
Q8MKB7_BOVIN	73	Na ⁺ /glucose cotransporter	98	98				
Q17QL7_BOVIN	49	KRT15 protein	63	100				
ANXA2_BOVIN	39	Annexin A2	63	80		100	22	
GIPC2_BOVIN	35	PDZ domain-containing protein GIPC2	100			100		
ANXA1_BOVIN	39	Annexin A1	63			51		100
A6QLH3_BOVIN	33	STX3 protein	100	57		51		99
Q2KIS4_BOVIN	34	Dehydrogenase/reductase (SDR family) member 1	98			100		
A5PKG9_BOVIN	59	FGR protein	63			100		
A7MBH9_BOVIN	40	GNAI2 protein	100	60				
Q0VCZ8_BOVIN	78	Acyl-CoA synthetase long-chain family member 1		98				99
ACHE_BOVIN	55	Acetylcholine receptor subunit epsilon						55
PIGR_BOVIN	59	Isoform Short of Polymeric immunoglobulin receptor			100	100	100	100
A7YWR0_BOVIN	36	Apolipoprotein E			98	100		73
A6H7D4_BOVIN	52	Lysophosphatidic acid acyltransferase, zeta			75	100	47	73
B5B0D4_BOVIN	20	Major allergen beta-lactoglobulin			75	100	100	99
CASA2_BOVIN	26	Alpha-S2-casein				100	100	
A6H7G0_BOVIN	34	CYB5R3 protein		80	75	100	100	
A6QNL0_BOVIN	40	CD14 protein		66	100	100	99	
ANXA5_BOVIN	36	Annexin A5			51	100		
CASK_BOVIN	21	Kappa-casein				100	100	
ALBU_BOVIN	69	Serum albumin				99	100	
LIPL_BOVIN	53	Lipoprotein lipase				100	97	
EF1A1_BOVIN	50	Elongation factor 1-alpha 1	100					29
NPT2B_BOVIN	76	Sodium-dependent phosphate transport-2B	100					
HSP7C_BOVIN	71	Heat shock cognate 71 kDa protein	99					

Accession Number	MW (kDa)	Identified Proteins in >35<55 kDa Region (III)*	Protein Probability					
			F	4°C	B	H	U	UV
K1C17_BOVIN	49	Keratin, type I cytoskeletal 17		98				
PIPNA_BOVIN	32	Phosphatidylinositol transfer protein alpha isoform		97				
5NTD_BOVIN	63	5'-nucleotidase						
A1L528_BOVIN	23	RAB1A, member RAS oncogene family			75			
D4QGC1_BOVIN	59	Tyrosine-protein kinase Lyn				100		
RAB18_BOVIN	23	Ras-related protein Rab-18						100
LACB_BOVIN	20	Beta-lactoglobulin						100
Q28049_BOVIN	14	Alpha lactalbumin (Fragment)						100
CASB_BOVIN	25	Beta-casein						98
Q3T101_BOVIN	25	IGL@ protein						98
Q0IIA4_BOVIN	59	Glycoprotein 2						100

F, Fresh; B, Batch; H, HTST; U, UHT; UV

A total of 58 proteins were identified

Table 19. Proteins identified in region IV (less than 35 kDa) of MFGM isolates through LC/MS.

Accession Number	MW (kDa)	Identified Proteins in <35 kDa Region (IV)*	Protein Probability					
			F	4°C	B	H	U	UV
BT1A1_BOVIN	59	Butyrophilin subfamily 1 member A1	100	100	100	100	100	100
PLIN2_BOVIN	49	Isoform 2 of Perilipin-2 (ADFP)	100	100	100	100	100	100
XDH_BOVIN	147	Xanthine dehydrogenase/oxidase	100	100	100	99	100	100
Q3T0K7_BOVIN	48	MFGE8 protein	100	100	100	100	78	100
Q3T101_BOVIN	25	IGL@ protein	100	100	100	100	100	100
FABPH_BOVIN	15	Fatty acid-binding protein, heart	100	100	100	100	100	100
RAB18_BOVIN	23	Ras-related protein Rab-18	100	100	100	100	100	100
B5B0D4_BOVIN	20	Major allergen beta-lactoglobulin	72	100	100	100	100	100
CASA1_BOVIN	25	Alpha-S1-casein	100	100	100	100	100	100
A4FV94_BOVIN	61	KRT6A protein	72	100	68	100	100	98
A1L528_BOVIN	23	RAB1A, member RAS oncogene family	100	100	100	100	100	100
CASA2_BOVIN	26	Alpha-S2-casein	72	100	100	100	100	100
CASK_BOVIN	21	Kappa-casein	98	100	100	100	100	100
K2C7_BOVIN	52	Keratin, type II cytoskeletal 7	100	100	98	70	78	70
RB11A_BOVIN	24	Ras-related protein Rab-11A	100	100	100	100	69	100
A8E4P3_BOVIN	31	STOM protein	100	100	100	100		100
Q861V9_BOVIN	16	Peptidyl-prolyl cis-trans isomerase (Frag)	100	100	100	100	62	100
CASB_BOVIN	25	Beta-casein	100	100		100	78	100
A4FUX1_BOVIN	25	CIDEA protein	100	100	100	100		100
Q2T9M8_BOVIN	23	Synaptosomal-associated protein	100	100	100	100		100
CTHL1_BOVIN	18	Cathelicidin-1	100	100	100	100		100
Q3SYR8_BOVIN	18	Immunoglobulin J chain	100	100	100	70		70
RAC1_BOVIN	21	Ras-related C3 botulinum toxin substrate 1	100	73	68	100		97
SAR1A_BOVIN	22	GTP-binding protein SAR1a	100	100	99	100		100

Accession Number	MW (kDa)	Identified Proteins in <35 kDa Region (IV)*	Protein Probability					
			F	4°C	B	H	U	UV
RAB7A_BOVIN	24	Ras-related protein Rab-7a	100	100	100	100		70
RAP1A_BOVIN	21	Ras-related protein Rap-1A	100	100	68	99		98
B6VAP7_BOVIN	21	CDC42 protein	100	100		100		
SEP15_BOVIN	18	15 kDa selenoprotein	72	73	99	100		70
B0JYL8_BOVIN	19	Cofilin 1	72	100	68	70		100
MUC1_BOVIN	58	Mucin-1	100	98	57			27
B0JYP6_BOVIN	26	IGK protein	100	100	100			100
ABCG2_BOVIN	73	ATP-binding cassette sub-family G-2	72	100	68			100
Q0IIA4_BOVIN	59	Glycoprotein 2		98				70
Q2TBH2_BOVIN	24	Related RAS viral (R-ras) oncogene homolog	100	100				100
CHP1_BOVIN	22	Calcium-binding protein p22	72	100				100
ARF1_BOVIN	21	ADP-ribosylation factor 1	100	73				70
GBB2_BOVIN	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	72	73	47			
YKT6_BOVIN	22	Synaptobrevin homolog YKT6	72	100		70		100
ARL15_BOVIN	23	ADP-ribosylation factor-like protein 15	99	99		100		70
Q6QRN7_BOVIN	34	PP1201 protein	72	100		70		100
A5D7E0_BOVIN	23	RAB35 protein		100		99	64	70
Q1RMN8_BOVIN	25	Immunoglobulin light chain, λ gene cluster		100	100		70	
A5D7R9_BOVIN	21	NRAS protein			99			100
PROF1_BOVIN	15	Profilin-1				70		99
A4FUH8_BOVIN	21	Chromosome 1 open reading frame 93 ortholog				100		
A6QQR3_BOVIN	36	APOL3 protein		73	28			
A7YWR0_BOVIN	36	Apolipoprotein E				28		
A6QNZ7_BOVIN	55	Keratin 10		99	34	100	100	98
GLCM1_BOVIN	17	Glycosylation-dependent cell adhesion molecule 1		100	100	100	98	
Q9TRB9_BOVIN	2	Enterotoxin-binding glycoprotein PP20K (Fragment)		53	31	100	100	
LACB_BOVIN	20	Beta-lactoglobulin			68	100	100	
Q28049_BOVIN	14	Alpha lactalbumin (Fragment)				100	100	
Q0VCZ8_BOVIN	78	Acyl-CoA synthetase long-chain family member 1		44		27		42
K2C71_BOVIN	57	Keratin, type II cytoskeletal 71		99		66		47
ACTB_BOVIN	42	Actin, cytoplasmic 1		100				100
A6H7G0_BOVIN	34	CYB5R3 protein		73				100
GNAS2_BOVIN	44	Alpha-S1 of Guanine nucleotide-binding protein G(s) subunit alpha isoforms short CKLF-like MARVEL transmembrane domain containing 7		73				47
Q1JQ95_BOVIN	18							100
A5D7N2_BOVIN	14	Histone H2B		99				99
RAB5C_BOVIN	23	Ras-related protein Rab-5C	72	100				
RAB1B_BOVIN	22	Ras-related protein Rab-1B	95	58		70		
RAB13_BOVIN	23	Ras-related protein Rab-13	72			100		
RHOC_BOVIN	22	Rho-related GTP-binding protein RhoC	72			100		

Accession Number	MW (kDa)	Identified Proteins in <35 kDa Region (IV)*	Protein Probability					
			F	4°C	B	H	U	UV
RAB8A_BOVIN	24	Ras-related protein Rab-8A	99					
Q3SX32_BOVIN	48	Mannose 6 phosphate receptor binding protein 1		100				
1433B_BOVIN	28	Isoform Short of 14-3-3 protein beta/alpha		95				
PERL_BOVIN	81	Lactoperoxidase		73				
Q9TSF1_BOVIN	14	Butyrophilin (Fragment)		96				
FABP7_BOVIN	15	Fatty acid-binding protein, brain			100			
A7E340_BOVIN	36	Mucin 15, cell surface associated					31	
RBM22_BOVIN	47	Pre-mRNA-splicing factor RBM22						98
A5D792_BOVIN	18	DCK protein						100
GBB1_BOVIN	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1						70
FAS_BOVIN	275	Fatty acid synthase						54

F, Fresh; B, Batch; H, HTST; U, UHT; UV
A total of 74 proteins were identified

E. MFGM Protein Profile from 2-Dimensional Gel Coupled with MALDI-TOF

1. Visualization of MFGM Proteins with Fluorescent Dye

SyproRuby is one of the most sensitive stains in the market, capable of detecting as little as 0.5-1 ng of protein per mm² (Candiano *et al.*, 2004). In this research, this fluorescent dye was utilized in preliminary studies to determine the MFGM protein pattern. Studies have shown that the sensitivity of SyproRuby is superior to colloidal Coomassie brilliant blue (Berggren *et al.*, 2000). It specifically binds to proteins by non-covalent interactions, avoiding impurities in samples such as lipids. As observed in Figure 34, SyproRuby resulted in similar 2-DE maps as the ones analyzed in the research.

Figure 34. Representative 2-DE map of Batch (left) and high temperature short time (HTST) pasteurization treatments dyed with fluorescent dye, Sypro-Ruby. A total of 100 μ g of protein was loaded and stained with a 1:5 dilution of the dye.

2. *Visualization of MFGM Protein with Coomassie Blue*

For a better understanding of the changes in the MFGM proteins during milk processing, 2DE gels were performed on the six treatments. Different concentrations were tested to optimize the protocol and obtain good resolution as well as sensitivity. The use of sensitive colloidal stain, which utilizes Coomassie Blue (Westermeyer, 2006), permitted loading only 100 μ g of protein. Figure 35 depicts the 2DE pattern for each treatment.

Figure 35. Representative 2-DE map of MFGM proteins during milk processing. A 12.5% SDS-PAGE stained w sensitive colloidal. A) Fresh, B) 4 °C, C) Batch, D) HTST, E) UHT, and F) UV.

All the treatments had good resolution with the exception of UHT, which resulted in streaks and spotting, probably due to the presence of complexes (Figure 35.E).

3. MFGM Protein Variation during Milk Processing

With the help of Delta2D, the 2-D gels were compared in a two-way ANOVA using the Fresh sample as a control to determine protein fluctuation ($p < 0.01$). Figure 36 shows a graphical representation of the percent protein change between each treatment and the

control, Fresh milk. As expected, UHT had the highest percent change among the comparisons followed by the batch treatment. The complete ANOVA-2 results are shown in Appendix K.

Figure 36. Protein expression change during milk processing. 2DE gels were compared using a statistical program. Each treatment was compared against the Fresh sample, and the change in protein expression is indicated as a percentage (Delta2D, $p < 0.01$).

The global change during the entire milk processing, including the cold treatment, can be visualized in Figure 37 ($p < 0.01$). An increase in temperature induced a 86% change in MFGM proteins; however, almost 20% of this fluctuation was due to UHT. Batch pasteurization also produced a high protein change, probably due to the prolonged time during the treatment. Cold pasteurization resulted in a protein fluctuation similar to 4 °C. Analysis of the treatments without heat (Fresh, 4 °C, and UV) resulted in a 52% change (Figure 37).

Figure 37. Protein expression change during milk processing. 2DE gels were compared using a statistical program. All the treatments were compared against the Fresh sample (6 Treat), without UHT (5 Treat), and no heat application (Fresh, 4 °C, UV). The change in protein expression is indicated as a percentage.

This data indicates that protein variation during milk processing can be visualized with 2DE gels and analyzed with Delta2D; but the latter is a tool that can be further used to help us interpret results.

4. Identification of MFGM Proteins during Milk Processing

Milk fat globule membrane proteins that are altered during milk processing were detected using Peptide Mass Fingerprinting (PMF). More than 300 spots were detected on the 2-dimensional gels with a pI range of 3 to 7 that differed significantly ($p < 0.01$) as a function of temperature. Region with high variation were chosen for further identification; however, only 65% of the these spots were successfully identified via MALDI-TOF. Limitations such as incompleteness of bovine protein databases and relatively high threshold parameters used to define a positive identification may have reduced the number of hits obtained. In addition, some proteins may have been irreversibly denatured and not rehydrated during the 2D sample preparation step, which avoided their final detection.

Among the identified proteins that vary during cold storage (4 °C), many isoforms of the major MFGM proteins were observed in accordance to previous studies (Fong *et al.*, 2007). Butyrophilin was detected in different areas of the composite gel image (proteome map) of the Fresh/4 °C set (Figure 35). These results corroborate previous observations that BTN is present in multiple forms. Indeed, Vanderghem, *et al.* (2008) identified three groups, 67 kDa, 62 kDa, and a third group at 80 kDa. The BTN present in this study has molecular weight of approximately 52 kDa.

Figure 38. Proteome map (Delta 2D) depicting 104 spots from the 4 °C milk sample versus the Fresh milk sample (control). The proteome map represents average pixel volumes for each protein spot. Labeled spots showed changes in abundance in response to cold storage temperature (two-way ANOVA , $p < 0.01$, total spots 305).

Lactadherin also varied in abundance during the storage conditions of milk, and it was observed as a train of spots at a molecular weight of approximately 90 kDa. Previous studies described two groups of lactadherin, with 60 kDa and 80 kDa (Vanderghem *et al.*,

2008). Other molecules that were altered in concentration during cold condition were adipophilin and perilipins. Mather (2000) reported two adipophilin with different isoelectric points (pI), 7.5 and 7.8. In another study, five isoelectric variants were found in 2DE gels. In this research, adipophilin with a predicted pI of 5.85 was observed, and the lower pI is probably due to phosphorylation (Table 20). The constant region of immunoglobulin heavy chain was identified indicating that immunoglobulins are altered and even denatured during cold temperatures. Fong *et al.* (2007) also identified this protein in the MFGM. For a positive identification, a minimum of two peptides was required along with a Mascot score of 50.

Table 20. Identified proteins in the proteome map from 4 °C milk sample versus the Fresh milk sample that vary during milk processing.

