to my family
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Functionality of
Milk Fat Globule Membrane Material

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in
Applied Biological Sciences
Dutch translation of the title:
Functionaliteit van fragmenten uit het melkvetglobulemembraan

Cover reference:
MFGM structure model: Dr. Roeland Rombaut
Others images: from the results of this PhD study

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<td>α-lactalbumin</td>
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<tr>
<td>ADPH</td>
<td>Adipophilin</td>
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<tr>
<td>AMF</td>
<td>Anhydrous milk fat</td>
</tr>
<tr>
<td>β-LG</td>
<td>β-lactoglobulin</td>
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<tr>
<td>BMP</td>
<td>Buttermilk powder</td>
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<tr>
<td>BM-MFGM</td>
<td>MFGM material obtained from MF of buttermilk</td>
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<tr>
<td>BRCA1</td>
<td>Breast cancer type 1 susceptible protein</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer type 2 susceptible protein</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BTN</td>
<td>Butyrophilin</td>
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<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
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<td>CD36</td>
<td>Cluster of differentiation 36</td>
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<td>CP</td>
<td>Cream phase</td>
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<td>Cryo-SEM</td>
<td>Cryo-Scanning electron microscopy</td>
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<tr>
<td>d₃₂</td>
<td>Sauter mean diameter</td>
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<tr>
<td>DF</td>
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<td>DSC</td>
<td>Differential scanning calorimetry</td>
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<td>ELSD</td>
<td>Evaporative light-scattering detector</td>
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<td>FID</td>
<td>Free induction decay</td>
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<td>FG(s)</td>
<td>Fat globule(s)</td>
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<tr>
<td>GluCer</td>
<td>Glucosylceramide</td>
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<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
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<td>HLB</td>
<td>Hydrophilic-lipophilic balance</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>LacCer</td>
<td>Lactosylceramide</td>
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<td>MF</td>
<td>Microfiltration</td>
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<td>MFGM</td>
<td>Milk fat globule membrane</td>
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<tr>
<td>MPa</td>
<td>Megapascal</td>
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<td>MUC1</td>
<td>Mucin1</td>
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<td>NC</td>
<td>Natural cream</td>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>O/W</td>
<td>Oil-in-water</td>
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<td>PASIII</td>
<td>Periodic acid Schiff III</td>
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<td>PC</td>
<td>Phosphatidylcholine</td>
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<tr>
<td>PCA</td>
<td>Principal component analysis</td>
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<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<td>PI</td>
<td>Phosphatidylinositol</td>
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<tr>
<td>PL(s)</td>
<td>Polar lipid(s)</td>
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<td>Proteose peptone 3</td>
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<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
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<td>Recombined cream(s)</td>
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<td>SC</td>
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<td>Sodium dodecyl sulfate</td>
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<td>Solid fat content</td>
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<td>SM</td>
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<td>SMP</td>
<td>Skimmed milk powder</td>
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<td>SMUF</td>
<td>Simulated milk ultrafiltrate</td>
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<td>SP</td>
<td>Serum phase</td>
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<tr>
<td>SSA</td>
<td>Specific surface area</td>
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<tr>
<td>T_{cr}</td>
<td>Crystallization temperature</td>
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<tr>
<td>t_{ch}</td>
<td>Churning time</td>
</tr>
<tr>
<td>t_{wh}</td>
<td>Whipping time</td>
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<td>t,T-program</td>
<td>Time-temperature program</td>
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<td>MFGM material obtained from MF of buttermilk whey</td>
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<td>WPC</td>
<td>Whey protein concentrate</td>
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<tr>
<td>XDH/XO</td>
<td>Xanthine dehydrogenase/oxidase</td>
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Summary

The milk fat globule membrane (MFGM) has been the subject of several studies over the years due to the growing interest in its nutritional and technological properties. MFGM material isolate from dairy by-products contains various proteins and polar lipids (PLs) which are efficient natural surface-active materials that could be used as emulsifiers. However, in view of this use, the functional properties of the MFGM-specific proteins and PLs need to be investigated. Currently, the role of MFGM components and their possible interaction at the oil-water interface during emulsification is lacking. In addition, the combined effects of MFGM proteins and PLs on the surface characteristics and adsorption behavior at the interface have, so far, not been addressed. Therefore, the main objective of this doctoral research was to gain a deeper insight in the technological potentials and the functional properties of different MFGM materials/components in oil-in-water emulsions, especially the role as emulsifiers of the PL moiety and protein moiety (i.e. MFGM-specific proteins, caseins and whey proteins) in the MFGM isolate.

Chapter 1 provides an overview of the origin, structure, composition (particularly PLs and membrane-specific proteins) and the biological values of MFGM materials as well as the techniques for isolation and purification of MFGM materials. Besides, an overview of dairy emulsion systems, particularly the stabilization, characterization of emulsions and the whipping properties of recombined cream (RC) are summarized. A detailed critical literature review on the technological functionalities of MFGM material is an important part of this chapter since these functionalities affect the stability of oil-in-water emulsions.

Buttermilk contains several components which are not found in other milk-derived fractions. The composition of buttermilk is rather similar to that of skimmed milk, with the exception of a considerable amount of materials (PLs and MFGM-specific proteins) that originate from the MFGM. The emulsifying properties of MFGM material isolated from reconstituted buttermilk using microfiltration (MF) (so-called BM-MFGM), in comparison with those of buttermilk powder (BMP), skimmed milk powder (SMP) and sodium caseinate (SC) were investigated in Chapter 2. Mixtures prepared with of 35% soybean oil were homogenized at different pressures. The particle size distribution and microscopic observation indicated the presence of aggregated emulsion droplets when BMP or SMP were used, especially at high homogenization pressures (15/2 MPa and above). With the use of BM-MFGM, no such aggregation was observed. Emulsions prepared with BM-MFGM at 9/2 MPa had a monomodal particle size distribution with an average droplet size of about 1.9µm, which was
significantly smaller than that of emulsions prepared with BMP and SMP at the same pressures. In addition, BM-MFGM emulsions had a much lower viscosity and Newtonian-like flow characteristics. Emulsions prepared with SC showed poor stability against separation during storage compared to the other emulsions. These results indicated the high potential of BM-MFGM as emulsifier.

Besides the use of buttermilk as a source for the isolation of MFGM, buttermilk whey, the by-product of cheese-making from buttermilk, is considered a more advantageous source than buttermilk for the concentration of MFGM material, since buttermilk whey contains virtually no casein micelles. Buttermilk whey still contains some residual fat and consists of lipoprotein particles, MFGM fragments, and small fat globules (FGs) which can be used as emulsifier. MFGM materials isolated from different dairy by-products can differ in composition and properties which may, therefore, influence the emulsifying properties of the materials. To compare the emulsifying properties of MFGM materials or the interaction between MFGM material (i.e. PLs and MFGM-specific proteins) and other components (caseins, whey proteins and minerals), the same BM-MFGM material used in Chapter 2 and MFGM material isolated by MF from buttermilk whey (so-called Whey-MFGM), were used to stabilize oil-in-water emulsions [35% (w/w) soybean oil]. In order to find out type and concentration of MFGM material with unique interfacial functionality, the emulsifying properties of the individual MFGM materials (BM-MFGM and Whey-MFGM) and their mixtures with BMP (4:6 by dry weighed) were compared with those of Lacprodan®PL-20, a commercial dairy ingredient which is rich in milk PLs and proteins. The particle size distribution, the viscosity, the surface protein load and surface PLs load of oil-in-water emulsions prepared using soybean oil were examined. The results are presented and discussed in Chapter 3. A pronounced droplet aggregation was observed with emulsions stabilized with Whey-MFGM or with a mixture of Whey-MFGM and BMP (4:6 w/w), whereas no aggregation was observed with emulsions stabilized with BM-MFGM, Lacprodan®PL-20 or a mixture of BM-MFGM and BMP. The surface protein load and PLs load were lowest with emulsions prepared with BM-MFGM, whereas the highest surface protein load and PLs load was observed with emulsions prepared with a mixture of Whey-MFGM and BMP. The reason for this could be the high ash content (minerals; e.g. Ca^{2+}) and the low casein to whey proteins ratio and the absence of MFGM-specific proteins (XO and BTN) of the Whey-MFGM. This indicated that the emulsifying ability of MFGM material isolated from dairy by-products were strongly dependent on the presence of MFGM components such as PLs, MFGM-specific
proteins, caseins, whey proteins and minerals, especially the concentration of these substances.

The influence of individual constituents of MFGM such as PLs, MFGM-specific proteins and non-MFGM proteins (i.e. caseins and whey proteins) in the stabilization of oil droplet emulsion or possible interaction between them at the interface during emulsification is presented in **Chapter 4 and Chapter 5**. In both chapters, MFGM protein and PLs concentrate was separated from whole BM-MFGM (i.e. MFGM material isolated from the reconstituted buttermilk using MF as described in Chapter 2) using solvent extraction. The emulsifying properties of MFGM protein and PLs concentrate, individually or in a combination, at various concentrations of proteins (0.3; 1.3 and 2.3 wt%) and PLs (0.3; 1.3 and 2.3 wt%), were investigated. The results of the physicochemical properties of the emulsions, more particularly the particle size distribution, flow behavior and creaming stability are presented and discussed in **Chapter 4**. It was found that at low emulsifier concentrations (< 2.3%), there was an interacting effect between proteins and PLs on the droplet size. The phase separation of emulsions prepared with a combination of 0.3% proteins and 0.3% PLs was similar to that of emulsions containing 0.3% proteins. The PLs in this system did not retard the phase separation in emulsions prepared with a combination of proteins and PLs. All emulsions showed pseudoplastic flow behavior, as the apparent viscosity decreased with an increasing shear rate. In conclusion, the use of MFGM protein concentrate had better emulsifying/stabilizing properties compared to those of the PLs concentrate. However, at low emulsifier concentrations (< 2.3%), combination of both proteins and PLs resulting in an enhancement in their emulsifying properties, especially in the formation of emulsions with a small droplets size and a low viscosity.

In **Chapter 5** the roles and the effects of MFGM protein and PLs concentrate, at various concentrations of proteins and PLs, on the surface characteristics and adsorption behavior or on a possible competitive adsorption at the interface of the emulsion is elucidated. The adsorption behavior of proteins and PLs at the oil-water interface as well as the surface protein load and PLs load were examined. In addition, cryo-SEM was used to elucidate the oil-surfactant-water interaction in the emulsion. The results showed that PLs concentrate had less emulsifying ability than MFGM protein concentrate. In emulsions prepared with both MFGM protein and PLs concentrate, proteins were more preferentially adsorbed at the emulsion droplet surface compared to PLs. The adsorbed proteins were not replaced by addition of polar lipids. The cryo-SEM micrographs showed that at 2.3% of emulsifier
concentration, the emulsions prepared with MFGM protein concentrate had smoother droplet surfaces with only the imprint of the structure created by the ice crystals, than emulsions prepared with PLs concentrate. In addition, almost all droplets in emulsions containing 2.3% proteins were broken across the interfacial layer after freeze-fracture while the droplets in emulsion containing PLs were broken along the interfacial layer and the droplets were almost always intact. The mechanism of this phenomenon needs to be further investigated.

Chapter 6 presents the preliminary findings about the use of MFGM materials to improve the whipping properties of RC. All MFGM materials of Chapter 3 (BM-MFGM, Whey-MFGM, Lacprodan®PL-20 and BMP) which were different in composition, were used to make RC. RC was prepared by homogenizing 35% (w/w) anhydrous milk fat into an aqueous phase with the same concentration of total protein (i.e. 2.3g 100g\(^{-1}\) RC). An aqueous phase was prepared by dispersing individual BM-MFGM, Whey-MFGM, Lacprodan®PL-20, or BMP or a mixture of MFGM materials and BMP (4:6, w/w) in water. The physicochemical and whipping properties of the RC were determined and compared with those of natural cream (NC). Amongst the four emulsifier materials used in this study, Whey-MFGM revealed a similar crystallization behavior to NC. After 5 days of storage, the solid fat content (SFC) of RC Whey-MFGM40, BM-MFGM40, Lacprodan40 and Lacprodan100 was similar to that of NC, whereas the SFC of BM-MFGM100 and BMP100 was higher than that of NC. RC made with a mixture of Whey-MFGM and BMP (4:6 w/w) showed improved whipping properties, namely a high overrun, no serum loss and reasonable firmness, as compared to NC. Shear-induced partial coalescence of whey-MFGM40 was slower compared to that of NC. The results indicated that a mixture of Whey-MFGM and BMP has promising features for being used as an ingredient in the production of RC by improving the whipping properties of the cream.

Finally in Chapter 7, conclusions from the results obtained in the whole PhD research were formulated. The key findings of the experimental works showed that the emulsifying properties of MFGM material depended on the sources or the dairy by-products for isolating the material and types of applied products. Amongst the emulsifier materials used in the whole PhD thesis, BM-MFGM had the highest emulsifying/stabilizing in oil-in-water emulsion. In contrast, Whey-MFGM had a high potential to improve the whipping properties of RC. Among the two main fractions of MFGM material isolated from reconstituted buttermilk (MFGM protein and PLs concentrate), MFGM protein concentrate had superior emulsifying ability compared to PLs concentrate. However, at low total emulsifier
concentrations (< 2.3%), there was an interaction between proteins and PLs resulting in an enhancement of their emulsifying properties. The optimum emulsifier concentration for coverage and stabilization of droplets is 2.3% proteins or a combination of 1.3% proteins with varying concentrations of PLs from 0.3 to 2.3%, and in such case surface protein load reached approximately 2.0 mg m$^{-2}$. 
Samenvatting

Door toenemende interesse in de nutritionele en technologische eigenschappen van het melkvetglobule membraan (MFGM) is dit reeds vele jaren het onderwerp van wetenschappelijk onderzoek. MFGM kan geïsoleerd worden uit verschillende nevenproducten van de zuivelindustrie en bevat verschillende types eiwitten en polaire lipiden. Deze kunnen gebruikt worden als natuurlijke emulgator omwille van hun efficiënte oppervlakte-actieve eigenschappen. Bijgevolg is het van cruciaal belang dat deze functionele eigenschappen van specifieke fragmenten uit het MFGM, i.e. eiwitten en polaire lipiden, verder worden onderzocht. Momenteel ontbreekt inzicht in de rol van MFGM fragmenten aan de olie-water interfase tijdens emulsievorming en in mogelijkse interacties tussen de verschillende componenten. Daarenboven werden de gecombineerde effecten van de MFGM eiwitten en polaire lipiden op de interfase eigenschappen alsook hun absorptiegedrag nog niet opgehelderd. Bijgevolg is het hoofddoel van dit doctoraatsonderzoek, meer inzicht te verwerven in het technologisch potentieel en de functionele eigenschappen van MFGM fragmenten in olie-in-water emulsies en meer specifiek de emulgerende rol van de eiwitten (i.e. MFGM-specifieke eiwitten, caseïne en wei-eiwitten) en de polaire lipiden van het geïsoleerde MFGM.

Hoofdstuk 1 biedt een overzicht van de oorsprong, structuur, samenstelling (voornamelijk polaire lipiden en membraan-specifieke eiwitten) en biologische waarde van MFGM fragmenten alsook van de technieken die kunnen aangewend worden voor isolatie en opzuivering van deze fragmenten. Verder wordt ook een overzicht gegeven van emulsiestabilisatie, de karakterisering van emulsies en de opklopeigenschappen van gerecombineerde room. Een kritische, gedetailleerde literatuurstudie met betrekking tot de technologische functionaliteit van MFGM fragmenten is een belangrijk deel van dit hoofdstuk aangezien deze functionaliteit de stabiliteit van olie-in-water emulsies sterk zal beïnvloeden.

Karnemelk bevat verscheidene componenten die niet worden teruggevonden in andere nevenstromen van de zuivelindustrie. De samenstelling van karnemelk is gelijkaardig aan deze van magere melk met uitzondering van de aanzienlijke hoeveelheid MFGM fragmenten (polaire lipiden en MFGM-specifieke eiwitten). In Hoofdstuk 2 werd onderzoek verricht naar de emulgerende eigenschappen van MFGM fragmenten die via microfiltratie geïsoleerd werden uit gereconstituïerde karnemelk (i.e. BM-MFGM) en deze werden vergeleken met fragmenten verkregen uit andere bronnen met name karnemelkpoeder (BMP), mager melkpoeder (SMP) en Na-caseïnaat (SC). Olie-in-water emulsies op basis van 35% sojaolie
werden gehomogeniseerd onder verschillende drukken. De deeltjesgroottedistributie en microscopische observaties duiden de aanwezigheid van geaggregeerde druppels aan in het geval van BMP en SMP en voornamelijk bij emulsies geproduceerd bij een hoge homogenisatiedruk (15/2 MPa en hoger). Emulsies op basis van BM-MFGM vertoonden deze aggregatie niet, bij een homogenisatiedruk van 9/2 MPa kon meer bepaald een monomodale deeltjesgroottedistributie met een gemiddelde druppelgrootte van 1.9 μm waargenomen worden. Dit was significant kleiner dan de gemiddelde druppelgrootte in emulsies op basis van BMP en SMP geproduceerd bij dezelfde druk. Bovendien vertoonden de BM-MFGM emulsies een veel lagere viscositeit en Newtoniaans vloeigedrag. Emulsies op basis van SC waren weinig stabiel waardoor fasescheiding duidelijk kon waargenomen worden tijdens bewaring. Deze resultaten benadrukken het potentieel van BM-MFGM als emulgator. Naast het gebruik van karnemelk als bron voor de isolatie van MFGM, wordt karnemelk wei, het nevenproduct van de kaasproductie uit karnemelk, aanzien als een meer voordelige bron voor de isolatie van MFGM omwille van de virtuele afwezigheid van caseïnemicellen. Karnemelk wei bevat een kleine hoeveelheid residueel vet en bestaat uit lipoproteïne deeltjes, MFGM fragmenten, en kleine vetdruppels met emulgerende eigenschappen. MFGM fragmenten geïsoleerd uit verschillende nevenstromen van de zuivelindustrie kunnen verschillen in samenstelling en eigenschappen en bijgevolg ook in hun emulgerende werking. Om dit te bevestigen werd het BM-MFGM dat reeds werd onderzocht in Hoofdstuk 2 vergeleken met MFGM geïsoleerd via microfiltratie uit karnemelk wei (i.e. Whey-MFGM). De emulgerende eigenschappen en de interacties tussen verschillende MFGM fragmenten (i.e. polaire lipiden en MFGM-specifieke eiwitten) en andere componenten (caseïne, wei-eiwitten en mineralen) werden onderzocht door beide fracties toe te passen in olie-in-water emulsies (35% w/w sojaolie). Met als doel inzicht te verwerven in het type en de concentratie van de MFGM fragmenten met unieke oppervlakte-actieve eigenschappen, werden de emulgerende eigenschappen van de individuele fracties (BM-MFGM en Whey-MFGM) en mengsels met BMP (4:6 w/w) vergeleken met een commercieel zuivel-gebaseerd ingrediënt, i.e. Lacprodan®PL-20, dat rijk is aan polaire lipiden en eiwitten. De deeltjesgrootteverdeling, viscositeit en adsorptie van eiwitten en polaire lipiden aan de interfase van olie-in-water emulsies op basis van sojaolie werden geanalyseerd en beschreven in Hoofdstuk 3. Een uitgesproken aggregatie van vetdruppels werd vastgesteld in emulsies gestabiliseerd met Whey-MFGM of met mengsels van Whey-MFGM en BMP (4:6 w/w), terwijl geen aggregatie werd vastgesteld in emulsies gestabiliseerd met BM-MFGM, Lacprodan®PL-20 of een
mengsel van BM-MFGM en BMP. In emulsies gestabiliseerd met BM-MFGM werden minder eiwitten en polaire lipiden geadsorbeerd dan in emulsies gestabiliseerd met een mengsel van Whey-MFGM en BMP. Mogelijks liggen het hoog asgehalte (mineralen, bv. Ca\(^{2+}\)), de lage verhouding van caseïne op wei-eiwitten en de afwezigheid van MFGM-specifieke eiwitten (XO en BTN) in Whey-MFGM aan de basis van dit verschil. Hieruit kon geconcludeerd worden dat de emulgerende werking van MFGM fragmenten geïsoleerd uit nevenproducten uit de zuivelindustrie sterk afhankelijk is van de aanwezigheid en vooral de concentratie van MFGM-specifieke componenten zoals polaire lipiden, MFGM-specifieke eiwitten, caseïne, wei-eiwitten en mineralen.

De werking van individuele MFGM componenten zoals polaire lipiden, MFGM-specifieke eiwitten en non-MFGM eiwitten (i.e. caseïne en wei-eiwitten) in de stabilisatie van olie druppels en mogelijke interacties aan de interfase tijdens emulgering worden toegelicht in Hoofdstuk 4 en Hoofdstuk 5. In beide hoofdstukken wordt gebruik gemaakt van een concentraat van MFGM eiwitten en een concentraat van MFGM polaire lipiden. Deze werden via solventextractie uit BM-MFGM bekomen (i.e. MFGM fragmenten via microfiltratie geïsoleerd uit gereconstitueerde karnemelk, zoals besproken in Hoofdstuk 2). De emulgerende eigenschappen van MFGM eiwitten en MFGM polaire lipiden, afzonderlijk of in combinatie, bij verschillende concentraties aan eiwitten (0.3; 1.3 en 2.3 wt%) en polaire lipiden (0.3; 1.3; 2.3 wt%), werden onderzocht. Hoofdstuk 4 beschrijft de fysicochemische eigenschappen van deze emulsies, meer bepaald de deeltjesgrootteverdeling, het vloeigedrag en de stabiliteit tegen oproming. Bij een lage emulgatorconcentratie (< 2.3%) werd een interagerend effect tussen eiwitten en polaire lipiden op de deeltjesgrootteverdeling opgemerkt. Echter, eiwitten vertonen een grotere impact op de deeltjesgrootteverdeling dan de polaire lipiden. De fasescheiding in emulsies gestabiliseerd met 0.3% eiwitten en 0.3% polaire lipiden en emulsies gestabiliseerd met 0.3% eiwitten was vergelijkbaar. De aanwezigheid van polaire lipiden had dus geen vertragend effect op de fasescheiding. Alle emulsies vertoonden pseudoplastisch vloeigedrag daar de schijnbare viscositeit daalde met een toenemende afshuiving. Er kon geconcludeerd worden dat MFGM eiwitten beter emulgerende/stabiliserende eigenschappen vertoonden dan MFGM polaire lipiden. Nochtans werd een verbetering in emulgerende eigenschappen waargenomen voor emulsies met een lage emulgatorconcentratie (< 2.3%) die zowel eiwitten als polaire lipiden bevatten, en voornamelijk bij de vorming van emulsies met kleine deeltjesgrootte en een lage viscositeit.
Samenvatting

In Hoofdstuk 5 wordt een licht geworpen op de effecten van MFGM eiwitten en MFGM polaire lipiden, in verschillende concentraties, op het adsorptiegedrag en de oppervlakte-activie eigenschappen alsook op een mogelijks competitieve adsorptie aan de interfacie van olie-in-water emulsies. De geadsorbeerde hoeveelheid en het adsorptiegedrag van eiwitten en polaire lipiden aan de olie-water interfacie werden opgehelderd. Bovendien werd cryo-SEM gebruikt om interacties tussen olie, water en emulgerende componenten aan te tonen. De resultaten bevestigen dat MFGM polaire lipiden een lagere emulgerend e werking hebben dan MFGM eiwitten. Deze laatste worden bij voorkeur geadsorbeerd aan de interfacie van emulsies waar zowel polaire lipiden als eiwitten aanwezig zijn en bovendien kon in deze emulsies geen competitieve verdringing tussen eiwitten en polaire lipiden aan de interfacie worden aangetoond. In emulsies gestabiliseerd met 2.3% emulgator kon aan de hand van cryo-SEM worden gedemonstreerd dat in de aanwezigheid van enkel MFGM eiwitten de druppels een gladder oppervlak vertoonden, met enkel een zichtbare afdruk van ijskristallen, dan wanneer enkel polaire lipiden aanwezig waren. Bovendien kon opgemerkt worden dat in beide emulsies de druppels op een andere manier worden gebroken tijdens bevriezing. In het geval van de eiwit-gestabiliseerde emulsies werd een breukvlak waargenomen dwars doorheen de interfacie terwijl in de emulsies gestabiliseerd met polaire lipiden het breukvlak langsheen de interfacie liep en de druppels grotendeels intact bleven. Het mechanisme dat aan de basis ligt van deze verschillen dient verder onderzocht te worden.

Hoofdstuk 6 focuseert op het gebruik van MFGM fragmenten ter verbetering van het opklopgedrag van gerecombineerde room. Alle MFGM fracties uit Hoofdstuk 3 (BM-MFGM, Whey-MFGM, Lacprodan®PL-20, BMP of mengsels van MFGM en BMP (4:6, w/w)), allen verschillend in samenstelling, werden gebruikt voor het maken van gerecombineerde room. In emulsies met Whey-MFGM analog aan natuurlijke room, werd een gelijkaardige hoeveelheid vast vet waargenomen in gerecombineerde room gestabiliseerd met Whey-MFGM40, BM-MFGM40, Lacprodan40 en Lacprodan10 in vergelijking met natuurlijke room. Wanneer de vier afzonderlijke MFGM fracties worden vergeleken, kon opgemerkt worden dat vetkristallisatie in emulsies met Whey-MFGM opploopt aan natuurlijke room. Na bewaring van de stalen gedurende vijf dagen, werd een gelijkmatige hoeveelheid vast vet waargenomen in gerecombineerde room gestabiliseerd met Whey-MFGM40, BM-MFGM40, Lacprodan40 en Lacprodan10 in vergelijking met natuurlijke room. Wanneer de vier afzonderlijke MFGM fracties worden vergeleken, kon geregistreerd worden dat vetkristallisatie in emulsies met Whey-MFGM analog verloopt aan natuurlijke room. Na bewaring van de stalen gedurende vijf dagen, werd een gelijkmatige hoeveelheid vast vet waargenomen in gerecombineerde room gestabiliseerd met Whey-MFGM40, BM-MFGM40, Lacprodan40 en Lacprodan10 in vergelijking met natuurlijke room.
room terwijl de hoeveelheid vast vet in emulsies gestabiliseerd met BM-MFGM100 en BMP100 hoger was. Een mengsel van Whey-MFGM en BMP (4:6 w/w) had een positief effect op de opklopeigenschappen van gerecombineerde room, namelijk meer luchtinslag, geen afscheiding van serum en een aanvaardbare stevigheid in vergelijking met natuurlijke room. Partiële coalescentie geïnduceerd door afschuiving in emulsies stabiliseerd met Whey-MFGM40 toonde een trager verloop in vergelijking met natuurlijke room. Deze resultaten impliceren dat een mengsel van Whey-MFGM en BMP een veelbelovend ingrediënt kan zijn voor gerecombineerde room met als doel het verbeteren van de opklopeigenschappen.