Spot ID	Protein ID	MW (kDa)	pI	GenBank ID	Mascot Score	Pep	Seq. (%)
5	Butyrophilin	59.21	5.11	gil2266432	283	6	25
2	Polymeric immunoglobulin receptor precursor	82.47	7.68	gil296479365	69	2	9
6	Perilipin-2	49.31	8.72	gil296484843	276	7	30
9	rho-related GTP-binding protein ThoC-like	21.93	6.20	gil327271431	246	2	23
10	Perilipin-2	49.31	8.72	gil296484843	472	8	36
11	Perilipin-2	49.31	8.72	gil296484843	414	9	36
12	ADFP protein	49.04	5.85	gil74354901	82	3	11
13	ADFP protein	49.04	5.85	gil74354901	49	4	17
16	ADFP protein	49.04	5.85	gil74354901	115	4	15
17	ADFP protein	49.04	5.85	gil74354901	114	4	13
20	rab GDP dissociation inhibitor beta	50.46	5.94	gil76253900	47	2	11
22B	Perilipin-2	49.31	8.72	gil296484843	204	6	27
24	ADFP protein	49.31	5.85	gil74354901	122	5	13
26	Perilipin-2	49.31	8.72	gil296484843	94	4	19
27	Perilipin-2	49.31	8.72	gil296484843	357	9	36
28	Butyrophilin-Fragment *	14.13	4.99	gil40388469	44	1	2
29	Butyrophilin	59.21	5.11	gil2266432	285	5	21
30	Butyrophilin subfamily 1 member A1 precursor †	59.22	5.10	gil27806233]	194	5	32
32	Protein phosphatase 1J	87.05	8.94	gil296208863	204	3	26
35A	Fatty acid-binding protein, heart	14.77	6.73	gil27805809	280	5	28
35B	Fatty acid-binding protein, heart	14.77	6.73	gil27805809	316	5	28
36	CDC42 protein	21.23	5.5	gil210062862	173	3	21

Spot ID	Protein ID	MW (kDa)	pI	GenBank ID	Mascot Score	Pep	Seq. (%)
38	immunoglobulin heavy chain constant region §	49.91	5.09	gil34538498	86	3	25
39	immunoglobulin heavy chain §	49.91	5.09	gil34538498	122	3	25
40	immunoglobulin heavy chain §	49.91	5.09	gil34538498	74	2	30
41	Butyrophilin	59.21	5.11	gil2266432	248	4	26
42	Butyrophilin	59.21	5.11	gil2266432	281	5	21
43	Butyrophilin	59.21	5.11	gil2266432	176	5	21
44	Butyrophilin	59.21	5.11	gil2266432	260	4	26
45	Butyrophilin	59.21	5.11	gil2266432	149	4	27
47	Butyrophilin	59.21	5.11	gil2266432	159	3	20
48	Butyrophilin	59.21	5.11	gil2266432	377	6	39
49	Butyrophilin	59.21	5.11	gil2266432	276	5	34
50	Butyrophilin	59.21	5.11	gil2266432	322	6	34
53	Alpha S1 casein*			gil159793193	68	1	8
54	Butyrophilin subfamily 1 member †	59.22	5.10	gil27806233	71	2	11
55	Butyrophilin subfamily 1 member †	59.22	5.10	gil27806233	143	4	21
56	Butyrophilin subfamily 1 member †	59.22	5.10	gil27806233	165	5	27
59A	Butyrophilin subfamily 1 member †	59.22	5.10	gil27806233	148	5	27
59B	Butyrophilin subfamily 1 member †	59.22	5.10	gil27806233	179	5	27
60	Butyrophilin subfamily 1 member †	59.22	5.10	gil27806233	108	4	22
63	Lactadherin	47.82	6.80	gil296475529	385	8	28
63	Lactadherin	47.82	6.80	gil296475529	423	8	28
65	Lacaderhin	47.82	6.80	gil296475529	536	10	35
68	ADFP protein	49.04	5.85	gil74354901	143	5	13
74	Perilipin-2	49.31	8.72	gil296484843	185	7	34
78	Perilipin-2*	49.31	8.72	gil296484843	49	1	13
91	Butyrophilin	59.21	5.11	gil2266432	173	5	21

* Marginal identification

† Butyrophilin subfamily 1 member A1 precursor; § Immunoglobulin heavy chain constant region
Pep. match, Peptide matches; Seq. (%), sequence coverage (%); MW, molecular weight.

After milking, milk goes into cold storage where it remains from hours to days before pasteurization. In this research, three types of pasteurization were performed on the milk after storage. Batch pasteurization (65 °C, 30 m) resulted in dramatic protein alterations. In fact, 344 spots were detected, from which 175 were significantly different ($p < 0.01$) as shown in Figure 39. The main proteins that change in concentration during batch treatment are alpha casein, CSN2, CSN3, β -lactoglobulin, and α -lactalbumin. The results from the 1D-LC/MS analysis highlighted the same findings (Figure 36).

Figure 39. Proteome map (Delta 2D) depicting 175 spots from Batch milk sample versus the Fresh milk sample (control). The proteome map represents average pixel volumes for each protein spot. Labeled spots showed changes in abundance in response to thermal treatment (65 °C, 30 m; two-way ANOVA , p < 0.01, total spots 344).

As in the 4 °C sample, butyrophilin, ADFP, and perilipins were also altered. A change in lactoferrin was specific to this thermal treatment as well as in the folate receptor 1-like protein (Table 21). A train of spots in the 90 kDa region was identified as polymeric immunoglobulin receptor (PIGR), which was in an area close to the lactadherin protein, which also fluctuated during this treatment.

Table 21. Identified proteins in the proteome map from Batch milk sample versus the Fresh milk sample that vary during milk processing.

Spot ID	Protein ID	MW (kDa)	pI	GenBank ID	Mascot Score	Pep	Seq. (%)
15	CSN3 protein- Kappa casein	21.22	6.83	gil36988716	128	4	24
16	CSN3 protein- Kappa casein	21.22	6.83	gil36988716	81	2	16
18	Peptidyl-prolyl cis-trans isomerase A †	17.86	8.34	gil47523764	139	3	23
23	Peptidyl-prolyl cis-trans isomerase A †	17.86	8.34	gil47523764	55	2	17
25	Perilipin-2	49.31	8.72	gil296484843	522	9	38
26	Perilipin-2	49.31	8.72	gil296484843	443	9	38

Spot ID	Protein ID	MW (kDA)	pI	GenBank ID	Mascot Score	Pep	Seq. (%)
29	Folate receptor 1-like	52.81	8.09	gil297460991	311	7	32
38	Lactoferrin	78.03	8.73	gil9650974	179	6	27
40	Lactoferrin	78.03	8.73	gil9650974	139	5	27
65	Folate receptor 1-like	52.81	8.09	gil297460991	253	5	26
78	Butyrophilin	59.21	5.11	gil2266432	151	5	27
86	Alpha S1 casein	18.67	5.23	gil159793193	375	6	28
87A	CSN1S2 protein-alpha-S2-casein	26.07	8.55	gil92097520	125	3	17
87B	CSN1S2 protein-alpha-S2-casein	26.07	8.55	gil92097520	150	4	22
88	Alpha S1 casein	18.67	5.23	gil159793193	343	6	28
90A	CSN1S2 protein-alpha-S2-casein	26.07	8.55	gil92097520	125	4	22
91	CSN2 protein-beta casein	25.20	5.53	gil83406093	49	2	16
94	CSN2 protein-beta casein	25.20	5.53	gil83406093	127	3	16
95	CSN2 protein-beta casein	25.20	5.53	gil83406093	96	3	16
100	Beta lactoglobulin	17.16	4.75	gil162748	283	6	31
101	Beta lactoglobulin	17.16	4.75	gil162748	309	6	35
103A	Beta lactoglobulin	17.16	4.75	gil162748	283	6	31
103B	Alpha lactalbumin	16.21	4.92	gil7542833	76	2	10
104A	Keratin, Type 1 cytoskeletal- Bovine	54.50	4.97	gil296476308	126	3	12
104B	Fatty acid-binding protein, heart	14.77	6.73	gil27805809	324	6	28
105	CSN3- kappa casein	21.22	6.83	gil36988716	115	4	24
106	CSN3- kappa casein	21.22	6.83	gil36988716	140	4	24
107	CSN3- kappa casein	21.22	6.83	gil36988716	96	3	24
108	CSN3- kappa casein	21.22	6.83	gil36988716	117	3	24
111	Actin, beta cytoplasmic	41.68	5.29	gil148744172	90	2	16
115	Butyrophilin	59.21	5.11	gil2266432	98	3	16
118	PIGR	82.46	7.08	gil151555693	98	3	18
119	PIGR	82.46	7.08	gil151555693	142	7	11
120	PIGR	82.46	7.08	gil151555693	144	4	25
121	PIGR	82.46	7.08	gil151555693	143	2	9
122	PIGR	82.46	7.08	gil151555693	129	3	18
124	PIGR	82.46	7.08	gil151555693	68	2	9
127	5'-nucleotidase †	62.84	6.22	gil194035289	90	3	13
128	5'-nucleotidase †	62.84	6.22	gil194035289	99	2	10
129	Lactadherin	47.82	6.80	gil296475529	133	4	12
133	IgG1 Heavy Chain constant region	35.83	6.09	gil7547266	120	2	22
143	ADFP protein	49.04	5.85	gil74354901	43	2	17
147	Lactadherin	47.82	6.80	gil296475529	42	2	14
154	PIGR	82.46	7.08	gil151555693	140	7	7
155	PIGR	82.46	7.08	gil151555693	115	4	6
161	ALB-serum albumin	69.19	5.88	gil74267962	56	3	18
163	Serotransferrin precursor	77.69	6.75	gil114326282	104	3	14
164	Perilipin-2	49.31	8.72	gil296484843	384	8	31
167	Butyrophilin	59.21	5.11	gil2266432	42	1	6

Pep. match, Peptide matches; Seq. (%), sequence coverage (%); MW, molecular weight.

† Sus scrofa

In the high temperature short time (HTST) pasteurization treatment, 350 spots were identified, and 128 spots were temperature significant ($p < 0.01$) (Figure 40). The

proteome map is similar to the 4 °C sample, but with other alterations as in the batch milk. Lactoferrin also fluctuated during this treatment.

Figure 40. Proteome map (Delta 2D) depicting 128 spots from high temperature short time (HTST) milk sample versus the Fresh milk sample (control). The proteome map represents average pixel volumes for each protein spot. Labeled spots showed changes in abundance in response to thermal treatment (72 °C, 16 s; two-way ANOVA , $p < 0.01$, total spots 350).

The main proteins that change in concentration during HTST are β -lactoglobulin and α -lactalbumin but in a smaller degree compared to batch treatment. A GTP-binding protein SAR1a varied during this treatment (Table 22). GTP-binding proteins play a role in regulating exocytosis.

Table 22. Identified proteins in the proteome map from HTST milk sample versus the Fresh milk sample that vary during milk processing.

Spot ID	Protein ID	MW (kDa)	pI	GenBank ID	Mascot Score	Pep	Seq. (%)
2	GTP-binding protein SAR1a	22.39	6.22	gil72535188	124	2	12
9	Butyrophilin	59.21	5.11	gil2266432	182	3	20

Pep. match, Peptide matches; Seq. (%), sequence coverage (%); MW, molecular weight.

Ultra high temperature (UHT) pasteurization resulted in 186 spots that were temperature significant ($p < 0.01$). The casein profile underwent a new pattern in the proteome map compared to batch and HTST pasteurization (Figure 41).

Figure 41. Proteome map (Delta 2D) depicting 186 spots from ultra high temperature (UHT) milk sample versus the Fresh milk sample (control). The proteome map represents average pixel volumes for each protein spot. Labeled spots showed changes in abundance in response to high thermal treatment (121 °C, 3 m; two-way ANOVA , $p < 0.01$, total spots 277).

Streaking diminished the number of identified proteins due to the complexity in matching spots between the UHT samples and Fresh milk. Nevertheless, the proteome map depicts clear changes in the casein, β -lactoglobulin, and α -lactalbumin proteins. The major milk allergen, β -lactoglobulin fragment, was identified in this thermal treatment (Table 23).

Table 23. Identified proteins in the proteome map from UHT milk sample versus the Fresh milk sample that vary during milk processing.

Spot ID	Protein ID	MW (kDa)	pI	GenBank ID	Mascot Score	Pep	Seq. (%)
1A	CSN3 protein- Kappa casein	21.22	6.83	gil36988716	62	2	15
1B	CSN3 protein- Kappa casein	21.22	6.83	gil36988716	150	3	24
2A	CSN3 protein- Kappa casein	21.22	6.83	gil36988716	88	4	24
2B	CSN3 protein- Kappa casein	21.22	6.83	gil36988716	115	3	23
3A	CSN3 protein- Kappa casein	21.22	6.83	gil36988716	156	4	24
3B	CSN3 protein- Kappa casein	21.22	6.83	gil36988716	87	4	22
4A	CSN3 protein- Kappa casein	21.22	6.83	gil36988716	118	4	24
4B	CSN3 protein- Kappa casein	21.22	6.83	gil36988716	56	2	16
10	Major Allergen Beta-lactoglobulin	19.96	4.85	gil195957138	71	3	21
11	CSN3 protein- Kappa casein	21.22	6.83	gil36988716	104	4	24
158A	Major Allergen Beta-lactoglobulin	19.96	4.85	gil195957138	211	4	23
158B	Major Allergen Beta-lactoglobulin	19.96	4.85	gil195957138	157	4	17
160B	Beta lactoglobulin	17.16	4.75	gil162748	186	5	22
165	Alpha lactalbumin	16.21	4.92	gil7542833	146	3	20
166	Alpha lactalbumin	16.21	4.92	gil7542833	74	1	8

Pep. match, Peptide matches; Seq. (%), sequence coverage (%); MW, molecular weight.

The last variant in this study was cold pasteurization via pulse ultraviolet treatment (UV). The proteome map permitted the detection of 344 spots from which 116 were temperature significant ($p < 0.01$, Figure 42). The percent protein change (37%) in the UV treatment versus Fresh milk is in the same range as cold storage and HTST thermal treatment; however, visually the proteome map looks more like the 4 °C sample.

Figure 42. Proteome map (Delta 2D) depicting 116 spots from cold pasteurization (pulsed ultraviolet, UV) milk sample versus the Fresh milk sample (control). The proteome map represents average pixel volumes for each protein spot. Labeled spots showed changes in abundance in response to thermal treatment (1.0 Joules/cm²/pulse; two-way ANOVA , $p < 0.01$, total spots 344).

Proteins with high carbohydrate content were not identified as altered proteins during milk processing, probably due to inhibitory action from the carbohydrate moieties, which may inhibit trypsin digestion and/or impair ionization of glycopeptides during mass spectrometry analyses.

V. DISCUSSION

A. Effects of Milk Processing on the Physical Properties of the MFGM

The particle size distribution in milk was between 0.4 to 10 μm in diameter in accordance to literature (Gassi *et al.*, 2008; Fong *et al.*, 2007; Mènard *et al.*, 2010). The Fresh milk has the highest volume-surface diameter ($d_{3,2}$), indicating the presence of small fat globules with larger surface area in unprocessed milk (Table). Cooling, thermal

treatment, and cold pasteurization did not have a pronounced influence on the particle size of milk; however, the fat globule size distribution width (Span) in these samples is wider, implying the presence of smaller vesicles and larger fat globules. Milk processing might induce the formation of smaller vesicles, which were first described by Waninge *et al.* (2004) utilizing cryogenic transmission electron microscopy (Cryo-TEM). They proposed that milk procedures such as cooling and homogenization lead to phospholipid release from the membrane and gives birth to phospholipid vesicles. These vesicles could also originate from milk fat globule coalescence and consist of lost membrane material (Waninge *et al.*, 2004). Walstra (1969) highlighted the presence of small fat globules (< 200 nm) even in milk after milking (Walstra, 1969); however, Michalski *et al.* (2002) later concluded that small fat droplets (<500 nm) start to appear in early processing stages but not right after milking. Using confocal microscopy, Gallier *et al.* confirmed variation in fat globule size in milk upon milk processing (Gallier *et al.*, 2010a).

With respect to the fat globules in cream, there is more variation in the size distribution, which ranges from 0.4 μm to 200 μm . Four peak areas are visible, which correspond to small globules (0.4 -1.0 μm), mid-large globules (1.5-10 μm), out of range (20-60 μm), and aggregated globules (70-200 μm). Gassi *et al.* (2008) observed fat globules from 0.02 μm to 200 μm with three peaks centering at 0.5 μm , 4 μm , and 45 μm . The large fat globules (>20 μm) were more pronounced in cream with medium and high heat treatments, and the results were ascribed to the fusion of smaller globules to form larger ones due to coalescence. Addition of SDS decreased the third peak (45 μm) indicating that it corresponds to aggregates of fat globules. In this study, it is expected that the third and four distributions represent fat globule aggregates of large globules since large fat globules are more predisposed to coalescence and lipolysis during milking

operations such as pumping and agitation (Wiking *et al.*, 2004). In fact, these areas are very prominent in milk that underwent heat pasteurization, especially in HTST, Batch, and UHT, whereas the samples that did not undergo heat treatment exhibit a higher percent volume in the normal size fat globule range. Prolonged and higher temperature treatments, such as in Batch and UHT, may have deactivated lipases and contributed to the stability of fat globules and therefore to less coalescence as compared to the HTST milk. Lipases are not always completely inactivated during regular pasteurization temperatures, so lipid breakdown continues during the milk's lifespan after heat treatment as well as of free fatty acid production. Furthermore, thermal treatment resulted in smaller fat globules ($< 0.5 \mu\text{m}$), which may correspond to vesicles formed by MFGM fragments composed of phospholipids and proteins as previously discussed.

B. Effects of Milk Processing on the Chemical Composition of the MFGM

1. Changes in Milk Composition

No significant difference was seen in the fat, lactose, total solid, and protein content of milk ($p > 0.05$). However, the casein content was marginally higher for UHT milk ($p = 0.05$). Complexes between β -lactoglobulin and casein may explain this observation. In buttermilk, higher casein content has been attributed to thermal treatment since whey proteins are titrated with the casein fraction, making casein value higher (Gassi *et al.*). The citric acid content also is higher in UHT milk compared to the other milk treatments ($p = 0.05$). Such findings were ascribed to the detection limits of FTIR as research indicates that citric acid does not vary with heating (Sommer and Hart, 1918). The urea content in UHT milk was higher ($p < 0.0001$) probably due to induced protein catabolism at 121°C at 15 psi as urea naturally decomposes into ammonia and carbon dioxide

(Metwalli *et al.*, 1996; Metwalli and van Boekel, 1998). This may also explain the higher urea content in the Fresh sample that gradually decreases in concentration in milk. The Fresh and UHT milk also exhibit a lower free fatty acid (FFA) content ($p < 0.0001$) in accordance to literature (Burton, 1988; Kintner and Day, 1965). Kintner and Day (1965) found that as the intensity of thermal treatment increases, the amount of FFA decreased. Higher FFA content in milk has been accredited to the survival of heat-resistant lipases of natural milk origins or produced by psychrotrophic bacteria during cold storage (Burton, 1988). The higher FFA content in 4 °C, Batch, HTST, and UV indicate instability of fat globules probably due to lipase activity from microbiological or natural origins or from fat globule damage during milk processing. In fact, lipoprotein lipase reaches optimal binding to MFGM after four hours of milking, and thereby, contributed to the high FFA in these samples (Dickow *et al.*, 2011; Wiking *et al.*, 2005).

2. *Effects on MFGM Isolates & Protein*

The MFGM isolates obtained, ranging from 171 to 315 mg of dry weight (pellet) per liter of milk, were higher than that previously described in literature, which correspond to approximately 95 mg dry weight per liter of fresh milk (Sánchez-Juanes *et al.*, 2009). These high values may have resulted from omission of cream washes that contribute to more material in the final product. Furthermore, the amount of MFGM isolate decreased as a function of thermal treatment as observed in previous research (Kim and Jiménez-Flores, 1995). According to their findings, higher temperatures resulted in less membrane and more insoluble product. The insolubility of the MFGM was not measured, but it was noticed that MFGM material from UHT sample had a difficult time going into solution. In addition, the isolated MFGM pellet in pasteurized samples was lower in protein content than those samples without thermal treatment ($p < 0.0001$). The amount of

protein in these isolates was lower than those previously reported, 48.7 mg of protein per 100 mg of MFGM (Sánchez-Juanes *et al.*, 2009). In this research, the protein in the isolates varies from 13.3 to 17.7 mg of protein per 100 mg of MFGM isolates, explaining the high amount of MFGM in the final pellets, which may contain other milk components. Thermal treatment resulted in higher protein content in the isolates compared to Fresh milk, indicating interactions between MFGM proteins and milk components, especially proteins, as temperature increased.

Discoloration was also observed in MFGM isolates as thermal treatment increased in accordance to other authors (Morin *et al.*, 2007b; Corredig and Dalgleish, 1998). Milk without thermal treatment such as Fresh and UV were reddish-brown compared to yellow-white from HTST milk. This result is believed to be caused by a decrease in the amount of iron in the MFGM. Corredig and Dalgleish actually measured the iron content by atomic absorption in samples that underwent heat treatment, concluding that the amount of iron in the MFGM decreased as a function of heating temperature even in temperatures as low as 62 °C. They hypothesized that heat induces the release of iron from xanthine oxidase, which has four residues holding an iron molecule. Iron release coupled with structural changes in XO make the protein available for disulfide exchange with other cyteine-containing proteins causing permanent denaturation and iron loss. Morin *et al.* (2007b) supported this hypothesis with a pronounced lower ash content in pasteurized cream compared to raw cream. We hypothesized that other iron-containing proteins such as lactoferrin denaturation may contribute to the discoloration, which becomes more prominent in ultra high temperature milk.

3. *Effects on the Phospholipid Profile of the MFG*

Separation and quantification of milk polar lipids were in accordance to literature (Rombaut *et al.*, 2005). The five major entities were isolated as single peaks with the exception of sphingomyelin (SM), which depicted three peaks. Peak 2 and 3 have been interpreted as derivatives of SM with extra hydroxyl groups (Rombaut *et al.*, 2006a). In other studies, three peaks were reported corresponding to different sphingoloid bases and fatty acids (Lopez *et al.*, 2008; Fong *et al.*, 2007). Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) containing different acyl groups were separated resulting in elution of phospholipids with longest to shortest acyl chain (18:0, 12:0, and 6:0) (Fagan and Wijesundera, 2004). With this evidence, it is suspected that the three peaks of SM correspond to derivatives with varying acyl chain length. PC also depicted different peaks, but the effect was not as pronounced as SM. These peaks have previously been correlated to changes in the fatty acid composition of polar lipids due to cow diet (Lopez *et al.*, 2008).

The amount of total polar lipids isolated in this study is in accordance with previous studies where PLs in milk range from 9.4 to 35.5 mg per 100 g of product (Christie *et al.*, 1987; Fong *et al.*, 2007). In this research, 7.7 to 20.5 mg of polar lipid were detected per 100 g of milk, which equals to 0.21 to 0.57 g of polar lipid per 100 of total lipids. The individual concentrations of polar lipids was not significantly differently ($p > 0.05$), implying that milk processing such as cooling and thermal treatment does not influence the concentration of PLs. In literature, conflicting results on the effect of temperature on polar lipids from whole milk fat globules have been reported. According to Rombaut *et al.* (2006), variations in polar lipids can be ascribed to differences in the methodology used in the analysis. The Mojonnier procedure may not extract all the individual polar

lipids, including lecithins, and cephalins (Morin *et al.*, 2007b), which contribute to the polar lipid variation. In fact, the lower concentrations of PI and PS observed in the samples are probably due to the use of ammonium to dissociate the MFGM matrix, as indicated in previous studies (Avalli and Contarini, 2005). Their acidic characteristics might have solubilized them in the aqueous phase.

With the experimental design utilized in this research, it was difficult to discern how milk processing affects the concentration of individual polar lipids in the milk fat globule membrane. The literature reported for this topic is also inconsistent. According to Houlihan *et al.* (1992), heating alone had little or no effect on polar lipids whereas Christie *et al.* (1987) did not detect PLs in UHT powders, attributing such observations to the effect of heat during processing, especially oxidation of polyunsaturated fatty acids (PUFA). Buttermilk produced from pasteurized cream resulted in lower phospholipid concentration, especially PE, PS, and PI (Morin *et al.*, 2007b). The authors hypothesized that during cream churning the inner membrane of the fat globules is exposed to milk serum, inducing possible interactions as well as dislocation of the MFGM. Gassi *et al.* (2008) found no variation in total PL content of creams that underwent different heat treatments while the PL content increased in their respective buttermilk by a factor of seven. During cream churning, more PE and less SM are observed in skim milk predicting that loosely bound polar lipids appear in skim milk due to high shear forces that destroy the surface of fat globules (Morin *et al.*, 2008).

Migration of lipids from MFGM to serum only occurs in the presence of serum components (Houlihan *et al.*, 1992). Residual MFGM material is found in skim milk, and since SM and PC are located in the outer leaflet, they are more likely to migrate into the milk serum upon centrifugation (Anderson and Brooker, 1974). Gallier *et al.* (2010a)

confirmed these observations in her PL quantification where SM and PC were less abundant in cream than in raw milk (Christie *et al.*, 1987). PE was three times less abundant in buttermilk and more concentrated in cream. Since PE is more hydrophobic than PC and SM, it might partition into the butter phase during butter manufacture and MFGM preparation.

Larger fat globules are more prone to coalescence due to changes in the MFGM (Mulder and Walstra, 1974) while small fat globules are more stable at high temperatures, enriching high-heat buttermilk in PL content (Gassi *et al.*, 2008). Studies indicate that proteins and glycoproteins play a role in stabilization of fat globules by interrupting clustering (Shimizu *et al.*, 1980). Removal of phospholipids' polar heads diminished the stability of cream by coalescence of fat globules and promoting oiling-off. Shimizu *et al.* (1980) concluded that PLs interrupt coalesce by repulsive forces derived from the ionogenic groups of the phospholipids. According to Lopez *et al.* (2010a), glycolipids and glycoproteins are heterogeneously present in the MFGM among glycerophospholipids, contributing to the stabilization of globules. The liquid-ordered domains, lipid rafts, are devoid of carbohydrate-containing molecules and enriched in SM and cholesterol. Only the outer layer of the bilayer allows lateral plane movement (Lopez *et al.*, 2010b) where SM may disengage from the MFGM throughout milk processing. On the other hand, similar phospholipid organization was seen in processed cream and native globules, indicating membrane component reorganization after drastic processing (Gallier *et al.*, 2010a).

The lower amounts of polar lipids in the first trials denote autooxidation of the polyunsaturated fatty acids during sample preparation or storage. The appearance of a polar peak, expected to be phosphatidyl acid (after the elution of SM) suggests

phospholipid degradation. LysoPC was not quantified in this research but in the literature more lysoPC is indicative of PC degradation during milk processing (Gallier *et al.*, 2010a). Moreover, Fong *et al.* (2007) reported levels of lyso-PE and lyso-PC even when lipids were stored at -80 °C. Gallier *et al.* (2010) concluded that milk processing leads to difference in phospholipid composition (Gallier *et al.*, 2010a); however, her samples did not originate from the same milk line. Since polar lipids change as a function of the fat content, season, stage of lactation, feeding, and breed (Jensen, 2002; Bitman and Wood, 1990), the absence of statistical differences in phospholipid concentration during milk processing might be a result of the effects discussed previously as well as the contribution of PL oxidation in our samples.

4. *Changes in the Sulfhydryl (SH) and Disulfide (SS) Groups*

Thermal treatment is indirectly proportional to the amount of free sulfhydryl (SH) groups and directly proportional to the amount of disulfide (SS) groups. This study indicates that heat treatments such in UHT result in lower free sulfhydryl groups and therefore more disulfide-bonding indicative of protein interactions and denaturation. Cold pasteurization on the contrary has similar values to the ones observed in Fresh milk. An interesting finding is the higher amount of free SH and SS in 4 °C compared to Fresh milk. Cooling may promote the association between milk and whey proteins via other interactions besides covalent bonding, which may explain the higher free sulfhydryl groups present in storage milk. These interactions were strong enough to maintain protein association during MFGM extraction, especially in centrifugation for cream collection. During storage, milk is constantly agitated to prevent creaming, so this process introduced air bubbles in the milk, and thereby, promoted the formation of disulfide bonds from the oxidation of free sulfhydryl groups or from thiol-disulfide exchange reactions. It must be

emphasized that these observations are only preliminary studies with regard to SH and SS quantification.

C. Effects of Milk Processing on the MFGM Proteins from IDE Perspectives

1. Quantification of Protein Variation during Milk Processing

The proteins of the milk fat globule fluctuate during milk processing; however, the introduction of milk and whey proteins is more pronounced. Thermal treatment resulted in the absorption of β -lactoglobulin as a function of heat intensity, as previously seen in literature (Fong *et al.*, 2007). The amount of β -casein and α -lactalbumin in MFGM isolates also increased in heat-treated samples, especially in UHT milk. In the contrary, the amount of α -casein decreased in thermally treated milks, probably due to interactions with other proteins. Figure 43 illustrates the relative abundance of these proteins in the MFGM from SDS-PAGE densitometry ($p < 0.0001$).

Figure 43. Fluctuation of milk and whey proteins in the MFGM during milk processing. Results obtained from SDS-PAGE densitometry ($p < 0.0001$, three independent trials, and three replicates).

Thermal treatment of milk can induce desorption of protein and lipids from the MFGM. The complex formed by β -lactoglobulin and κ -casein during heat treatment may displace the proteins from the MFGM (Houlihan *et al.*, 1992). In fact, xanthine oxidase, butyrophilin, and ADFP are less abundant in UHT milk compared to the other treatments (Figure 44). These three proteins are the main MFGM proteins involved in fat globule excretion and are located within the trilayer, indicating complete disruption of the membrane during UHT pasteurization.

Figure 44. Fluctuation of the main MFGM proteins during milk processing. Results obtained from SDS-PAGE densitometry ($p < 0.0001$, three independent trials, and three replicates).

Other MFGM proteins such as MFG-E8, Mucin 15, annexin, and another protein with an apparent molecular weight of 69 kDa (denoted as X, Figure 45) fluctuate highly under intensive heat treatments. At this molecular weight, proteins such as serum albumin and heat shock cognate 71 kDa may be present; nevertheless, this band could encompass other proteins with similar weight. UHT causes the disappearance of Mucin 15, monocyte differentiation antigen (CD14), and κ -casein in their apparent MW region while high

molecular weight bands are observed even in reducing conditions (Figure 32). Also, UHT milk depicted more streaking, which originates from polypeptides resulting from protein denaturation.

Figure 45. Fluctuation of MFGM proteins during milk processing. Results obtained from SDS-PAGE densitometry. X unidentified protein band ($p < 0.0001$, three independent trials, and three replicates).

2. Identification of Proteins that Change in Milk Processing

a) Proteins in Global Composition

Heat induces protein interactions and displacement as well as adsorption on the milk fat globule membrane (Singh, 2006; Evers, 2004a). LC/MS identified changes in the MFGM protein profile in each treatment and permitted the functional classification of these. From this work, the 100 identified proteins were categorized into 13 groups, with the lipid transport/metabolism as the highest section followed by the immunity & defense section (Figure 46).

Figure 46. Functional category assignments for identified proteins. A total of 13 categories were assigned based on information in Uniprot and NCBI.

To better understand the changes that milk undergoes during regular milk processing, the frequency of proteins in each treatment was compared in each category. The following spider graphs represent a treatment for each angle, and only the proteins that showed variation are graphed. The protein probability is given as a percentage; this is a straightforward means to evaluate the relative abundance change of each protein for each processing treatment. The following graphs depict each group of proteins by function, and its variation given the processing steps. The categories for function are:

- Lipid transport / metabolism (Figure 47)
- Immunity / Defense (Figure 48)
- Membrane / Protein trafficking (Figure 49)
- G-protein mediated signaling (Figure 50)
- Protein synthesis, binding and/or folding (Figure 51)

- Homeostatic regulators (Figure 52)

General transporters, Cell Structure, Cell surface receptor mediated signal transduction, Milk proteins, Whey proteins, and Unknown function proteins are better understood in a table (Table 15).

Figure 47. Variation of proteins involved in lipid transport/metabolism during milk processing. BTN (Frag), Butyrophilin (Fragment); AUP-1, Ancient ubiquitous protein 1; LS, Lanosterol synthase; 17-b HSDH, 17-beta hydroxysteroid dehydrogenase; S-4-a-C-3-D, Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating; CYB5R3, CYB5R3 protein; LPAAT, Lysophosphatidic acid acyltransferase, zeta; AP E, Apolipoprotein E; LPL, Lipoprotein lipase; FABP (brain), Fatty acid-binding protein, brain

A total of 20 proteins involving lipid transport/metabolism were identified, but nine of them are present in all the samples with high probabilities including the two main MFGM proteins, XO and BTN. Mannose-6- phosphate receptor binding protein 1 was absent from UHT milk while CDEA protein, which plays a role in fat accumulation, had a low probability (16%) in the same sample. CIDEA also inhibits triglyceride hydrolysis and promotes lipid storage (Christianson *et al.*, 2010). The remaining nine proteins fluctuated as indicated in Figure 47. More proteins are observed as thermal treatment

increases, indicating association of lipid transport/metabolism proteins during heating and explaining the higher levels of SH and SS groups observed in 4 °C milk, which gradually decreases as heat intensity augments.

Fourteen proteins associated with immunity and defense were identified, of which four are constantly present throughout milk processing such as MFG-E8 (lacthaderin), immunoglobulin receptor, and mucin 15. Cathelicidin-1, mucin-1, and immunoglobulin J chain disappeared in UHT milk. Six proteins varied among the treatments (Figure 48), including Annexin A1, a calcium-dependent membrane aggregatin protein that mediates inflammation (Perretti and D'Acquisto, 2009). The toll-like receptor 2, implicated in pathogen recognition, was uniquely present in HTST.

Figure 48. Variation of proteins involved in immunity & defense during milk processing.
 ANX-A1, Annexin A1; IGK , IGK protein; GDCA, Glycosylation-dependent cell adhesion molecule 1; CD14, Monocyte differentiation antigen CD14; Ig (), Immunoglobulin light chain, lambda gene cluster; LPO, Lactoperoxidase.

Proteins involved in membrane/protein trafficking were also a major group in the MFGM isolates. A total of 13 proteins were identified, including two Ras-related proteins (Rab-18 and Rab 11A) persistently observed in all the samples whereas three proteins

implicated in vesicular transport (SNAP23A and SAR1a) and late endocytic transport (Rab-7a) were missing exclusively in the UHT milk. Seven proteins seem to fade in the thermal treatment, Batch and UHT (Figure 49). Ras-related protein Rab-8A was observed solely in Fresh milk.

Figure 49. Variation of proteins involved in membrane protein trafficking during milk processing. YKT6, Synaptobrevin homolog YKT6; ADP-RF, ADP-ribosylation factor 1; STX3, STX3 protein; Rab-13, Ras-related protein Rab-13; R-ras, Related RAS viral (R-ras) oncogene homolog; Rab-5C, Ras-related protein Rab-5C; RAB35, RAB35 protein; Rab-8A, Ras-related protein Rab-8A.