Tenslotte werden de conclusies uit dit doctoraatsonderzoek geformuleerd in Hoofdstuk 7. De belangrijkste bevindingen van het experimenteel werk bewijzen dat de emulgerende werking van MFGM fragmenten afhankelijk is van de bron en dus het type nevenproduct van de zuivelindustrie alsook het product waarin deze fragmenten worden toegepast. Van alle MFGM fragmenten gebruikt in dit onderzoek, heeft BM-MFGM het hoogste potentieel inzake emulgerende/stabiliserende werking in olie-in-water emulsies. Whey-MFGM, daarentegen, toont het meest potentieel ter verbetering van de opklopeigenschappen van gerecombineerde room. Wanneer de twee grote fracties aanwezig in MFGM (eiwitten en polaire lipiden) worden vergeleken, kan gesteld worden dat MFGM eiwitten een superieure emulgerende capaciteit vertonen ten opzichte van de polaire lipiden. Echter, bij lage emulgatorconcentratie (< 2.3%), werden de emulgerende eigenschappen van polaire lipiden verbeterd door aanwezigheid van eiwitten en meer specifiek door de interactie tussen beide fracties. De optimale emulgatorconcentratie om voldoende adsorptie en bijgevolg stabilisatie te hebben is 2.3% eiwitten of een combinatie van 1.3% eiwitten met een variabele concentratie polaire lipiden tussen 0.3 en 2.3%. In deze gevallen wordt een belading van 2.0 mg eiwitten per m² bekomen aan het de interfase van de druppels.
Outline of the Research

This manuscript describes the functionality of milk fat globule membrane material. The outline of the subjects of research is summarized in Figure 0-1. All chapters are connected to some extent. The outline comprises a literature review in Chapter 1, an experimental research section, including research strategies and the research findings in Chapter 2 – Chapter 6, and a general conclusion in Chapter 7.

Chapter 1 presents some background information related to MFGM, with special focus on the technological functionalities of MFGM material. Emulsion formation, stability and characteristics of dairy emulsions are also subjects of the review.

Chapter 2 describes the behavior of emulsions prepared with BM-MFGM material and soybean oil. The emulsifying properties of BM-MFGM material are compared to those of BMP, SMP and SC at different homogenization pressures.

In Chapter 3, the emulsifying properties of MFGM materials isolated from two dairy by-products (buttermilk powder and buttermilk whey) and a commercial Lacprodan®PL-20 are presented.

Chapters 4 and 5 describe the effect of PLs and MFGM proteins, separately or in combination, on the physicochemical properties and on the competitive adsorption behavior between the two component groups, at the interface of oil-in-water emulsions.

Chapter 6 presents preliminary data on the use of the MFGM materials isolated from dairy by-products, to improve the whipping properties of RC.

Chapter 7 contains general conclusions and some recommendations for future research.
Outline of the research

Chapter 1: Literature review

Isolation of MFGM materials from dairy by-products using MF

Chapter 2. Emulsifying properties of a MFGM material isolated from reconstituted buttermilk

Chapter 3. Comparison of emulsifying properties of MFGM materials isolated from different dairy by-products

Chapter 6. Potential of MFGM materials to improve the whipping properties of RC

Separation of BM-MFGM material into MFGM protein and PLs concentrate using solvent fractionation

Chapter 4. Combined effects of MFGM proteins and PLs on the physicochemical properties of O/W emulsions

Chapter 5. Adsorption behavior of combined MFGM proteins and PLs at the interface of oil-in-water emulsions

Chapter 7. Conclusions and perspectives

Figure 0-1. Schematic outline of the research strategy. Arrows indicate the interrelation of the chapters
Chapter 1. Literature Review

Part of this chapter is redrafted after the accepted chapter-in-book manuscript:


1.1 Introduction

The dispersion of milk FGs in milk is not a simple oil-in-water emulsion. The FGs are surrounded by a complex membrane, which cannot be considered as a simple monomolecular film of surface active material. Instead, the membrane has several distinct layers that are laid down during synthesis in the secretory cells of the mammary gland. This membrane is known as the MFGM. This membrane is different from that of either milk or plasma and represents a unique biophysical system (Singh, 2006). The origin, composition and structure of the MFGM have been recently elucidated and reported by numerous reviewers. This membrane offers protection against enzymatic attack and prevents the globules to coalesce and stabilizes the globules in the milk serum (Danthine et al., 2000, Evers, 2004). The MFGM and its components are considered beneficial due to their health, nutrition- and technology-related properties.

Origin, structure and composition of MFGM are discussed in the first part of this chapter. Next, the techniques for isolation and purification of MFGM from raw milk and dairy by-products are presented. Knowing the mechanisms of emulsion destabilization will help select the suitable process parameters, composition or the formulation for emulsion preparation. An overview of dairy emulsion systems, particularly the factors influencing stability and characteristics of dairy emulsions are also discussed. The chapter goes on with presenting the mechanism of structure built-up and the characteristics of whipped cream since it is important to determine the whipping properties of RC. A review of up-to-date knowledge concerning the technological functionalities and the possible applications of MFGM material for food products is another important part of this chapter. Finally, the biological values of MFGM components are given.
1.2 Origin and structure of MFGM

1.2.1 Origin

The membrane surrounding milk lipid globules is essentially a tripartite structure that originates from the apical plasma membrane, the endoplasmic reticulum and possibly from other intracellular compartments. The MFGM fraction originating from the apical membranes has a typical bilayer appearance and is termed the primary membrane. The material derived from the endoplasmic reticulum has the appearance of a monolayer of proteins and PLs that covers the triacylglycerol-rich core lipids of the globules before secretions (Keenan and Mather, 2006).

The triglycerols are synthesized in or on the surfaces of rough endoplasmic reticulum membranes and accumulate in the form of micro-lipid droplets in the cytoplasm. These intracellular droplets are covered by a diffuse interfacial layer, which consists of phospholipids, glycosphingolipids, cholesterol and proteins. Micro-lipid droplets grow in volume by fusion with each other to form cytoplasmic lipid droplets of various sizes, which are then transported to the apical pole of the cell through the cytoplasm and are secreted from the epithelial cell. During secretion, the droplets are coated with the outer plasma membrane from the cell. The composition of the outer bilayers of fat globule membrane is similar to that of the apical plasma membrane of secretory cells (Keenan and Dylewski, 1995, Heid and Keenan, 2005). Apart from the MFGM, secretory cell fragments can also be secreted into the lumen. They are rich in PLs, have a similar composition as the MFGM and comprise only 4% of the total milk fat (Deeth, 1997, Keenan et al., 1999).

1.2.2 Structure

The MFGM is highly structurized and contains unique PLs and membrane specific proteins. The natural MFGM consist of three distinct layers. Viewed from the inside lipid core outwards following elements can be distinguished: a monolayer of proteins and PLs surrounding the intracellular fat droplet, an intermediate electron dense proteinaceous coat on the inner face of the bilayer and finally, a true bilayer membrane of PLs and proteins. The entrained cytoplasmic materials between the inner coat and outer bilayer membrane form the cytoplasmic crescents (Danthine et al., 2000, Michalski et al., 2002, Evers, 2004, Keenan and Mather, 2006) (Figure 1-1). The MFGM originates from several distinct layers with a total thickness varying from 10 to 20 nm (Wooding, 1971, Walstra et al., 1999)
Chapter 1. Literature review

Figure 1-1. Structure of the fat globule with detailed arrangement of the main MFGM. From Dewettinck et al. (2008).

The bilayer membrane of the MFGM is derived from the apical plasma membrane of the secretory cell and the most widely accepted model for this type of membrane is the fluid mosaic model. This suggests that the phospholipid bilayer serves as the backbone of the membrane, which exists in a fluid state. Peripheral membrane proteins are partially embedded or loosely attached to the bilayer. Trans-membrane proteins extend through the lipid bilayer. Carbohydrate moieties from glycolipids and glycoproteins are oriented outwards. Cholesterol is present in the PL bilayer (Dewettinck et al., 2008).

In a more recent review, Lopez et al. (2011a) has proposed new 2-dimensional models for the structure of the MFGM (Figure 1-2).

Figure 1-2. Schematic representation of the MFGM suggested by Lopez et al. (2011a)
These models describe the coexistence of at least two lipid phases in the MFGM: the fluid matrix in a liquid-disordered (Ld) phase and liquid-ordered (Lo) phase domains. The former is composed of the glycerophospholipids (PE, PC, PI, PS), proteins, glycoproteins, glycolipids and the latter is the segregation of SM and cholesterol. The presence of Lo phase SM-rich domains either across the two leaflets of the MFGM bilayer or only on the outer leaflet of the MFGM, remains to be elucidated (Lopez, 2011b).

1.2.3 Composition of MFGM

The amount and composition of the MFGM varies considerably depending on the fat globule size and the fat content of milk, which is in turn, is influenced by several others factors such as the type of feed, breed, age, health and stage of lactation of cows (Keenan and Dylewski, 1995, Keenan, 2001). The major components of the MFGM comprise membrane specific proteins (mainly glycoproteins), phospho- and sphingolipids. Protein and phospholipids together account for over 90% of the membrane dry weight but the relative proportions of lipids and proteins may vary widely (Singh, 2006). The average composition of the MFGM is given in the Table 1-1.

Table 1-1. Estimated average composition of the MFGM (Walstra et al., 2006)

<table>
<thead>
<tr>
<th>Component</th>
<th>mg/100g FGs</th>
<th>g/100g MFGM dry matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>1800</td>
<td>70</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>650</td>
<td>25</td>
</tr>
<tr>
<td>Cerebrosides</td>
<td>80</td>
<td>3</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>Monoglycerides</td>
<td>+(^a)</td>
<td>?</td>
</tr>
<tr>
<td>Water</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Carotenoids + Vit. A</td>
<td>0.04</td>
<td>0.0</td>
</tr>
<tr>
<td>Fe</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Cu</td>
<td>0.01</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>&gt; 2570</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^a\) +; present, but quantity unknown

The composition of the milk fat globule membrane also changes when subjected to different treatments such as cooling, agitation, heating and aging (Evers, 2004). Kirst, (1996) divided the factors that affect the composition of the MFGM into 3 groups: physiological,
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chemical/enzymatic and physical/mechanical. Physical/mechanical factors are related to milk handling during and after milking. Pre-factory milk handling includes air inclusion, pumping and stirring of the milk, changes in temperature and changes in time. Milk handling at the factory involves aging, agitation, heat treatment, separation, homogenization and changes in water content (McPherson and Kitchen, 1983).

1.2.3.1 Lipids in MFGM

Milk fat globule membranes consist mainly of PLs and a negligible amount of neutral lipids (triglycerides, diglycerides, monoglycerides, cholesterol and its esters). High melting triglycerides are the major fraction of the neutral lipids in the MFGM (Wooding and Kemp, 1975). However these triglycerides appear to originate from contamination by the core of the FGs during the isolation process of the membrane (Walstra, 1985). Hence, the method of isolation has great influence on the triglyceride content of the MFGM. Sterols and sterol esters account for 0.3 to over 2% of the total membrane-associated lipids. Cholesterol is most likely incorporated into the MFGM as a constituent of the plasma membrane-derived outer bilayer (Mather, 2011).

The phospholipids and sphingolipids are the PL fractions (Dewettinck et al., 2008). In bovine milk, about 60% of the PLs are associated with the milk FGs and the rest is located in the membrane material of skim milk (Patton and Keenan, 1975). The major PL fraction present in the MFGM consists of phosphatidylcholine (PC), 35%; phosphatidylethanolamine (PE), 30%; sphingomyelin (SM), 25%; phosphatidylinositol (PI), 5%; phosphatidylserine (PS), 3%; glucosylerceramide (GluCer), lactosylerceramide (LacCer). Gangliosides (Gang) are present in trace amounts (Deeth, 1997, Danthine et al., 2000). An example of HPLC separation pattern of milk PLs is given in Figure 1-3.

The MFGM phospholipids contain high levels of long chain (> C₁₄) fatty acids; short and medium chain (C₄-C₁₄) fatty acids are virtually absent. Especially, PE is highly unsaturated followed by PI and PS. PC is rather saturated as compared to other glycerophospholipids. SM is very uncommon in its fatty acid pattern and consists of around 70-97% of saturated fatty acid and of a high proportion (40-50%) of fatty acids having a chain length of C₂₀ or more. These two physical features contribute to the lower fluidity and the retained rigidity of the MFGM (Bitman and Wood, 1990).
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1.2.3.2 Proteins in MFGM

Depending on the source of the milk and the isolation method, the content of proteins present in the MFGM varies from 25-70% (Danthine et al., 2000, Walstra et al., 2006, Fong et al., 2007). Historically, polyacrylamide gel electrophoresis (PAGE) had been used to elucidate the protein composition of the MFGM and the major MFGM proteins were designated according to their relative mobility in sodium dodecyl sulfate (SDS)-PAGE. The major proteins, in order from the top of the SDS gels, are mucin 1 (MUC1), xanthine dehydrogenase/oxidase (XDH/XO), periodic acid Schiff III (PASIII), cluster of differentiation 36 (CD36), butyrophilin (BTN), periodic acid Schiff 6/7 (PAS 6/7) and adipophilin (ADPH) (Mather, 2000). In addition, the minor proteins of the MFGM are polymeric immunoglobulin receptor proteins, apolipoprotein E, apolipoprotein A1, 71 kg/mol heat shock cognate protein, clusterin, lactoperoxidase, immunoglobulin heavy chain, peptidylpropyl isomerase A, actin, fatty acid binding protein (FABP) and breast cancer type 1 susceptibility protein (BRCA1) (Fong et al., 2007). Furthermore, parts of the proteose peptone fraction like proteose peptone 3 (PP3) originate from the MFGM (Campagna et al., 2001). These are summarized in Table 1-2. A detailed discussion of the biochemical properties of the major MFGM proteins can be found in the review by Mather (2000).
Table 1-2. Protein components of the MFGM (Singh, 2006).

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Molecular weight (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucin 1 (MUC 1)</td>
<td>160,000 – 200,000</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>150,000</td>
</tr>
<tr>
<td>Pass III</td>
<td>95,000 – 100,000</td>
</tr>
<tr>
<td>CD36 or PAS IV</td>
<td>76,000 – 78,000</td>
</tr>
<tr>
<td>Butyrophilin</td>
<td>67,000</td>
</tr>
<tr>
<td>Adipophilin (ADPH)</td>
<td>52,000</td>
</tr>
<tr>
<td>PAS 6/7</td>
<td>48,000 – 54,000</td>
</tr>
<tr>
<td>Fatty acid binding protein (FABP)</td>
<td>13,000</td>
</tr>
<tr>
<td>BRCA 1</td>
<td>210,000</td>
</tr>
</tbody>
</table>

Figure 1-4. SDS-PAGE of different dairy products. (1) raw milk; (2) skimmed milk; (3) acid buttermilk; (4) butter serum, aqueous fraction obtained from churning of cream; (5) acid buttermilk whey, soluble fraction obtained from acidification of sweet-cream buttermilk; (6) acid buttermilk quark, coagulated fraction obtained from the acidification of the sweet-cream buttermilk; (7) MFGM-isolate; and (8) mark 12 molecular weight standard. On each lane, 250 ng of protein was loaded (Rombaut et al., 2006b). Cited from Dewettinck et al., 2008.

The protein composition is highly dependent on the isolation method and the analytical procedure. Moreover, all proteins are not equally connected with the MFGM. Some are integral proteins, some are peripheral proteins and others are only loosely attached. With
proteomic analysis, more than 250 proteins were identified to associate with MFGM (Le et al., 2013). However, upon separation by SDS-PAGE the MFGM material is resolved into 7-8 major bands (Figure 1-4).

In electrophoresis, after immobilization on gels, proteins must be visualized by staining with dyes. Coomassie blue, silver staining and Periodic acid/Schiff (PAS) reagents are often used for MFGM proteins. However, proteins have different staining sensitivity to dyes. Coomassie blue staining is widely applied to detect, non-specifically, all proteins. Coomassie blue stains micrograms of proteins. Silver staining can be 100 times (nanograms) more sensitive than Coomassie blue, making silver staining useful for detecting proteins in trace quantities and for staining two-dimensional gels (Morrissey, 1981, Echan and Speicher, 2007). Morrissey (1981) found that there is a considerable variation from protein to protein in the sensitivity and that some proteins which stain well with Coomassie blue do not stain at all with silver stains. PAS staining is mainly used to detect structures containing a high proportion of carbohydrates in the molecule, and hence is specifically applied to visualize glycoproteins (Mather, 2000). As seen in Figure 1-5, MUC1 and PASIII, heavily glycosylated proteins, are clearly observed after PAS staining while XDH/XO, BTN, ADPH, PAS 6/7 and FABP stained strongly with Coomassie blue.

![Figure 1-5](image)

**Figure 1-5.** Separation of bovine MFGM proteins by SDS-PAGE. Gels were stained either with Coomassie blue (CB) (left) or the periodic acid/Schiff reagent (PAS) (right) (Mather, 2000).
1.3 Isolation and purification of MFGM material

1.3.1 Isolation of MFGM from raw milk

Milk contains as little as 2 g L\(^{-1}\) of MFGM material. At lab-scale production level, MFGM from raw milk can be obtained after a four-steps isolation procedure (Singh, 2006). Firstly, the separation of FGs is carried out from whole milk by centrifugation to obtain cream and skim milk. The cream is then washed to remove contaminating components including caseins and whey proteins. Further disruption of the FGs and the release of the membrane material can be achieved using a number of processes, including freezing and thawing, agitation or churning, or treatment with detergents or bile salts. The membrane fractions are subsequently recovered by means of several methods such as ultra-centrifugal sedimentation (100,000 g), freeze-drying or MF. The yield and purity of the final MFGM material are influenced by the type of washing solutions, the washing temperature, the number of washing steps, and the volume of the washing solution used for each washing (Le et al., 2009). The lower the loss during isolation, the more representative is the MFGM material obtained compared with that of natural MFGM in terms of composition and functional (emulsifying) properties (Le et al., 2009).

1.3.2 Production of MFGM from dairy by-products

Next to raw milk, dairy industrial by-products like buttermilk, butter serum and buttermilk whey are considered interesting sources for the isolation of MFGM material because of their low cost and their richness in MFGM components (Rombaut et al., 2007a). Buttermilk is the main by-product obtained during butter making. It is rich in MFGM fractions and also contains all water soluble material present in milk e.g. caseins, whey proteins and lactose. Buttermilk whey is the by-product of the cheese and casein manufacturing processes from buttermilk. It still contains some residual fat which consists of lipoprotein particles, MFGM and small FGs (Rombaut et al., 2007a). Several techniques have already been developed to separate MFGM material from these by-products (Figure 1-6).

Separation of the MFGM material from dairy by-products can be achieved by cross-flow MF. This technique results in a successive separation and fractionation of milk FGs (Goudédranche et al., 2000). The filtration parameters such as pH, temperature, type of buttermilk, pore size and type of membrane material will influence the ultrafiltration or MF process of buttermilk (Morin et al., 2004, Rombaut et al., 2007a).
Figure 1-6. Various techniques and possible pathways for isolation of MFGM from dairy by-products (Le, 2012)

Prior to MF, the similarity in size of casein micelles and MFGM components is the first major consideration taken into account to achieve an effective fractionation of buttermilk (Sachdeva and Buchheim, 1997). This similarity has to be overcome to achieve an effective fractionation of buttermilk and butter serum. Corredig et al. (2003) used citrate to dissociate the casein micelles of buttermilk; the MFGM material is then recovered by high speed centrifugation. In later studies the above authors observed that MF of citrate treated buttermilk through a membrane of 0.1 µm nominal pore size was more suitable for isolating MFGM materials than high speed centrifugation. Rombaut et al. (2006b) also reported that the addition of trisodium citrate was necessary to dissociate casein micelles into casein components that were small enough to permeate the membrane during the filtration process. However, the high sodium
citrate concentrations may result in loss of PLs (64% recovery) during filtration due to blocking and fouling of the filter membrane with MFGM particles (Rombaut et al., 2006b).

In another approach, the removal of caseins from reconstituted buttermilk has been achieved by precipitation of the caseins and subsequent filtration (through 0.2-µm membranes) of the phospholipids extracts (Sachdeva and Buchheim, 1997). However, the loss of PLs in the curd is dependent on the coagulation method used. Sachdeva and Buchheim (1997) obtained a recovery of 53, 79 and 83% of PLs from a reconstituted buttermilk in the resulting whey by coagulation of caseins with lactic acid bacterial, citric acid, and rennet treatment, respectively. Morin et al. (2007) investigated another process to eliminate the caseins, by washing the cream prior to churning. When compared to normal cream buttermilk, the permeation fluxes during MF were two-fold higher when the cream buttermilk was washed. Caseins and whey proteins were the main classes of proteins removed during washing. Buttermilk obtained from washed cream will be a good substrate for the isolation of MFGM by MF because it contains less caseins (Morin et al., 2007).

Whey contains 0.4 to 0.5% residual fat which is the main source of small FGs, lipoprotein particles and milk fat globule membrane fragments (Rombaut et al., 2007a). Isolation of the MFGM from whey (buttermilk whey, acid buttermilk whey) by filtration is favorable because whey is free of caseins. Absence of caseins facilitates to obtain concentrated MFGM material by filtration, although the MFGM concentration of whey is limited compared with other dairy fractions such as buttermilk or butter serum. Morin et al. (2006) reported that the expected transmission of MFGM proteins through the membrane was lower when using buttermilk whey compared to regular buttermilk.

To increase the purity of MFGM material, filtration is normally performed in combination with DF. There are two manners in which DF can be carried out. Batch DF is performed by adding a solvent to the retentate followed by reconcentration, while in continuous DF the solvent is added continuously to the retentate at a rate equal to the permeate flow rate. Corredig et al. (2003) reported that increasing the number of DFs from 2 to 6, reduces casein contamination in the retentate from 30% to 6%. However, increasing the DF steps has the disadvantage of loss of MFGM material (Sachdeva and Buchheim, 1997, Rombaut et al., 2007a). The purity of MFGM material isolated from dairy by-products would not be comparable with MFGM isolated from raw milk, even with more extensive DF (Le, 2012).
After MF, several techniques can be applied to further purify MFGM material. In MFGM, the PLs interact strongly with membrane-specific proteins which makes it difficult to extract PLs completely from proteins. Therefore, solvent extraction and fractionation techniques have been developed and the purities of different fractions depend on the nature of the raw material and on the techniques used. This is a challenging step. In addition, the products obtained using these methods may not be acceptable for human consumption, unless the solvent is completely removed. Solvent extraction can be carried out using special instruments that use supercritical carbon dioxide (rather than organic lipids) as the solvent. Supercritical carbon dioxide is excellently suited as an extraction solvent due to its low critical parameters, its relative low cost, its non-toxicity, its chemical inertness, and because the resulting products are free from organic solvent residues. Astaire et al. (2003) succeeded in removing the triglycerides present in microdiafiltrated buttermilk retentate powder using supercritical carbon dioxide, hereby increasing the PLs content from 31.02 to 83.15 g 100 g\(^{-1}\) total lipids. Most likely, the inclusion of a second supercritical carbon dioxide extraction step, with addition of a modifier like ethanol, would exclusively remove the PLs, hereby resulting in a 100% pure fraction. This was actually achieved with grinded soybeans by Montanari et al. (1999). This technique can also be used for the purification of MFGM proteins, as the remaining fraction, after removal of triglycerides and PLs, are mainly MFGM proteins and minerals. The latter can even be further removed by DF or (electro)dialysis under acid conditions, yielding probably pure MFGM proteins.

On a lab scale, whole MFGM material after MF can be extracted and separated into different fractions by using organic solvent extraction (Le, 2012). A combination of chloroform and methanol at various ratios, derived from the methods described by Folch et al. (1957) and Bligh and Dyer (1959), is considered as most efficient for the extraction of PLs. Methanol disrupts hydrogen bounds between the lipids and the surrounding polar molecules, (i.e. water) and then, non-polar solvents (i.e. chloroform) access freely to the lipids. However, chloroform is under strict rules for use at some laboratories and therefore, dichloromethane was used as the alternative solvent (Le, 2012). The separated phase, containing the MFGM proteins and the PLs, was evaporated using a rotary evaporator (Le, 2012). The major MFGM proteins have been purified using serial chromatography and their properties have been characterized using different biochemical techniques (Mather, 2000). A PLs mixture can be fractionated into different fractions/classes by using certain solvent extraction methods and
chromatographic techniques (van Nieuwenhuyzen and Tomas, 2008, Rombaut and Dewettinck, 2009).

1.4 Dairy emulsion system

1.4.1 Introduction

An emulsion is a colloidal system prepared by the mixing two immiscible liquids. In an emulsion, droplets of one liquid are dispersed into another liquid. These droplets are called the dispersed phase and the liquid that contains these droplets is called the continuous phase. Water and oil are the two liquids commonly used in food emulsions. If the emulsion contains water as the continuous phase then it is said to be an oil-in water (O/W) emulsion (e.g. milk, cream) whereas a water-in-oil emulsion contains oil as the continuous phase (e.g. butters).

1.4.2 The stability of dairy emulsion

The stability of milk fat emulsions is related to the physical and chemical characteristic of milk and dairy products. Thermodynamically, emulsions are unstable. Stability is a time dependent kinetic phenomenon and is largely dependent on the size distribution of the globules. A wide variety of emulsifiers, either synthetic or natural, are used in food emulsions. There are also some dairy by-products commonly used as emulsifiers such as whey protein isolate, sodium caseinate, milk protein isolate and MFGM isolate (Kanno, 1989, Dalgleish, 1997, Dickinson and Golding, 1997, Damodaran, 2005, Dewettinck et al., 2008). The surface active components or surfactants such as proteins or small molecular surfactants are used in emulsions to increase their kinetic stability and to reduce the surface or interfacial tension. Use of these surface active components in an emulsion will protect the emulsion droplets by forming an interfacial layer. In addition, the mobility of the emulsion droplets can be decreased by adding thickeners or gelling agents. The emulsion can be formed by mixing the oil and water phases together with an emulsifier. It is important that one of the phases (either oil phase or water) breaks up into small droplets which remain dispersed within the liquid. This can be achieved by using a mixer or homogenizer. All these components (emulsifiers and gelling agents) will be distributed in the dispersed oil phase, the continuous aqueous phase and the oil-water interfacial layer at the time of homogenization or emulsification. Various types of instability mechanisms are at the basis of changes in the number, the size and the arrangements of droplets in the O/W emulsion which can be observed in emulsion systems under the form of Ostwald ripening, creaming, flocculation/aggregation, coalescence and breaking (Dickinson and Tanai, 1992, Lopetinsky
et al., 2006, Vaclavik and Christian, 2008, Fredrick et al., 2010). The different methods by which an emulsion can become unstable or break down are outlined in Figure 1-7.

**Figure 1-7. Schematic representation of the instability mechanisms in oil-in-water emulsions (Lopetinsky et al., 2006)**

**Creaming/sedimentation:** Creaming or sedimentation is caused by the difference in the mass density between the dispersed oil phase and the continuous water phase. If the droplets have a lower density, they tend to move upward to form a layer of emulsion droplets at the surface of the emulsion. This phenomenon is known as creaming. On the contrary, if the droplets have a higher density, they tend to move downwards to form a layer at the bottom of the emulsion. This phenomenon is known as sedimentation. Generally, the density of oil and fat is lower than the density of water, thus droplets of O/W emulsions tend to cream, while those of W/O emulsions tend to sediment. The rate of creaming of a single droplet is defined by the Stokes’ law (see detail in section 1.4.4.2). Sufficiently large droplets suspended in an aqueous medium of low viscosity will cream. Creaming in itself does not destabilize an emulsion, but the high concentration of oil droplets in the creamed layer promotes interactions that lead to flocculation, aggregation or coalescence (Dalgleish, 1997). The rate of creaming and the thickness of the cream layer are very markedly influenced by the processing operations. Creaming is faster and more complete at low temperature because of the temperature
dependent precipitation of the cryoglobulins. Gentle agitation during the initial stages of creaming promotes and enhances cluster formation and creaming. Homogenization prevents creaming due to the reduction of fat globule size and the increase in the surface area.