In the G-protein mediated cell signaling group, 11 proteins were identified including two subunits of the guanine nucleotide-binding protein G(I)/G(S)/G(T) constantly identified throughout milk processing and seven proteins that varied highly in all the samples with the exception of UHT milk where they were completely absent (Figure 50).

Figure 50. Variation in proteins involved in G-mediated cell signaling during milk processing. Ras-related C3, Ras-related C3 botulinum toxin substrate 1; Rap-1A, Ras-related protein Rap-1A; ADP-RF15, ADP-ribosylation factor-like protein 15; CDC42, CDC42 protein; NRAS, NRAS protein; RhoC, Rho-related GTP-binding protein RhoC; GNB G(i) α-1, Guanine nucleotide-binding protein G(i) subunit alpha-1.

Nine proteins implicated in protein synthesis, binding, and/or folding were recognized, from which a fragment of a protein similar to peptidylprolyl isomerase A (cyclophilin A) was picked in all the samples. As in previous groups, UHT was depleted of many proteins observed in the other milk treatments (Figure 51). This data in conjunction with other analysis are indicative of protein denaturation and exclusion in the UHT MFGM isolate. The elongation factor 1-alpha1 was present almost exclusively in Fresh, but a low-probability is also seen in UV milk. The latter was also the host of DCK protein, which is a core component of nucleosome.

Figure 51. Variation of proteins involved in protein synthesis, binding, and/ or folding during milk processing. 15 kDa SP, 15 kDa selenoprotein; Cofilin 1, Cofilin 1 (Non-muscle); HSC, 71 kDa, Heat shock cognate 71 kDa protein; RRL, Ribonucleotide reductase large subunit; HIST2H2BE, HIST2H2BE protein; Ankyrin, Ankyrin repeat domain 22; EF 1-a 1, Elongation factor 1-alpha 1; DCK , DCK protein.

Homeostatic regulators were also identified in the MFGM isolates with high variation throughout milk processing (Figure 52). A total of seven proteins were detected. One of them, cytoplasmic aconitate hydratase, an iron sensor, was solely identified in Fresh milk. This group of proteins was more abundant in thermal treated samples.

Figure 52. Variation in proteins involved in homeostatic regulation during milk processing. cGMP II PK, cGMP-dependant type II protein kinase; PP1201, PP1201 protein; SDR-1, Dehydrogenase/reductase (SDR family) member 1; ANX A5, Annexin A5; SA, Serum albumin.

The general transporter and cell structure groups are not illustrated in spider graphs, but less variation was observed in these proteins (Table 15). Only three proteins were associated with cell surface receptor mediated signal transduction, which are mainly present in Fresh and HTST milk. The milk proteins α -S1-casein, α -S2-casein, and κ -casein were adsorbed onto the MFGM throughout milk processing. On the other hand, whey proteins, α -lactalbumin and β -lactoglobulin, were more abundant in intense thermal treatments such as HTST and UHT. Four proteins with unknown or unspecified roles were detected whereas Reinhard and Lipolis (2010) identified more than 20 proteins in this category, including MUC proteins, which in this research have been classified in the Immunity & Defense group. The fragment of β -lactoglobulin, enterotoxin-binding glycoprotein PP20K, was seen in thermally treated samples, and it has been shown that this protein binds to MFGM during heating (Shida *et al.*, 1994).

b) Proteins Identification in Molecular Weight Regions

Four regions were analyzed in 1-DE gels that encompass a higher than 150 kDa (Region I), higher than 55 and less than 150 kDa (Region II), higher than 35 and less than 55 kDa (Region III), and less than 35 kDa (Region IV). In region I (Table 16), only three proteins with high molecular weight were observed, including XO (155 kDa), acetyl-CoA carboxylase 1 (265 kDa), and fatty acid synthase (275 kDa).

Figure 53. Protein identified in region I (> 150 kDa). Molecular weight is shown in parenthesis. cGMP-PK (87), cGMP-dependant type II protein kinase; LP (81), Lactoperoxidase; Acyl-CoA Syn (78), Acyl-CoA synthetase long-chain family member 1; Na-DPTP, Sodium-dependent phosphate transport protein 2B; SA (69), Serum albumin; MA-b-LG (20), Major allergen beta-lactoglobulin; GDCAM-1 (17), Glycosylation-dependent cell adhesion molecule 1; FABP (15), Fatty acid-binding protein.

The other 45 proteins have lower molecular weights than the expected region, indicating complex formations among them. Therefore, HTST and especially UHT produce more low-molecular weight proteins such as FABP, SA, PP20K extrinsic protein, and β -casein. UHT was exclusively enriched in lactoferrin, β -lactoglobulin, α -lactalbumin in this region. Figure 53 illustrates some of proteins with high variation in

this region, embracing the homeostatic regular serum albumin and lactoperoxidase, an antimicrobial agent believed to protect cow's udder from infection.

In region II (Table 17), a total of 46 proteins were identified of which 22 proteins had a lower-molecular weight outside the range for this region. Figure 54 denotes some of the region two proteins that fluctuated during milk processing. The toll-like receptor 2 (TLR-2) and alkaline phosphatase, an enzyme utilized to verify pasteurization, were identified specifically in this region in the HTST sample whereas cytoplasmic aconitate hydratase, an iron sensor, was exclusive to Fresh milk. As observed before, whey proteins were adsorbed onto UHT MFGM isolates.

Figure 54. Protein identified in region II (>55 and < 150 kDa). Molecular weight is shown in parenthesis. GNB-2 (37), Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2; cGMP-KP (87), cGMP-dependant type II protein kinase; LS (83), Lanosterol synthase; ACR (55), Acetylcholine receptor subunit epsilon; 5'-Nase (63), 5'-nucleotidase; Na+/Glu T (73), Na+/glucose cotransporter; BTN (F, 14), Butyrophilin (Fragment).

Region III (Table 18) resulted in the recognition of 60 proteins, including nine and twelve outside the upper limit and lower limits, respectively. Seven proteins varied highly among the treatments and are shown in Figure 55. Phosphatidylinositol transfer protein

alpha form (32 kDa) was identified in this region in the 4 °C milk but was not picked in the global identification. CD14 was observed in thermally treated samples including 4 °C but not in Fresh and UV milks.

Figure 55. Proteins identified in region III (> 35 and < 55 kDa). Molecular weight is shown in parenthesis. GNBP-11 (42), Guanine nucleotide-binding protein subunit alpha-11; S-4-a-CD (40), Sterol-4-alpha-carboxylate 3-dehydrogenase; GNAI2 (40), GNAI2 protein; CD14 (40), CD14 protein; ANX- A2 (39), Annexin A2; ANX- A1 (39), Annexin A1; 17-b-HSD (38), 17-beta hydroxysteroid dehydrogenase; CYB5R3 , CYB5R3 protein; STX3, STX3 protein.

Region IV (Table 19) resulted in 74 proteins mainly from protein denaturation and the recognition of polypeptides in this section. A total of 58 proteins were identified in the proper molecular weight.

D. Effects of Milk Processing on the MFGM Proteins from 2DE Perspectives

From the time milk leaves the cow udder, it undergoes changes in temperature as well as mechanical processing such as agitation and thermal treatment, causing interactions among MFGM, milk, and whey proteins. Using 2-DE gels coupled with MALDI-TOF, protein variation was observed along milk processing.

As the majority of the milk proteins have pI in the range of 4 to 7, a linear pH 3 to 10 was expected to resolve more protein from the MFGM. Cooling is the least invasive process that influences the association of α - and β -casein to MFGM proteins as well as changes in butyrophilin and adipophilin. Cryatallization of the fat causes volume changes in the fat globules and thus renders the MFGM to the adsorption of other proteins as shown in Figure 56 (Dickow *et al.*, 2011). BTN seems to be damaged more readily than other MFGM because it is exposed on the surface of fat globules due to its glycosylated nature (Dewettinck *et al.*, 2008). Adipophilin on the other hand locates specifically within inner phospholipid layer of the MFGM, and variation can be ascribed to exposed monolayer regions as well as damaged bilayer (Robenek *et al.*, 2006a). XO is embedded between the inner and bilayers, which may preclude denaturation due to milk processing. It cannot be ruled out that the protocol used in this analysis prevented the identification of XO.

VI. CONCLUSIONS & LIMITATIONS

In milk processing, the most abrupt thermal treatment can be considered UHT pasteurization since the intensity of the heat disturbs the native structure of fat globules and MFGM constituents. In fact, Christie *et al.* (1987) did not detect PLs in UHT powders, attributing such findings to autoxidation and the harsh treatment. Also, thermal treatment promotes fat globule aggregates as observed in previous studies (Gassi *et al.*, 2008). In this research, fewer aggregates were observed in non-thermal processes such as Fresh, 4 °C, and UV. Among these, Fresh resulted in the smallest globules, and thereby, was expected to be enriched in more polar lipids. The latter molecules remained

unaffected during milk processing, but these findings add to the plethora of conflicting PL research that attempted to characterize the effect of thermal treatment on polar lipids.

Figure 56. Milk processing effects on milk fat globules and milk fat globule membrane proteins. Globules are smaller in Fresh milk, and as processing proceeds, some triacylglycerides are released in cooling as well MFGM membrane. Thermal treatment enhances rearrangements between proteins and lipids of the membrane and promotes aggregation of globules and vesicle formation. Casein micelles and whey proteins are the main proteins that are adsorbed to the globule surface.

Even though the procedure utilized in this research was not the exact UHT protocol, the environment was simulated enough to observe reactions. In real UHT milk, mechanical stress also plays a role in milk processing and probably adds to the effect of heat treatment. In general, it can be stated that as the intensity of heat increases, more milk and whey proteins absorbed onto the milk fat globule membrane and augmented MFGM proteins displacement. In addition, a decrease in the amount of free sulfhydryl

(SH) and an increase in disulfide bonds (SS) in the line production of milk correlates with protein interactions. Even cooling promoted the association of proteins, probably through hydrophobic bonding, contributing to the amount of SH and SS levels. Figure 56 represents the main effects of milk processing on the milk fat globule membrane as milk proceeds in the dairy industry from the dairy unit, cooling, and thermal treatment.

In summary, pulsed UV light is clearly effective in inactivating microorganisms in vitro, but its potential in real foods and efficacy still need research despite the minimal effects observed in this study. Further research to separate MFGM from milk sources is also necessary (Singh, 2006); however, this task will not be easy as MFGM is sensitive to processing and handling. Nevertheless, with our findings it can be concluded that HTST milk is an optimal source of MFGM to supplement formulated foods that attempt to satisfy consumer's expectation of health-oriented and nutritious food products.

VII. DIRECTIONS FOR FUTURE RESEARCH

The interaction between polar lipids and proteins are important aspects of the MFGM that deserve more research in order to help us understand their functions (Gubbens *et al.*, 2009). Characterizing the alterations of these molecules during the regular path that milk takes from the dairy unit to our table is even more important to help us establish parameters that will preserve the integrity of polar lipids and proteins that have been ascribed with health-promoting effects. Another process to consider in milk production is spray-drying, as milk powder is the most common source of milk in developing countries, and so, the need to preserve the MFGM is also required. As Fong *et al.* (2007) suggested, some MFGM proteins may be used as alternates to synthetic anti-bacterial and anti-viral agents on top of their use as supplements; however, further work is required on

the optimization of food-grade downstream production processes for MFGM isolates that results in products similar to native state (El-Loly, 2011) and thereby allowing us to benefit from the effects that neonates received in milk.

VIII. REFERENCES

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IX. APPENDIX

A. Protocols for Buffers

All the reagents were of analytical grade and kept at room temperature unless otherwise stated. With regard to 2D protein analysis, reagents were of electrophoresis purity grade. Nanopure water of $18\text{M}\Omega/\text{cm}^2$ was utilized to make solutions.

1. 10X Phosphate Buffer Saline (PBS)

To make a 10X stock solution, 87 g of NaCl, 18.2 g of K_2HPO_4 , and 2.3 g of KH_2PO_4 were placed into a one-liter bottle and filled with deionized water up to 900 mL. The solutes were dissolved completely, and the pH was adjusted with 1N NaOH to 7.2. Then, the final volume was adjusted to one liter using a graduated cylinder.

2. Formic Acid Buffer

A 1M formic acid (06440, Fluka) buffer was made and neutralized with triethylamine (TEA) (HPLC grade, 04884, Fisher Scientific) to a final pH of 3. In a small beaker, 80% of the final buffer volume was placed with nanopure water. The 1M formic acid was made by adding the equivalent concentrated formic acid ($V_i = (C_f \times V_f) / C_i$ $i=\text{initial}, f=\text{final}$) followed by dropwise addition of TEA. Neutralization point was checked with litmus paper, and for the final pH, a small volume was measured with a pH meter to avoid introducing contamination. The buffer could be used for one week.

3. *2-DE-gel Buffers*

a) Homogenization Buffer

This was a 7M urea, 2M thiourea, 1% ASB-14, 40mM Tris base, 0.001% bromophenol blue, 0.5% ampholyte and 40mM DTT. Reagents were purchased from BioRad unless indicated. In a 250 mL beaker, 42g of urea (161-0731), 15.2g of thiourea (T8656, Sigma-Aldrich, MO), 0.484g of Tris base (161-0719), 1 g of amidosulfobetaine-14 (ASB-14) (182750, Calbichem), and 1 mL of 0.1% bromophenol blue were combined. The solution's volume was brought up to 100 mL with nanopure water and stored at 4°C. Immediately before use, 6mg dithiothreitol (DTT, 161-0611) and 5µl immobilized pH gradient (IPG) buffer pH 3-10 (17-6000-87, GE Healthcare, NJ) were added per mL of working homogenization buffer. ASB-14, bromophenol blue, DTT, and IPG buffer were kept at 4°C.

b) Immobilized pH Gradient (IPG) Rehydration Buffer

This was a 7M urea, 2M thiourea, 2% CHAPS, 2% NP-40, 0.002% bromophenol blue, 0.5% ampholyte and 100mM DTE. In a 200 mL beaker, 42.4g of urea, 15.2g thiourea, 2 mL 100% nonidet P-40 (Fluka), 2.0g CHAPS (C3023, Sigma-Aldrich, MO), 0.5 mL 0.1% Bromophenol Blue. The volume was brought up to 100mL with nanopure water, and the solution was filtered with a 0.2 micron filter. The final solution was stored at 4°C, and before use, 20mg dithioerythritol (DTE, D8255, Sigma-Aldrich, MO) and 6.25µl IPG buffer pH 3-10 were added per 1.25 mL of rehydration buffer. DTE was kept at -20 °C.

c) Equilibration Buffer

This was a 375mM Tris pH 8.8, 6M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue. In a 400 mL beaker, 50 mL of 1.5M Tris-HCl, pH8.8 (161-0798), 72 g urea, 60 mL 99% glycerol (BP229, Fisher Scientific), 20 mL 20% SDS (161-0418), and 400 μ L 0.1% bromophenol blue were combined. The volume was brought up to 200mL with nanopure water, and the solution was stored at 4 °C. To make working equilibration buffer, the solution was brought to room temperature, and 250 mg of DTT and 625 mg of iodoacetamide (163-2109) were added to 25 mL of equilibration buffer in separate tubes.

d) 10X SDS-PAGE Running Buffer

In 1L bottle, 50mL of 20% SDS, 30g of Tris-base, and 144g of glycine were mixed and dissolved with nanopure water to fill one liter. The solution was stored at 4°C.

e) Molecular Weight Standards for 2-D gels

All the reagents were molecular grade from BioRad and kept at 4 °C unless otherwise stated. An aliquot of 950 μ L of Laemmli sample buffer (161-0737), 50 μ L of beta-mercaptoethanol, and 27 μ L of SDS-PAGE molecular weight standards low range (161-0304, stored at -20°C) were combined. Aliquots of 40 μ L were kept in microcentrifuge tube and stored at -20°C. To load gels, an aliquot was heated at 100°C for 3 min, and 5 μ L of standard solution was pipetted onto a Biorad IEF electrode wick square (165-4071) placed over parafilm. The wicks were dried completely before placing in cassettes.

f) Agarose (0.8%)

This was a 25 mmol/L Tris-base, 192 mmol/L glycine, 0.1% SDS. In a 250mL Erlenmeyer flask, 0.8g of low melt agarose (161-3111), 100 mL of 1x running buffer, and

300 μ L of 0.1% bromophenol blue were mixed and heated in a microwave. Once the agarose melted, it was stored at -20°C. The agarose was reheated for gel electrophoresis and allowed to cool to 40-50°C before loading over the IPG strips.

g) 10% TCA/Acetone

In a beaker, 20 g of trichloroacetic acid (TCA, BP555, Fisher Scientific) was placed into 100 ml of 100% acetone and dissolved completely. The volume was then adjusted with a graduated cylinder up to 200 ml with more 100% acetone and stored at -20 °C.

h) Bromophenol Blue (0.1%)

In a 250 mL Erlenmeyer flask, 0.61g of Tris base was added to 100ml nanopure water and mixed with a stir bar until dissolved. Then 0.1g of bromophenol blue was added and stirred until completely solubilized. Solution was kept at 4 °C.