**Ostwald ripening**: Ostwald ripening in emulsion is a process of gradual growth of the larger droplets at the expense of smaller ones due to mass transport of the soluble dispersed phase (oil) through the continuous phase (water) (Kabalnov and Shchukin, 1992, Taylor, 1995). Liquids that are referred to as being immiscible often have a not negligible mutual solubility. With emulsions, which are usually polydisperse, the smaller droplets will have a higher solubility when compared with the larger ones (due to curvature effects). With time, the smaller droplets disappear and their molecules diffuse to the bulk and become deposited onto the larger droplets.

**Aggregation**: Droplets frequently approach each other due to Brownian motion. Flocculation and aggregation of the oil droplets in an emulsion arise from the inability of the adsorbed surfactant layer to prevent the close approach of the droplets in case there is insufficient surfactant to cover the entire oil-water interface created by the homogenization (Dickinson, 1989). This depends on the forces between the droplets, which include Van der Waals interactions, electrostatic forces and a variety of short-range forces. The composition of both phase and the presence of non-adsorbing polymers in the continuous phase can cause an attractive depletion force. The main repulsive forces are electrostatic, caused by identical electric charges on both droplets, mostly due to substances adsorbed onto the interface of the droplets. The pH and the ionic strength of the continuous phase are important variables determining the extent of electron repulsion. The particles tend to aggregate because of inter-atomic attraction forces, but aggregation is opposed by repulsive charge interactions (or enhanced by attractive charge interaction) which arise from the adsorbed material. The net charge of the proteins, and consequently of the adsorbed protein layer, is highly dependent on pH: if the pH is close to the isoelectric point of the protein, its net charge approaches zero, which favors aggregation of the emulsion droplets. A stable emulsion will have a charge that is large enough to prevent a close approach of the suspended droplets (Dalgleish, 1997). Steric stabilization arises from the adsorption of flexible macromolecules onto the emulsion droplets. Such molecules can prevent the particles from a close approach by a combination of mechanical and thermodynamic effects (Dalgleish, 1997). Although the two terms are almost synonymous, flocculation is generally regarded as being weaker than aggregation. And if gentle stirring suffices to achieve disruption, the aggregation is considered to be reversible,
and it is often called flocculation, while coagulation refers to aggregation where strong forces are needed to disrupt the aggregates.

**Coalescence:** Coalescence occurs when two droplets were in close contact either by aggregation or in the cream layer for some time, so that a very thin film of the continuous phase prevents oil-oil contact. The rate of film rupture depends on the film thickness and on its mechanical properties, in particular on the Gibbs elasticity of the film and the stress at which the film breaks (Borwankar et al., 1992, Urbina-Villalba et al., 2005). Emulsion droplets are moving constantly and so collision time may be very short. However, if this time is long compared to the time required for the film to break, coalescence is likely to occur. The film rupture mechanisms are largely dependent on the continuous phase properties and on the properties of the emulsifiers adsorbed at the droplets. Indeed, emulsifier molecules form a layer, usually called interfacial membrane, around the droplets that protects them from rupture. These mechanisms are very complicated and almost unique for each emulsion as they strongly depend on the system properties (McClements, 2004).

### 1.4.3 Factors influencing stability of dairy emulsions

#### 1.4.3.1 Effects of dairy emulsifiers

##### a. Effects of milk proteins

Milk proteins are well known for their emulsifying and emulsion stabilizing properties because they are amphiphilic molecules and are predominantly present at the interface (Morr, 1982, Mulvihill and Fox, 1989). In general, proteins stabilize the interface by forming a strong viscoelastic film and giving stability to an emulsion by offering protection against external factors. Proteins are commonly used as foaming and emulsifying agents in the food industry. The stability of emulsions and foams depends on the mechanical properties of the above mentioned layer. The surface hydrophobicity and charge will determine the extent of protein adsorption. To expose the hydrophobic residues to the hydrophobic phase, the adsorbed protein will unfold and rearrange its secondary and tertiary structures (Bos and van Vliet, 2001). When a high amount of proteins is present at the surface, they aggregate and form interactions. The higher the protein concentration, the less susceptible the emulsion is to partial coalescence (Goff, 1997b).

During emulsification, milk proteins become rapidly adsorbed at the O/W interface as individual molecules or in the form of aggregates (Walstra and Smulders, 2004). The newly formed layer results in steric stabilization and protects finely dispersed droplets against
recoalescence. Consequently, the emulsion is more physically stable during storage and processing (Dickinson and Stainsby, 1982).

The differences in the type and concentration of proteins affect the degree of stabilization. Caseins from milk are highly surface active and adsorb readily the whey proteins. This is because the caseins contain many hydrophobic amino acids which make the proteins to possess regions of high hydrophobicity (Swaisgood, 1992). The casein molecule is composed of four distinct proteins, $\alpha_{s1}$, $\alpha_{s2}$, $\beta$- and $\kappa$-caseins, all of which are phosphoproteins. Among these proteins, $\beta$-casein has the highest tendency to be adsorbed at the interface while $\kappa$-caseins have the lowest tendency (Hunt and Dalgleish, 1995). Caseins are very flexible molecules which are amphiphilic in their primary structure. The casein molecule is a polypeptide which is hydrophobic and contains charged residues which are not uniformly distributed along the chain. Casein monomers cannot sufficiently move away their hydrophobic part from contact with water, therefore, caseins tend form a micellar structure. However, rearrangement of caseins into micelles leads to the reduction of their emulsifying activity in the micelluar structure; the hydrophobic groups are gathering at the core while a shortage of them at the surface cause a reduction in the tendency of micelles to adsorb. Caseins are used by the dairy industries to improve emulsification. Through solubilization of caseins in acid media, and the addition of NaOH, the native structure of caseins is modified to become sodium caseinates. These processes affect the extent of protein-protein interaction, resulting in different levels of aggregation (Lucey et al., 2000).

The whey proteins comprise $\alpha$-LA, $\beta$-LG, blood serum albumin and immunoglobulins. Whey proteins have a globular structure that is stabilized via intermolecular disulfide bonds between the cysteine residues (Das and Kinsella, 1990). When compared to caseins, the whey proteins are less surface active and adsorb slowly as a result of their low hydrophobicity. On adsorption the whey proteins are unfolded i.e. undergo surface denaturation (Shimizu et al., 1981, Das and Kinsella, 1990). During unfolding, free sulfhydryl groups are exposed and interact with other protein free sulfhydryl groups and form a protective layer against flocculation (Corredig and Dalgleish, 1999). The emulsifying properties of whey proteins depend on both emulsion composition such as mineral content, surfactant and polysaccharide content, and also physical factors including temperature, pH and pressure (Dickinson and Stainsby, 1982, Dickinson and Tanai, 1992, Singh and Waungana, 2001). The effects of processing on their emulsifying and emulsion stabilizing properties will be further discussed in section 1.6.5.
MFGM proteins, although being only a very small portion of the total milk proteins, are fundamental for the stability of milk FGs (McPherson and Kitchen, 1983). Several MFGM proteins have been identified, mainly by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Mather and Keenan, 1975, Basch et al., 1985) (discussed in section 1.2.3.2). The high hydrophobicity of certain regions of their structure seems to play a role in stabilizing the milk FGs (Basch et al., 1985). Most studies on MFGM proteins have been carried out with membranes released and collected from washed, unheated milk FGs (Keenan and Patton, 1995). However, the functional properties of proteins derived from commercial buttermilk need to be characterized by carefully preparing the samples to eliminate skim milk proteins (Corredig and Dalgleish, 1998a). The heat treatment strongly affects the functional properties of MFGM isolates. This will be further discussed in section 1.6.5.2.

b. Effects of PLs

PLs are amphiphilic molecules which contain a hydrophobic tail and a hydrophilic head group. The head group contains the negatively charged phosphate group whereas the tail group consists of fatty acids (Rombaut and Dewettinck, 2006c). This unique feature largely contributes to the emulsifying properties of the membrane. The chemical structure for the most common phospholipids is shown in Figure 1-8, left.

Figure 1-8. Chemical structure of a phospholipid (X is choline, ethanolamine, serine, inositol or glycerol) (left), depiction of the head and tail part in a phospholipid (middle) and a schematical illustration of the orientation of phospholipids at the interface of an oil droplet (right) (Horn, 2012).
The phosphate group is either esterified with amino alcohols (choline, ethanolamine or serine) or polyols (inositol or glycerol), as depicted by “X” in the figure. SM which is an important milk PL, differs slightly in structure, as the glycerol part is substituted by an amino alcohol, sphingosine. Commercially available phospholipids from e.g. milk or, soy contain a combination of the different PLs. In soy lecithin PC, PE and PI are the major constituents, in milk the main constituents are PC, PE and SM (Le et al., Submitted). As shown in Figure 1-8 (right), phospholipids adsorb at the oil droplet surface with the hydrophilic head facing the water phase, and the fatty acid tails projecting into the oil droplet.

When compared to proteins, the PLs are more capable to reduce the interfacial tension, whereas at the interface the protein molecules can be anchored at various sites. Once the PLs are gathered at the interface, they will form a fluid-adsorbed layer at temperatures above their transition temperature (Hasenhuettl and Hartel, 2008). PLs are classified in two groups according to their head group charge: ionic and non ionic PLs. PLs are also classified based on the difference in their solubility in water or oil: water soluble PLs with a high HLB versus the oil soluble ones with a low HLB (Bos and van Vliet, 2001). These properties affect PLs adsorption at the interface (Peng et al., 1993). Generally water soluble surfactants are more surface active than the oil soluble ones. However, emulsions stabilized with PLs mostly show less stability against coalescence (Bos and van Vliet, 2001).

c. Interaction between proteins and small-molecular surfactants

The presence of PLs, beside proteins, may alter the stability of the emulsions. PLs are able to displace the proteins from the fluid-fluid interface by two mechanisms (Nylander et al., 1997):

- Replacement: in this mechanism PLs are adsorbed at the interface according to their strong surface activity and low Gibbs interfacial energy and displace proteins from the interface.

- Solubilization: in this mechanism PLs (mainly water soluble one) bind to the protein and form a protein-surfactant complex. The PL does not need to adsorb at the interface, but it must interact strongly with the protein.

Interaction of PLs with proteins may change the conformation and interactions of proteins and eventually influence their functionality (Dickinson and Ritzoulis, 2000, Kelley and McClements, 2003).
In a system that contains both small molecular surfactants (e.g. PLs) and proteins, small molecular surfactants may bind to proteins through hydrophobic and electrostatic interaction, for ionic surfactants, and hydrophobic interaction, for non-ionic surfactants (Coke et al., 1990, McClements, 2004). Whey proteins induce more internal interactions than caseins, leading to the formation of a viscoelastic layer, therefore, making them more capable to interact with PLs (Clark et al., 1991). XO and BTN also may be capable of forming a viscoelastic layer via disulfide bonding because of the presence of sulfur residues in their amino acid chain (Massey and Harris, 1997, Mather and Keenan, 1998). Studies by Dickinson and Hong (1994) as well as Chen and Dickinson (1993) showed that proteins, by forming a more viscoelastic protein film, are more difficult to displace from the interface. Wilde et al. (2004) reported that when comparing $\beta$-casein and $\beta$-LG, $\beta$-casein was displaced from the interface at a lower surfactant concentration due to its lower collapse pressure point and lower resistance against surface pressure resulting from the adsorption of surfactants at the interface. The capability of proteins to interact with PLs determines the mechanism of protein removal from the interface. Caseins have less affinity to interact with PLs which results in their removal from the interface by a replacement mechanism while whey proteins, especially $\beta$-LG, mainly interact with PLs, leading to a solubilization mechanism. The interaction of PLs and proteins may increase or decrease the surface activity of proteins as well as that of PLs. A complex of small molecular surfactant/protein formation was also involved in the competition of protein and PL in this system (Clark et al., 1991, Nylander et al., 1997, Bos and van Vliet, 2001, McClements, 2004). Proteins are reversibly adsorbed at the fluid-fluid interface and can be replaced by PLs. PLs also can interact with proteins and change their functionality. The combination of both PLs and proteins can alter the emulsion stability (Dickinson and Ritzoulis, 2000, Kelley and McClements, 2003, Gunning et al., 2004, Pugnaloni et al., 2004, Wilde et al., 2004).

### 1.4.3.2 Effects of fat volume

The protein-fat ratio in emulsions also influences the degree of stabilization. At a lower ratio of protein and fat, the amount of proteins is not high enough to cover the overall O/W interface and as a result not all FGs are stabilized by proteins. During emulsification these uncovered FGs may aggregate and form clusters (Fredrick et al., 2010). Van Camp et al. (1996) observed the effect of the fat-protein ratio on emulsions stabilized by whey protein concentrate and sodium caseinate and reported that an increase in the fat-protein ratio resulted...
in a decreased whipping time, overrun and increased firmness and stability of the whipped emulsion.

### 1.4.3.3 Effects of homogenization

Homogenization is the most common method to improve the stability of a dairy and food emulsions. It results in the formation of smaller particle sizes with more uniform size distributions, hence limiting the rate of phase separation (Biasutti et al., 2010). During homogenization, proteins are absorbed from the continuous phase onto the newly created fat globule membrane. The composition and the structure of this absorbed layer influence the stability and the rheological properties of emulsions (Dickinson and Golding, 1998).

Phipps (1975), and Mulder and Walstra (1974) reported that, for homogenization pressures between 0.25 – 40.5 MPa, the average fat globule diameter \( d \) of emulsions decreases with emulsification pressure \( P \) in a relation \( d \propto P^{-0.6} \). Robin et al. (1992) used a microfluidizer to prepare butter oil-in-water emulsions and observed that the average size of the particles decreased with an increase in pressure, and reached a minimum value at around 60 MPa. Martin-Gonzalez et al. (2009) reported that an emulsion containing 30% oil and 2-3.5% caseins showed a gel-like structure when homogenized at pressures between 20 to 100 MPa.

The main effect of homogenization is the reduction in droplet size and consequently in the increase of the O/W surface area. The increase in homogenization pressures reduces the droplet size of the emulsions and thus improves the shelf life of the products by reducing the creaming rate. High pressure homogenization resulted in the increase of the surface activity of emulsifying molecules; it may improve the efficiency of the product e.g. coating ability or penetration action (Floury et al., 2000).

### 1.4.4 Characteristics of dairy emulsions

As already described earlier, emulsions consist of at least one immiscible liquid dispersed in another liquid in the form of droplets. Emulsions are unstable systems tending to reach a stable state by separating two immiscible liquids into individual phases. However, it is possible to make a stable emulsion from a kinetic point of view. In general, emulsion stability depends on the four following factors: (i) the particle size of dispersed phase in emulsion, (ii) the viscosity of continuous phase and its rheological behavior, (iii) the density difference between dispersed and continuous phases and, (iv) the presence of surfactants (Abismail et al., 1999). The latter factor is very important regarding the adsorption at the interface, the effective role in the particle size and the viscosity of emulsions (Abismail et al., 1999).
1.4.4.1 Particle size distribution

Stability or/and shelf-life, which is a kinetic concept, of an O/W emulsion, stabilized by milk protein as emulsifier is strongly influenced by the initial droplet size distribution. Stable emulsions have no distinguishable change in the droplet size distribution over the time-scale of the observation (Dickinson, 1982). A sufficient amount of emulsifier such as proteins and PLs in a system can cover all oil droplets and, in this way, avoiding droplet coalescence (van Aken and van Vliet, 2002). Changing the droplet size distribution and the flocculation of droplets leads to visible separation of cream from the bulk dispersed phase. The use of buttermilk and MFGM isolate in recombined oil-in-water emulsions considerably reduced the particle size (Roesch et al., 2004, Sodini et al., 2006) and enhanced the creaming stability (Scott et al., 2003b, Roesch et al., 2004).

The rheological and textural properties of dairy products are greatly influenced by the size of the FGs. Michalski et al. (2002) concluded that there are three types of particles present in homogenized milk: (i) disrupted FGs from the main population the surface fraction of which is covered by casein micelles, (ii) the original small FGs which belong to an isolated population in milk (size around 100nm) and (iii) the newly formed lipid-protein complexes surrounded by a new membrane containing caseins. In an emulsion, several mechanisms (e.g. flocculation, coalescence, or Ostwald ripening) might be responsible for the growth of particles. Various parameters such as initial droplet size, droplet concentration, and continuous phase rheology will normally determine the growth rate of particles. The above mentioned parameters may vary according to the nature of the system.

Many techniques have been developed to measure droplet size distribution; the most used techniques are microscopy (Davis, 2005), light scattering (Lindner et al., 2001, Kiokias et al., 2004, Richardson, 2005), ultrasonic methods (McClements and Coupland, 1996, Coupland and McClements, 2001, Smyth et al., 2005) and more recently NMR (Hollingsworth et al., 2004, Kiokias et al., 2004, Johns, 2009).

1.4.4.2 Creaming/sedimentation characterization

Under undisturbed conditions, creaming is the most obvious evidence for instability of an O/W emulsion. (Pinfield et al., 1997, McClements, 2000, Dickinson, 2001). The rate of creaming is affected by the oil droplets size, the continuous phase viscosity and the density difference between the dispersed phase and the continuous phases. The rate of creaming (in dilute emulsions) follows Stokes’ law equation:
\[ \nu = \frac{2r^2(\Delta \rho)g}{9\eta_o} \]  \hspace{1cm} [1.1]

Where \( \nu \) is the rate of creaming, \( r \) is the radius of the oil droplets, \( \Delta \rho \) is the difference in the densities of the dispersed and the continuous phases, \( g \) the gravity acceleration, and \( \eta_o \) the viscosity of the continuous phase.

According to equation [1.1], the rate of creaming can be greatly reduced if the density difference between the dispersed oil phase and the aqueous continuous phase is minimized, if the radius of the oil droplets is reduced, and if the viscosity of the continuous phase is increased.

Surfactants can increase emulsion stability in terms of creaming in three ways: (i) by preservation against coalescence through the formation of an electrostatic repulsion (i.e. anionic PLs), (ii) by introducing steric repulsion (i.e. proteins), (iii) by increasing the viscosity of emulsions (i.e. high molecular weight polymers such as proteins) (Tadros, 2013).

Creaming or sedimentation in an emulsion can be easily accessed by optical observations. Indeed, in most cases, creaming is characterized by a whitish/yellowish layer at the top of the emulsion, while when a layer appears at the bottom of an emulsion, sedimentation occurs. Creaming/sedimentation rate can be determined by measuring the volume of cream/sediment in the emulsion with time. This may be done by placing the emulsion in a calibrated beaker or tube and measuring the height of the cream/sediment at pre-determined time intervals. Emulsion stability was measured for the first time by Dagorn-Scaviner et al. (1987) through measuring the separated aqueous phase volume as a function of time. In some cases, visual observations are not accurate enough to measure the creaming/sedimentation rate; creaming or sedimentation can occur very quickly or the distinction between continuous phase and cream/sediment layer is difficult to visualize. More sophisticated techniques are then used to measure the creaming rate, using light scattering (Chanamai and McClements, 2000), ultrasonic imaging (Basaran et al., 1998) or shear rheology (see 1.4.4.3).

1.4.4.3 Rheological characteristics

Rheology is a study to investigate the flow characteristics of emulsions. Rheology studies the flow behavior of emulsions in terms of shear stress and shear rate.

\[ \eta = \frac{\sigma_s}{\dot{\gamma}} \quad \text{with } \eta = f(\dot{\gamma}') \]  \hspace{1cm} [1.2]
Where, \( \eta \) is the viscosity of the emulsion, \( \sigma_s \) (N.m\(^{-2}\)) as the shear stress applied and \( \gamma' \) (s\(^{-1}\)) is the shear rate.

The following factors are affecting rheological behavior of the emulsion: (i) the viscosity of the continues phase, (ii) the volume fraction of dispersed phases, (iii) the characteristics of particles (i.e. particle size distribution concentration or volume fraction) and (iv) the nature of the interactions between droplets which is affected by concentration and the nature of emulsifiers (Barnes, 1994).

The effect of viscosity of the continuous phase on the viscosity of emulsions is described by following equation:

\[
\eta = \eta_c \left(1 - \frac{\phi}{\phi_m}\right)^{-2} \quad [1.3]
\]

Whereby, \( \eta \) is the viscosity of the emulsion, \( \eta_c \) is the viscosity of the continuous phase, \( \phi \) is the volume fraction of the dispersed phase and \( \phi_m \) is the maximum phase volume at which phases are diverged.

As shown in equation [1.3], \( \phi \) has a direct relation with viscosity. Viscosity is increased by increasing the volume fraction of the dispersed phase while \( \phi_m \) is negatively correlated with viscosity. \( \phi_m \) for dispersed particles is measured by the flow induced movement from a three dimensional, isotropic, random distribution of particles at rest towards a more two-dimensional ordered distribution at higher shear rate, with the latter being a system of stirrings and layers of particles. Since \( \phi_m \) indicates the way particles fill space, particle size distribution affects \( \phi_m \) and consequently the viscosity. Wide particle size distributions lead to an increase in \( \phi_m \) and eventually in a decrease in the viscosity. Moreover, since deformability of particles affects the \( \phi_m \) toward the distortion of neighbor particles, smaller particles are less deformable. Therefore, smaller particles lead to higher viscosity (Barnes, 1994).

The interactions between adsorbed proteins at the interface of different droplets, affect the state of aggregation, coalescence and viscosity of emulsions. Interaction between adsorbed proteins in turn, is influenced by factors such as the protein surface coverage, the adsorbed layer thickness, the surface charge density, and the aqueous phase conditions (mostly pH, ionic strength and calcium ion content). The rates of aggregation and coalescence of protein-stabilized emulsions, are accelerated by flow fields especially in the presence of phospholipids (Dickinson et al., 1993b).
Surfactants, according to their nature, could form a double layer around droplets (i.e. anionic surfactants such as some PLs) or a viscoelastic film layer (i.e. proteins) around oil droplets and introduce a repulsion force between droplets (Tadros, 2004).

Proteins are able to stabilize emulsions in two different ways. Proteins reduce surface tension by their surface activity. However, the essential stabilizing function of proteins is the formation of a viscoelastic film around droplets resulting in resistance of fluid interface against tangential stresses produced by the adjoining flowing liquids (Lucassen-Reynders and Benjamin, 1999). Proteins are also able to stabilize emulsions by increasing the viscosity of bulk emulsions. The excess of proteins from coverage of droplets remain in the emulsions and increase emulsion viscosity.

The rheological behavior of emulsions is also an important property influenced by the state of aggregation of droplets and hence of the colloidal interactions (Dickinson and Golding, 1998). Some information on the strength of the bonds between the globules in a cluster may be obtained from the rheological behavior of dilute emulsions by determining the shear rate at which the viscosity is no longer time independent but declines irreversibly. Information about the nature of the bonds in a cluster may be obtained from the breaking-up of the clusters in different reagents (Melsen, 1987). Breakdown processes resulting from emulsion instability (i.e. creaming/sedimentation, flocculation, Ostwald ripening and coalescence) (see 1.4.2) can be investigated by various rheological techniques. Techniques that can be applied are (i) measuring the shear stress as a function of shear rate (i.e. steady state), (ii) strain as a function of time while constant stress is applied (i.e. creep) and (iii) oscillatory techniques (Tadros, 2004).

To investigate creaming or sedimentation a rheometer with constant shear stress is applied. When the viscosity of emulsions exceeds a certain value creaming or sedimentation is totally removed. In this method emulsions are placed in a gap between two concentric cylinders or cone-plane geometry and strain (i.e. reformation) is followed as a function of time (Goodwin and Hughes, 2008).

Coalescence measurement is carried out using all three methods. In the creep method critical stress and residual viscosity is measured as a function of time. The oscillatory method measures change of the storage and loss modulus with applied amplitude and frequency (Tadros, 2004). To assess emulsion flocculation also all these rheological techniques can be used.
1.4.4.4 Interfacial surface of emulsion

Surface active agents are adsorbed at the interface depending on their amphiphilic properties. Surfactants form an adsorbed layer around droplets and produce a repulsive force between particles, giving rise to a stabilized emulsion. In emulsions containing different types of surface active molecules, the surfactants are adsorbed according to their surface-active capacity. Hence, those with a higher surface activity are adsorbed faster at the interface and also may displace other surfactants. Therefore, the surface coverage is affected by the type and concentrations of the emulsifiers in the system. Moreover, the properties of the adsorbed layer influence the bulk emulsion stability (Damodaran, 2005).

In dairy emulsions, milk proteins and PLs are the main surfactants. PLs or small molecular surfactants are also highly surface active, lower the interfacial tension to a great extent and have a high adsorption energy per unit area (m$^2$). Proteins, being high molecular weight surfactants, lower the interfacial tension to a lesser extent than PLs. However, emulsions stabilized with proteins are more stable against coalescence because of the steric repulsion between droplets formed by the protein film around the droplets (Damodaran, 2005). Proteins in unfolded form have more surface activity regarding to exposure of their hydrophobic parts (Dickinson, 1999). In emulsions containing both caseins and whey proteins, caseins are adsorbed at a faster rate at the interface. Some processes such as high pressure homogenization or heating lead to denaturation, unfolding of whey proteins and a change in their surface activity. However, whey proteins have more ability to form protein-protein interaction through disulfide bonding (McClements, 2004, Wilde et al., 2004). Hence, they are able to form a more viscous layer and increase the surface protein load. Therefore, composition of the surface protein load affects the interfacial properties of droplets.

In a study using 20% soy O/W emulsions with caseins obtained from skim milk (0.2-2.0%), it was suggested that caseins had different conformations depending on the concentrations used. At low concentrations casein was expected to stretch over the surface, whereas at high concentrations, the casein molecules were expected to form a more compact structure. A compact structure of the casein molecules would provide additional space for more casein at the surface, whereby individual molecules would protrude further out into the aqueous phase and create a thicker interfacial layer (Fang and Dalgleish, 1993a). In comparison, the droplet surface adsorption behavior of whey proteins was suggested to be a little different, owing to the globular nature of the whey proteins (Hunt and Dalgleish, 1994). Since more whey proteins were needed to obtain a stable emulsion (1.5 mg m$^{-2}$ compared to 1 mg m$^{-2}$ for the
casein), these proteins were not expected to be able to stretch over the droplet surface to the same extent as caseins. However, later studies on the adsorption of individual whey protein components (β-LG or α-LA prepared from whey protein isolate (WPI) products) by Fourier transform infrared spectroscopy revealed some concentration dependent structural changes upon adsorption (Fang and Dalgleish, 1997, 1998). In the study on β-LG-stabilized emulsions, the authors suggested that when present at low concentration (1% β-LG to 20% oil) the protein molecules were stretched over the interface whereby they changed conformation. In contrast, when proteins were present in excess (2% β-LG to 20% oil) they did not have to stretch to cover the interface, and therefore did not differ in conformation from the native proteins in solution (Fang and Dalgleish, 1997). Thus, a concentration dependent conformational behavior of whey proteins was suggested, similarly to the one suggested for caseins. In 30% soy O/W emulsions prepared with a combination of sodium caseinate and whey protein concentrate (1:1), whey proteins adsorbed preferentially to caseins at total protein concentrations below 3%, whereas the opposite was observed at total protein concentrations above 3% (Ye et al., 2008). In homogenization studies on milk, the adsorption of the different milk proteins and their conformations at the interface have been shown to depend on the homogenization equipment used (Dalgleish et al., 1996).