4. SDS-PAGE Gel Preparation

All the reagents were of electrophoresis purity grade from BioRad and stored at room temperature unless indicated. To make 12 gels at 12.5% acrylamide, 120mL of milliQ H₂O, 58.5mL of 1.5M Tris-HCl pH 8.8, 75mL of 40% acrylamide/bisacrylimide solution (161-0148, stored at 4 °C), and 1.463 mL of 20% SDS were combined in a 500 mL Erlenmeyer vacuum flask. This solution was de-gassed for about 15 min with a vacuum pump. A solution of 10% ammonium persulfate (APS) (161-0700) was prepared by dissolving 0.10g of APS into 1.00mL of MilliQ water. Once degassed, 675 μ L of the 10% APS solution, the catalyst, and 292.5 μ L of tetramethylethylenediamine (TEMED, 161-0801, kept at 4 °C) were added to polymerize the gels. To remove bubbles and smooth the surface, 95% isopropanol was added to the top of the gels with a glass Pasteur pipette. Gels were set on a leveled surface for 30 min, and the ethanol was decanted off top. The

gels were then rinsed with nanopure water and dried before placing the equilibrated IPG strips.

5. *Sensitive Colloidal Staining Solution*

This was a 34% (v/v) methanol, 3% (v/v) phosphoric acid, 17% (w/v) ammonium sulfate. In a 1L bottle, 400 mL of water and 30 mL of 85% H_3PO_4 were mixed. Then, 170 g of $(\text{NH}_4)_2\text{SO}_4$ were added to the solution slowly. If $(\text{NH}_4)_2\text{SO}_4$ is added too quickly, it becomes granulated and hard to dissolve. Once all the $(\text{NH}_4)_2\text{SO}_4$ was dissolved, 340 mL of methanol were added slowly to prevent localized precipitation of $(\text{NH}_4)_2\text{SO}_4$. Once the solution was homogenous, the volume was adjusted to one liter.

6. *Protein Extraction Buffers for MALDI-TOF*

Reagents were of LC/MS grade and kept at room temperature. Glassware was used Small beakers cleaned well and rinsed with the appropriate solvent.

a) *Destaining Buffer (25mM NH_4HCO_3 /50% acetonitrile)*

In a 50ml beaker, 0.059 g of NH_4HCO_3 was added to 15mL of 100% acetonitrile. The volume was adjusted to 30mL by adding 15mL HPLC grade water.

b) *Analyte Solution (0.1% TFA/ Acetonitrile 2:1)*

First a 0.1% TFA solution was prepared by adding 20 μL of TFA (kept at -20 C) to 20 mL of HPLC Grade Water (W5-4, Fisher Scientific). Then, the analyte solution was prepared by adding 10 mL of 0.1%TFA and 5 mL of acetonitrile. The solution was mixed in a glass beaker rinsed with acetonitrile.

c) Promega Mass Spectrometry Grade Porcine Trypsin Gold

Aliquots of Promega V5280 trypsin (100ug/vial) were prepared and stored at -70°C. First, 50 mM acetic acid was made by diluting 29 µL of concentrated acetic acid to 10 mL with HPLC grade water in a graduated cylinder. Then, 100 µL of this solution was added to the vial containing the powdered trypsin. Then, the trypsin solution was mixed and put in 5 µL aliquots. To prepare working trypsin solution, a 40 mM NH_4HCO_3 /10% acetonitrile solution was prepared by dissolving 31.6 mg of NH_4HCO_3 into 9 mL of HPLC Grade water in a graduated cylinder followed by the addition of one mL of acetonitrile. Before digestion, an aliquot was thawed, and 450 µL of 40mM NH_4HCO_3 /10% acetonitrile were added for a final 165µg/15µL trypsin concentration per well.

d) Mass Spectrometry Peptide Standard

Aliquots of Peptide Calibration Standard (206195, Bruker Corp.) were prepared by adding 0.1%TFA/water. First, 2mL of HPLC grade water was measured in a graduated cylinder, and 2 µL of TFA were added. The peptide mixture was dissolved completely in 125 µL of 0.1%TFA/water, and 5µL aliquots were stored at -70°C.

e) Matrix Solution (0.2mg HCCA/mL 0.1%TFA/ Acetonitrile)

In a 1.5mL Eppendorf tube, 0.2mg of α -hydroxycyanocinnamic acid (HCCA, 201344 mass-spectrometry grade, Bruker Corp., stored at -20°C) was measured, or enough powder was put in the tube to fill up to the bottom line. To acidify HCCA, 50 µL of 0.1% TFA was added and mixed. Then, 950 µL of LC/MS grade acetonitrile was added and vortexed until HCCA was in solution.

f) Wash Buffer (10mM Ammonium Phosphate in 0.1%TFA)

In a 50mL beaker, 20 μ L of TFA (kept at -20°C was added to 20 mL of HPLC water. Then, 5.75mg of $\text{NH}_4\text{H}_2\text{PO}_4$ was added to 5 mL of 0.1% TFA. The beaker was rinsed with HPLC water.

g) Recrystallization Buffer (Ethanol/acetone/0.1% TFA, 6:3:1 v/v/v)

This buffer must be prepared right before use (at the most five min). In a 50ml glass beaker rinsed with ethanol, 6mL of ethanol, 3mL of acetone, and 1mL of 0.1%TFA were combined.

h) Tris-glycine buffer

This was a 91.9 mM glycine, 4 mM EDTA. In a 1L bottle, the Tris-glycine buffer was prepared by adding 10.4 g of Tris (hydroxymethyl) aminomethane and 1.2 g of EDTA in nanopure water. The pH was adjusted to 8, and the final volume to one liter.

i) Tris-glycine/8M urea/ 17mM SDS buffer

This was a 91.9 mM glycine, 4 mM EDTA, 8M urea, 17mM. To a liter of Tris-glycine buffer, 480.5 g of urea was added and allowed to dissolve completely before adding 5 g of SDS.

B. Microbial Reduction

Table 24. Microbial reduction in heat and cold pasteurization. Aerobic and coliforms petrifilms were incubated for 48 and 24 hrs respectively.

Process	CFU/mL		% Reduction	
	Aerobic	Coliform	Aerobic	Coliform
Raw	7.43E+04	174	0	0
5ml,2s	4.15E+04	153	44	12
7ml,2s	4.80E+04	151	35	13
7ml,3s	5.30E+04	148	29	15
7ml,5s	5.25E+04	140	29	20
7ml,7s	5.15E+04	140	31	20
7ml,9s	5.35E+04	135	28	22
10ml,5s	6.85E+04	146	8	16
Batch	2.65E+02	0	100	100
HTST	1.05E+02	0	100	100
UHT	0	0	100	100

Table 25. Microbial reduction in UV pulsed light treatment. Aerobic and coliform petrifilms were incubated for 48 and 24 hrs respectively.

Process	CFU/mL		% Reduction	
	Aerobic	Coliform	Aerobic	Coliform
Raw	6.40E+05	565	0	0
5ml,5s	4.25E+05	270	34	52
7ml,2s	4.95E+05	330	23	42
7ml,3s	3.65E+05	330	43	42
7ml,5s	1.95E+05	320	70	43
7ml,7s	4.15E+05	390	35	31
7ml,9s	3.25E+05	380	49	33
10ml,5s	5.55E+05	380	13	33

C. MFGM Extraction

Table 26. MFGM material (pellet) from samples.

Treat	MFGM Trials, pellet mg per liter of milk				
	I	II	III	IV	Mean
Fresh	350	325	322	261	315 ^a
4C	328	343	273	257	300 ^a
Batch	262	311	208	229	252 ^{a,b}
HTST	194	223	184	275	219 ^{b,c}
UHT	160	208	167	150	171 ^c
UV	338	329	274	239	295 ^a

D. MFGM Protein Quantification

Table 27. Effect of milk processing on MFGM proteins.

Treat	Protein Trials, mg per 100 mg of MFGM pellet			
	II	III	IV	Mean $\pm$ STD
Fresh	13.28	11.56	15.18	13.34 $\pm$ 1.81
4 °C	13.04	14.64	14.68	14.12 $\pm$ 0.93
Batch	17.90	17.08	15.86	16.95 $\pm$ 1.03
HTST	17.89	17.83	17.27	17.66 $\pm$ 0.34
UHT	16.35	19.83	15.45	17.21 $\pm$ 2.31
UV	14.55	14.51	15.93	15.00 $\pm$ 0.81

E. Milk Composition

Table 28. Milk composition for each independent trial.

MP	Treat	g/100 mL milk							Acidity °TH	Urea mg/dL	Density SG	FPD mC	FFA mEkv/l
		Fat	Prot	Cas	Citric	Lac	TS	SNF					
II	Fresh	4.29	3.49	2.60	0.18	4.74	13.55	9.11	16.63	385.00	1028.47	516	0.313
	4-C	4.01	3.33	2.42	0.18	4.79	13.15	9.01	15.83	328.00	1028.33	520	0.403
	Batch	4.11	3.43	2.59	0.18	4.94	13.49	9.25	15.29	360.67	1029.67	533	0.514
	HTST	4.00	3.33	2.47	0.18	4.79	13.13	9.01	15.87	346.67	1028.33	518	0.405
	UHT	4.03	3.39	2.71	0.19	4.81	13.25	9.09	16.14	479.33	1028.80	532	0.261
	UV	3.98	3.34	2.46	0.18	4.81	13.12	9.03	15.46	354.00	1028.73	517	0.519
III	Fresh	2.99	3.14	2.29	0.19	4.84	11.92	8.49	15.86	338.00	1028.10	512	0.240
	4-C	4.03	3.30	2.39	0.18	4.71	13.07	8.90	15.72	305.00	1027.90	507	0.386
	HTST	3.69	3.03	2.06	0.17	4.35	12.05	8.23	14.80	283.50	1025.30	455	0.353
	Batch	4.05	3.35	2.48	0.19	4.79	13.22	9.03	15.67	328.00	1028.65	515	0.432
	UHT	4.13	3.39	2.60	0.20	4.74	13.29	9.00	15.51	399.50	1028.90	521	0.292
	UV	3.99	3.33	2.45	0.18	4.73	13.06	8.93	15.57	317.00	1028.25	507	0.488
IV	Fresh	4.19	3.41	2.50	0.15	4.76	13.35	9.03	16.39	372.50	1028.65	520	0.328
	4-C	3.70	3.41	2.49	0.16	4.79	12.90	9.05	16.04	329.50	1029.00	514	0.562
	Batch	3.69	3.43	2.55	0.16	4.85	12.95	9.13	15.93	352.00	1029.45	519	0.606
	HTST	3.95	3.40	2.53	0.16	4.77	13.15	9.04	16.39	352.00	1028.90	514	0.558
	UHT	3.67	3.40	2.65	0.17	4.69	12.75	8.92	15.85	432.50	1028.85	511	0.420
	UV	3.73	3.42	2.55	0.16	4.79	12.94	9.06	16.10	325.00	1029.18	515	0.597

MP, milk processing (trial); Prot, protein; Cas, casein; Citric, citric acid; Lac, lactose; TS, total solid; SNF, solid nonfat; FPD, freezing point depression; FFA, free fatty acid; °TH, Thorner degrees; SG, specific gravity; mEkv/l, milliequivalents/l
S/•(N), standard deviation of the mean, which was calculated from three independent trials
Subscripts within a column indicate statistically significant values (p< 0.05)

F. Phospholipids in the MFGM

Table 29. Phospholipid content⁺ indicated as mg of phospholipid per gram of milk

Trial	PL	Treatment, mg PL per gram of milk					
		Fresh	4C	Batch	HTST	UHT	UV
II	PE	3.04 ± 0.06	1.83 ± 0.04	3.14 ± 0.04	2.68 ± 0.06	3.55 ± 0.07	2.38 ± 0.04
	PI	0.47 ± 0.02	0.41 ± 0.02	0.62 ± 0.01	0.34 ± 0.02	0.48 ± 0.01	0.80 ± 0.09
	PS	0.04 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.04 ± 0.01	0.02 ± 0.01	0.11 ± 0.09
	PC	5.49 ± 0.04	5.59 ± 0.18	6.18 ± 0.06	5.80 ± 0.05	6.52 ± 0.17	6.91 ± 0.41
	SM	6.96 ± 0.16	6.47 ± 0.12	7.65 ± 0.36	7.75 ± 0.15	7.30 ± 0.21	8.71 ± 0.36
III	PE	3.01 ± 0.07	2.15 ± 0.11	2.06 ± 0.06	3.39 ± 0.14	3.43 ± 0.04	1.90 ± 0.13
	PI	0.82 ± 0.05	0.63 ± 0.01	0.65 ± 0.05	0.51 ± 0.10	0.58 ± 0.10	0.77 ± 0.10
	PS	0.50 ± 0.03	0.08 ± 0.00	0.06 ± 0.0	0.12 ± 0.05	0.07 ± 0.0	0.17 ± 0.01
	PC	4.59 ± 0.15	6.64 ± 0.09	6.92 ± 0.28	6.70 ± 0.21	7.52 ± 0.07	6.82 ± 0.25
	SM	7.44 ± 0.17	8.61 ± 0.22	8.27 ± 0.25	8.07 ± 0.14	8.59 ± 0.09	8.57 ± 0.22
IV	PE	4.61 ± 0.31	2.69 ± 0.04	2.99 ± 0.02	2.89 ± 0.03	2.05 ± 0.03	0.72 ± 0.02
	PI	0.42 ± 0.0	0.41 ± 0.01	0.39 ± 0.01	0.46 ± 0.01	0.47 ± 0.00	0.41 ± 0.01
	PS	0.02 ± 0.01	0.28 ± 0.02	0.26 ± 0.01	0.18 ± 0.01	0.12 ± 0.01	0.05 ± 0.0
	PC	5.09 ± 0.52	3.13 ± 0.04	2.83 ± 0.10	3.49 ± 0.00	3.60 ± 0.03	2.94 ± 0.01
	SM	5.48 ± 0.38	3.86 ± 0.06	3.62 ± 0.04	4.33 ± 0.07	4.74 ± 0.02	3.63 ± 0.03

+ Mean and standard deviation of three HPLC measurements

Table 30. Polar lipids quantification in milk during processing (trials II & III).

Treat	Polar lipids, mg per 100 g of milk					
	PE	PI	PS	PC	SM	Total
Fresh	3.02	0.64	0.27	5.04	7.20	16.18
4°C	1.99	0.52	0.05	6.12	7.54	16.22
Batch	2.60	0.64	0.05	6.55	7.96	17.79
HTST	3.03	0.43	0.08	6.25	7.91	17.70
UHT	3.49	0.53	0.05	7.02	7.95	19.04
UV	2.14	0.79	0.14	6.87	8.64	18.58
S/SR(N)	0.31	0.07	0.08	0.39	0.41	0.83

S/•(N), standard deviation of the mean, which was calculated from two independent trials

Table 31. Polar lipid quantification in milk during processing (trials II, III, and IV).

Treat	Polar lipids, mg per 100 g of milk					
	PE	PI	PS	PC	SM	Total
Fresh	3.55	0.57	0.19	5.06	6.62	15.99
4°C	2.22	0.48	0.13	5.12	6.32	14.27
Batch	2.73	0.55	0.12	5.31	6.51	15.22
HTST	2.99	0.44	0.11	5.33	6.72	15.58
UHT	3.01	0.51	0.07	5.88	6.88	16.35
UV	1.67	0.66	0.11	5.56	6.97	14.97
S/SR(N)	0.44	0.06	0.08	0.54	0.44	1.23

S/•(N), standard deviation of the mean, which was calculated from three independent trials

Table 32. Phospholipids in total lipids (mg/100 g total lipids).

Trial	PL	Treatments mg PL per 100 gram total lipids					
		Fresh	4C	Batch	HTST	UHT	UV
II	PE	71.97	44.72	74.87	66.46	87.01	59.45
	PI	11.08	10.11	14.76	8.48	11.89	20.04
	PS	0.92	0.58	0.82	1.08	0.41	2.78
	PC	129.88	136.33	147.31	144.00	160.06	172.53
	SM	164.64	158.01	182.55	192.34	179.00	217.40
	Total	378.49	349.75	420.31	412.35	438.38	472.20
III	PE	104.28	53.66	51.16	90.82	83.69	47.85
	PI	28.34	15.65	16.24	13.69	14.07	19.37
	PS	17.48	1.95	1.40	3.11	1.81	4.39
	PC	159.35	165.57	171.83	179.60	183.45	172.11
	SM	258.08	214.47	205.36	216.44	209.54	216.05
	Total	567.52	451.30	445.99	503.65	492.56	459.76
IV	PE	113.71	74.73	83.08	74.63	57.01	19.89
	PI	10.38	11.31	10.80	11.75	13.02	11.29
	PS	0.57	7.89	7.26	4.53	3.42	1.41
	PC	125.52	87.06	78.60	89.98	99.86	81.59
	SM	135.04	107.48	100.36	111.76	131.43	100.79
	Total	385.23	288.47	280.09	292.66	304.74	214.96

Table 33. Polar lipids quantification in cream during processing (trials II & III).