A few studies have been carried out on emulsions with combinations of milk proteins and PLs. Using a combination of caseins and lecithin in 20% O/W emulsions, lecithin was found to enhance the emulsion stability when the caseins concentration was low (< 0.3%). This was suggested to be due to a better coverage of the oil droplet surface by the emulsifiers, since the PLs supplemented the caseins at the interface, whereby the caseins could protrude more into the water phase and stretch less over the surface (Fang and Dalgleish, 1993a). However, when protein concentrations were 0.4%, a displacement of protein at the interface rather than a supplementation occurred (Courthaudon et al., 1991a, Dickinson and Iveson, 1993a, Fang and Dalgleish, 1993a).

1.5 Dairy cream

1.5.1 Natural cream

Cream is the fluid milk product comparatively rich in fat, in the form of an emulsion of fat-in-skimmed milk (oil-in-water emulsion), obtained by physical separation from raw milk by centrifugation. Based on the fat content, the cream and cream products were classified into different types. The cultured products and various liquid types of cream products contain 10-
50% of fat. The other types such as coffee, half, light and single creams contain 10 to 30% of fat and the whipped cream contains 30 to 40% of fat while 45 to 50% of fat is present in the double cream. The cream is used as a major ingredient in other dairy and non-dairy products. Cream is the material for the production of butter and butter oil (Hoffmann, 2002). The products made from NC show better physicochemical and sensory characteristics than the recombined dairy creams (Fredrick et al., 2013).

1.5.2 Recombined cream

RC is cream obtained by recombining milk ingredients with or without the addition of potable water and with the same end product characteristics as NC (Codex, 1976). RC is an alternative to NC and is used in the production of some dairy products such as pasteurized cream, whipped cream, cheese and ice cream. For the manufacture of RCs, ingredients from both dairy and non-dairy origin are used. Skim milk powder (SMP) and anhydrous milk fat (AMF) are the most common dairy components used. Emulsifiers (non-dairy components) like monoglycerides and di-glycerides and stabilizers (non-dairy ingredients) like carrageenan are also used in cream production. However, the creams produced using non-dairy ingredients are not considered as 100% dairy creams. It has been reported that some surface active components of milk (milk fat globule membrane suspension, buttermilk and butter-derived aqueous phase) and modified milk fat have been successfully combined to obtain reformulated cream comparable with NC (Vanderghem et al., 2007).

Over the last decade, creams recombined from milk ingredients have received increasing interest because of their obvious advantages in industrial production. Firstly, the storage costs of the raw materials for RC are lower, and secondly, the composition and desired properties of RC can be standardized and adapted and are independent of the milking seasons (van Lent et al., 2008).

However, the functional properties of RC are affected by both the composition and the processing parameters (van Lent et al. (2008). The stability of the emulsion and the physical properties of cream can be affected by processing techniques and surface active components such as proteins and phospholipids (Scott et al., 2003b).

1.5.3 Whipped cream
1.5.3.1 Introduction

Due to whipping of cream, air bubbles are introduced and FGs aggregate. This leads to the formation of bubbles and a FGs aggregation network which hardens the cream and forms finally the whipped cream (Ihara et al., 2010). It was pointed out by Prentice (1992) that the whipping of creams with a fat content of 30-40% will result in products with good quality. Moreover, these creams are good for whipping and they create more foam. In addition, they have a high overrun, good stability and a long storage time. Noda and Shiinoki (1986) observed changes in rheological properties to investigate the mechanism of foam formation. They concluded that most of the air was introduced in the first stage of whipping and that whipped cream shows viscoelastic behavior. A rigid three dimensional structure is formed because of the decrease in the size of the air bubbles. The decrease in size of the air bubbles is due to the fat globule aggregates.

It has been reported by Needs et al. (1988) that the distance between the air bubbles decreases during whipping. This permits an increase of the network of FGs between the air bubbles. It delivers stiffness to the whipped cream. If fewer crystals penetrate the air/water interface of certain air bubbles, the whipped cream will be of better quality. In the case that all the air bubbles and their air/water interfaces are penetrated by a large number of fat crystals, the formation of defective creams occurs (Brooker, 1990). Different mechanisms may affect the stability of whipped cream: Ostwald ripening, leakage, collapse of the foam and sagging (Ward et al., 2006).

1.5.3.2 Mechanism of structure build-up

During whipping, cream is converted from a two-phase (fat-in-water emulsion) liquid system to a three-phase solid system in which air is incorporated and trapped in a network of FGs (Needs and Huitson, 1991). Two types of partial coalescence are at the basis of this structure build-up: surface mediated (perikineti c) and shear induced (orthokinetic) partial coalescence (Hotrum et al., 2005). Both mechanisms are illustrated in Figure 1-9.

a. Surface-mediated partial coalescence

The crucial forerunner for the surface-mediated partial coalescence is the adhesion of FGs to the air/water interface. The air bubbles perform a vital role in surface-mediated partial coalescence (Figure 1-9a). To a larger extent, the surface-mediated partial coalescence process will control the whipping time (Hotrum et al., 2005). During the whipping of NC, the partial crystallized FGs will stick to the surface of the air bubbles. As a result of surface-
mediated partial coalescence, these adsorbed FGs will form clumps. Afterwards, the clumped FGs will form a three dimensional network due to further continuation of the surface-mediated partial coalescence. Furthermore, the network of clumped FGs will hold and deliver stability to the incorporated air bubbles. In addition, it contributes to the desired texture and gives the mechanical strength to the final whipped cream (Dickinson, 2010). The wetting characteristics of the oral mucosa and the characteristics of the interfacial layer offer stability to the FGs in an emulsion and will be the reason for the surface-mediated partial coalescence in the mouth (Dresselhuis et al., 2007).

![Diagram](image)

**Figure 1-9.** The mechanism of (a) surface mediated partial coalescence and (b) shear-induced partial coalescence (Hotrum et al., 2005).

### b. Shear-induced partial coalescence

This type of partial coalescence is independent of the incorporated air. As a result of convection, partial coalescence will occur. It means that by the approach of two FGs next to each other, the fat crystals inside these globules will pierce the thin film between them. As a result, FGs will stick to other globules and this will result in the formation of large clumps (Boode et al., 1993) as shown in Figure 1-9b. Convection makes the emulsion droplets to attach to each other which leads to the shear induced partial coalescence. The process of shear induced partial coalescence ensues an orthokinetic like flocculation under the conditions of laminar flow (Walstra, 2003).
There are four parameters on which partial coalescence depends: the gravity vs. surface tension, the viscosity in both fluids vs. surface tension and the relative density difference between the two fluids. To have partial coalescence, the surface tension should be the dominant force, otherwise it results in total coalescence. This means that the gravity vs. surface tension and the viscosity vs. surface tension should be below a critical value (Gilet et al., 2007). In addition, partial coalescence is extremely influenced by the amount and the type of surfactant on the surface of the droplets and by the solid fat content (SFC) (Darling, 1982, Goff et al., 1987). Partial coalescence is enhanced by applying a velocity gradient, by increasing the lipid content, by carefully manipulating the concentration of solid lipids and by minimizing the repulsion provided by the globule membrane (Walstra et al., 1999). Specific parameters such as mechanical shear forces (flow conditions), the processing time and temperature will highly influence the occurrence and the speed of partial coalescence in an O/W emulsion (Fredrick et al., 2010).

According to Hotrum et al. (2005) the whipping process consists of three stages. In the first stage, most of the air is incorporated because of foam formation and these foams are stabilized by proteins. In this stage, via turbulent flow, the large air bubbles break up into small ones to obtain a stable foam. In the second stage, a layer of FGs is coated around the air bubbles. This continues until there are no free FGs anymore in the continuous phase. Then, the air bubbles destabilize very fast together with an expulsion of the fat globule aggregates. This is the last stage of whipping. Further whipping would lead to a rapid decrease of air and the creation of butter grains (van Aken, 2001). The detailed mechanism of structure built-up and factors governing the whipping creams were discussed by Fredrick (2011).

1.5.3.3 Characterization of whipped cream

Whipped creams are thermodynamically unstable. When cream is subjected to whipping, numerous factors will influence the whipping properties and the texture and stability of the end product. It is difficult to control these physical properties and they affect the characteristics of whipped cream such as flavor, appearance and mouth feeling (Walstra et al., 2006). Consumers and dairy industries expect whipping cream to have certain desirable qualities such as taste, shelf-life and whipping characteristics. The dairy industries express whipping characteristics in terms of shorter whipping time, high overrun, good firmness and high stability (Bruhn and Bruhn, 1988).
**Whipping time:** Whipping time is the time needed to whip cream until it reaches maximum volume. To achieve whipping, the interfacial layer should not be too strong (Fredrick, 2011). When small molecular surfactants are used, the whipping time decreases. In fact, small molecular surfactants promote partial coalescence (van Lent et al., 2008). Camacho et al. (1998) explained the effect of the fat content on the texture and stability of whipped cream. Hydrocolloidal stabilizers modify the whipping time, overrun, texture and stability of whipped cream. In an emulsion, the speed and ease of the fat droplets to attach and spread at the air water interface will determine the whipping time (Hotrum et al., 2005).

**Overrun:** Overrun is an important parameter to estimate the volume of air incorporated or the proportion of air hold up in the whipped cream (Jakubczyk and Nirajn, 2006). It has been reported by Birkett (1983) that extreme stability and stiffness of the foam relies on a high overrun. Increased volume measurements after whipping are used to determine the overrun (Nesaretnam et al., 1993). For traditional dairy whipped cream the desirable overrun is around 100-120% (Graf and Müller, 1965).

**Serum loss:** The amount of aqueous phase released from the whipped cream after a certain period of time, is called the serum loss. To have a stable whipped cream, the serum loss should be as minimal as possible. This measurement characterizes the stability of the whipped cream (Graf and Müller, 1965). Serum loss is measured by transferring a certain amount of whipped cream into a filter or sieve, hold in a flask and, after keeping it for some hours at a constant temperature, measuring the mass of serum fed through the flask via the filter or sieve (van Aken, 2001, Allen et al., 2006).

**Firmness:** The structural rigidity of the whipped cream is explained by the firmness (Goff, 1997a). The puncture test is used to determine the firmness of the whipped cream. This test is based on the insertion of a probe in the whipped cream at a constant speed until it reaches a defined depth. The puncture test is performed using probes connected to a texture analyzer (Van Camp et al., 1996, van Aken, 2001, Shamsi et al., 2002).

1.6 **Technological functionalities of MFGM material for food application**

MFGM materials are found in quite significant amounts in different dairy products especially in cream, butter, buttermilk, cheese and cheese whey. The unique functionality of MFGM materials have led to research and developing technologies for isolation, separation and use in different food emulsions (Singh, 2006). The MFGM enriched dairy products are often preferred for their emulsifying properties and capacity to improve texture especially for
reduced fat food products (Ward et al., 2006). The functionalities of the MFGM largely depend on the type of processing of the milk e.g. heating, cooling, homogenization, evaporation, drying etc. (Evers, 2004) and the method of isolation and separation of the MFGM from raw materials (Singh, 2006).

1.6.1 Emulsifying properties in food emulsion systems

1.6.1.1 Emulsifying properties of MFGM material

Concerning the emulsifying properties, proteins and PLs are the two major surface active components of the MFGM that could be considered as efficient and natural emulsifying agents for food applications due to their amphiphilic nature (Vanderghem et al., 2010). Extensive investigations on the emulsifying properties of the membrane were conducted from 1980 until present. The MFGM materials isolated from the dairy by-products are complex mixtures of proteins, PLs and other contaminated components. The processes of the isolation and conversion into protein powder modify the native protein structures and may consequently influence their functional properties, including the emulsifying properties.

One of the first studies about the emulsifying properties of MFGM protein and PLs in the stabilization of the FGs in milk was done by Shimizu et al (1980). They digested MFGM proteins, obtained from washed cream with papain and then used them in cream. The decrease in stability of the cream due to clustering of FGs was observed. It was concluded that proteins and glycoproteins in MFGM may play an important role in the stabilization of FGs. In addition, removal of the phospholipid’s polar head group of PLs of MFGM, extracted from washed cream, by phospholipase C resulted in a remarkable decrease in the stability of cream emulsions. Shimizu et al. (1980) concluded that these groups deliver a repulsive force that disturbs the coalescence of FGs. Later, Kanno (1989) proved the ability of MFGM isolated from fresh raw milk to stabilize the butter oil emulsion. The whipping ability and foam stability were maximal when MFGM was used at 2%, equivalent to 80 mg MFGM per g fat (Kanno, 1989). According to Phipps and Temple (1982), the emulsifying properties of MFGM could be explained by its effect on surface tension at the emulsion interface.

Corredig and Dalgleish (1998b) prepared a model soybean O/W emulsion with MFGM materials from fresh raw cream and found that the newly formed oil droplets covered by the MFGM material, behaved differently from emulsions stabilized by other milk proteins i.e. no displacement occurred on the addition of small molecular weight surfactant (Tweens and Triton X-100) and the droplets were not affected by the presence of proteins such as an
addition of β-LG or caseins. This is due to fact that a strong interaction may exist at the interface and phospholipids component of the MFGM may lower the interfacial tension (Corredig and Dalgleish, 1998c).

The type of raw material, pretreatment and method of separation have a significant effect on the composition of MFGM isolates and consequently on their emulsifying properties. In reconstituted milk fat emulsion (20-80 mg MFGM/g fat), it was observed that MFGM can stabilize 25 times its mass of milk fat (Kanno, 1989). Kanno et al. (1991) reported that by increasing the MFGM concentrations, the viscosity increased linearly between 40 and 80 mg/g fat. Droplets size decreases linearly with increasing membrane concentration which is comparable to those of homogenized milk. The surface protein coverage also increases at acid pH with the increase of MFGM concentrations in the emulsion; by using > 80mg MFGM material/g fat it is possible to prepare stable milk FGs that have similar stability as the natural milk FGs (Kanno, 1989, Kanno et al., 1991). However, Wong and Kitts (2003); Corredig and Dalgleish (1997b) found that commercial buttermilk has inferior emulsifying and stabilizing capacity than non-fat dried milk.

Sodini et al. (2006) studied the compositional and functional properties of sweet, sour and whey buttermilk and reported that sweet and cultured buttermilk exhibit lower emulsifying properties and higher viscosity and lower pH whereas the functional properties of whey buttermilk were independent of pH. The emulsifying properties of those three types of buttermilk are better than milk and whey but have lower foaming capacity. However, among the above listed buttermilk samples, whey buttermilk was found to have the highest emulsifying properties and the lowest foaming capacity. Possible reasons for this could be the higher ratio of phospholipids to protein in whey buttermilk compared with sweet or cultured buttermilk (Sodini et al., 2006). Roesch et at. (2004) studied the emulsifying properties of the MFGM fraction obtained by MF from commercial buttermilk and buttermilk concentrate (BMC). An emulsion prepared with 10% soybean oil and with > 0.25% MFGM isolate (60% w/w proteins) from buttermilk, showed good stability against creaming and the small particle size distribution increased with MFGM concentrations, whereas a similar emulsion prepared with BMC showed extensive flocculation. The instability of emulsions prepared with low concentrations of MFGM isolate was caused by the incomplete coverage of the oil/water interface by large clumps of membrane material, which formed bridges between the emulsion droplets (Roesch et al., 2004). These findings are in disagreement with the previous work by Corredig and Dalgleish (1997b). These authors observed that the MFGM isolate is a poor
emulsifier compared to a whole isolate of buttermilk (containing whey proteins, caseins, and MFGM specific protein). Emulsions with 10% soybean oil needed a much higher percentage (> 8%) of MFGM isolate to produce a droplet size distribution similar to that found for the emulsions prepared with 1-2% (w/v) whole isolate of buttermilk.

Thompson and Singh (2006b) described that liposomes, a type of vesicle formed through the self-assembly of amphiphilic molecules, can be produced from phospholipids. During microfluidization, hydrophobic molecules can be incorporated into the lipid bilayer and hydrophilic molecules become entrapped in the aqueous core. The composition of the MFGM phospholipids material is different from the commonly used soy or egg derived phospholipids, which influences the properties and structure of the liposomes (Thompson et al., 2006a). The unique composition of the MFGM phospholipids can be used to protect sensitive components such as sensitive ingredients or can be applied to confine undesirable flavors (Singh, 2006).

The differences in the functionality and major differences in the efficiency of MFGM material in food emulsion preparation may be explained by the source of the MFGM material, the intensity and frequency of the heat treatment, the type of fractionation procedure as well as the preparation condition.

1.6.1.2 Emulsifying properties of milk PLs concentrate

After a first step of isolation of MFGM fragments from industrial sources, several techniques can be applied to further purify PLs (see 1.3.2). Several dairy functional ingredients which contain mainly PLs have been launched on the commercial market. Lacprodan-20 and -75 of Arla Foods Ingredients amba (Denmark) contain 20 and 75% phospholipids, respectively and Phospholac 600, produced by Fonterra Cooperative Group Ltd. (New Zealand) contains more than 70% phospholipids. One interesting result from the use of such enriched PL isolates has been reported by the research group of Snow Brand Milk Products (Japan) where bovine milk lecithin (85% PLs) was found to stabilize soy lecithin (95% PLs) solidified cream reconstituted from butter oil (Miura et al., 2006). Figure 1-10 shows the difference in particle size distribution with the use of bovine or soy lecithin as emulsifying agents. Among the PL species, PC but not PE or SM, regardless their origin, was found to be the determinant of the emulsifying effect (Miura et al., 2004, Miura et al., 2006). PLs from milk differ from soy lecithin in both the classes of PLs as well as FA composition (Boyd et al., 1999).
Zhu and Damodaran (2013) used ethanol to extract PLs from cheese-whey derived MFGM and obtained a dairy lecithin containing 31% total PLs. Oil-in-water emulsions made with less than 2% of this lecithin (relative to the total emulsion weight) were unstable; however, emulsions made with more than 4% dairy lecithin were very stable for more than 60 days at room temperature (Zhu and Damodaran, 2013).

1.6.2 Texture improving properties in cheese and yogurt

The gel structure in milk products can be obtained by the interactions between FGs and milk proteins through the MFGM. The newly formed MFGM in homogenized fresh milk or recombined milk would cross-link (structure promoter) with the protein network and reinforce it in either rennet or acid gels (Lucey et al., 1998, Lopez and Dufour, 2001, Michalski et al., 2002). Several studies regarding the texture improving properties of MFGM on cheese reviewed the positive effects.

*Reduction of fat loss:* Lopez et al. (2007) studied the influence of the MFGM on the microstructure of ripened Emmental cheese. Results showed that smaller fat losses were found during the pressing of the cheese curd with the use of rennet-induced coagulation.

*Enhancement of texture and water holding capacity:* Emmental cheese produced from small MFGs, containing a higher amount of MFGM material, retained more moisture than the cheese made from large MFGs after ripening (Michalski et al., 2003, Michalski et al., 2004). Furthermore, the addition of buttermilk decreased hardness, increased the body and improved the texture of cheeses (Mayes et al., 1994, Mistry et al., 1996, Poduval and Mistry, 1999, Turcot et al., 2002).

For the production of non-fat and low fat yoghurt, several attempts have been made to improve the syneresis defects due to a reduced solids content by using buttermilk powder and...
ultrafiltered buttermilk as the sources of MFGM material (Trachoo and Mistry, 1998). The addition of up to 4.8% sweet buttermilk powder to low fat yoghurt enhanced its sensory qualities by softening the product and making it smoother (Trachoo and Mistry, 1998). Confirmation by confocal microscopy of yoghurt prepared with sweet buttermilk supplement showed that the membrane-like material may function as bridges, which link the protein matrix together, improving the yoghurt texture (Trachoo, 2003). A study carried out by Le et al. (2011b) on the potential of using MFGM enriched material isolated from reconstituted buttermilk as supplement ingredient for yoghurt production showed the ability of improving consistency and adhesiveness of the yoghurt gel.

**Improvement of cheese yield:** Due to the binding of β-LG to the MFGM during heat treatment, the increase in cheese yield by incorporating whey protein in the gel structure can be obtained (Molina et al., 2000). Lysophospholipids released from the MFGM by phospholipase treatment before pressing the curds may act as surface-active agents which improves water and fat emulsification during processing, leading to their increased retention (Lilbæk et al., 2006). Addition of sweet buttermilk as a rich source of MFGM material significantly reduced free oil escape and increased cheese yield (Mistry et al., 1996, Govindasamy-Lucey et al., 2006). Reduced fat Mozzarella cheese produced with the addition of ultrafiltered buttermilk may improve the moisture retention and contributed to a further reduction of the free oil content during homogenization (Poduval and Mistry, 1999).

### 1.6.3 Flavour enhancing capacity

An enhancement of flavor development has also been reported after the addition of buttermilk to reduced-fat cheese. Several explanations have been suggested:

- Moisture captured by the MFGM in the serum phase could act as an environment for microbial enzymes related to the flavor development (El-Loly, 2011).

- Hydrolyzed MFGM components (glycoproteins) may act as a carbon source for some lactic acid bacteria in Cheddar cheese during ripening (Laloy et al., 1996, Lopez et al., 2007).

- Proteolysis and lipolysis by MFGM enzymes together with those by starter may contribute to the more intense flavour in cheeses (Laloy et al., 1996, Ma and Barbano, 2000, Michalski et al., 2003, Lopez et al., 2007).

- The larger fat globule surface area is likely to enhance aroma perception due to a greater contact surface of fat in the mouth (Michalski et al., 2003).
1.6.4 Other applications

Not only in dairy application MFGM plays a role. MFGM fragments are employed as a baking improver to ameliorate fat dispersion and anti staling, especially the PLs present in MFGM. As an additive in chocolate they decrease the viscosity and prevent crystallization. Another innovative utilization of buttermilk is the supplementation of natural fruit beverages with this by-product (Farah and Bachmann, 1981, Shukla et al., 2004). These fruit beverages combine the high protein and mineral content of buttermilk with the vitamins of the fruit in a cheaper product. Furthermore, PLs are used as wetting enhancers in instant products by improving the wetting stability. As a stabilizer, they prevent spattering and browning in margarine (Dewettinck et al., 2008). In contrast with plant lecithin, MFGM or milk PLs contain a substantial part of sphingolipids, which can be used as functional ingredient for food application (Dewettinck et al., 2008).

Some non food applications include drug delivery carrier (choline supplier), fat liquoring for leather fatting, pigment dispersion in paints etc. (Vannieuwenhuyzen, 1981). PLs in dairy products contain more sphingolipids than products of plant origin, which could potentially be used as source of ceramides for the cosmetic industry (Becart et al., 1990). Ceramides play a major role in the water retaining properties of the epidermis (Elimelech, 2002) hence it is of great commercial importance in cosmetic and pharmaceutical industries, such as in hair care and skin care products (Zhang et al., 2006). MFGM phospholipids could also be used for the production of emulsions for drug delivery (Yuasa et al., 1994).

Thompson and Singh (2006b) produced a liposome from a phospholipid rich MFGM fraction using microfluidization. Pharmaceutical and cosmetic industries use liposomes for the entrapment and controlled release of drugs or nutraceuticals and even for special technique such as gene delivery (Lasic, 1998). Liposomes have many potential applications in food industries ranging from the protection of sensitive ingredients to increase the efficacy of food additives and to confine undesirable flavours (Thompson and Singh, 2006b)

1.6.5 Effect of processing on the functionality of the MFGM

Different studies on the functionality of MFGM material may have inconsistent results, as they possibly depend on various factors, such as the dairy sources for MFGM isolation, the intensity and frequency of processing (e.g. agitation, ageing, homogenization and heat treatment). This is considered to be an important reason for the not always superior functionality of MFGM from industrial sources (Corredig and Dalgleish, 1998b). Such
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changes in functionality of the MFGM with heat treatment and other processing need to be understood to explain the variability of the sources of buttermilk available commercially. The effects of heat treatment and some other processing treatments on the functionality of the MFGM are described separately.

1.6.5.1 Effect of heat treatment

In dairy processing, heat treatment is used as an operational unit to inactivate microorganisms and enzymes and hence to increase the stability of milk products. However, heat treatment causes changes in the membrane which will alter interactions with other components, hence also the emulsifying properties of the MFGM material.

a. Effect of heat treatment on proteins

During heat treatment of milk at a temperature above 70°C several changes take place, including denaturation of whey proteins and formation of complexes between whey proteins, caseins and FGs (Corredig and Dalgleish, 1999).

However, the casein micelle fraction is almost not affected by temperatures up to 100°C, since micelles are present in milk as a stable association of colloids. Conversely, these temperatures have a significant effect on the whey protein fraction of milk (Vasbinder and de Kruif, 2003).

A number of factors influence the degree of interaction between the whey proteins and the caseins micelles, including the temperature, the pH and the concentration of the whey proteins present in milk. From the numerous changes which take place during heat treatment of milk, the denaturation of the whey proteins is the most noticeable. It has been demonstrated that the most heat labile whey proteins are the immunoglobulins and the serum albumin, the β-LG is intermediately stable whereas the α-LA is the most heat stable (Rüegg et al., 1997).

The susceptibility of whey proteins to heat denaturation in whey is influenced by several factors such as pH, concentrations of Ca²⁺, concentrations of proteins, presence of sugars, alcoholic groups and protein modifying agents. On the contrary, caseins, due to their random coil organization, are not very susceptible to denaturation processes (Dalgleish, 1996).

The heating of milk results in a complex of native whey proteins and denatured whey proteins present as whey protein aggregates, casein-whey protein aggregates and whey protein coated casein micelles (Vasbinder and de Kruif, 2003). The most important complex formed during heat treatment of milk is believed to be the β-LG/κ-casein complex (Corredig and Dalgleish, 1999).
Figure 1-11 shows the interaction between β-LG and κ-casein during heat treatment. The free sulphydryl group which is present in β-LG seems to play a fundamental role in this reaction. The free cysteine residue contained in the β-LG structure seems to be of high importance in the denaturation process, which includes several steps: dissociation of the quaternary structure, changes in the conformation of the polypeptide chain and aggregation by disulfide bridging (Relkin and Launay, 1990). More precisely, thiol groups can form disulfide bonds with other cysteine-containing proteins, like β-LG or BSA, or with proteins having S-S bridges, like α-LA, κ- and αs2-casein. Interaction of β-LG with κ-casein, which is present at the outer part of the casein micelle like a hairy brush, leads to coating of the micelles with β-LG. Interactions of β-LG with cysteine-containing serum caseins might lead to casein-whey protein aggregates. Additionally, interactions of β-LG with cysteine-containing whey proteins like α-LA and β-LG molecules result in the formation of whey protein aggregates (Vasbinder and de Kruif, 2003).