Treat	Polar lipids, mg per 100 g of total lipids					Total
	PE	PI	PS	PC	SM	
Fresh	88.13	19.71	9.20	144.61	211.36	224.91
4C	49.19	12.88	1.26	150.95	186.24	128.41
Batch	63.02	15.50	1.11	159.57	193.95	96.16
HTST	78.64	11.08	2.09	161.80	204.39	93.36
UHT	85.35	12.98	1.11	171.76	194.27	97.55
UV	53.65	19.70	3.58	172.32	216.73	101.58

S/•(N), standard deviation of the mean, which was calculated from two independent trials

G. SH & SS Quantification

Table 34. Effect of heat treatment on the amounts of free and total sulfhdryl (SH) and disulfide (SS) groups in the milk fat globule membrane.

Sample	μmol per gram of protein		
	FreeSH	TotalSH	SS
Fresh	52.94 ^b	153.78 ^b	50.42 ^c
4 °C	71.40 ^a	298.98 ^a	113.79 ^a
Batch	37.73 ^c	206.92 ^b	84.60 ^b
HTST	31.86 ^c	159.14 ^b	63.64 ^b
UHT	19.04 ^d	280.98 ^a	130.97 ^a
UV	53.39 ^b	179.16 ^b	62.88 ^{b,c}

H. Particle Size Analysis

Figure 57. Fat globule (FG) size distribution of 40% cream as a function of particle diameter (μm). Data for Trial III.

Figure 58. Fat globule (FG) size distribution of 40% cream as a function of particle diameter (μm). Data for Trial II.

I. SDS-PAGE Quantification

Table 35. Quantification of MFGM proteins by SDS-PAGE densitometry.

Band #	Relative Concentration (Intensity x mm)+						
	Fresh	4°C	Batch	HTST	UHT	UV	STD
308	2707						
284	4743	4834					
252	10796	5580	7794	9813			1690
217	10506	6181	5999	5540	35239	21148	
181	10370	12283	11782	13813	16848	10837	1228
174	9340	10978				18197	
151	89227 ^a	92421 ^a	82725 ^a	84773 ^a	19588 ^b	84899 ^a	3413
140	14169	13844	8301	10534	10270	11204	1471
131	9620	5167	3313	4826		6327	1199
124					6532		
120	6508	6327	4703	6827	ND	5590	859
110			3025			2817	
106			2162				
101	1636	1392	1802	1335		1975	200
89-87	7530	2729	12864	6481	4066	1226	1360
80	13363	12493				15980	2947
76-77		19139	14721	8896	12925	12913	4095
70	45472 ^a	58776 ^a	60092 ^a	49616 ^a	5132 ^b	53127 ^a	4085
63	12984	17455	18158	17058	ND	16307	5114
59	107741 ^a	116856 ^a	104980 ^a	113828 ^a	51726 ^b	104538 ^a	4420
55-56	21114	16874	14302	6929	3810	9062	3455
53	6296	4979	8867	13673	16791	4846	2968
51-52	38187 ^a	49789 ^a	43582 ^a	48326 ^a	7457 ^b	43408 ^a	3220
49-50	19506 ^a	26504 ^a	20307 ^a	25785 ^a	2900 ^b	23182 ^a	1734
41-43	2905	8350	5819	4957		13441	2256
39+	20923 ^a	22870 ^a	6745 ^b	7225 ^b	20640 ^a	13094 ^{ab}	2149
38	21746 ^a	17659 ^b	6059 ^c	6834 ^c		14004 ^b	605
33-34	6650	6445	5267	6044	7977	5623	579
31	3057	3539	5951	3791		3046	1595
29			8347	6193			
25-26	9695 ^c	15745 ^{bc}	26934 ^b	13215 ^c	60784 ^a	12751 ^c	3466
21-22++	13307 ^a	11312 ^{ab}	4982 ^{bc}	6010 ^{abc}	2378 ^c	11182 ^{ab}	1577
20	9737	6872	8017	7335		7682	751
18	7978 ^c	5168 ^c	45421 ^b	44815 ^b	90121 ^a	4391 ^c	6805
13	17031 ^{bc}	17935 ^{bc}	25333 ^b	21306 ^{bc}	40186 ^a	10577 ^c	2833
10	6424	8859	9542	4586	6860	17465	3577

Relative concentration is the band trace, which is determined by peak intensity and band length

Band number indicates the MW

Groups within a row are statistically different, p <0.000. + p <0.001, ++ p<0.004.

J. 1DE & Mass Tandem Spectrometry

LC/MS data from 1DE was analyzed using Scaffold (TM). The Scaffold's settings were 95% probability, min 2 peptide, and 70% peptide coverage. The analysis resulted in 123 identified proteins from which 100 have been identified, and the rest are uncharacterized proteins. Tables 38 and 39 show the results as the percent protein probability, peptides recognized, and peptide coverage. Table 40 presents the functional classification of MFGM proteins.

Table 36. LCMS results for Fresh, 4 C, and Batch treatments.

Accession Number	MW	Identified Proteins*	Fresh			4 °C			Batch		
			Pro	Pep	%C	Pro	Pep	%C	Pro	Pep	%C
BT1A1_BOVIN	59	Butyrophilin subfamily 1 member A1	100	73	72	100	54	65	100	42	53
PLIN2_BOVIN	49	Perilipin-2	100	66	83	100	55	71	100	50	77
XDH_BOVIN	147	Xanthine dehydrogenase/oxidase	100	84	59	100	80	55	100	67	50
Q3T0K7_BOVIN	48	MFGE8 protein	100	23	51	100	19	48	100	20	46
ABCG2_BOVIN	73	ATP-binding cassette sub-family G member 2	100	19	29	100	15	21	100	9	9
PIGR_BOVIN	82	Polymeric immunoglobulin receptor	100	10	19	100	14	19	100	20	25
FABPH_BOVIN	15	Fatty acid-binding protein, heart	100	16	86	100	15	75	100	14	68
CD36_BOVIN	53	Platelet glycoprotein 4	100	9	15	100	7	12	100	6	12
RAB18_BOVIN	23	Ras-related protein Rab-18	100	12	60	100	11	57	100	11	60
CASK_BOVIN	21	Kappa-casein	98	1	5	100	4	24	100	5	23
Q0VCZ8_BOVIN	78	Acyl-CoA synthetase long-chain family member 1	100	10	17	100	11	19	100	3	9
CASA1_BOVIN	25	Alpha-S1-casein	100	6	31	100	8	37	100	5	29
A1L528_BOVIN	23	RAB1A, member RAS oncogene family	100	5	31	100	10	63	100	9	55
Q3T101_BOVIN	25	IGL@ protein	100	5	26	100	8	38	100	7	39
ACACA_BOVIN (+1)	265	Acetyl-CoA carboxylase 1	100	8	5	100	3	2	100	5	3

Accession Number	MW	Identified Proteins*	Fresh			4 °C			Batch		
			Pro	Pep	%C	Pro	Pep	%C	Pro	Pep	%C
Q861V9_BOVIN	16	Similar to peptidylprolyl isomerase A (Cyclophilin A) (Fragment)	100	6	33	100	7	67	100	2	15
GBB2_BOVIN	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	100	7	24	100	3	10	100	3	12
ENPP3_BOVIN	100	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	100	6	9	100	6	9	100	3	5
FAS_BOVIN	275	Fatty acid synthase	100	4	3	100	4	2	100	2	1
B5B0D4_BOVIN	20	Major allergen beta-lactoglobulin	73	1	8	100	2	19	100	10	68
NPT2B_BOVIN	76	Sodium-dependent phosphate transport protein 2B	100	14	17	100	8	13	100	6	10
A6QNZ7_BOVIN (+1)	55	Keratin 10 (Epidermolytic hyperkeratosis; keratosis palmaris et plantaris)	100	5	9	100	6	8	71	1	2
A4FV94_BOVIN	61	KRT6A protein	73	1	4	100	2	5.4	79	1	2
CASA2_BOVIN	26	Alpha-S2-casein	73	1	4	100	2	9	100	2	9
GBB1_BOVIN (+1)	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	100	4	23	83	1	10	83	1	7
A5D7M6_BOVIN (+2)	63	KRT5 protein	99	2	4	100	3	6			
A7E340_BOVIN (+2)	36	Mucin 15, cell surface associated	58	1	3	71	1	3	79	1	3
RB11A_BOVIN (+1)	24	Ras-related protein Rab-11A	100	3	16	99	2	11	100	3	16
A6H7J7_BOVIN	25	Putative uncharacterized protein	73	1	30	73	1	41	100	2	30
ACTB_BOVIN (+3)	42	Actin, cytoplasmic 1	100	4	15	100	2	7	100	3	10
A8E4P3_BOVIN	31	STOM protein	100	9	32	100	7	31	100	4	18
A4FUX1_BOVIN	25	CIDEA protein	100	3	22	100	5	27	100	3	22
Q9TSF1_BOVIN	14	Butyrophilin (Fragment)	42	1	62	98	1	31	97	1	45
GNAS2_BOVIN	46	Guanine nucleotide-binding protein G(s) subunit alpha isoforms short	100	2	5	73	1	3	83	1	3
Q2T9M8_BOVIN	23	Synaptosomal-associated protein 23 isoform SNAP23A	100	5	34	100	5	34	99	2	18
Q0IIA4_BOVIN	59	Glycoprotein 2 (Zymogen granule membrane)	100	9	20	100	11	22	100	8	16
CTHL1_BOVIN	18	Cathelicidin-1	100	3	27	100	7	44	100	2	23
A5D7Q2_BOVIN	52	Putative uncharacterized protein	100	3	7	98	1	2	100	5	14
SAR1A_BOVIN	22	GTP-binding protein SAR1a	100	5	36	100	3	30	98	1	13
RAB7A_BOVIN	24	Ras-related protein Rab-7a	100	3	19	100	3	14	100	3	22
MUC1_BOVIN	58	Mucin-1	100	3	3	100	4	5	71	2	2

Accession Number	MW	Identified Proteins*	Fresh			4 °C			Batch		
			Pro	Pep	%C	Pro	Pep	%C	Pro	Pep	%C
Q3SYR8_BOVIN	18	Immunoglobulin J chain	100	2	17	100	2	17	100	2	17
RAC1_BOVIN	21	Ras-related C3 botulinum toxin substrate 1	100	2	13	73	1	5	73	1	5
Q3SX32_BOVIN	48	Mannose 6 phosphate receptor binding protein 1	100	5	18	100	2	6	83	1	6
RAP1A_BOVIN (+1)	21	Ras-related protein Rap-1A	100	2	14	100	2	14	73	1	8
SEP15_BOVIN	18	15 kDa selenoprotein	73	1	10	73	1	10	99	2	15
B0JYL8_BOVIN (+2)	19	Cofilin 1 (Non-muscle)	73	1	17	100	2	24	73	1	17
B0JYP6_BOVIN	26	IGK protein	100	2	15	100	3	15	100	5	36
A9XTK4_BOVIN	38	17-beta hydroxysteroid dehydrogenase	100	5	26	99	2	13			
YKT6_BOVIN	22	Synaptobrevin homolog YKT6	35	1	4	100	2	12			
ARL15_BOVIN	23	ADP-ribosylation factor-like protein 15	98	1	6	97	2	10			
Q8MKB7_BOVIN (+1)	73	Na ⁺ /glucose cotransporter	73	1	4	83	1	4			
B6VAP7_BOVIN (+1)	21	CDC42 protein	100	3	22	100	2	17			
Q17QL7_BOVIN	49	KRT15 protein	73	1	7	100	2	11			
5NTD_BOVIN	63	5'-nucleotidase	19	1	2	71	1	3			
NSDHL_BOVIN	40	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	73	1	5						
D5HSX1_BOVIN	87	cGMP-dependant type II protein kinase	100	2	3	64	1	2			
Q6QRN7_BOVIN	34	PP1201 protein	73	1	4	100	2	8			
Q2TBH2_BOVIN	24	Related RAS viral (R-ras) oncogene homolog	100	2	11	99	2	11			
CHP1_BOVIN	22	Calcium-binding protein p22	73	1	8	100	2	13			
ARF1_BOVIN (+2)	21	ADP-ribosylation factor 1	100	4	29	73	1	6			
A6QLH3_BOVIN	33	STX3 protein	100	2	15	51	1	3			
ANXA1_BOVIN	39	Annexin A1	73	1	5						
A5PKG9_BOVIN	59	FGR protein	73	1	6						
GIPC2_BOVIN	35	PDZ domain-containing protein GIPC2	100	2	9						
Q2KIS4_BOVIN	34	Dehydrogenase/reductase (SDR family) member 1	97	1	5						
RHOC_BOVIN	22	Rho-related GTP-binding protein RhoC	73	1	6						

Accession Number	MW	Identified Proteins*	Fresh			4 °C			Batch		
			Pro	Pep	%C	Pro	Pep	%C	Pro	Pep	%C
HSP7C_BOVIN	71	Heat shock cognate 71 kDa protein	100	4	5						
RAB13_BOVIN	23	Ras-related protein Rab-13	73	1	12						
Q3ZC65_BOVIN (+1)	46	Ancient ubiquitous protein 1	30	1	3						
ERG7_BOVIN	83	Lanosterol synthase	58	1	2				74	1	1.4
ANXA2_BOVIN	39	Annexin A2	73	1	5	83	1	5			
Q99BK3_9ALPH	88	Ribonucleotide reductase large subunit	61	1	1	100	2	3	79	1	1
GNAI1_BOVIN	40	Guanine nucleotide-binding protein G(i) subunit alpha-1	99	2	8						
RAB5C_BOVIN	23	Ras-related protein Rab-5C	73	1	7	100	2	13			
A6H7G0_BOVIN (+1)	34	CYB5R3 protein				83	1	6	83	1	6
A5D7E0_BOVIN	23	RAB35 protein				100	2	21			
GLCM1_BOVIN	17	Glycosylation-dependent cell adhesion molecule 1				100	3	18	100	2	17
A6QNL0_BOVIN (+2)	40	Monocyte differentiation antigen CD14				63	1	3	100	3	17
Q1RMN8_BOVIN	25	Immunoglobulin light chain, lambda gene cluster				100	2	41	100	2	30
A6QM09_BOVIN	25	Putative uncharacterized protein				100	2	38	100	2	26
PERL_BOVIN	81	Lactoperoxidase				63	1	3			
Q9TRB9_BOVIN	2	Enterotoxin-binding glycoprotein PP20K (Fragment)							28	1	40
ANXA5_BOVIN	36	Annexin A5							38	1	3
LACB_BOVIN	20	Beta-lactoglobulin							73	1	78
B3VTM3_BOVIN (+4)	78	Lactoferrin							100	4	6
A6H7D4_BOVIN (+1)	52	1-acylglycerol-3-phosphate O-acyltransferase 6 (Lysophosphatidic acid acyltransferase, zeta)							83	1	3
A7YWR0_BOVIN (+2)	36	Apolipoprotein E							83	1	5
A5D7R9_BOVIN	21	NRAS protein							97	2	12
A5D7N2_BOVIN (+7)	14	HIST2H2BE protein				99	2	21			
A2VDV1_BOVIN	22	Ankyrin repeat domain 22				73	1	7			
Q28049_BOVIN	14	Alpha lactalbumin (Fragment)									

Accession Number	MW	Identified Proteins*	Fresh			4 °C			Batch		
			Pro	Pep	%C	Pro	Pep	%C	Pro	Pep	%C
ALBU_BOVIN	69	Serum albumin									
LIPL_BOVIN	53	Lipoprotein lipase									
RAB8A_BOVIN	24	Ras-related protein Rab-8A	99	2	21						
EF1A1_BOVIN	50	Elongation factor 1-alpha 1	100	2	9						
ACOC_BOVIN	98	Cytoplasmic aconitate hydratase	100	2	3						
FABP7_BOVIN	15	Fatty acid-binding protein, brain							100	2	22
D4QGC1_BOVIN (+1)	59	Tyrosine-protein kinase Lyn									
PPBT_BOVIN	57	Alkaline phosphatase, tissue-nonspecific isozyme									
A4FUH8_BOVIN (+1)	21	Chromosome 1 open reading frame 93 ortholog									
B5T263_9CETA (+6)	90	Toll-like receptor 2 OS=Bos indicus x Bos taurus GN=TLR2 PE=4 SV=1									
A5D792_BOVIN (+5)	18	DCK protein									

Pro, probability of protein; Pep, peptides identified; %C, percent peptide coverage

The number of peptides that could be specifically assigned to a single protein using a peptide false discovery rate of 0.1% is indicated.

Table 37.B. LC/MS results for HTST, UHT, and UV treatment

Accession Number	MW	Identified Proteins*	HTST			UHT			UV		
			Pro	Pep	%C	Pro	Pep	%C	Pro	Pep	%C
BT1A1_BOVIN	59	Butyrophilin subfamily 1 member A1	100	83	82	100	53	65	100	51	65
PLIN2_BOVIN	49	Perilipin-2	100	84	88	100	42	77	100	51	76
XDH_BOVIN	147	Xanthine dehydrogenase/oxidase	100	118	70	100	67	52	100	73	57
Q3T0K7_BOVIN	48	MFGE8 protein	100	32	53	100	18	35	100	21	44
ABCG2_BOVIN	73	ATP-binding cassette sub-family G member 2	100	23	29	100	16	23	100	10	10
PIGR_BOVIN	82	Polymeric immunoglobulin receptor	100	29	43	100	22	24	100	6	13

Accession Number	MW	Identified Proteins*	HTST			UHT			UV		
			Pro	Pep	%C	Pro	Pep	%C	Pro	Pep	%C
FABPH_BOVIN	15	Fatty acid-binding protein, heart	100	17	90	100	6	37	100	9	64
CD36_BOVIN	53	Platelet glycoprotein 4	100	15	26	100	8	13	100	5	9
RAB18_BOVIN	23	Ras-related protein Rab-18	100	17	79	100	5	19	100	13	64
CASK_BOVIN	21	Kappa-casein	100	7	33	100	12	56	100	3	24
Q0VCZ8_BOVIN	78	Acyl-CoA synthetase long-chain family member 1	100	16	28	100	9	12	100	7	8
CASA1_BOVIN	25	Alpha-S1-casein	100	8	37	100	3	19	100	4	22
A1L528_BOVIN	23	RAB1A, member RAS oncogene family	100	8	47	100	3	11	100	5	32
Q3T101_BOVIN	25	IGL@ protein	100	5	20	100	4	20	100	7	37
ACACA_BOVIN (+1)	265	Acetyl-CoA carboxylase 1	100	18	11	100	3	2	100	3	2
Q861V9_BOVIN	16	Similar to peptidylprolyl isomerase A (Cyclophilin A) (Fragment)	100	9	69	43	1	7	100	4	24
GBB2_BOVIN	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	100	5	20	100	5	19	100	2	8
ENPP3_BOVIN	100	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	100	6	9	100	5	7	100	5	8
FAS_BOVIN	275	Fatty acid synthase	100	5	3	98	1	0.5	98	1	0.3
B5B0D4_BOVIN	20	Major allergen beta-lactoglobulin	100	32	87	100	39	89	100	4	27
NPT2B_BOVIN	76	Sodium-dependent phosphate transport protein 2B	100	11	12	100	9	16			
A6QNZ7_BOVIN (+1)	55	Keratin 10 (Epidermolytic hyperkeratosis; keratosis palmaris et plantaris)	100	7	7	100	7	7	98	1	2
A4FV94_BOVIN	61	KRT6A protein	100	2	5	100	2	5	74	1	4
CASA2_BOVIN	26	Alpha-S2-casein	100	8	26	100	4	10	100	3	13
GBB1_BOVIN (+1)	37	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	100	5	21	100	2	11	99	3	11
A5D7M6_BOVIN (+2)	63	KRT5 protein	99	2	4	100	2	4	20	1	4
A7E340_BOVIN (+2)	36	Mucin 15, cell surface associated	99	2	4	98	2	3	51	1	3
RB11A_BOVIN (+1)	24	Ras-related protein Rab-11A	100	5	31	70	1	6	100	4	20
A6H7J7_BOVIN	25	Putative uncharacterized protein	71	1	23	58	1	20	74	1	36

Accession Number	MW	Identified Proteins*	HTST			UHT			UV		
			Pro	Pep	%C	Pro	Pep	%C	Pro	Pep	%C
ACTB_BOVIN (+3)	42	Actin, cytoplasmic 1	100	4	15	58	1	3	100	5	12
A8E4P3_BOVIN	31	STOM protein	100	3	10	50	1	6	100	5	24
A4FUX1_BOVIN	25	CIDEA protein	100	6	32	16	1	8	100	5	27
Q9TSF1_BOVIN	14	Butyrophilin (Fragment)	100	3	80						
GNAS2_BOVIN	46	Guanine nucleotide-binding protein G(s) subunit alpha isoforms short	100	5	19	32	1	4			
Q2T9M8_BOVIN	23	Synaptosomal-associated protein 23 isoform SNAP23A	100	5	42				100	5	32
Q0IIA4_BOVIN	59	Glycoprotein 2 (Zymogen granule membrane)	100	2	6				100	6	9
CTHL1_BOVIN	18	Cathelicidin-1	100	2	23				100	5	40
A5D7Q2_BOVIN	52	Putative uncharacterized protein	100	4	9				100	2	5
SAR1A_BOVIN	22	GTP-binding protein SAR1a	100	2	19				100	4	30
RAB7A_BOVIN	24	Ras-related protein Rab-7a	100	3	19				74	1	5
MUC1_BOVIN	58	Mucin-1	50	1	2				100	4	5
Q3SYR8_BOVIN	18	Immunoglobulin J chain	71	1	10				74	1	10
RAC1_BOVIN	21	Ras-related C3 botulinum toxin substrate 1	100	4	33				99	2	9
Q3SX32_BOVIN	48	Mannose 6 phosphate receptor binding protein 1	100	7	24				100	3	8
RAP1A_BOVIN (+1)	21	Ras-related protein Rap-1A	99	2	14				98	1	8
SEP15_BOVIN	18	15 kDa selenoprotein	100	2	17				74	1	10
B0JYL8_BOVIN (+2)	19	Cofilin 1 (Non-muscle)	71	1	7				100	2	24
B0JYP6_BOVIN	26	IGK protein							100	2	15
A9XTK4_BOVIN	38	17-beta hydroxysteroid dehydrogenase	100	5	22				41	1	5
YKT6_BOVIN	22	Synaptobrevin homolog YKT6	71	1	5				100	3	16
ARL15_BOVIN	23	ADP-ribosylation factor-like protein 15	100	3	19				74	1	6
Q8MKB7_BOVIN (+1)	73	Na+/glucose cotransporter				100	2	6	51	1	4
B6VAP7_BOVIN (+1)	21	CDC42 protein	99	2	16						
Q17QL7_BOVIN	49	KRT15 protein				50	1	7			

Accession Number	MW	Identified Proteins*	HTST			UHT			UV		
			Pro	Pep	%C	Pro	Pep	%C	Pro	Pep	%C
5NTD_BOVIN	63	5'-nucleotidase	100	3	10	100	3	10			
NSDHL_BOVIN	40	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	100	3	12	58	1	5	100	2	8
D5HSX1_BOVIN	87	cGMP-dependant type II protein kinase	100	2	3				66	1	2
Q6QRN7_BOVIN	34	PP1201 protein	71	1	4				100	2	5
Q2TBH2_BOVIN	24	Related RAS viral (R-ras) oncogene homolog							100	2	17
CHP1_BOVIN	22	Calcium-binding protein p22							100	2	14
ARF1_BOVIN (+2)	21	ADP-ribosylation factor 1							74	1	12
A6QLH3_BOVIN	33	STX3 protein	50	1	5				71	1	5
ANXA1_BOVIN	39	Annexin A1	50	1	5				100	3	10
A5PKG9_BOVIN	59	FGR protein	100	3	9						
GIPC2_BOVIN	35	PDZ domain-containing protein GIPC2	100	2	9						
Q2KIS4_BOVIN	34	Dehydrogenase/reductase (SDR family) member 1	100	4	15						
RHOC_BOVIN	22	Rho-related GTP-binding protein RhoC	100	3	23						
HSP7C_BOVIN	71	Heat shock cognate 71 kDa protein	50	1	2						
RAB13_BOVIN	23	Ras-related protein Rab-13	100	2	17						
Q3ZC65_BOVIN (+1)	46	Ancient ubiquitous protein 1	99	2	9						
ERG7_BOVIN	83	Lanosterol synthase	100	2	4	100	3	5			
ANXA2_BOVIN	39	Annexin A2	99	2	8	45	1	5			
Q99BK3_9ALPH	88	Ribonucleotide reductase large subunit									
GNAI1_BOVIN	40	Guanine nucleotide-binding protein G(i) subunit alpha-1				31	1	5			
RAB5C_BOVIN	23	Ras-related protein Rab-5C									
A6H7G0_BOVIN (+1)	34	CYB5R3 protein	100	5	23	100	2	8	74	1	6
A5D7E0_BOVIN	23	RAB35 protein	71	1	17	39	1	16	74	1	18
GLCM1_BOVIN	17	Glycosylation-dependent cell adhesion molecule 1	100	6	44	80	1	7			
A6QNL0_BOVIN (+2)	40	Monocyte differentiation antigen CD14	100	6	31	98	1	9			
Q1RMN8_BOVIN	25	Immunoglobulin light chain, lambda gene cluster				75	1	23			

Accession Number	MW	Identified Proteins*	HTST			UHT			UV		
			Pro	Pep	%C	Pro	Pep	%C	Pro	Pep	%C
A6QM09_BOVIN	25	Putative uncharacterized protein	100	2	19						
PERL_BOVIN	81	Lactoperoxidase	50	2	1.3	99	3	3			
Q9TRB9_BOVIN	2	Enterotoxin-binding glycoprotein PP20K (Fragment)	100	2	70	100	2	70			
ANXA5_BOVIN	36	Annexin A5	100	3	14						
LACB_BOVIN	20	Beta-lactoglobulin	100	6	87	100	5	85			
B3VTM3_BOVIN (+4)	78	Lactoferrin	100	10	19	100	10	13			
A6H7D4_BOVIN (+1)	52	1-acylglycerol-3-phosphate O-acyltransferase 6 (Lysophosphatidic acid acyltransferase, zeta)	100	3	9	40	1	3	71	1	3
A7YWR0_BOVIN (+2)	36	Apolipoprotein E	100	3	14				53	1	3
A5D7R9_BOVIN	21	NRAS protein							100	2	12
A5D7N2_BOVIN (+7)	14	HIST2H2BE protein							98	1	12
A2VDV1_BOVIN	22	Ankyrin repeat domain 22	99	2	13				74	1	6
Q28049_BOVIN	14	Alpha lactalbumin (Fragment)	100	2	20	100	5	37			
ALBU_BOVIN	69	Serum albumin	100	22	49	100	18	29			
LIPL_BOVIN	53	Lipoprotein lipase	100	2	5	21	1	4			
RAB8A_BOVIN	24	Ras-related protein Rab-8A									
EF1A1_BOVIN	50	Elongation factor 1-alpha 1							28	1	5
ACOC_BOVIN	98	Cytoplasmic aconitate hydratase									
FABP7_BOVIN	15	Fatty acid-binding protein, brain									
D4QGC1_BOVIN (+1)	59	Tyrosine-protein kinase Lyn	100	2	6						
PPBT_BOVIN	57	Alkaline phosphatase, tissue-nonspecific isozyme	99	2	4						
A4FUH8_BOVIN (+1)	21	Chromosome 1 open reading frame 93 ortholog	99	2	25						
B5T263_9CETA (+6)	90	Toll-like receptor 2 OS=Bos indicus x Bos taurus GN=TLR2 PE=4 SV=1	97	2	5						
A5D792_BOVIN (+5)	18	DCK protein							100	2	12

Pro, probability of protein; Pep, peptides identified; %C, percent peptide coverage

Table 38. Functional classification of MFGM proteins

MW	Accession Number	Uniprot	Identified Proteins	Function	Functional Classification
275	FAS_BOVIN	Q71SP7	Fatty acid synthase	A multifunctional protein with 7 catalytic activities. It catalyzes the formation of long-chain fatty acids from acetyl-CoA, malonyl-CoA and NADPH.	Lipid transport/metabolism
265	ACACA_BOVIN	Q9TTS3	Acetyl-CoA carboxylase 1	Catalyzes the rate-limiting reaction in the biogenesis of long-chain fatty acids. It also carries out three functions: biotin carboxyl carrier protein, biotin carboxylase and carboxyltransferase	Lipid transport/metabolism
147	XDH_BOVIN	P80457	Xanthine dehydrogenase/oxidase	Complexes with butyrophilin and adipophilin in fat globule secretion. Also, it plays a role in purine degradation, oxidation of hypoxanthine to xanthine, oxidation of xanthine to uric acid, and contributes to the generation of reactive oxygen species	Lipid transport/metabolism/secretion
100	ENPP3_BOVIN	P15396	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	Hydrolyzes extracellular nucleotide and cleaves a variety of phosphodiester and phosphosulfate bonds including deoxynucleotides, nucleotide sugars, and NAD.	Unknown
98	ACOC_BOVIN	Q0VCU1	Cytoplasmic 9 aconitate hydratase	Iron sensor. It functions as mRNA binding protein that regulates uptake, sequestration and utilization of iron when cellular iron levels are low.	Homeostatic regulator
90	B5T263_9CETA	B5T263	Toll-like receptor 2 (TLR2)	Mediates recognition of microbial patterns to eliminate pathogens	Immunity & Defense
88	Q99BK3_9ALPH	Q99BK3	Ribonucleotide reductase large subunit	Catalyzes the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides	Protein synthesis, binding, and/or folding
87	D5HSX1_BOVIN	D5HSX1	cGMP-dependant type II protein kinase	Phosphorylates proteins for up or down regulation	Homeostatic regulator
83	ERG7_BOVIN	P84466	Lanosterol synthase	Catalyzes the cyclization of (S)-2,3 oxidosqualene to lanosterol, a reaction that forms the sterol nucleus.	Lipid transport/metabolism
82	PIGR_BOVIN	P81265	Polymeric immunoglobulin receptor	Binds polymeric IgA and IgM at the basolateral surface of epithelial cells.	Immunity & Defense
81	PERL_BOVIN	P80025	Lactoperoxidase	Antimicrobial agent that might protect cow's udder from infection and promote growth in newborn calves.	Immunity & Defense
78	Q0VCZ8_BOVIN	Q0VCZ8	Acyl-CoA synthetase long-chain family member 1	Catalytic activity	Lipid transport/metabolism
78	B3VTM3_B	B3VTM3	Lactoferrin	Maintains cellular iron homeostasis, transports iron, and	Homeostatic regulator

MW	Accession Number	Uniprot	Identified Proteins	Function	Functional Classification
76	OVIN NPT2B_BO VIN	Q27960	Sodium-dependent phosphate transport protein 2B	peptidase activity Involved in actively transporting phosphate into cells via Na ⁺ cotransport	General transporter
73	ABCG2_BO VIN	Q4GZT4	ATP-binding cassette sub-family G member 2	Plays an important role in the exclusion of xenobiotics from the brain. May be involved in brain-to-blood efflux	General transporter
73	Q8MKB7_B OVIN	Q8MKB7	Na ⁺ /glucose cotransporter	Transport sodium ion/glucose membrane	General transporter
71	HSP7C_BO VIN	P19120	Heat shock cognate 71 kDa protein	Acts as a repressor of transcriptional activation and as a chaperone	Protein synthesis, binding, and/or folding
69	ALBU_BOV IN	P02769	Serum albumin	The main protein of plasma, also present in milk, with a good binding capacity for water, Ca ²⁺ , Na ⁺ , K ⁺ , fatty acids, hormones, bilirubin and drugs.	Homeostatic regulator
63	A5D7M6_B OVIN	A5D7M6	KRT5 protein	Structure molecule activity	Cell structure
63	5NTD_BOV IN	Q05927	5'-nucleotidase	Hydrolyzes extracellular nucleotides into membrane permeable nucleosides.	General transporter
61	A4FV94_BO VIN	A4FV94	KRT6A protein	Intermediate filament provides structure molecule activity	Cell Structure
59	BT1A1_BO VIN	A4FV94	Butyrophilin subfamily 1 member A1	Complexes with xanthine oxidase for fat globule secretion & MFGM function	Lipid transport/metabolism
59	Q0IIA4_BO VIN	Q0IIA4	Glycoprotein 2 (Zymogen granule membrane)	A major membrane protein in the pancreatic zymogen granule, which is cleaved and released into the pancreatic duct along with exocrine secretions. Its function is unknown (Dittie and Kern, 1992).	Unknown/secretion
59	A5PKG9_B OVIN	A5PKG9	FGR protein	Also known as Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog. It is involved in protein phosphorylation, defense response to Gram (+) bacteria, nucleotide binding, protein kinase activity, ATP binding; transferase activity; transferring phosphorous-containing groups	Cell surface receptor mediated signal transduction
59	D4QGC1_B OVIN	D4QGC1	Tyrosine-protein kinase Lyn	Protein phosphorylation; response to DNA damage stimulus; + regulation of cell proliferation; response to hormone stimulus; phosphorylation; erythrocyte differentiation; intracellular signal transduction;	Cell surface receptor mediated signal transduction
58	MUC1_BOV IN	Q8WML4	Mucin-1	Provides a protective layer on epithelial cells against bacterial.	Immunity & Defense