By increasing the time of treatment, more extensive reactions take place and the higher temperature of the treatment, the faster the protein-protein interactions. Corredig and Dalgleish (1996) reported that a faster interaction with casein micelles occurred at the lowest pH values, as well as an increased amount of whey protein complexed. However, β-LG seems to be more dependent on pH and temperature changes than α-LA. The amounts of whey proteins associated with FGs are low compared to the amounts bound to casein micelles during heating of milk (Corredig and Dalgleish, 1996). The possibility that caseins attach to the MFGM via the bridge caseins - β-LG - MFGM proteins needs to be elucidated. The formation of complexes between skim milk derived proteins and MFGM proteins is of significance in milk processing. Complex formation occurs with heat treatment and strongly affects the functional properties of MFGM isolates when used as ingredients in foods (Ward et al., 2006).

In the presence of whey proteins, large amounts of β-LG and α-LA associate with the MFGM (Corredig and Dalgleish, 1996, Ye et al., 2004). Direct evidence of heat-induced covalent disulfide interactions between whey proteins and MFGM proteins can be obtained by electrophoresis and isoelectric focusing of the heated MFGM (Kim and Jimenez-flores, 1995, Corredig and Dalgleish, 1996, Ye et al., 2002). The heat-induced formation of protein complexes on the surface of the MFGM may include denaturation of the individual proteins with the formation of aggregates containing MFGM proteins alone (BTN, XO, PAS6/7) or MFGM proteins with whey proteins.
In the work of Iametti et al. (1997), the pasteurization of cream in an industrial plant resulted in the incorporation of caseins into MFGM to even a much higher extent than the incorporation of whey proteins into the latter. Interaction of caseins with the MFGM after heating whole milk was also observed in the work of Ye et al. (2004).

The stability of oil-in-water emulsion prepared with MFGM material depends on the heat treatment of the cream from where the MFGM material is isolated (Corredig and Dalgleish, 1998b). Several studies showed that the surface properties of MFGM can be modified by heat treatment. These changes were suggested to be associated with the percentage of crystalline triglycerides and the PLs transition. (Innocente et al., 1997, Danthine et al., 2000, Karray et al., 2006).

MFGM materials isolated from industrial buttermilk are poor emulsifiers of soybean oil-in-water emulsions as compared to those isolated from fresh cream (Corredig and Dalgleish, 1997b). This is related to the intensity of membrane protein denaturation and association with β-LG during heat treatment and the churning process following the manufacturing of buttermilk. Innocente et al. (1997) studied the change in surface properties of the soluble fraction of the MFGM at different temperatures (4 – 40°C) and observed that during preparation of emulsions, temperature affects their functional properties. A temperature of even as low as 65°C results in loss of emulsifying properties of the membrane fraction. The heat treatment (80°C for 2.5-20 min) and churning had an influence on the behavior of the
membrane and could have caused excessive denaturation of membrane proteins which influences the association of whey protein with MFGM (Houlihan et al., 1992). The solubility of MFGM materials is influenced by the degree of denaturation of the MFGM proteins, the intensity of complex formation between MFGM proteins and the lipid fractions and the association of whey proteins with the MFGM caused by the heat treatment (Corredig and Dalgleish, 1998b). These changes at molecular level may have an impact on protein functionality which sometimes is desirable and other times can be detrimental (Singh and Creamer, 1992). Ye et al. (2002) showed that, when FGs are heated in the absence of serum proteins, there is a tendency to form a complex between BTN and XO by disulfide bonds at temperature as low as 60°C. At higher temperature (80°C) a significant amount of serum proteins, particularly β-LG, interacts with the MFGM (Lee and Sherbon, 2002).

b. Effect of heat treatment on phospholipids

Very little is known about the changes that occur in the phospholipids fraction of MFGM with thermal processing (Ward et al., 2006). McCrae (1999) showed that the heat stability depends on the type of protein-lecithin interactions. Two mechanisms were considered: (i) lecithin increased the heat stability by displacement of or by interaction with surface-adsorbed protein and (ii) lecithin interacted with free protein in solution thereby influencing heat-induced free protein/bound protein interactions. Several studies have demonstrated an interaction of certain phospholipids with milk protein (including β-LG). Kristensen et al. (1997) reported that the interaction between phospholipids and β-LG leads to a stabilization of the proteins against thermally induced unfolding. The effect was found to be dependent on the type of phospholipid polar head group as well as on the fatty acid composition. From these observations, the lecithin effect is thought to result from the binding of lecithin to whey proteins, which induces their tendency to denaturation and/or binding to caseins micelles, either bound or free (Van der Meeren et al., 2005).

1.6.5.2 Effect of other processing treatments

Large FGs are more susceptible to shear stress than the smaller ones. Wiking et al. (2003) reported that the resistance of the FGs membrane against coalescence during agitation is determined by the size of the fat globule, the fat content, the milk temperature and the shear rate. Mechanical treatments such as agitation, pumping and high shear can cause changes in the composition of the MFGM, as well as changes in the size of the FGs (McPherson and Kitchen, 1983).
Homogenization is often used to reduce the size of the FGs, delay creaming and improve emulsion stability. During homogenization, the interfacial area increases significantly. Rearrangement of the original MFGM material occurs and considerably more protein is necessary to cover the newly formed interface. For this reason, casein micelles are adsorbed on the milk FGs (Figure 1-12). This effect explains the observation that the FGs in homogenized milk have a much higher protein load than untreated FGs (Sharma and Dalgleish, 1993). Homogenization and heating are unit operations that are usually combined during milk processing. Differences in homogenization as well as the conditions of thermal treatment result in differences in the protein load on the MFGM surface and alter the ratio of whey proteins to caseins (Sharma and Dalgleish, 1993). Homogenization of emulsions prepared using skim milk will result in a competitive adsorption between the casein and whey proteins. The adsorption of casein fractions was preferential in homogenized dairy creams (Dickinson, 2006). Elling et al. (1996) stated that the amount of phospholipids incorporated in the reconstituted membrane could vary if the homogenization pressure is controlled. Figure 1-12 shows schemes of the organization of the native MFGM and the homogenized fat droplet.

The addition of CaCl$_2$ as process additive during cheese making could affect the emulsification properties. Srinivasan et al. (1996) and Dickinson and Davies (1999) indicated that the addition of CaCl$_2$ prior to emulsification increased the surface protein coverage; this could be attributed to an aggregation of the caseins, due to binding with Ca$^{2+}$, and subsequent adsorption of the aggregated caseins at the droplet surface. In addition, Ca$^{2+}$ binding reduces charge repulsion between casein molecules and hence can increase protein packing at the interface.
1.6.6 Concluding remarks

From all information presented above, it becomes clear that MFGM material has many advantageous technological functionalities. The MFGM material can be isolated from raw milk or from dairy by-products. While MFGM material isolated from raw milk is, so far, mainly used for research purposes, the potential applications lie in the MFGM material isolated from the side products derived from milk. As presented in section 1.3., many investigators have been successful in isolating MFGM materials, which contain a high concentration of PLs and specific membrane proteins from by-products of dairy industrial processing. This opens opportunities to make use of such cheap sources to manufacture added-value ingredients with special functionalities. However, the treatment during milk processing affects the composition of the isolated materials and hence their technological functionalities. More studies are needed on the effect of processing (e.g. heat treatment) on the PL moiety of MFGM material. Whether or not the changes in phospholipids composition,
chemical structure or alteration in interaction of phospholipids together or with other components of the membrane or of the milk serum phase would change the technological properties is still to be explored.

When talking about technological aspects of MFGM material, one refers directly to its emulsifying properties, which have been assumed to be due to the PL fraction. It is noteworthy that PLs in MFGM fragments strongly interact with other components such as proteins and cholesterol (see 1.2.2). Proteins also have an amphiphilic characteristic and can act as surface-active compounds (Singh, 2006). However, surface-active properties of glycoproteins, MFGM-specific proteins, have not been studied. When MFGM, as a whole material, is applied to stabilize emulsions the contribution part of the protein moiety needs to be investigated.

1.7 Biological values of MFGM

A great deal of knowledge on the possible roles of proteins and PL components of MFGM in relation to its beneficial physiological effects on human health has been gathered during the last 20 years (Spitsberg, 2005). The MFGM and its components are linked with various health beneficial properties since they influence several biological functions in humans (Fong et al., 2007). In a review by Spitsberg (2005), it was described that bovine MFGM phospholipids play a role in cell function and transport systems. The consumption of phospholipids supplements by humans during their physical training will alter the neuroendocrine function. This is one of the beneficial effects of phospholipids. Especially, these phospholipids will avoid muscle soreness and influence the well-being. In the gastrointestinal tract of humans, some phospholipids will deliver an antimicrobial activity after being digested (van Hooijdonk et al., 2000). Upon digestion, sphingolipids undergo sequential cleavage to ceramide and sphingosine in the small intestine and colon, and these are subsequently adsorbed by intestinal cells and degraded into fatty acids, or reincorporated into sphingolipids (Schmelz et al., 1994). In experiments on rats, sphingolipids were found to inhibit both the early and the late stages of colon carcinogenesis (Dillehay et al., 1994, Schmelz et al., 1996). This protective effect against colon carcinogenesis of dietary sphingolipids was confirmed by feeding weanling Fischer-344 Rats with MFGM (Snow et al., 2010). A possible mechanism of action is that exogenously supplied sphingolipids bypass a sphingolipid signaling defect that is important in cancer (for example, a loss of cellular sphingomyelin turnover to produce ceramide and sphingosine) (Berra et al., 2002). The role of sphingolipids in relation to colon
health has been reviewed recently (Kuchta et al., 2012). An overview of the PLs and their nutritional aspects in the MFGM is shown in Table 1-3.

The MFGM proteins play an important role in various cell functions and defense mechanisms in newborns (Schroten et al., 1992, Peterson et al., 1998b, Wang et al., 2001). Some important functions of the bioactive major protein of the MFGM are described by Park (2009). To mention a few: butyrophilin is known for its receptorial functions and aid in modulating the encephalitogenic T cell response. Lactoferrin inhibits the classical pathway of complement activation and bacteriostatic action by competing with bacteria for iron. Carbonic anhydrase plays an essential role in the gastrointestinal tract development of the newborn. Lactadherin promotes cell adhesion and the protective function against fimbriated microorganisms is promoted by Mucin I. The growth of cancer cells was inhibited by the minor high molecular weight MFGM proteins BRCA1 (breast cancer type 1 susceptibility protein) and BRCA2 (breast cancer type 2 susceptibility protein). A small peptide with 11 amino acids residues identical to the C-terminal of bovine mammary gland fatty acid binding protein (FABP) was found to be a potent inhibitor of breast cancer cell growth in vitro at extremely low concentration (Spitsberg and Gorewit, 1997, Peterson et al., 1998a). Spitsberg (1995, 2005) stated that after consumption of MFGM fragments, a certain number of inhibitory peptides could be released and subsequently absorbed in the digestive tract. The absorbed peptides would enter the bloodstream, and after reaching the organs or tissues, they could exert their inhibitory action on the cells undergoing carcinogenic transformation.

Bovine MFGM contains a component, presumably of protein origin, which could inhibit in vitro the purified Escherichia coli β-glucuronidase (Ito et al., 1993), the enzyme involved in the intestinal degradation of glucuronides. Glucuronyl transferase neutralizes the toxic compounds in liver cells through the formation of glucuronides, which are subsequently excreted. Some bacteria in the intestine have β-glucuronidases, which degrade the glucuronides. As a result, toxic agents which might be carcinogenic are released, and stimulate the formation of e.g. colon cancer (Spitsberg, 2005). Therefore, the consumption of MFGM could prevent colon cancer due to the presence of an inhibitor of β-glucuronidase (Ito et al., 1993). In Table 1-4, the most important MFGM proteins are given together with their functions and health aspects.
Table 1-3. Overview of the PLs and other MFGM components with their nutritional properties (Dewettinck et al., 2008).

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<thead>
<tr>
<th>Component</th>
<th>Nutritional aspects</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLs</td>
<td>Reduction of number of aberrant crypt foci and adenocarcinomas</td>
</tr>
<tr>
<td>Sphingolipids and metabolites</td>
<td>Shift in tumor type (malignant → benign)</td>
</tr>
<tr>
<td></td>
<td>Anticholesterolemic</td>
</tr>
<tr>
<td></td>
<td>Protection of the liver from fat and cholesterol induced steatosis</td>
</tr>
<tr>
<td></td>
<td>Suppression of gastrointestinal pathogens</td>
</tr>
<tr>
<td></td>
<td>Neonatal gut maturation</td>
</tr>
<tr>
<td></td>
<td>Myelination of the developing central nervous system</td>
</tr>
<tr>
<td></td>
<td>Endogenous modulators of vascular function</td>
</tr>
<tr>
<td></td>
<td>Associated with age-related diseases and the development of Alzheimer</td>
</tr>
<tr>
<td>Sphingosine 1-phosphate</td>
<td>Mitogenic</td>
</tr>
<tr>
<td>Phosphatidylserine (PS)</td>
<td>Restore normal memory on a variety of tasks</td>
</tr>
<tr>
<td></td>
<td>Positive effects on Alzheimer patients</td>
</tr>
<tr>
<td></td>
<td>Improve exercise capacity of exercising humans</td>
</tr>
<tr>
<td>Phosphatidylcholine (PC)</td>
<td>Support liver recovery from toxic chemical attack or viral damage</td>
</tr>
<tr>
<td></td>
<td>Protects the human GI mucosa against toxic attack</td>
</tr>
<tr>
<td></td>
<td>Reduction of necrotizing enterocolitis</td>
</tr>
<tr>
<td>Lysophosphatidylcholine (lysoPC)</td>
<td>Bacteriostatic and bacterial capacity</td>
</tr>
<tr>
<td>Other components</td>
<td>Strong gastro protective role in the duodenal mucosa</td>
</tr>
<tr>
<td>Vitamin E and carotenoids</td>
<td>Antioxidants</td>
</tr>
</tbody>
</table>
Table 1-4. Overview of the important MFGM proteins and other components with their molecular weight (MW), iso electric point (IEP), function and health aspects (Dewettinck et al., 2008).

<table>
<thead>
<tr>
<th>Component</th>
<th>MW (kDa)</th>
<th>IEP</th>
<th>Function</th>
<th>Health aspects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid binding protein (FABP)</td>
<td>13</td>
<td>5-5.5</td>
<td>Transport of fatty acids</td>
<td>Cell growth inhibitor anticancer factor (FABP as selenium carrier)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Regulation of lipid metabolism</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Increase of lipid droplets in cytoplasm</td>
<td></td>
</tr>
<tr>
<td>Butyrophilin (BTN)</td>
<td>66-67</td>
<td>5</td>
<td>MFG secretion</td>
<td>Suppression of multiple sclerosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Belong to Ig</td>
<td>Induces or modulates experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>Xanthine oxidase (XDH/XO)</td>
<td>146 (300)</td>
<td>7.7</td>
<td>Structural, lipid secretion</td>
<td>Bacterial agent</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Role in purine metabolism</td>
<td>Redox reaction /anti-inflammatory</td>
</tr>
<tr>
<td>Mucin 1 (MUC1)</td>
<td>160-200</td>
<td>&lt; 4.5</td>
<td>Protection from physical damage</td>
<td>Protection effect against rotavirus infection</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Protection from invasive pathogens</td>
<td></td>
</tr>
<tr>
<td>Breast cancer type 1 susceptibility protein (BRCA 1)</td>
<td></td>
<td></td>
<td>Cancer suppressor</td>
<td>Inhibition of breast cancer</td>
</tr>
<tr>
<td>Breast cancer type 2 susceptibility protein (BRCA2)</td>
<td></td>
<td></td>
<td>Direct regulation of cytokinesis</td>
<td>Inhibition of breast cancer</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cancer suppressor</td>
<td></td>
</tr>
</tbody>
</table>
### Chapter 1. Literature review

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW/PI</th>
<th>pH</th>
<th>Function</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactadherin</td>
<td>47</td>
<td>6-6.6</td>
<td>Member of cadherins</td>
<td>Protection from viral infection in the gut</td>
</tr>
<tr>
<td>(PAS VI/VII)</td>
<td></td>
<td></td>
<td>Ca-dependent adhesive properties</td>
<td>Role in epithelialization, cell polarization, cell movement and rearrangement, neurite outgrowth</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phospholipid binding</td>
<td>Synaptic activity in the central nervous system</td>
</tr>
<tr>
<td>Proteose Peptone 3 (PP3)</td>
<td>18-30/14</td>
<td>6.3</td>
<td>Membrane associated</td>
<td>Expressed in lactating mammary gland</td>
</tr>
<tr>
<td>Adipophilin</td>
<td>52</td>
<td>7.5-7.8</td>
<td>Uptake and transport of fatty acids/triacylglycerol (TAG)</td>
<td>Marker of the secretory and ductal epithelium</td>
</tr>
<tr>
<td>Periodic acid (PAS III)</td>
<td>95-100</td>
<td>&lt; 4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cluster of differentiation (CD36)</td>
<td>76-78</td>
<td>&lt; 7</td>
<td></td>
<td>Macrophages marker</td>
</tr>
<tr>
<td>Other components</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-glucuronidase inhibitor</td>
<td></td>
<td></td>
<td></td>
<td>Inhibition of colon cancer</td>
</tr>
<tr>
<td>Helicobacter pylori inhibitor</td>
<td></td>
<td></td>
<td></td>
<td>Prevention of gastric diseases</td>
</tr>
<tr>
<td>Phospoproteins</td>
<td></td>
<td></td>
<td></td>
<td>Organic phosphorus/Ca-phosphate source</td>
</tr>
<tr>
<td>MFGM antigens</td>
<td></td>
<td></td>
<td></td>
<td>Coronary atherogenic effects</td>
</tr>
</tbody>
</table>
The MFGM contains more than 25 different enzymes and most of them are members of the class of hydrolases followed by oxidoreductases and transferases. The most abundant enzymes present are alkaline phosphatase and xanthine oxidase (Keenan, 2001). There are many more proteins and enzymes present in bovine MFGM preparations than those discussed above. The majority are peripheral proteins loosely adsorbed to the MFGM and could exert important biological functions.
Chapter 2. Emulsifying Properties of a MFGM Material Isolated From Reconstituted Buttermilk

This chapter is redrafted after:


Abstract

The MFGM contains proteins and PLs with potential technological functionalities. The emulsifying properties of MFGM material isolated from reconstituted buttermilk using MF (BM-MFGM) were compared to those of BMP, SMP and SC. Oil-in-water emulsions stabilized with MFGM material, at identical total protein/soybean oil ratios, were prepared using a two-step homogenizer at pressures of 0/2, 3/2, 9/2, 15/2 and 21/2 MPa. Above 15/2 MPa, droplet aggregation was observed with BMP or SMP emulsions, whereas no aggregation was observed with BM-MFGM emulsions. BM-MFGM emulsions prepared at 9/2 MPa showed a monomodal particle size distribution with an average droplet size of about 1.9 µm, significantly smaller compared to BMP and SMP emulsions. Emulsion viscosity depended on emulsifying materials and homogenization pressures. Sodium caseinate emulsions showed poor stability. These results indicate the potential of BM-MFGM for its use as emulsifier.

2.1 Introduction

The FGs in raw milk are surrounded by the MFGM, a true biological membrane, composed mainly of PLs and unique membrane specific proteins (Walstra et al., 2006). The MFGM acts in milk as a natural emulsifying agent which protects the FGs against coalescence. Besides very specific proteins, it also contains PLs which are amphiphilic in nature, and are therefore considered as good emulsifiers (Kanno, 1989, Dewettinck et al., 2008). The MFGM is of
particular importance considering the various health-related properties that have been reported for its different constituents. Among these are anticholesterolemic effects, inhibition of cancer cell growth, and bactericidal properties (Spitsberg, 2005, Dewettinck et al., 2008).

When cream is churned during butter making, buttermilk is formed. During churning and agitation, the FGs rupture and the MFGM is released into the aqueous phase. Consequently, buttermilk is some of the dairy by-products which are rich sources of MFGM material (Rombaut et al., 2005). The manner in which cream is handled, treated or stored can affect the composition of the isolated MFGM (Keenan and Dylewski, 1995). Most reported studies have been focused on examining the characteristics of the MFGM. In these studies, to obtain valid analytical data, the milk FGs had to be retreated to remove any absorbed serum constituents. Therefore, cream was abundantly washed with buffered solutions prior to isolation of MFGM (Mather and Keenan, 1975, Le et al., 2009). However, the water phase which results from the industrial process of buttermaking contains, together with the MFGM components, high levels of skim milk protein contaminants (Malin et al., 1994). Therefore, results obtained in a laboratory using washed creams are not necessarily representative for the MFGM-derived material from industrial buttermilk.

The separation of MFGM fragments from dairy constituents can be achieved by MF (Morin et al., 2007). The properties of the isolated MFGM largely depend on the type of treatment of the milk during processing, e.g. heating, cooling, homogenization, evaporation, drying (Evers, 2004), and on the method of isolation and separation of the MFGM from the source materials (Singh, 2006). According to the results of Corredig and Dalgleish (1997b) MFGM-enriched materials isolated from industrial buttermilk were poor emulsifiers for soybean oil-in-water emulsions as compared to those isolated from fresh cream. The type of starting material, pretreatment and method of separation have a significant effect on the composition of MFGM isolates and consequently on their emulsifying properties (Kanno, 1989, Kanno et al., 1991). Wong and Kitts (2003) as well as Corredig and Dalgleish (1997b) found that commercial buttermilk had inferior emulsifying and stabilizing capacity as compared to non-fat dried milk. Roesch et al. (2004) studied the emulsifying properties of commercial BMP and the MFGM fragments obtained by MF of reconstituted buttermilk. The emulsions made with the MFGM isolate were found to have a good stability against creaming; the small particle size distribution pattern increased with MFGM concentration, whereas a similar emulsion prepared with buttermilk concentrate showed extensive flocculation.
Although buttermilk is rich in MFGM derived fractions, the use of buttermilk in food systems is closely related to its particular composition in emulsifying components. The proteins of the MFGM isolated from untreated cream mainly contained MFGM-specific proteins whereas the MFGM material isolated from industrial buttermilk still contained high ratios of non-MFGM proteins, such as caseins and whey proteins, e.g. β-LG and α-LA. Whether or not the alteration in interaction of PLs together or with other components of the membrane or of the milk serum phase would change the emulsifying properties needs still to be explored. To get a better understanding of the participation of MFGM components isolated from reconstituted buttermilk, it is necessary to further investigate the properties of emulsions with different milk protein compositions.

The objective of this work package was to evaluate the use of MF to concentrate MFGM material from buttermilk (the obtained material was called BM-MFGM), and to investigate the efficacy of this MFGM material as an emulsifier. The oil-in-water (O/W) emulsions were prepared with the BM-MFGM material and the emulsifying properties of this material were compared to those of BMP, SMP and SC.

### 2.2 Materials and methods

#### 2.2.1 Materials and chemicals

Buttermilk powder was obtained from FrieslandCampina (Lummen, Belgium). Skim milk powder was purchased from Hochdorf Swiss Milk AG (Hochdorf, Switzerland), sodium caseinate from Acros-Organic (Geel, Belgium) and soybean oil was purchased from a local supermarket.

Chemicals for analysis were obtained from Chem-Lab (Zedelgem, Belgium) and Sigma–Aldrich (Steinheim, Germany). For the analysis of PLs, high-performance-liquid-chromatography (HPLC)-grade dichloromethane, supra-gradient methanol and HPLC grade water were obtained from Biosolve (Valkenswaard, The Netherlands).

#### 2.2.2 Isolation of the MFGM material

Buttermilk was reconstituted at 4% total solids of BMP in deionized water (Millipore SA, Malsheim, France) under agitation for 1 h. Trisodium citrate (1% w/w) was added and the solution was stored overnight at 4°C to ensure full hydration. A cross-flow MF method with continuous DF was used to isolate the MFGM materials. For each batch, 25 kg of the reconstituted buttermilk was microfiltered and diafiltered with 70 kg of deionized water. MF
was carried out according to Le et al. (2011b) with some modifications. The MF unit consisted of a Millipore frame with Pellicon® 2 cassette filter (PVGVPC05), a hydrophilic PVDF Durapore® membrane with a pore size of 0.22 µm and a membrane surface of 0.5 m² (Millipore, Brussels, Belgium). The feed-pump was a compressed air-operated diaphragm pump (Chemcor series of Almatec, Kamp-Lintfort, Germany). The dry matter content of the final retentate was about 9%; it was adjusted to pH 6.8 using 1 N HCl and stored at < –20°C until further analysis and emulsion preparation. The MFGM enriched material obtained after the MF was called BM-MFGM.

### 2.2.3 Compositional analysis

The dry matter content of the samples was determined by gravimetric difference after heating at 105°C (IDF, 2004). The total protein content of the samples was determined by the Kjeldahl method (IDF, 1993) using 6.38 as the conversion factor. The total fat content was determined by gravimetric determination using the Röse–Gottlieb method (IDF, 1986). Total ash content was determined by heating and igniting the samples in an electric muffle furnace at 550°C (Williams, 1984) and the lactose content was determined by subtraction.

The protein separation system and all reagents for SDS-PAGE were obtained from Invitrogen (Merelbeke, Belgium). Samples were prepared as described by Le et al. (2009). The wet gel was scanned at 400 dpi using a high-resolution transmission scanner (UMAX Powerlook III, Taipei, Taiwan) and was analyzed by Imagemaster Totallab Software (GE Healthcare, Diegem, Belgium). Separation was carried out on gradient (4-12%) polyacrylamide gels with an Xcell Surelock system. The name giving of the major proteins was done according to Mather (2000): XO, CD36, BSA, BTN, PAS 6/7, ADPH. The relative weight proportion of caseins and whey proteins of samples was estimated using densitometry on SDS-PAGE according to following equation:

\[
\text{Caseins/Whey} = \frac{\text{Band intensity of caseins}}{\text{Band intensities of (lactoferrin+BSA+ β-LG+ α-LA)}} [2.1]
\]

It is important to note that color intensity of protein band increases linearly with increasing protein load and finally levels off in the calibration curve of band intensity vs. concentrations (Fishbein, 1972). When the protein load is above the saturation point, the band intensity remains constant and the analysis using densitometry on SDS-PAGE in not an accurate quantitative method without using standards. However, the results may have some indicative values and help for discussion and comparison purpose.
PLs were solvent-extracted and analyzed by a Shimadzu HPLC System (Tokyo, Japan) with an evaporative light-scattering detector (ELSD; Alltech-3300, Alltech Associates Inc., Lokeren, Belgium). The procedure of extraction and analysis was according to the method of Le et al. (2011a). All chemical analyses were carried out in duplicate.

### 2.2.4 Emulsion preparation

Mixtures of 35% soybean O/W were prepared and supplemented with BM-MFGM, BMP, SMP or SC at the same concentration of total protein (2.3 g per 100 g of product). The composition of the emulsions is shown in Table 2-1. Powders were diluted in water under gentle stirring at room temperature and stored overnight at 4°C to allow full hydration. After adding soybean oil, the samples were heated and maintained at 50°C and pre-homogenized at 13000 rpm for 2 minutes using an Ultra-Turrax T25 (IKA® – Werke GmbH & Co. KG, Staufen, Germany). The emulsions were prepared using a two-step laboratory-scale high-pressure homogenizer (APV Cooling Systems, Albertslund, Denmark). For each emulsion formulation, five homogenization pressures, namely 0/2, 3/2, 9/2, 15/2 and 21/2 MPa were applied. After the homogenization, the emulsions were stored at 4°C to prevent microbial growth before the characterization tests.

<table>
<thead>
<tr>
<th>Emulsions&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Emulsifying materials (% dry matter)</th>
<th>Soybean oil (%)</th>
<th>Water (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM-MFGM</td>
<td>3.30</td>
<td>35</td>
<td>61.7</td>
</tr>
<tr>
<td>BMP</td>
<td>6.71</td>
<td>35</td>
<td>58.3</td>
</tr>
<tr>
<td>SMP</td>
<td>6.69</td>
<td>35</td>
<td>58.3</td>
</tr>
<tr>
<td>SC</td>
<td>2.37</td>
<td>35</td>
<td>62.6</td>
</tr>
</tbody>
</table>

### 2.2.5 Emulsion characterization

#### 2.2.5.1 Measurement of particle size distribution

After one day of storage at 4°C, 1% of the emulsion was dispersed in water or in 1% SDS solution (Ye et al., 2004) before the measurement of the particle size distribution. The measurement was carried out using a long bench Malvern Mastersizer S (Malvern Instruments Ltd., Malvern, UK) equipped with a MS17 automated sample dispersion unit. The
polydispersity analysis of the samples was carried out using a 300RF lens. The standard presentation code (1.5295, 0.01, 1.3300 for the particle refractive index, particle absorption, and dispersant refractive index, respectively) was used according to the procedure of Roesch et al. (2004). The $d_{3,2}$ was used to compare the emulsion droplet sizes. At least two emulsions, independently prepared, were measured for each treatment and average values were taken.

2.2.5.2 Microscopic observation

Microscopic observation of the emulsions was carried out at room temperature after one day of storage at 4°C. Samples were first diluted 10 times in deionized water to reduce the density of emulsions and a drop of the sample was carefully placed on a glass slide, covered with a cover slip and observed at 50x magnification using a Leitz Diaplan microscope (Leitz diaplan Leica, Solms, Germany). The images were recorded with a built-in Olympus Color View camera and processed with cell D software (Olympus, Aartselaar, Belgium).

2.2.5.3 Emulsion stability

After the homogenization, 10 mL of emulsion was poured into graduated tubes of 10 mm diameter and stored undisturbed at 4°C for 10 days. The volume of the serum layer formed at the bottom of the tube was recorded every two days. The emulsion separation was carried out in triplicate, and expressed as a percentage as follows:

$$\text{Separation (\%)} = \left( \frac{\text{volume of serum}}{\text{volume of emulsion}} \right) \times 100 \quad [2.2]$$

2.2.5.4 Rheological characteristics

The rheological characteristics of the samples were measured after one day of storage at 4°C using a TA instruments AR2000 controlled-stress rheometer (TA instruments Inc., New Castle, DE), equipped with a conical concentric cylinder geometry (28 mm diameter) and a cup (30 mm diameter). For the characterization of the flow curves, emulsion samples were mixed gently and passed through a syringe to ensure homogeneity of the sample. After this step, 20g of each sample emulsion was weighted into the cup of the rheometer for the measurements.

Flow curves were measured with increasing shear rates, 0.1 to 100 s$^{-1}$ (31 measuring points), and at a temperature of 20°C. The experimental data were fitted to the power law equation:

$$\text{Shear stress} = K \times (\text{Shear rate})^n \quad [2.3]$$

where $K$ is the consistency index and ‘$n$’ is the flow behavior index.
2.2.6 Statistical analysis

Statistical analysis was performed using the SPSS version 21 (IBM Inc., Chicago, IL., USA). One-way analysis of variance (ANOVA) and Tukey’s test were used for multiple comparison of means. The differences were considered statistically significant at $P \leq 0.05$.

2.3 Results and discussion

2.3.1 Composition of the experimental materials

The composition of different experimental materials is shown in Table 2-2. Results of multiple comparisons of the means showed that the total protein and ash content of BM-MFGM and SC were significantly different from that of BMP and SMP. BMP and SMP contained similar concentrations of total protein and ash. Significant differences in the relative ratio of caseins and whey proteins, total lipid, lactose and PLs content were observed among different materials.

Table 2-2. Composition of experimental materials, expressed as % on dry-matter basis.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Total protein ± SD</th>
<th>Casein/whey protein ratio</th>
<th>Total lipids ± SD</th>
<th>Ash ± SD</th>
<th>Lactose ± SD</th>
<th>PLs ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP</td>
<td>34.39 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.72 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.93 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.14 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>56.53 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.16 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMP</td>
<td>34.25 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.75 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.37 ± 0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.13 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50.25 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.27 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-MFGM</td>
<td>69.60 ± 1.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.02 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.8 ± 0.60&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.25 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.30 ± 1.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.30 ± 0.31&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SC</td>
<td>96.85 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;3&lt;/sup&gt;</td>
<td>ND</td>
<td>2.25 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>1</sup> Data are expressed as mean values ± SD of three replicates. Different superscript letters within a column refer to statistically significant differences (Tukey’s test, $P \leq 0.05$).

<sup>2</sup> Estimation of relative ratio of casein to whey proteins using densitometry.

<sup>3</sup> ND: Not determined.

PLs can be used as a parameter to indicate the enrichment of MFGM fragments in the BM-MFGM. Compared to the initial material, BMP, the PLs content of BM-MFGM increased about 2.84 times (Table 2-2). The total protein and total lipids also increased 2 and 2.8 times, respectively. Morin et al. (2006) reported an increase in PLs and total protein of 2.25 and 1.83 times, respectively, using MF with a 0.4 µm ceramic membrane in combination with 2x DF. Le et al. (2011b) reported an increase in total lipid and total proteins of 2.41 and 1.95 times, respectively, using MF with a hydrophilic PVDF multi-flat sheet membrane module with pore
size of 0.22 µm in combination with three DF steps. The lactose and ash content was found to decrease by a factor of 15 and 2.2, respectively. This was as expected since during MF, the majority of lactose and minerals will pass through the filtering membrane (Corredig et al., 2003, Rombaut et al., 2006b). On the other hand, MFGM fragments/particles were concentrated in the retentate, leading to the higher total lipid and PLs content in the BM-MFGM. The results were agreement with the data published by Le et al. (2011b).

Upon separation by SDS-PAGE, the major MFGM proteins, in order from starting at the the top, include MUC1, XO, PASIII, CD36, BTN, PAS6/7, ADPH and PP3 (Mather, 2000). However, MUC1 and PASIII, being heavily glycosylated proteins, are not or poorly stained with Coomassie blue (Mather, 2000). Among the major MFGM proteins, BTN and XO are the most abundant MFGM proteins with an estimated amount of 34 – 43% and 13 – 20% of the total MFGM proteins, respectively (Waud et al., 1975, Mather and Jack, 1993, Mondy and Keenan, 1993). Therefore, BTN and XO have been taken as an example for the discussion in this chapter. Based on densitometry, band intensities of XO and BTN of BM-MFGM (lane 2) were found to be 1.2 and 1.4 times higher than those of BMP, respectively (Figure 2-1). In addition to MFGM-specific proteins, BM-MFGM contained a considerable amount of non-MFGM protein such as caseins and whey proteins e.g. β-LG and α-LA. However, the ratio of caseins to whey proteins in BM-MFGM was found to decrease by a factor of 1.7 compared to

---

**Figure 2-1.** SDS-PAGE patterns of molecular weight standards (lane 1); BM-MFGM (lane 2); BMP (lane 3); SC (lane 4); and SMP (lane 5). The load on each sample lane was 16µg of total protein.
that in BMP (Table 2-2), because caseins would pass through the membrane during the MF process after dissociation of the casein micelles (Corredig et al., 2003, Rombaut et al., 2006b). To further decrease the concentration of the non-MFGM proteins in the final product, more DF steps can be considered (Corredig et al., 2003). However, it is noted that with even more extensive DF, the purity of the filtered product, in term of MFGM proteins, would not be comparable with that of MFGM material isolated from raw (unheated) milk. Cano-Ruiz and Richter (1997), Corredig and Dalgleish (1998a), Ye et al. (2004) and Morin et al. (2007) reported that heat treatment during buttermilk powder production may induce an interaction between β-LG, α-LA, κ-casein and the surface of FGs.

As expected, skim milk powder showed the lowest total lipid (1.93 g 100 g⁻¹) and PLs (0.16 g 100 g⁻¹) content and the highest lactose (56.53 g 100 g⁻¹) content compared to BMP and BM-MFGM (Table 2-2). These results are similar to those published by Le et al. (2011b). The ratio of caseins to whey proteins of SMP was high (3.72) compared to that of BMP (1.76) because caseins were a major part of the proteins in the latter. In addition, SDS-PAGE of BMP showed the presence of a considerable amount of MFGM-specific proteins such as XO and BTN compared to that of SMP (Figure 2-1). Sodium caseinate has the highest total protein content (96.46 g 100 g⁻¹) among all the materials (Table 2-2). Lipid, lactose and PLs content of SC was not analyzed, however their levels are expected to be negligible. As expected, casein bands were dominant in SC (Figure 2-1).

2.3.2 Particle size distribution

The effect of different materials and homogenization pressures on the droplet size distribution is shown in Figure 2-2. After diluting in water, the emulsions prepared with BM-MFGM and SC had a narrower particle size distribution compared to that of emulsions prepared with BMP and SMP. This may indicate that the oil droplets in BMP and SMP emulsions were aggregated, probably due to the fact that their surfaces may only be partially covered and that there were bridges between neighboring droplets (Roesch et al., 2004). Euston and Hirst (1999) found that the emulsifying properties of milk protein products were strongly influenced by the aggregation state of the constituent proteins. The presence of casein aggregates strongly influenced the emulsifying behavior, resulting in a less efficient functionality as emulsifiers. When present at sufficiently high concentration, small surfactants, due to their structure and high reorientation ability, can diffuse rapidly to the interface and inhibit protein adsorption to the oil droplet (Bos and van Vliet, 2001, Pugnalonia
et al., 2004). In the present study, SMP contained the lowest MFGM fractions, as evidenced by the low presence of PLs and MFGM proteins in the SDS-PAGE (Figure 2-1). This can probably explain the lower emulsifying capacity of SMP compared to BM-MFGM. BMP has a 20 times higher PLs content, but a 2.1 times lower ratio of caseins to whey proteins compared to SMP (Table 2-1 and Table 2-2). Cano-Ruiz and Richter (1997) showed that caseins and whey proteins cover the surface of FGs when they are homogenized, leading to an increase in their surface area, but caseins decrease the surface tension at a faster rate than whey proteins and exhibit better emulsifying properties. However, casein micelles are less surface active than caseinates (Courthaudon et al., 1999). Besides that, the buttermilk powder used in this study underwent heat treatment during the production. Excessive heat denaturation may impair the emulsifying properties by rendering the proteins insoluble (Voutsinas et al., 1983).

Emulsions prepared with BM-MFGM and SC at higher homogenization pressure (> 9/2 MPa) showed a bimodal distribution with one population of about 0.5µm droplet diameter and the second larger group with an average droplet size of about 2.5µm. The bimodal distribution of particles might be attributed to the formation of clusters or aggregates of droplet re-coalescence under high homogenization intensity (Floury et al., 2000). It was also found that a higher homogenization pressure led to higher turbulent and shear flow intensity, thus causing a higher coalescence efficiency as well as a higher rate of droplets collision, resulting in a higher coalescence level (Jafari et al., 2008, Hakansson et al., 2009). Another possible reason was that a higher pressure led to smaller droplets, more intense Brownian motion and easier collision and assembling during the subsequent process (e.g. storage) (Long et al., 2012).

For emulsions prepared with SMP and BMP, the measured $d_{3.2}$ after dilution in SDS 1% was significantly lower than that after dilution in water. For emulsions prepared with BM-MFGM and SC, there was no significant difference in the $d_{3.2}$ when the emulsions were either diluted in SDS 1% or in water (Table 2-3). In the presence of SDS, disruption of bridging flocculation occurs, displacing the protein aggregates with the surfactant molecules. When an emulsion is diluted in an SDS solution, it is possible to determine the size distribution of the individual, rather than aggregated or flocculated fat droplets (Roesch et al., 2004).
Figure 2-2. Particle size distribution of emulsions prepared with different emulsifying materials at different homogenization pressures. The measurements were carried out after dilution in water (left column) or in 1% SDS (right column).
Table 2-3. Sauter mean diameter, $d_{3,2}$, of emulsions prepared with different emulsifiers and at different homogenization pressures after dilution in water or in 1% SDS solution$^1$.

<table>
<thead>
<tr>
<th>Pressure (MPa)</th>
<th>SMP in water</th>
<th>SMP in SDS</th>
<th>BMP in water</th>
<th>BMP in SDS</th>
<th>BM-MFGM in water</th>
<th>BM-MFGM in SDS</th>
<th>SC in water</th>
<th>SC in SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/2</td>
<td>6.44 ± 0.42&lt;sub&gt;c&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.34 ± 0.47&lt;sub&gt;B&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>8.33 ± 0.67&lt;sub&gt;d&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.79 ± 0.25&lt;sub&gt;AB&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.74 ± 0.16&lt;sub&gt;AB&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.53 ± 0.06&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.07 ± 0.46&lt;sub&gt;d&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.91 ± 0.47&lt;sub&gt;AB&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>3/2</td>
<td>4.58 ± 1.23&lt;sub&gt;B&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.93 ± 0.05&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.67 ± 1.97&lt;sub&gt;C&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.68 ± 0.12&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.66 ± 0.12&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.63 ± 0.06&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.69 ± 0.08&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.64 ± 0.06&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>9/2</td>
<td>4.63 ± 0.93&lt;sub&gt;B&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.22 ± 0.09&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.36 ± 0.83&lt;sub&gt;C&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.14 ± 0.09&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.89 ± 0.11&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.92 ± 0.07&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.00 ± 0.19&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.98 ± 0.15&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>15/2</td>
<td>6.14 ± 1.24&lt;sub&gt;C&lt;/sub&gt;&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.65 ± 0.25&lt;sub&gt;B&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.96 ± 1.27&lt;sub&gt;D&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.58 ± 0.03&lt;sub&gt;B&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.76 ± 0.06&lt;sub&gt;b&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.76 ± 0.05&lt;sub&gt;b&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.60 ± 0.03&lt;sub&gt;a&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.63 ± 0.09&lt;sub&gt;a&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>21/2</td>
<td>5.38 ± 1.37&lt;sub&gt;B&lt;/sub&gt;&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.12 ± 0.30&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.48 ± 1.97&lt;sub&gt;C&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.11 ± 0.12&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.57 ± 0.07&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62 ± 0.09&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.40 ± 0.04&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.39 ± 0.03&lt;sub&gt;a&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

$^1$ Data are expressed as mean values ± SD of three replicates. Values in the same column which share a common lower case superscript did not differ significantly. Values in the same row which share a common upper case subscript do not differ significantly (Tukey’s test, $P \leq 0.05$).
Table 2-3 shows that the average particle size of emulsions prepared with different materials decreased significantly with increasing homogenization pressures after dilution with SDS 1%. The results are in agreement with Mulder and Walstra (1974), Phipps (1975) and Robin et al. (1992). Dalgleish (1997) showed that during milk homogenization, the surface area of the FGs is increased and amphiphilic molecules are adsorbed onto the surfaces. Due to the globule size reduction, more surface active material is required to cover the oil-water interface and provide for emulsion stability. Tomas and Paquet (1994) reported that an inadequate amount of proteins could cause some aggregation of fat droplets. In general, it can be concluded that emulsions prepared with different materials showed different emulsifying behavior. The difference in concentration of PLs, and the difference in protein composition between emulsifying materials, were probably responsible for the differences in the droplets size of emulsions.

2.3.3 Microscopic observation

Differences in the degree of flocculation of emulsions prepared with different materials can be seen in Figure 2-3. Microscopic examination showed that emulsions prepared with SMP contained many large aggregates (Figure 2-3B). This could be due to the fact that caseins were adsorbed in an aggregated form to the droplet surfaces and to the fact that aggregated proteins are less effective emulsifiers (Roesch et al., 2004). This was in agreement with the data on the particle size distribution.

Microscopic comparison of the emulsions prepared with BM-MFGM and with the initial BMP material (Figure 2-3A and C) showed that BM-MFGM is a better emulsifier than BMP due to the presence of the higher amount of PLs. In addition, BM-MFGM has a high amount of MFGM-specific proteins such as XO and BTN as compared to BMP. The specific membrane proteins may have good emulsifying properties and are responsible for emulsion stability (Vanderghem et al., 2010). Sodini et al. (2006) also showed that better emulsifying properties can be obtained with a higher ratio of PLs to protein. However, when MFGM material is used, the contribution of each moiety i.e. PLs and specific membrane proteins and their potential interaction, remains to be investigated (see Chapter 4 and Chapter 5). Corredig et al. (2003) reported that stable soy O/W emulsions could be made with MFGM material isolated from raw, untreated cream, and that these emulsions were less susceptible to exchanges at the interface. Strong interaction within the MFGM may exist at the interface and the PLs of the MFGM may lower the interfacial tension more effectively compared to dairy proteins (Corredig and Dalgleish, 1998a).
Sodium caseinate, a widely-used ingredient because of its good solubility and emulsifying properties and its heat stability, is mainly composed of caseins with negligible amounts of PLs. Microstructural observation of emulsions prepared with SC showed individual oil droplets and no sign of aggregation, not much different from that of BM-MFGM (Figure 2-3C and D). It is noted that the characteristics of the emulsifying materials required to stabilize oil-in-water emulsions probably depend not only on the ratio of PLs to proteins, but also on the difference in protein composition between various materials.

### 2.3.4 Stability of emulsions

Emulsions prepared with BM-MFGM showed no visible phase separation (Figure 2-4). The excellent stability of these emulsions could be attributed to the presence of more milk fat membrane constituents, such as PLs (Scott et al., 2003b, Roesch et al., 2004). Emulsions
prepared with sodium caseinate were the least stable among the four types of emulsions, even at higher homogenization pressures (e.g. 21/2 MPa, data not shown). Singh (2011) reported that the creaming stability of sodium caseinate emulsions showed a complex dependency on the protein content. At low protein content, the globules are joined by bridging flocculation, whereas the presence of large amounts of unadsorbed casein molecules causes phase separation due to depletion flocculation (Dickinson and Golding, 1997). In this study, separation of emulsions made with 2.3% sodium caseinate could be caused by the presence of an excessive amount of protein resulting in creaming. This was confirmed by the data on the average droplet size and by microscopic observation as well. Ye (2008) also reported that emulsions prepared with sodium caseinate > 1% showed a decreased creaming stability.

**Figure 2-4.** Stability of emulsions prepared with BMP (---), SMP (……), BM-MFGM (—), and SC (——) at homogenization pressures of 9/2 MPa during quiescent storage at 4°C.

Emulsions prepared with BMP and SMP showed creaming when a lower homogenization pressure was applied (data not shown) but showed better stability only at higher homogenization pressures (> 9/2 MPa). SMP and BMP have a similar protein content, but the casein to whey protein ratio was different (Table 2-2). The casein and whey protein content of those materials play an important role in stabilizing the emulsion. High pressure homogenization may result in further unfolding of the globular proteins and the exposure of hydrophobic sites. This influences the attachment of proteins at the interface, reduces interfacial tension and stabilizes the emulsions (Roesch and Corredig, 2003, Martin-Gonzalez et al., 2009). Euston and Hirst (1999) reported that milk protein concentrate and SMP were more stable to
creaming than those made with SC. This is because the protein aggregates in milk protein concentrate and SMP were too large to cause depletion flocculation and thus form very viscous emulsions, which will reduce creaming. This is clearly supported by the data in section 2.3.5.

### 2.3.5 Rheological behavior

The curves of shear stress versus shear rates are shown in Figure 2-5. The power law model fitted the data well ($P < 0.05$). The parameters of different emulsifying materials for the preparation of emulsions contributed to a change in rheological behavior with the coefficients of determination ($R^2$) listed in Table 2-4.

Emulsions prepared with BM-MFGM (regardless of the homogenization pressures) showed a flow behavior more similar to a Newtonian flow behavior and a very low shear stress against shear rate. Van Lent et al. (2008) reported that all samples of reconstituted dairy creams made with skim milk powder had a much higher apparent viscosity compared to samples of fresh cream and cream prepared with cream residue powder. These authors found that skim milk-stabilized RCs were characterized by the formation of clusters, probably as a consequence of the low amount of free proteins in the serum phase, because the fat droplets were covered with a thick layer of proteins.

![Figure 2-5. Flow curves of the emulsions prepared with BMP (---), SMP (……), BM-MFGM (—), and SC (– – –) at homogenization pressures of 9/2 MPa after one day of storage.](image)

As shown in Table 2-4, the consistency index ($K$) of SMP emulsion was significantly higher than that of the other materials. Furthermore, the power law indices of emulsions prepared with BMP, SMP and SC were significantly lower than that of BM-MFGM. No significant difference in the power law index was observed among the emulsions with BMP and SMP.
Table 2-4. Flow curve parameters of emulsions prepared with different materials at homogenisation pressures of 9/2 MPa\(^1\).

<table>
<thead>
<tr>
<th>Materials</th>
<th>( K ) (Pa.s(^n))</th>
<th>( n )</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP</td>
<td>3.78 ± 1.62(^b)</td>
<td>0.54 ± 0.10(^b)</td>
<td>0.998</td>
</tr>
<tr>
<td>SMP</td>
<td>5.04 ± 0.50(^c)</td>
<td>0.49 ± 0.07(^b)</td>
<td>0.993</td>
</tr>
<tr>
<td>BM-MFGM</td>
<td>0.02 ± 0.00(^a)</td>
<td>0.83 ± 0.01(^a)</td>
<td>0.994</td>
</tr>
<tr>
<td>SC</td>
<td>0.32 ± 0.02(^a)</td>
<td>0.36 ± 0.01(^c)</td>
<td>0.987</td>
</tr>
</tbody>
</table>

\(^1\) Data are expressed as mean values ± SD of three replicates. Different superscript letters within a column refer to statistically significant differences (Tukey’s test, \( P \leq 0.05 \)).

A similar trend was observed in the consistency and the power law indices for different emulsions. Emulsions with BM-MFGM showed a lower consistency index and a higher power law index indicating less shear thinning behavior, whereas the opposite was found for emulsions prepared with BMP and SMP. The higher shear stress observed for the emulsions prepared with SMP and BMP indicated that these emulsions had a higher viscosity compared to BM-MFGM emulsions. The shear stress increased with the homogenization pressures (data not shown). Emulsions prepared with SMP were apparently more viscous compared to other emulsions. Similar results were also reported by Scott, Duncan, Summer, Waterman, and Kaylegian (2003b). These authors showed that cream containing SMP and low-melt butter oil was more viscous than the cream containing a sweet buttermilk and butter-derived aqueous phase. Among the experimental materials in the current study, BM-MFGM stabilized emulsions had very low shear stress and fluid like flow behavior (index \( n \) is close to 1), possibly due to the higher PLs content of BM-MFGM. Scott et al. (2003b) also reported that a higher PLs and unsaturated fatty acid content of buttermilk and butter-derived aqueous phase may contribute to an increased fluidity of the emulsions.

2.4 Conclusions

The use of different materials in the preparation of emulsions had a significant influence on their properties, such as particle size distribution, microstructure, rheology and stability. Emulsions prepared with BM-MFGM and SC materials had narrow particle distribution patterns with a smaller mean diameter. Microscopic observation also confirmed the smaller particle sizes of emulsions prepared with BM-MFGM and SC material compared to emulsions prepared with BMP and SMP. In addition, BM-MFGM emulsions had good stability, very low
viscosity and Newtonian-like flow behavior, whereas SC-stabilized emulsions had the highest creaming rate. These observations can be explained by the difference in composition of the four materials, where BM-MFGM contained the highest concentration of PLs and SC consisted mostly of caseins. In conclusion, BM-MFGM had better emulsifying/stabilizing properties compared to BMP, SMP and SC.
Chapter 3. Comparison of Emulsifying Properties of MFGM Materials Isolated From Different Dairy by-Products

This chapter is redrafted after:

**Abstract**

Emulsifying properties of MFGM materials isolated from reconstituted buttermilk (BM-MFGM) and buttermilk whey (Whey-MFGM), individually or in mixtures with BMP were compared with those of a commercial dairy ingredient (Lacprodan®PL20), a material rich in milk PLs and proteins. Particle size distribution, viscosity, interfacial protein and PLs load of O/W emulsions prepared using soybean oil were examined. Pronounced droplet aggregation was observed with emulsions stabilized with Whey-MFGM or with a mixture of Whey-MFGM and BMP. No aggregation was observed for emulsions stabilized with BM-MFGM, Lacprodan®PL20 or a mixture of BM-MFGM and BMP. The surface protein load and PLs load was lowest in emulsions with BM-MFGM. Highest protein load and PLs load was observed for emulsions prepared with a mixture of Whey-MFGM and BMP. The difference in composition of MFGM materials, such as in whey proteins, caseins, MFGM-specific proteins, PLs, minerals, and especially their possible interactions determine their emulsifying properties.

**3.1 Introduction**

Emulsifying properties of milk derived components influence the physicochemical characteristic of dairy emulsions (see Chapter 2). In recent years, there has been increasing interest in gathering knowledge on the composition and properties of MFGM materials. MFGM materials are found in quite significant amounts in different dairy products such as cream, butter, buttermilk, buttermilk whey and cheese. The unique functionality of MFGM-enriched materials has led to research and development of techniques to isolate, purify and apply the materials in different food emulsions (Singh, 2006). The serum phase as by-product from churning cream into butter is known as buttermilk. It is rich in MFGM fragments and contains all water-soluble components from the milk such as lactose, caseins and whey proteins (see
Chapter 3. Emulsifying properties of MFGM materials isolated from dairy by-products

2.1). Buttermilk whey is obtained from buttermilk after coagulating and removing casein micelles, a process applied in the production of some special cheese and in caseinate manufacturing. Buttermilk whey still contains some residual fat and consists of lipoprotein particles, MFGM fragments and small FGs (Rombaut and Dewettinck, 2007b). Proper utilization of these cheap by-products in order to isolate the functional MFGM material and subsequent application of the material in the development of new products has great economical and technological value. Besides some by-products of the dairy industry, Lacprodan®PL20 is a dairy formulation enriched with PLs and proteins. It has potential as a market alternative to the semi-synthetically head group exchanged soy phospholipids because it is a natural source for PS. Moreover, Lacprodan®PL20 can be used as an active health ingredient in functional foods such as drinks, ice creams and chocolate (Burling and Graverholt, 2008).