MW	Accession Number	Uniprot	Identified Proteins	Function	Functional Classification
57	PPBT_BOVIN	P09487	Alkaline phosphatase, tissue-nonspecific isozyme	Catalytic activity: A phosphate monoester + H ₂ O = an alcohol + phosphate.	Unknown/indicator
55	A6QNZ7_BOVIN	A6QNZ7	Keratin 10 (Epidermolytic hyperkeratosis; keratosis palmaris et plantaris)	Intermediate filament, provides structural activity	Cell structure
53	CD36_BOVIN	P26201	Platelet glycoprotein 4	Binds to collagen, thrombospondin, anionic phospholipids and oxidized LDL. Binds long chain fatty acids and may function in the transport and/or as a regulator of fatty acid transport.	Lipid transport/metabolism
53	LIPL_BOVIN	P11151	Lipoprotein lipase	Hydrolyzes triglycerides of circulating chylomicrons and very low density lipoproteins (VLDL).	Lipid transport/metabolism
52	A6H7D4_BOVIN	A6H7D4	1-acylglycerol-3-phosphate O-acyltransferase 6 (Lysophosphatidic acid acyltransferase, zeta)	Involved in acyl-CoA metabolic process, lipid biosynthetic process, regulation of multicellular organism growth, and transferase activity	Lipid transport/metabolism
50	EF1A1_BOVIN	P68103	Elongation factor 1-alpha 1	Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis.	Protein synthesis, binding, and/or folding
49	PLIN2_BOVIN	Q9TUM6	Perilipin-2	May be involved in development and maintenance of adipose tissue	Lipid transport/metabolism
49	Q17QL7_BOVIN	Q17QL7	KRT15 protein	Intermediate filament	Cell structure
48	Q3T0K7_BOVIN	Q3T0K7	MFGE8 protein	Involved in cell adhesion, phagocytosis, engulfment, integrin binding, and phosphatidylethanolamine binding.	Immunity & Defense
48	Q3SX32_BOVIN	Q3SX32	Mannose 6 phosphate receptor binding protein 1	Coat protein for lipid droplets. Shares homology with perilipin and adipophilin	Lipid transport/metabolism
46	GNAS2_BOVIN	P04896	Guanine nucleotide-binding protein G(s) subunit alpha isoforms short	Involved as modulators or transducers in various transmembrane signaling systems. The G(s) protein is involved in hormonal regulation of adenylate cyclase: it activates the cyclase in response to beta-adrenergic stimuli.	G-protein mediated cell signaling
46	Q3ZC65_BOVIN	Q3ZC65	Ancient ubiquitous protein 1	Involved in acyltransferase activity and metabolic process	Lipid transport/metabolism
42	ACTB_BOVIN	P60712	Actin, cytoplasmic 1	Involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.	Cell structure
40	NSDHL_BOVIN	Q3ZBE9	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	Catalyzes following reaction: 3-beta-hydroxy-4-beta-methyl-5-alpha-cholest-7-ene-4-alpha-carboxylate + NAD(P) ⁺ = 4-alpha-methyl-5-alpha-cholest-7-en-3-one + CO ₂ + NAD(P)H.	Lipid transport/metabolism

MW	Accession Number	Uniprot	Identified Proteins	Function	Functional Classification
40	GNAI1_BOVIN	P63097	Guanine nucleotide-binding protein G(i) subunit alpha-1	Involved as modulators or transducers in various transmembrane signaling systems.	G-protein mediated cell signaling
40	A6QNL0_BOVIN	A6QNL0	Monocyte differentiation antigen CD14	Cooperates with MD-2 and TLR4 to mediate the innate immune response to bacterial lipopolysaccharide (LPS).	Immunity & Defense
39	ANXA1_BOVIN	P46193	Annexin A1	It is a calcium/phospholipid-binding protein, which promotes membrane fusion and is involved in exocytosis. Annexin A1 is released from apoptotic neutrophils and acts on macrophages to promote phagocytosis and removal of apoptotic cells (Perretti and D'Acquisto, 2009).	Immunity & defense
39	ANXA2_BOVIN	P04272	Annexin A2	It is a calcium-regulated membrane-binding protein, which binds two Ca^{+2} ions and whose affinity for calcium is greatly enhanced by anionic phospholipids. It is a cell surface receptor that induces the expression cell adhesion molecules (Yoder; Lennon <i>et al.</i> ; Zhang and McCrae).	Cell surface receptor mediated signal transduction
38	A9XTK4_BOVIN	A9XTK4	17-beta hydroxysteroid dehydrogenase	Enzyme involved in oxidation-reduction process, binding, and metabolic process	Lipid transport/metabolism
37	GBB2_BOVIN	P11017	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	Involved as a modulator or transducer in various transmembrane signaling systems. The beta and gamma chains are required for the GTPase activity, for replacement of GDP by GTP, and for G protein-effector interaction.	G-protein mediated cell signaling
37	GBB1_BOVIN	P62871	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	Modulator or transducer in various transmembrane signaling systems.	G-protein mediated cell signaling
36	A7E340_BOVIN	A7E340	Mucin 15, cell surface associated	Provides protection to neonate by binding to bacteria	Immunity & Defense
36	ANXA5_BOVIN	P81287	Annexin A5	An anticoagulant protein that acts as an indirect inhibitor of the thromboplastin-specific complex. Recently, it was demonstrate to contribute to membrane repair (Bouter <i>et al.</i> , 2011).	Homeostatic regulator
36	A7YWR0_BOVIN	A7YWR0	Apolipoprotein E	Involved in negatively regulation of endothelial cell proliferation, lipid metabolic process, cholesterol catabolic process, lipid transport, induction of apoptosis, and anti-apoptosis;	Lipid transport/metabolism
35	GIPC2_BOVIN	Q1JQD4	PDZ domain-containing protein GIPC2	Found in cytoplasm, provides structure	Cell structure
34	Q6QRN7_BOVIN	Q6QRN7	PP1201 protein	Also known as RECS1 is expressed in higher levels in response to laminar shear stress (Shukla <i>et al.</i>)	Homeostatic regulator

MW	Accession Number	Uniprot	Identified Proteins	Function	Functional Classification
34	Q2KIS4_BO VIN	Q2KIS4	Dehydrogenase/reductase (SDR family) member 1	Involved in oxidation-reduction process, binding, and metabolic process	Homeostatic regulator
34	A6H7G0_B OVIN	P07514	CYB5R3 protein	Involved in desaturation and elongation of fatty acids, cholesterol biosynthesis, drug metabolism, and, in erythrocyte, methemoglobin reduction.	Lipid transport/metabolism
33	A6QLH3_B OVIN	A6QLH3	STX3 protein	Involved in intracellular protein transport, neurotransmitter transport, SNAP receptor activity, exocytosis; cell-cell junction;	Membrane/protein trafficking
31	A8E4P3_BO VIN	A8E4P3	STOM protein	Associates with membrane and involved in cation conductance	General transporter
26	CASA2_BO VIN	P02663	Alpha-S2-casein	Important role in the capacity of milk to transport calcium phosphate. Casocidin-I inhibits the growth of E.coli and S.carnosus.	Milk protein
26	B0JYP6_BO VIN	B0JYP6	IGK protein	Immunoglobulin light chain kappa v	Immunity & defense
25	CASA1_BO VIN	P02662	Alpha-S1-casein	Important role in the capacity of milk to transport calcium phosphate. Antioxidant peptide has 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity	Milk protein
25	Q3T101_BO VIN	Q3T101	IGL@ protein	Immunoglobulin lambda locus	Immunity & defense
25	A4FUX1_B OVIN	A4FUX1	CIDEA protein	A member of CIDE (cell-death-inducing DNA-fragmentation-factor-45-like effector) family of proteins, which are highly enriched in brown adipose tissue playing a critical role in adaptive thermogenesis and fat accumulation (Christianson <i>et al.</i>).	Lipid transport/metabolism
25	Q1RMN8_B OVIN	Q1RMN8	Immunoglobulin light chain, lambda gene cluster	Involved in immunity	Immunity & defense
24	RB11A_BO VIN	Q2TA29	Ras-related protein Rab-11A	Modulates endosomal trafficking. Acts as a major regulator of membrane delivery during cytokinesis	Membrane/protein trafficking
24	RAB7A_BO VIN	Q3T0F5	Ras-related protein Rab-7a	Involved in late endocytic transport. Contributes to the maturation of phagosomes (acidification)	Membrane/protein trafficking
24	Q2TBH2_B OVIN	Q2TBH2	Related RAS viral (R-ras) oncogene homolog	GTP-binding; nucleotide-binding; small GTPase mediated signal transductitn; intracelleluar; membrane	Membrane/protein trafficking
24	RAB8A_BO VIN	A4FV54	Ras-related protein Rab-8A	May be involved in vesicular trafficking and neurotransmitter release	Membrane/protein trafficking

MW	Accession Number	Uniprot	Identified Proteins	Function	Functional Classification
23	RAB18_BOVIN	Q0IIG8	Ras-related protein Rab-18	Plays a role in apical endocytosis/recycling. May be implicated in transport between the plasma membrane and early endosomes	Membrane/protein trafficking
23	A1L528_BOVIN	A1L528	RAB1A, member RAS oncogene family	GTP-binding; nucleotide-binding; protein transport; small GTPase mediated signal transduction	G-protein mediated cell signaling
23	Q2T9M8_BOVIN	Q2T9M8	Synaptosomal-associated protein 23 isoform SNAP23A	Forms a complex which serves as a binding site for the general membrane fusion machinery. Synaptobrevin/VAMP and syntaxin are believed to be involved in vesicular transport. A member of the ARF family of GTP-binding proteins	Membrane/protein trafficking
23	ARL15_BOVIN	Q5EA19	ADP-ribosylation factor-like protein 15		G-protein mediated cell signaling
23	RAB13_BOVIN	Q58DS5	Ras-related protein Rab-13	Could participate in polarized transport, in the assembly and/or the activity of tight junctions	Membrane/protein trafficking
23	RAB5C_BOVIN	Q58DS9	Ras-related protein Rab-5C	Involved in protein transport, probably involved in vesicular traffic.	Membrane/protein trafficking
23	A5D7E0_BOVIN	A5D7E0	RAB35 protein	GTP-binding; nucleotide binding; protein transport; small GTPase mediated signal transduction	Membrane/protein trafficking
22	SAR1A_BOVIN	Q3T0D7	GTP-binding protein SAR1a	Involved in transport from the endoplasmic reticulum to the Golgi apparatus. SAR1A-GTP-dependent assembly of SEC16A on the ER membrane forms an organized scaffold defining endoplasmic reticulum exit sites (ERES).	Membrane/protein trafficking
22	YKT6_BOVIN	Q3T000	Synaptobrevin homolog YKT6	It is a vesicular soluble NSF attachment protein receptor (v-SNARE) mediating vesicle docking and fusion to a specific acceptor cellular compartment.	Membrane/protein trafficking
22	CHP1_BOVIN	Q3SYS6	Calcium-binding protein p22	Required for constitutive membrane traffic. Inhibits GTPase-stimulated Na ⁺ /H ⁺ exchange. Also inhibits calcineurin phosphatase activity. Required for activity of SLC9A1/NHE1	General transporter/ Ca ⁺² in milk
22	RHOC_BOVIN	Q1RMJ6	Rho-related GTP-binding protein RhoC	Regulates a signal transduction pathway linking plasma membrane receptors to the assembly of focal adhesions and actin stress fibers	G-protein mediated cell signaling
22	A2VDV1_BOVIN	A2VDV1	Ankyrin repeat domain 22	Assists in protein-protein interaction	Protein interaction
21	CASK_BOVIN	P02668	Kappa-casein	Kappa-casein stabilizes micelle formation, preventing casein precipitation in milk. Casoxins A, B and C have opioid antagonist activity. Casoxin C causes biphasic ileal contractions through the binding to the complement C3a receptors. Casoplatelin inhibits platelet aggregation.	Milk protein

MW	Accession Number	Uniprot	Identified Proteins	Function	Functional Classification
21	RAC1_BOVIN	P62998	Ras-related C3 botulinum toxin substrate 1	A plasma membrane-associated small GTPase, which cycles between active GTP-bound and inactive GDP-bound states. In its active state, binds to a variety of effector proteins to regulate cellular responses such as secretory processes, phagocytosis of apoptotic cells, epithelial cell polarization and growth-factor induced formation of membrane ruffles.	G-protein mediated cell signaling
21	RAP1A_BOVIN	P62833	Ras-related protein Rap-1A	Induces morphological reversion of a cell line transformed by a Ras oncogene.	G-protein mediated cell signaling
21	B6VAP7_BOVIN	B6VAP7	CDC42 protein	Small GTPase mediated signal transduction; cell-cell adhesion; acin cytoskeleton organization; positive regulation of protein phosphorylation;	G-protein mediated cell signaling
21	ARF1_BOVIN	P84080	ADP-ribosylation factor 1	A GTP-binding protein involved in protein trafficking among different compartments. It modulates vesicle budding and uncoating within the Golgi complex. Deactivation induces the redistribution of the entire Golgi complex to the endoplasmic reticulum, suggesting a crucial role in protein trafficking.	Membrane/protein trafficking
21	A5D7R9_BOVIN	A5D7R9	NRAS protein	Neuroblastoma RAS viral (v-ras) oncogene homolog, a small GTPase mediated signal transduction protein that shuttles between Golgi apparatus and the plasma membrane	G-protein mediated cell signaling
21	A4FUH8_BOVIN	Q58CY6	Chromosome 1 open reading frame 93 ortholog	Unsure	Unknown
20	B5B0D4_BOVIN	B5B0D4	Major allergen beta-lactoglobulin	Fractionation in whey protein correlates with allergen	Whey protein
20	LACB_BOVIN	P02754	Beta-lactoglobulin	Primary component of whey.	Whey protein
19	B0JYL8_BOVIN	B0JYL8	Cofilin 1 (Non-muscle)	Cofilin causes depolymerization at the minus end of filaments, which prevents their reassembly.	Protein synthesis, binding, and/or folding
18	CTHL1_BOVIN	P22226	Cathelicidin-1	It has a potent microbicidal activity and is active against <i>S.aureus</i> and <i>E.coli</i> .	Immunity & defense
18	Q3SYR8_BOVIN	Q3SYR8	Immunoglobulin J chain	Immunity	Immunity & defense
18	SEP15_BOVIN	A8YXY3	15 kDa selenoprotein	It may contribute to the quality control of protein folding in the endoplasmic reticulum	Protein synthesis, binding, and/or folding

MW	Accession Number	Uniprot	Identified Proteins	Function	Functional Classification
18	A5D792_BO VIN	A5D792	DCK protein	It is a core component of nucleosome.	Protein synthesis, binding, and/or folding Immunity & defense
17	GLCM1_BO VIN	P80195	Glycosylation-dependent cell adhesion molecule 1	It is a mucin-like endothelial glycoprotein that acts as an adhesive ligand for L selectin, a leukocyte cell surface protein (Dowbenko <i>et al.</i> , 1993)	
16	Q861V9_BO VIN	Q5EA19	Similar to peptidylprolyl isomerase A (Cyclophilin A) (Fragment)	Also known as PPIA accelerates protein folding	Protein synthesis, binding, and/or folding
15	FABPH_BO VIN	P10790	Fatty acid-binding protein, heart	Plays a role in the intracellular transport of long-chain fatty acids and their acyl-CoA esters..	Lipid transport/metabolism
15	FABP7_BO VIN	Q09139	Fatty acid-binding protein, brain	Plays a role in the intracellular transport of long chain fatty acids and their acyl-CoA esters.	Lipid transport/metabolism
14	Q9TSF1_BO VIN	Q9TSF1	Butyrophilin (Fragment)	Complexes with xanthine oxydase in fat globule secretion & MFGM function	Lipid transport/metabolism
14	A5D7N2_B OVIN	A5D7N2	HIST2H2BE protein	It is a core component of nucleosome.	Protein synthesis, binding, and/or folding
14	Q28049_BO VIN	Q28049	Alpha lactalbumin (Fragment)	A whey protein, which binds calcium ions.	Whey protein
2	Q9TRB9_B OVIN	Q9TRB9	Enterotoxin-binding glycoprotein PP20K (Fragment)	A <i>E. coli</i> heat-labile enterotoxin that binds to caseins, whey proteins, and milk fat globule membrane (Shida <i>et al.</i> , 1994)	Whey protein

K. Two-Dimensional Statistical Data from Delta2D

Table 39. ANOVA-2 results utilizing Delta2D for all treatments against Fresh milk as a control ($p < 0.01$).

Parameter	3 Week_6Treat		3 Week_5Treat		Fresh/4C/UV	
	#	%	#	%	#	%
Temperature significant	286	86	199	65	169	52
Week significant	211	64	194	63	168	52
Interaction significant	184	55	144	47	94	29
Temperature non-significant	46	14	109	35	155	48
Week non-significant	121	36	114	37	156	48
Interacion non-significant	148	45	164	53	230	71
Non-significant for all effects	18	5	50	16	90	28
Total Spots	332		308		324	

#, Total protein in category; %, Percent variation

Table 40. ANOVA-2 results utilizing Delta2D for each treatment against Fresh milk as a control ($p < 0.01$).

Parameters	4 C		UV		Batch		HTST		UHT	
	#	%	#	%	#	%	#	%	#	%
Temperature significant	104	34	116	37	171	50	128	37	186	67
Week significant	138	45	112	37	145	43	127	36	101	36
Interaction significant	54	18	54	17	100	29	83	24	92	33
Temperature non-significant	201	66	195	63	170	50	222	63	91	33
Week non-significant	167	55	199	64	196	57	223	64	176	64
Interacion non-significant	251	82	257	83	241	71	267	76	185	67
Non-significant for all effects	109	36	132	42	102	30	133	38	60	22
Total Spots	305		311		341		350		277	

#, Total protein in category; %, Percent variation