As discussed in section 1.6.5, it appears that processing conditions in dairy processing influence differently the nature of MFGM materials in the derived by-products and that their MFGM isolates differ in composition (of PLs and proteins) and in their technological functionalities. When speaking about technological aspects of MFGM material, one refers directly to its emulsifying properties, which have been assumed to be due to the PL fraction. Sodini et al. (2006) studied the compositional and functional properties of sweet, sour and whey buttermilk and reported that the emulsifying properties of those three types of buttermilk are better than milk and whey but have a lower foaming capacity. Among the above-listed buttermilk samples, whey buttermilk was found to have the highest emulsifying properties and the lowest foaming capacity. Possible reasons for this could be the higher ratio of PLs to protein in whey buttermilk as compared to sweet or cultured buttermilk. The results in Chapter 2 also showed that MFGM fragments, obtained by MF from reconstituted buttermilk, had better emulsifying and stabilizing properties as compared to BMP, SMP and SC, probably due to a higher concentration in PLs. However, proteins also are amphiphilic and thus can act as surface-active compounds (Singh, 2011). Many investigators have been successful in isolating MFGM fragments, which contain high concentrations of PLs and specific membrane proteins, from by-products of dairy industrial processing. However, whether proteins or PLs or both are responsible for the emulsifying activity is unclear. It is also unclear how the emulsifying properties are modified by possible interactions between MFGM materials and other components (e.g., whey proteins, caseins and minerals). To get a better understanding of the participation of MFGM components, in this study the properties of emulsions prepared with
different MFGM materials and mixtures of MFGM and the initial BMP material was investigated.

3.2 Materials and methods

3.2.1 Materials

Buttermilk whey, the by-product of the manufacturing processes of cheese and caseins, was obtained from Büllinger Butterei (Büllinger, Belgium). Lacprodan®PL20, a spray-dried powder rich in milk phospholipids and proteins, was kindly provided by Arla Foods (Viby, Denmark). This product is a registered trademark. It is obtained during butter oil production (40% fat) by using centrifugation and membrane filtration processes (Burling and Graverholt, 2008). The final concentrate, containing over 20% phospholipids in total solids was spray-dried. The Lacprodan®PL20 production procedure is patent pending WO2006/128465 A1 (Arla Foods). BMP and soybean oil used in this study are the same materials as used in the study described in Chapter 2.

3.2.2 Isolation of the MFGM materials

Reconstituted buttermilk was prepared as described in section 2.2.2. For buttermilk whey, the pH was adjusted to 7.5 by adding 1N KOH before MF (Rombaut et al., 2007a). Cross-flow MF was performed in combination with continuous DF (rate of water addition was equal to the permeation rate) to separate the MFGM fragments from the two materials (reconstituted buttermilk and buttermilk whey) (see 2.2.2). The materials obtained from reconstituted buttermilk and buttermilk whey after MF were called BM-MFGM (as in Chapter 2) and Whey-MFGM, respectively. BM-MFGM and Whey-MFGM were freeze-dried (Vaco 5-D, Zirbus Technology GmbH, Bad Grund, Germany) to obtain a water-free MFGM-enriched powder. For further analysis and emulsion preparation the MFGM-enriched powder was stored below –20°C.

3.2.3 Compositional analysis

The content of total protein, total lipids, ash, PLs and dry matter were determined as described in section 2.2.3.

The method and the calculations used to determine protein using SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) have been described in section 2.2.3.

3.2.4 Emulsion preparation
To compare the emulsifying properties of MFGM materials and the interaction between MFGM material and other components (caseins, whey proteins) following materials were used to stabilize O/W emulsion (35%, w/w, soybean oil): BM-MFGM, Whey-MFGM, Lacprodan®PL20 or mixtures of MFGM material and BMP. For emulsions prepared with a mixture of the MFGM material and BMP, a ratio of 4:6 on dry weighed basis was used due to practical considerations. Results of a screening test showed no significant difference between the ratio 4:6 and 5:5. The supplement of the emulsifying materials was calculated to have the same concentration of total protein (2.3g 100g⁻¹ of product). Table 3-1 illustrates the composition of the emulsions prepared for experiments.

Table 3-1. Composition (weight percentage) of emulsions (all contained 35% w/w of soybean oil)

<table>
<thead>
<tr>
<th>Emulsions</th>
<th>Isolated MFGM/BMP ratio</th>
<th>Isolated MFGM (% DM)</th>
<th>BMP (% DM)</th>
<th>Total PLs (g 100g⁻¹ product)</th>
<th>Water (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM-MFGM100</td>
<td>10</td>
<td>3.30</td>
<td>0</td>
<td>0.33</td>
<td>61.70</td>
</tr>
<tr>
<td>Whey-MFGM100</td>
<td>10</td>
<td>8.52</td>
<td>0</td>
<td>0.91</td>
<td>56.48</td>
</tr>
<tr>
<td>Lacprodan100</td>
<td>10</td>
<td>4.41</td>
<td>0</td>
<td>0.95</td>
<td>60.59</td>
</tr>
<tr>
<td>BM-MFGM40</td>
<td>4</td>
<td>1.32</td>
<td>3.82</td>
<td>0.29</td>
<td>59.86</td>
</tr>
<tr>
<td>Whey-MFGM40</td>
<td>4</td>
<td>3.44</td>
<td>3.82</td>
<td>0.46</td>
<td>54.74</td>
</tr>
<tr>
<td>Lacprodan40</td>
<td>4</td>
<td>1.76</td>
<td>3.82</td>
<td>0.52</td>
<td>59.42</td>
</tr>
</tbody>
</table>

BM-MFGM100 = emulsion with BM-MFGM alone as the emulsifier; Whey-MFGM100 = emulsion with Whey-MFGM alone as the emulsifier; Lacprodan100 = emulsion with Lacprodan®PL20 alone as the emulsifier; BM-MFGM40 = emulsion with mixture of BM-MFGM and BMP (4:6 w/w); Whey-MFGM40 = emulsion with mixture of Whey-MFGM and BMP (4:6 w/w); Lacprodan40 = emulsion with mixture of Lacprodan®PL20 and BMP (4:6 w/w).

The procedure for the preparation of the emulsions was described in section 2.2.4. In Chapter 2, for each emulsion formulation, different homogenization pressures were compared. In this study, homogenization was performed at 9 and 2 MPa for the first and second step, respectively. After homogenization, the emulsions were stored at 4°C before analysis. Emulsions were prepared in triplicate.
3.2.5 Emulsion characterization

3.2.5.1 Measurement of particle size distribution

Similar procedures and equipment as described in section 2.2.5.1 were applied.

3.2.5.2 Microscopic observation

Similar procedures and equipment as described in section 2.2.5.2 were applied.

3.2.5.3 Rheological characteristics

Similar procedures and equipment as described in section 2.2.5.4 were applied.

3.2.5.4 Determination of surface protein load, PLs load and the adsorption behavior of proteins at oil/water interface

Emulsions (25g) were weighed into a centrifugation plastic tube, capped and centrifuged at 12,000 x g for 45 min at 20°C using a Sigma 4K15 centrifuge (Sartorius AG, Göttingen, Germany). The serum phase (bottom layer) was carefully removed using a syringe. The cream phase (top layer) was dispersed into deionized water and re-centrifuged at 12,000 x g for 45 min. The serum phase was again removed. The combined serum phases were filtered through 0.45µm and 0.2µm cellulose acetate filter (Whatman™, Germany).

The surface protein load of emulsions was determined according to Ye (2010) with some modification. The adsorbed protein content (cream phase) (g) was determined by the Kjeldahl method using 6.38 as the conversion factor as described in 2.2.3.

To determine the surface PLs load, the PLs in the cream phase (g) were extracted and analyzed as described in 2.2.3.

The total fat surface area (m²) was derived from the d₃₂. The surface protein load (Γ_pro, mg m⁻²) and PLs load (Γ_Pl, mg m⁻²) were calculated from equation [3.1] and [3.2], respectively:

\[ \Gamma_{\text{Pro}} = \frac{\text{total protein adsorbed (mg)}}{\text{total fat surface area (m}^2) \]  \[ \Gamma_{\text{PL}} = \frac{\text{total PLs adsorbed (mg)}}{\text{total fat surface area (m}^2) \]  

The protein profile in the emulsions, the individual adsorbed protein (i.e. top layer) at the surface of the oil droplets and the non-absorbed protein in the serum phase (i.e. bottom layer) were determined using SDS-PAGE. The XCell SureLock Mini Cell Electrophoresis System, all reagents and the gradient gels (Novex NuPAGE® 4-12% Bis –Tris 1mm x 17 wells) were purchased from Invitrogen (Merelbeke, Gent). Samples were prepared as described by Le et al.
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(2009) with some modifications. Sample reduction was performed following the manufacturer’s protocol. To avoid vertical streaks formation on the gel after staining, the samples were partially defatted prior to reducing and loading on gel. Defatting was performed using a 20:1 (v/v) dichloromethane:methanol mixture to dilute the sample in an Eppendorf tube followed by repeated vortexing for approximately 20 s. The suspension was centrifuged at 1,680 x g for 60 s and the dichloromethane (lower phase) was removed. The sample was dried by flushing with nitrogen gas and re-dissolved in distilled water by sonication. The load on each sample lane was 16 µg of total protein. Electrophoresis was performed according the protocol of Invitrogen (Merelbeke, Gent) with Simply Blue™ safe stain (Coomassie Blue). The wet gel was scanned at 400 dpi using a high-resolution transmission scanner (UMAX PowerLook III, Taipei, Taiwan). The different types of proteins were identified by comparing the protein bands with the molecular weight (MW) of Mark12™ Protein Standard. Among the major MFGM proteins, BTN and XO are the most abundant as presented in section 2.2.3. Therefore BTN and XO were taken as an example for the calculation of the adsorption behavior of MFGM proteins at the oil-in-water interface. The band intensity of proteins in the emulsion, cream phase and serum phase was compared. The proportion of individual protein (XO, BTN, caseins, β-LG and α-LA) at the surface of the oil droplets (i.e. cream phase) and individual protein in the emulsion (i.e. in cream phase + in the serum phase) were calculated using densitometry on SDS-PAGE according to the following equation [3.3]:

\[
\text{Surface proportion of a specific protein (\%) = } \frac{\text{Band intensity of the protein in cream phase}}{\text{Total band intensity of the protein in cream and serum phases}} \times 100
\]  

[3.3]

3.2.6 Statistical Analysis

Statistical analysis was performed using the SPSS version 21 (IBM Inc., Chicago, IL., USA). One-way analysis of variance (ANOVA) and Tukey’s test were used for multiple comparison of means. The differences were considered statistically significant at \( P \leq 0.05 \).

3.3 Results and discussion

3.3.1 Composition of the experimental materials

The composition of the different experimental materials is shown in Table 3-2. It was found that the composition of the materials was significantly different from each other. Whey-MFGM contained the highest amount of total lipids (39.01 g 100 g\(^{-1}\)) while PLs were most abundant in
Lacprodan® PL20 (23.10 g 100 g\(^{-1}\)). According to the manufacturer of this material, it has a minimum phospholipids content of 16%, a maximum ash content of 6% and a protein content of 49-55%. The analyses data in this study were found to be similar (Table 3-2). Among the four materials, Whey-MFGM had the highest amount of ash (23.39 g 100 g\(^{-1}\)) and the lowest protein content (26.50 g 100 g\(^{-1}\)). The low protein content was due to the fact that large amounts of proteins, mainly caseins, were already coagulated and precipitated during cheese making (Vanderghem et al., 2010). The addition of calcium chloride as process additive during cheese making results in the high ash content of the buttermilk whey as well as the derived MFGM isolate (Rombaut et al., 2007a). The data in Table 3-2 also show that the ratio of caseins to whey proteins in the Whey-MFGM material was clearly the lowest among all materials.

**Table 3-2. Composition of the emulsifying materials, expressed as % on dry-matter basis\(^1\)**

<table>
<thead>
<tr>
<th>Materials</th>
<th>Total protein</th>
<th>Casein/whey protein ratio(^2)</th>
<th>Total lipids</th>
<th>Ash</th>
<th>PLs</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP</td>
<td>34.25 ± 0.14(^b)</td>
<td>1.75 ± 0.01(^d)</td>
<td>8.37 ± 0.30(^a)</td>
<td>7.13 ± 0.05(^c)</td>
<td>3.27 ± 0.13(^a)</td>
</tr>
<tr>
<td>BM-MFGM</td>
<td>69.63 ± 1.30(^d)</td>
<td>1.02 ± 0.02(^b)</td>
<td>23.80 ± 0.61(^b)</td>
<td>3.25 ± 0.17(^c)</td>
<td>9.30 ± 0.31(^b)</td>
</tr>
<tr>
<td>Whey-MFGM</td>
<td>26.50 ± 1.19(^a)</td>
<td>0.14 ± 0.01(^a)</td>
<td>39.01 ± 0.20(^d)</td>
<td>23.39 ± 0.31(^d)</td>
<td>12.10 ± 0.19(^c)</td>
</tr>
<tr>
<td>Lacprodan® PL20</td>
<td>52.22 ± 1.27(^c)</td>
<td>1.26 ± 0.08(^c)</td>
<td>31.10 ± 0.57(^c)</td>
<td>6.46 ± 0.10(^b)</td>
<td>23.10 ± 0.70(^d)</td>
</tr>
</tbody>
</table>

\(^1\)The values ± SD of three replicates; different superscript letters within a column refer to statistically significant differences (Tukey’s test, \(P \leq 0.05\)).

Compared to the starting material, BMP, BM-MFGM contained more proteins (69.63 g 100 g\(^{-1}\)), lipids (23.80 g 100 g\(^{-1}\)) and PLs (9.30 g 100 g\(^{-1}\)). The difference in composition of those materials was already discussed in section 2.3.1.

Based on densitometry, the band intensity of XO, PAS6/7 and ADPH of Lacprodan® PL20 (lane 2) was similar to that of BM-MFGM, while the band intensity of BTN of BM-MFGM (lane 3) was found to be 1.2 times higher than that of Lacprodan® PL20. Among the four materials, band intensities of the major MFGM-specific proteins of Whey-MFGM were faint or even not distinguishable (lane 4). The absence of MFGM proteins could be explained by the fact that the pI of BTN, FABP and MUC1 is around pH 4.5-5; they will probably be coagulated as well upon acidification. The pI of XO is 7.8. Possibly, the part of XO which is present in the buttermilk is the tightly MFGM-bounded part. It has been shown that XO is directly anchored to BTN, which could explain that although its pI is not reached, XO is recovered in the cheese part of the coagulated buttermilk as an anchor of the precipitated BTN. Rombaut (2006a)
reported that, after a separation of the buttermilk into whey and fresh cheese, all MFGM proteins were abundantly present in the cheese fraction. In addition to MFGM specific proteins, non-MFGM proteins such as caseins and whey proteins were found to be present, up to a considerable amount, in BMP, BM-MFGM and Lacprodan® PL20 (Figure 3-1). However, the ratio of caseins to whey proteins is significantly different among the four materials (Table 3-2).

**Figure 3-1.** SDS-PAGE patterns of different samples. Lane 1: BMP, Lane 2: Lacprodan® PL20, Lane 3: BM-MFGM, Lane 4: Whey-MFGM, Lane 5: molecular mass (kDa) standard. The load on each lane contained 16µg total protein.

### 3.3.2 Particle size distribution

In Figure 3-2, the droplet size distribution of different emulsions is shown. In Table 3-3, the $d_{3.2}$ of emulsions prepared with different emulsifying materials after dilution in water or in 1% SDS solution is given.

The effect of different MFGM materials and mixtures of individual MFGM material and BMP on droplet size distribution is shown in Figure 3-2. Measured after diluting in water, the emulsions with BM-MFGM100, Lacprodan100 and BM-MFGM40 had a narrower particle size distribution pattern compared to that of the emulsions with Whey-MFGM100, Whey-MFGM40 or Lacprodan40. Sodini et al.(2006) found that better emulsifying properties can be obtained with a higher ratio of phospholipids to proteins. In the present study, a wider particle size distribution was found in emulsions prepared with a high content of PLs (i.e. Whey-
MFGM100), whereas emulsions prepared with BM-MFGM (regardless the concentrations) had the lowest PLs content (Table 3-1) and a narrower particle size distribution. This may indicate that some other factors are interacting with the adsorption process at the droplet surface. Corredig and Dalgleish (1997b) found that emulsion stability strongly depends on the type of isolate that was used. The isolate that contained only MFGM fragments was a poor emulsifier compared to the isolate containing whey proteins, caseins and MFGM-specific proteins.

![Figure 3-2. Particle size distribution of emulsions prepared with different emulsifying materials. The measurements were carried out after dilution in water (left) or in 1% SDS (right).](image)

Whey-MFGM contains the lowest level of caseins and MFGM proteins, as evidenced by the MFGM protein pattern in the SDS-PAGE (Figure 3-1). The absence of caseins strongly influences the emulsifying behavior. Corredig and Dalgleish (1998a) reported that when buttermilk powder was used to prepare O/W emulsions, caseins made up to about 50% of the total proteins adsorbed at the interface. Cano-Ruiz and Richter (1997) also showed that caseins and whey proteins covered the surface of the FGs when they were homogenized, leading to an increase in their surface area, but caseins decreased the surface tension at a faster rate than whey proteins and were found to have good emulsifying properties. However, emulsions with Whey-MFGM100 had the highest ratio of PLs to protein, which could cause an increase in the emulsifying properties of emulsions. Whey-MFGM100 had also the highest ash content (minerals e.g., Ca$^{2+}$), due the addition of calcium chloride as process additive during the coagulation step in cheese making. Calcium (Ca$^{2+}$) being a bivalent cation, can bind any two
 neighboring negative charged groups and can bridge caseins together, or bridge two negatively
groups of a protein. This can give rise to protein aggregation. Moreover, no visible phase
separation was observed in emulsions containing Whey-MFGM100 (data not shown).

Table 3-3 shows that the $d_{3,2}$ of emulsions prepared with different emulsifying materials, after
dilution in water, was significantly different. The emulsions with of BM-MFGM100,
Lacprodan100 and BM-MFGM40 have a smaller droplet size compared to those with Whey-
MFGM100, Whey-MFGM40 and Lacprodan40.

Table 3-3. Sauter mean diameter\(^{1}\), $d_{3,2}$, of emulsions prepared with different emulsifying
materials after dilution in water or in 1\% SDS solution.

<table>
<thead>
<tr>
<th>Emulsions</th>
<th>In water</th>
<th>In SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM-MFGM100</td>
<td>1.44 ± 0.04(^a)</td>
<td>1.20 ± 0.30(^a)</td>
</tr>
<tr>
<td>Whey-MFGM100</td>
<td>5.87 ± 0.01(^d)</td>
<td>1.15 ± 0.01(^a)</td>
</tr>
<tr>
<td>Lacprodan100</td>
<td>2.09 ± 0.05(^b)</td>
<td>1.67 ± 0.02(^b)</td>
</tr>
<tr>
<td>BM-MFGM40</td>
<td>2.08 ± 0.03(^b)</td>
<td>1.63 ± 0.03(^b)</td>
</tr>
<tr>
<td>Whey-MFGM40</td>
<td>6.22 ± 0.04(^c)</td>
<td>2.10 ± 0.08(^c)</td>
</tr>
<tr>
<td>Lacprodan40</td>
<td>4.40 ± 0.15(^c)</td>
<td>1.69 ± 0.01(^b)</td>
</tr>
</tbody>
</table>

\(^{1}\)Data are expressed as mean values ± SD of three replicates. Different superscript letters
within a column refer to statistically significant differences (Tukey’s test, $P \leq 0.05$).

The $d_{3,2}$ of emulsions with either Whey-MFGM100 or Whey-MFGM40 or Lacprodan40 diluted
in water was found to be larger than that of emulsions diluted in SDS 1\% (Table 3-3). In the
presence of SDS, disintegration of the emulsion droplet aggregates occurs, displacing the
protein aggregates with the surfactant molecules (i.e. SDS). When an emulsion is diluted in an
SDS solution, it is possible to determine the size distribution of the individual fat droplets and
not the aggregated or flocculated ones (Roesch et al., 2004). The result was confirmed by the
data on the stability of emulsions. All emulsions prepared with different emulsifying materials
showed no separation after 10 days of storage at 4\°C (figure not shown).

In general, it can be concluded that emulsions prepared with different ingredients showed
different emulsifying behaviors. The droplet size of emulsions was not only affected by the
higher participation of PLs, but also by the presence of other components such as whey
proteins, caseins, MFGM-specific proteins and minerals.
3.3.3 Microscopic observation

Differences in the degree of flocculation of emulsions prepared with different MFGM materials or mixture of individual MFGM material and BMP (4:6 w/w) can be seen in Figure 3-3. Microscopic examination showed that emulsions Whey-MFGM100 contained many aggregates (Figure 3-3B). This result was in agreement with the data on the particle size distribution (section 3.3.2). Wong and Kitts (2003) reported that not the MFGM components but rather the ratio between the casein, whey, and MFGM content in buttermilk determines its functional properties. In addition, Whey-MFGM has a very low amount of MFGM-specific proteins, especially XO and BTN (Figure 3-1). Vanderghem et al. (2010) reported that the specific-membrane proteins may have good emulsifying properties and be responsible for emulsion stability.

**Figure 3-3.** Microscopic images of emulsions prepared with different emulsifying materials A: BM-MFGM100; B: Whey-MFGM100; C: Lacprodan100; D: BM-MFGM40; E: Whey-MFGM40 and F: Lacprodan40. The scale bar indicates 50µm.

Despite the fact that the level of MFGM proteins (mainly XO and BTN) and the casein/whey proteins ratio of emulsions Whey-MFGM40 was higher compared to that of emulsions Whey-MFGM100, many large aggregates were observed in the latter emulsion (Figure 3-3E). This
could probably be due to the fact that the binding of Ca\(^{2+}\) to the casein-coated oil droplets results in aggregation of emulsion droplets. Swaisgood (1992) reported that the binding of Ca\(^{2+}\) to phosphoserine in caseins reduces the electrostatic repulsions between the casein molecules, which could promote interactions between hydrophobic domains, leading to formation of aggregates.

Microscopic comparison of emulsions with Lacprodan100 and Lacprodan40 (Figure 3-3C and F) showed that Lacprodan100 is a better emulsifier than Lacprodan40, probably due to the difference in the level of PLs present (Table 3-1). In addition, the buttermilk powder used in this study was heat-treated during production. Excessive heat denaturation may impair the emulsifying properties by rendering the proteins insoluble (Voutsinas et al., 1983). With the same amount of BMP added, emulsions with Lacprodan40 contained more aggregates than emulsions with BM-MFGM40. According to Scholey et al. (2013), Lacprodan®PL20 is a milk protein concentrate rich in natural, non-synthetic milk phospholipids. It could probably contain more casein aggregates in the constituent proteins, resulting in a less efficient functionality as emulsifier.

Microstructural observation of emulsion prepared with BM-MFGM alone as emulsifier (i.e. BM-MFGM100) showed the presence of individual oil droplets and no sign of aggregation (Figure 3-3A), which was not much different from what was observed with Lacprodan100 and BM-MFGM40 emulsions (Figure 3-3C and D). It is noted that in emulsifying materials many other substances such as whey proteins, caseins, MFGM proteins, PLs and minerals are present in different concentrations, which may influence the stability of oil-in-water emulsions. The effect of these components as well as of their concentrations still needs further investigation.

### 3.3.4 Rheological behavior

The curves of shear stress versus shear rates are plotted in Figure 3-4. The power law model fitted the data well (\(P < 0.05\)). The parameters of the different emulsifying materials for the preparation of emulsions contributed to a change in the rheological behavior with a coefficient of determination (\(R^2\)) as listed in Table 3-4. Emulsions with BM-MFGM100, Lacprodan100 and BM-MFGM40 showed a flow behavior more similar to Newtonian flow (index \(n\) is close to 1) and a very low shear stress against shear rates. In contrast, emulsions with Whey-MFGM100 or Whey-MFGM40 or Lacprodan40 showed a shear-thinning behavior and a gradually increasing shear stress against shear rates (Figure 3-4). This could probably be due to the break-up of aggregates under the effect of a high shear rate (Dickinson and Golding, 1997).
These results were consistent with the changes in the average droplet size of the emulsions with different materials (Table 3-3).

![Flow curve of emulsions BM-MFGM100 (─ · ·), Whey-MFGM100 (....), Lacprodan100 (----), BM-MFGM40 (– · – ), Whey-MFGM40 (–―), Lacprodan40 (―–―).](image)

**Figure 3-4.** Flow curves of emulsions BM-MFGM100 (─ · ·), Whey-MFGM100 (....), Lacprodan100 (----), BM-MFGM40 (– · – ), Whey-MFGM40 (–―), Lacprodan40 (―–―).

**Table 3-4.** Flow curve parameters\(^1\) of emulsions prepared with different emulsifying materials

<table>
<thead>
<tr>
<th>Emulsions</th>
<th>K (Pa s(^b))</th>
<th>n</th>
<th>R(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM-MFGM100</td>
<td>0.01 ± 0.001(^a)</td>
<td>0.86 ± 0.021(^{cd})</td>
<td>0.9947</td>
</tr>
<tr>
<td>Whey-MFGM100</td>
<td>6.18 ± 0.471(^b)</td>
<td>0.29 ± 0.003(^a)</td>
<td>0.9700</td>
</tr>
<tr>
<td>Lacprodan100</td>
<td>0.04 ± 0.000(^a)</td>
<td>0.79 ± 0.002(^c)</td>
<td>0.9966</td>
</tr>
<tr>
<td>BM-MFGM40</td>
<td>0.03 ± 0.003(^a)</td>
<td>0.94 ± 0.051(^d)</td>
<td>0.9852</td>
</tr>
<tr>
<td>Whey-MFGM40</td>
<td>5.72 ± 0.516(^b)</td>
<td>0.33 ± 0.058(^a)</td>
<td>0.9682</td>
</tr>
<tr>
<td>Lacprodan40</td>
<td>0.63 ± 0.042(^a)</td>
<td>0.68 ± 0.007(^b)</td>
<td>0.9974</td>
</tr>
</tbody>
</table>

\(^1\)The values ± SD of three replicates; different superscript letters within a column refer to statistically significant differences (Tukey’s test, P ≤ 0.05).

### 3.3.5 The adsorption behavior of proteins at O/W interface, the surface protein load and PLs load
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The adsorption behavior of MFGM materials and mixtures of individual MFGM material and BMP in oil-in-water emulsions was studied by comparing the SDS-PAGE pattern of the cream phase with that of the serum phase (Figure 3-5).

Figure 3-5. SDS-PAGE patterns of different samples. Lanes 1 and 1': cream and serum phase of BM-MFGM100, respectively; Lanes 2 and 2': cream and serum phase of Whey-MFGM100, respectively; Lanes 3 and 3': cream and serum phase of Lacprodan100, respectively; Lanes 4 and 4': cream and serum phase of BM-MFGM40, respectively; Lanes 5 and 5': cream and serum phase of Whey-MFGM40, respectively; Lanes 6 and 6': cream and serum phase of Lacprodan40, respectively; Lane 7: molecular mass (kDa) standards. The load on each lane contained 16µg total protein.

As discussed in Chapter 1, the MFGM is highly complex in structure and composed of different protein and lipid components with specific technological and nutritional properties. Because of the complex nature of the MFGM proteins, it has not been possible to identify all specific protein components of MFGM adsorbed at the interface. No studies to date have been published on the adsorption behavior of MFGM proteins at the oil-in-water interface. As discussed in section 2.3.1, BTN and XO are the most abundant proteins in MFGM. In addition, among the visualized bands in Figure 3-5, band intensities of XO, BTN, caseins, β-LG and α-LA were clearly distinguishable. Therefore, the relative proportion of those individual adsorbed proteins at the surface (cream phase) and the sum of proteins in the cream phase and in the serum phase (i.e. in the original emulsion) was estimated and discussed in this report. The presence of XO,
BTN and caseins in Whey-MFGM was negligible (Figure 3-1). Therefore, the adsorbed XO, BTN and caseins in emulsions with Whey-MFGM100 were not taken into account. Among the remaining five emulsions, the BM-MFGM100 emulsions showed the lowest amount of XO and BTN in the adsorbed phase (lane 1’and 1’’, Figure 3-5) occupying 40.3 ±1.8% and 42.8±1.8% of total adsorbed proteins, respectively (Figure 3-6). With Whey-MFGM40 emulsions, all XO, BTN and caseins were adsorbed onto the oil droplets. The intensities of adsorbed caseins, β-LG and α-LA of Lacprodan100 and Lacprodan40 emulsions were similar, while BM-MFGM40 contained higher amounts of adsorbed caseins compared to that of BM-MFGM100 (Figure 3-6).

![Emulsions](image)

**Figure 3-6.** SDS-PAGE band intensity of XO ( ), BTN ( ), caseins ( ), β-LG ( ); α-LA ( ) in cream phase relative to the sum of those in cream and serum of different emulsions. CP ~ cream phase; SP~serum phase.

The $\Gamma_{\text{Pro}}$ and $\Gamma_{\text{PL}}$ of emulsions prepared with different MFGM materials is given in **Error! Not a valid bookmark self-reference.** The interfacial layer of emulsions probably consisted of a mixture of whey proteins, caseins, MFGM proteins and PLs. The $\Gamma_{\text{Pro}}$ of emulsions prepared at the same concentration of total protein (2.3g 100g$^{-1}$ of product), containing different MFGM proteins components was significantly different from each other. This may indicate that the composition regarding caseins, whey proteins and MFGM proteins in the emulsions was significantly different (Table 3-2 and Figure 3-1). Whereas, with an increasing PLs content in
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the emulsions (Table 3-1), the $\Gamma_{PL}$ increased, except for Whey-MFGM40 emulsions (Error! Not a valid bookmark self-reference.).

**Table 3-5. The surface protein load ($\Gamma_{Pro}$) and PLs load ($\Gamma_{PL}$) of emulsions prepared with different MFGM materials**¹

<table>
<thead>
<tr>
<th>Emulsions</th>
<th>$\Gamma_{Pro}$ (mg m⁻²)</th>
<th>$\Gamma_{PL}$ (mg m⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM-MFGM100</td>
<td>5.24 ± 0.12ᵃ</td>
<td>0.18 ± 0.01ᵃ</td>
</tr>
<tr>
<td>Whey-MFGM100</td>
<td>8.58 ± 0.05ᶜ</td>
<td>1.20 ± 0.11ᶜ</td>
</tr>
<tr>
<td>Lacprodan100</td>
<td>7.59 ± 0.11ᵇ</td>
<td>1.29 ± 0.03ᶜ</td>
</tr>
<tr>
<td>BM-MFGM40</td>
<td>9.65 ± 0.16ᵈ</td>
<td>0.36 ± 0.01ᵃ</td>
</tr>
<tr>
<td>Whey-MFGM40</td>
<td>17.96 ± 0.70ᶠ</td>
<td>1.93 ± 0.15ᵈ</td>
</tr>
<tr>
<td>Lacprodan40</td>
<td>11.37 ± 0.03ᶜ</td>
<td>0.71 ± 0.07ᵇ</td>
</tr>
</tbody>
</table>

¹The mean values ± SD of three replicates; different superscript letters within a column refer to statistically significant differences (Tukey’s test, $P \leq 0.05$).

Among the six emulsions, the $\Gamma_{Pro}$ of Whey-MFGM40 emulsions was highest compared to that of other emulsions, probably due to the presence of many large aggregates. Srinivasan et al. (1996) as well as Dickinson and Davies (1999) indicated that the addition of calcium chloride prior to emulsification increased the $\Gamma_{Pro}$; this could be attributed to the aggregation of caseins, due to binding with Ca²⁺, and subsequent adsorption of the aggregated caseins at the droplet surface. In addition, Ca²⁺ binding reduces charge repulsion between the casein molecules and hence can increase the protein packing at the interface. As mentioned, the concentration of Ca²⁺ could be high in Whey-MFGM due to the fact that large amounts of proteins, mainly caseins, were already coagulated and precipitated during cheese making. Similar to the $\Gamma_{Pro}$, the highest $\Gamma_{PL}$ was observed in Whey-MFGM40 emulsions, despite of the fact that the PLs content in this emulsion was lower as compared to Whey-MFGM100 emulsions, which also contained large aggregations. This can be explained by the difference in concentrations of PLs, minerals, MFGM-specific proteins and ratio of casein and whey proteins. However, this phenomenon needs to be further investigated. Compared to all emulsions, the lowest $\Gamma_{Pro}$ and $\Gamma_{PL}$ was observed for the BM-MFGM100 emulsions. This could be due to the strong interaction between MFGM material and other components (e.g., whey proteins and caseins) that may exist at the interface and that the PLs of MFGM may lower the interfacial tension; consequently, the
particles remain individually dispersed in the emulsion. Corredig and Dalgleish (1998a) found that the newly formed oil droplets covered by the MFGM material, behaved differently from emulsions stabilized by other milk proteins, i.e. no displacement occurred on the addition of small molecular weight surfactant and the droplets were not affected by the presence of proteins, e.g. an addition of β-LG or caseins. Data in the present study showed that no competitive displacement seemed to occur between proteins and PLs, but that the presence of components such as whey proteins, caseins, MFGM-specific proteins and minerals, and especially their concentrations, were affected by these substances on the interfacial layer of the emulsions. In order to get a full understanding of the effect of different concentrations of PLs and proteins or possible interaction between them at the interface during emulsification or on possible competitive adsorption at the interface of the emulsion, further research with the same isolated MFGM source is needed.

3.4 Conclusions

The emulsifying and emulsion-stabilizing properties of MFGM materials were strongly influenced by their composition. The use of BM-MFGM (regardless concentrations) and Lacprodan®PL20 as emulsifiers resulted in emulsions with similar properties characterized by a narrow particle size distribution patterns with a smaller mean diameter, a very low viscosity and a Newtonian-like flow behavior. Emulsions prepared with Whey-MFGM alone as emulsifier contained the highest amount of PLs together with the presence of many large aggregates of droplets and showed a shear-thinning behavior. Whey-MFGM did not have good emulsifying properties compared to other MFGM materials, despite of the fact that it contained a higher concentration of PLs. This could be due to the high concentration of minerals (especially Ca$^{2+}$) in the Whey-MFGM. All data demonstrated that not only the PLs content but also the presence of other components (e.g., whey proteins, caseins, MFGM-specific proteins and minerals), and especially the level of these substances and their possible interactions in the different MFGM materials, determined their emulsifying properties.
Chapter 4. Combined Effects of MFGM Proteins and PLs on the Physicochemical Properties of Oil-in-Water Emulsions

This chapter is redrafted after:

Abstract
MFGM contains proteins and PLs making this complex mixture a good emulsifier. In this study, MFGM material was isolated from reconstituted buttermilk using MF and was separated into a MFGM protein concentrate and a PLs concentrate using solvent fractionation. The emulsifying properties of those two surface-active materials, separately or in combination, were investigated. The MFGM protein concentrate and the PLs concentrate and their combinations were used as emulsifiers to prepare oil-in-water emulsions using a two-step homogenizer. The physicochemical properties of the emulsions, more particularly the particle size distribution, the flow behavior and the creaming stability were examined. Results showed that proteins have superior emulsifying properties compared to PLs.

4.1 Introduction
MFGM, a thin film surrounding the FGs in milk, contains mainly membrane-specific proteins and PLs (Walstra et al., 2006). Both MFGM proteins and PLs have technological functionalities and could be used as emulsifying agents due to their amphiphilic nature (Kanno et al., 1991, Roesch et al., 2004, Vanderghem et al., 2010). The use of such enriched PLs isolate as an emulsifier has been reported by Miura et al. (2006). These authors demonstrated that emulsions made with bovine milk PLs had a narrower distribution of droplet sizes than those containing soybean lecithin. In another study, Ihara et al. (2011) reported that reconstituted cream made with buttermilk containing high PLs concentration was more stable, had a higher droplet surface area and was more acid tolerant than that made with buttermilk containing low PLs concentration. These authors concluded that the acid tolerance of cream was not due to phospholipids or lipophospholipids but could also be partially attributed to complexes of PLs and proteins. Along with PLs, proteins also have amphiphilic characteristics and can act as
Chapter 4. Effects of MFGM proteins and PLs on the physicochemical properties of emulsions

surface-active compounds (Singh, 2011). They are able to stabilize emulsion droplets due to their ability to absorb and unfold rapidly at the oil-water interface. One of the first studies about the emulsifying properties of MFGM proteins and PLs regarding the stabilization of the FGs in milk was done by Shimizu et al. (1980). They digested MFGM proteins, obtained from washed cream with papain and then used them in cream. A decrease in the stability of the cream, due to clustering of FGs was observed. It was concluded that proteins and glycoproteins in MFGM may play an important role in emulsion stabilization.

MFGM material, which contains both MFGM proteins and PLs, has been investigated for the stabilization of O/W emulsions in many studies as presented in the survey of literature (section 1.6). However, in different studies, inconsistent results regarding the effect of MFGM components, especially the ratio of PLs and MFGM proteins, on the emulsifying properties were found due to the fact that the composition MFGM proteins and the concentration of PLs were not standardized as discussed in Chapter 2 and Chapter 3. The results of Wong and Kitts (2003) also suggested that not the MFGM components but rather the ratio between the level of casein, whey and MFGM in buttermilk determine the functional properties. Thus far, the role of individual constituents of MFGM (i.e. PLs, MFGM-specific proteins, caseins and whey proteins) in the stabilization of droplets has not been elucidated.

To evaluate the emulsification ability of these substances and the role of proteins and PLs in O/W emulsions, the BM-MFGM material used in Chapter 2 was separated into a MFGM protein concentrate and a PLs concentrate using solvent fractionation. The effect of the concentration of those two surface-active materials, individually or in combination, on the physicochemical properties of oil-in-water emulsions, more particularly the particle size distribution, the flow behavior and the creaming stability were examined.

4.2 Materials and methods

4.2.1 Materials and chemicals

BM-MFGM and soybean oil used in this work package were the same materials as used in the study described in Chapter 2. To separate the MFGM protein and the PLs moiety, BM-MFGM material was dispersed in dichloromethane/methanol (2/1 v/v) at a ratio of 1/10 (w/v). The mixture was shaken and left undisturbed for 15 min to allow phase separation. The lower phase containing the PLs was collected in a flask. The upper phase was washed twice with dichloromethane/methanol (20/1 v/v) at the same ratio of 1/10 (w/v). The lower phases were again collected in the same flask. The upper phase containing MFGM proteins was collected in
a separate flask. The solvents used in both extractions were evaporated at 36°C under vacuum using a rotary evaporator. The residue of solvents in the final extracts was completely removed under a flow of nitrogen gas. The extracts obtained from the upper phase and lower phase of the BM-MFGM material were called ‘MFGM protein concentrate’ and “PLs concentrate”, respectively. The composition of MFGM protein and PLs concentrate was analyzed as described in 2.2.3. The MFGM protein concentrate contained 83.98% total protein, on dry basis (w/w). The PLs concentrate contained 39.7% total PLs. The MFGM protein and PLs concentrate were stored at below –20°C prior to subsequent emulsion preparation.

4.2.2 Emulsion preparation

Oil-in-water emulsions were prepared with 35% soybean oil using different combinations of MFGM protein and PLs concentrate. Concretely, PLs concentrate (0, 0.3, 1.3 and 2.3 g PLs 100g⁻¹ emulsion) was dispersed in soybean oil using a magnetic stirrer. The aqueous phase containing MFGM proteins (0, 0.3, 1.3 and 2.3 g proteins 100g⁻¹ emulsion) was prepared by their dispersion in deionized water. Next, the oil and aqueous phase with all combinations of MFGM protein and PLs concentrate were mixed and heated to 50°C. Subsequently, the mixture was pre-homogenized and homogenized using the same equipments and the parameters as described in section 3.2.4. After homogenization, the emulsions were stored at 4°C before characterization. Three replicate emulsions were prepared for each combination of MFGM protein and PLs concentrate.

4.2.3 Determination of physicochemical properties of emulsions

4.2.3.1 Measurement of particle size distribution

Similar procedures and equipment as described in section 2.2.5.1. were applied.

4.2.3.2 Microscopic observation

Similar procedures and equipment as described in section 2.2.5.2. were applied.

4.2.3.3 Emulsion stability

Similar procedures and equipment as described in section 2.2.5.3. were applied.

4.2.3.4 Rheological characteristics

Similar procedures and equipment as described in section 2.2.5.4. were applied.

4.2.4 Statistical analysis

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Statistical analysis was performed using SPSS version 21 (IBM Inc., Chicago, IL., USA). One-way analysis of variance (ANOVA) and Tukey’s test were used for multiple comparison of means. A two-way ANOVA was performed to compare the interaction of the effects of various levels of two factors in a factorial experiment. The differences were considered statistically significant at $P \leq 0.05$.

4.3 Results and discussion

4.3.1 Particle size distribution

The droplet size distribution and the $d_{3,2}$ of emulsions prepared with the different combinations of proteins (0 to 2.3 wt%) and PLs (0 to 2.3 wt%) after dilution in water or in 1% SDS solution, are shown in Figure 4-1 and Table 4-1, respectively. Two-way ANOVA analysis of the data in Table 4-1 indicated that there was a statistically significant interacting effect between proteins and PLs on the droplet size of emulsion ($P = 0.013$). The interactive effect between various concentrations of proteins and PLs on the $d_{3,2}$ of emulsion is shown in Figure 4-5A.

Among all emulsions, a wider particle size distribution was observed in those prepared with a low concentration (i.e. 0.3%) of either proteins or PLs or a combination of both (Figure 4-1A and B), whereas emulsions containing a high concentration (i.e. 2.3%) of either proteins or PLs showed a narrow particle size distribution. Corredig and Dalgleish (1997a) reported that the droplet size distribution was strongly influenced by the concentrations of MFGM isolate (e.g. containing both proteins and PLs). When concentrations of proteins or/and PLs are low, the system contained insufficient surface active agents to cover all of the freshly created surface, and the formation of large-sized oil droplets might be due to coalescence (Urbina-Villalba and Garcia-Sucre, 2001). Moreover, the formation of large-sized droplets in emulsions containing a low level of proteins can be caused by bridging flocculation of the droplets, sharing a common protein between the newly formed neighboring droplets, as shown by Roesch et al. (2004).
Figure 4-1. Particle size distribution of emulsions prepared with different concentrations of proteins and PLs. The measurements were carried out after dilution in water (left) or in 1% SDS(right).
Table 4-1. Sauter mean diameter, $d_{3,2}$, of emulsions prepared with varying concentrations of proteins and PLs after dilution in water or in 1% SDS solution.

<table>
<thead>
<tr>
<th>Concentration of PLs (wt%)</th>
<th>Concentrations of proteins (wt%)</th>
<th>in water</th>
<th>in SDS</th>
<th>in water</th>
<th>in SDS</th>
<th>in water</th>
<th>in SDS</th>
<th>in water</th>
<th>in SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.3</td>
<td>1.3</td>
<td>2.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>7.00 ± 0.74&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.03 ± 0.38&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.87 ± 0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.29 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.54 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.53 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>10.63 ± 0.69&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.84 ± 0.63&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.76 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.61 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.77 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.88 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.64 ± 0.07&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.60 ± 0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>2.62 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.38 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.58 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.40 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.59 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.74 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.65 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
<td>2.28 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.14 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.81 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.69 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.79 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.73 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are expressed as mean values ± SD of three replicates (ND, not determined). Values in the same column which share a common lower case superscript didn’t differ significantly. Values in the same row which share a common upper case subscript weren’t significantly different (Tukey’s test, $P \leq 0.05$).
Table 4-1 also shows that emulsions prepared with proteins generally showed smaller droplet sizes than emulsions prepared with PLs at the same concentrations. The $d_{3,2}$ of emulsions decreased significantly with increasing concentrations of proteins (0.3% to 2.3%), whereas there was no significant difference in the $d_{3,2}$ between the emulsions prepared with 1.3% and 2.3% of PLs. On the other hand, the $d_{3,2}$ of emulsions prepared with 0.3% proteins alone or a combination of 0.3% proteins and 0.3% PLs, and diluted in water, was found to be larger than those diluted in SDS, while there was no significant difference in the $d_{3,2}$ when emulsions prepared with 0.3% PLs were diluted either in SDS or in water. The presence of a small molecular weight surfactant such as SDS, resulted in the displacement of proteins at the interface, leading to a disruption of bridging flocculation. According to Roesch et al. (2004), dilution of an emulsion with an SDS solution can result in a size distribution pattern similar to that of the individual droplets, instead of that of aggregated or flocculated droplets.

The use of 0.3% PLs in the emulsion containing 0.3% and 1.3% proteins resulted in a significant decrease of the $d_{3,2}$ compared to that of emulsions prepared with 0.3% and 1.3% proteins alone. Sodini et al. (2006) reported that a higher ratio of PLs to proteins increased the emulsion characteristics and, consequently, gives rise to a narrower size distribution. However, the results in the present study suggest that the presence of a level of PLs (0.3%) in emulsions containing both PLs and proteins, led to a decrease in the average droplet size, whereas with a continued increase in the PLs content in the system, the $d_{3,2}$ of emulsions remained unchanged. In contrast, in emulsions formulated with the same concentrations of PLs, increasing the protein concentrations from 0.3% to 1.3% led to a significant decrease in the $d_{3,2}$ (Table 4-1). This result was consistent with the results described in Chapter 3, namely that the droplet size of emulsions was not only affected by the higher participation of PLs, but also by the presence of other components, such as whey proteins, caseins, MFGM-specific proteins and minerals. However, at 2.3% proteins, increasing the PLs concentrations in the range between 0-1.3% increased the droplets sizes of emulsion (Table 4-1). The composition of the PLs adsorbed at the interface may affect the protein composition of the adsorbed layer as well. The surface load of proteins and PLs as well as their adsorption at the oil-in-water interface still needs to be further investigated. The results are presented and discussed in Chapter 5.
4.3.2 Microscopic observation

The observed differences in the degree of flocculation of emulsions as affected by the protein and PLs content can be seen in Figure 4-2.

Figure 4-2. Microscopic images of emulsions prepared with varying concentrations of proteins and/or PLs (A: 0.3% proteins; B: 1.3% proteins; C: 2.3% proteins; D: 0.3% PLs; E: 1.3% PLs; F: 2.3% PLs; G: 0.3% proteins-0.3% PLs; H: 1.3% proteins -1.3% PLs; I: 0.3% proteins -2.3% PLs). The scale bars indicate 50µm.

Microscopic imaging revealed that the emulsions prepared with PLs 0.3% were characterized by large droplets probably due to coalescence (Figure 4-2D), whereas flocculation occurred
in emulsions prepared with 0.3% proteins alone or in combination with 0.3% PLs (Figure 4-2A and G). Flocculation can occur due to bridging in emulsions prepared with a low concentration of proteins (Roesch et al., 2004). Tomas and Paquet (1994) also showed that the aggregation of fat droplets in emulsions is due to the presence of an insufficient amount of proteins. During emulsion preparation, homogenization or ultra-mixing are commonly applied to reach a uniform texture. High homogenization pressure reduces the droplet sizes and, hereby, increases the surface area. Consequently, surface active agents are necessary to be adsorbed at the interface in order to reduce the surface tension and to stabilize the emulsion; therefore, enough amphiphilic molecules have to be present to cover the surface area (Kanno et al., 1991). By increasing the concentration of proteins and/or PLs in the emulsion or both, the droplet size was decreased and no indication of aggregation was noticed (Figure 4-2B, C, F, H and I). This observation was in agreement with the particle size distribution data (section 4.3.1).

4.3.3 Emulsion stability

The emulsion stability as affected by the protein and/or PL concentration, and as a function of storage time, is shown in Figure 4-3.

All emulsions in this study showed no visible phase separation after 10 days of storage, except for the emulsions prepared with low concentrations of emulsifiers (i.e. 0.3% proteins; 0.3% and 1.3% PLs; a combination of 0.3% proteins and 0.3% PLs) as shown in Figure 4-3. The use of proteins seemed to result in more stable emulsions than those of PLs. Indeed, phase separation of emulsions containing PLs was not only found at 0.3%, but also at 1.3%. In addition, at low emulsifier concentration (0.3%), the degree and rate of phase separation of emulsions prepared with PLs were higher as compared to emulsions prepared with proteins. The difference in droplet size distribution of both types of emulsions can likely explain the dissimilarity in separation behavior. While the large-sized droplets present in emulsions containing only PLs might be due to the oil droplets’ coalescence, the larger ones in emulsions prepared with a limited level of proteins may be formed as a result of coalescence or bridging flocculation (Day et al., 2007). Along the same line, Guggisberg et al. (2012) reported that heat treatment has a great influence on the stability of emulsions prepared with buttermilk in terms of protein denaturation. These authors found that heat-treated buttermilk produced more stable emulsions due to protein interactions. These interactions are mainly disulfide bounds between sulphhydryl groups of amino acids (Howell and Taylor, 1995) and
result in the formation of a viscoelastic adsorbed layer around droplets, protecting them against flocculation via the creation of the steric and electrostatic repulsion between droplets (Murray and Dickinson, 1996). Heat treatment and high pressure homogenization result in an increased unfolding of XO and BTN and in an exposure of their reactive groups. The latter are sulphhydryl groups originally located in the inner part of proteins and induce protein-protein interactions (Wootton, 1994, Kim et al., 2002, Srinivasan et al., 2003, McSweeney et al., 2004). Moreover, Vanderghem et al. (2010) proposed that a high amount of MFGM-specific proteins such as XO and BTN are responsible for the observed good emulsifying properties of microfiltered BMP. Therefore, heat treatment may have a strong influence on the stability of emulsions prepared with proteins. The buttermilk powder used in this study underwent heat treatment during the production process and, therefore, the possibility that protein denaturation occurred is high.

**Figure 4-3.** Stability of emulsions prepared with different concentrations of proteins and PLs during quiescent storage at 4°C. Emulsion prepared with 0.3% proteins (……); 0.3% PLs (—·—); 1.3% PLs (—·—); combination of 0.3% proteins and 0.3% PLs (—). No phase separation was observed in the other emulsions.

By increasing the concentrations of either proteins or PLs, the stability of emulsions increased, e.g. phase separation in emulsions containing 0.3% PLs was 3.9 times higher than that of emulsion containing 1.3% PLs after 2 days of storage. These results are in agreement with the studies of Kanno (1989) and Roesch et al. (2004) in which it was shown that emulsions exhibiting good stability result from an increase in MFGM matter concentrations.
Chapter 4. Effects of MFGM proteins and PLs on the physicochemical properties of emulsions

On the other hand, the results in the present study showed that the phase separation behavior of emulsions prepared with a combination of 0.3% proteins and 0.3% PLs was similar to that of emulsions containing 0.3% proteins only (Figure 4-3). This might indicate that the stability of emulsions prepared with proteins or with a combination of proteins and PLs in equal amounts are similar and, hence, that the PLs in the system do not contribute to the emulsion stability.

4.3.4 Rheological behavior

The plots of apparent viscosity versus shear rates of the emulsions as affected by varying proteins and/or PLs content are shown in Figure 4-4 and flow curve parameters are summarized in Table 4-2. The interaction between various concentrations of proteins and PLs on the flow parameters of emulsions (i.e. apparent viscosity at fixed shear rate (100 s$^{-1}$, consistency index $K$ and flow behavior index $n$) are shown in Figure 4-5B, C and D, respectively.

All emulsions showed pseudoplastic flow behavior, as the apparent viscosity decreased with increasing shear rates (Figure 4-4). At 100 s$^{-1}$, the effect of PLs on the apparent viscosity of emulsions strongly depended on the concentration of the proteins used to prepare the emulsions. In the absence of proteins, the apparent viscosity of emulsions increased markedly with an increase in PL concentrations from 0.3% to 2.3% (Figure 4-5B). In emulsions prepared with proteins, whether or not combined with PLs, increasing the protein concentrations increased the apparent viscosity (Figure 4-5B and Table 4-2). This rheological behavior could be due to an increased adsorption of proteins at the interface (see details in Chapter 5). The lateral interaction among proteins at the interface leads to the formation of a viscoelastic layer and an increase in viscosity (Murray and Dickinson, 1996). In addition, Chanamai and McClements (2000) reported that the apparent viscosity of oil-in-water emulsions was strongly influenced by the sizes and concentrations of emulsion droplets. These authors showed that the apparent viscosity of emulsions increased with decreasing droplet sizes and by an increase in the droplet concentrations (Chanamai and McClements, 2000). Since an increase in the concentrations of either proteins or PLs, or both, caused a reduction in the average droplet size (Table 4-1), there was more interparticle resistance to flow. Hence, a higher concentration in proteins and/or PLs led to a higher apparent viscosity. At a low emulsifier concentrations, the emulsions prepared with 0.3% proteins showed a higher apparent viscosity than those prepared with 1.3% proteins (Figure 4-4A and Figure 4-
A similar behavior was observed with emulsions prepared with 0.3% proteins and 0.3% PLs compared to those prepared with other combinations. This may probably has due to the breakup of aggregates under the effect of a high shear rate (Dickinson and Golding, 1997). The flow data are consistent with these data on the $d_{3,2}$ and with the microscopic observations as well.

Figure 4-4. Apparent viscosity of the emulsions prepared with various concentrations of either proteins or PLs (A) and with some combinations of proteins and PLs (B).
Table 4-2. Flow parameters\(^{1}\) of emulsions as affected by varying proteins and PLs content

<table>
<thead>
<tr>
<th>Proteins (wt%)</th>
<th>PLs (wt%)</th>
<th>(K) (Pa.s(^{n}))</th>
<th>(n) (-)</th>
<th>(\eta^*) (mPa.s) at shear rate 100 s(^{-1})</th>
<th>(R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0</td>
<td>0.017 ± 0.003(^{abc})</td>
<td>0.90 ± 0.09(^{cd})</td>
<td>12.97 ± 0.98(^{ef})</td>
<td>0.980</td>
</tr>
<tr>
<td>1.3</td>
<td>0</td>
<td>0.014 ± 0.001(^{abc})</td>
<td>0.89 ± 0.05(^{cd})</td>
<td>8.85 ± 0.64(^{ed})</td>
<td>0.980</td>
</tr>
<tr>
<td>2.3</td>
<td>0</td>
<td>0.027 ± 0.005(^{c})</td>
<td>0.84 ± 0.08(^{ed})</td>
<td>14.45 ± 0.05(^{fg})</td>
<td>0.996</td>
</tr>
<tr>
<td>0</td>
<td>0.3</td>
<td>0.016 ± 0.001(^{abc})</td>
<td>0.67 ± 0.03(^{b})</td>
<td>5.96 ± 0.54(^{a})</td>
<td>0.988</td>
</tr>
<tr>
<td>0</td>
<td>1.3</td>
<td>0.120 ± 0.016(^{e})</td>
<td>0.46 ± 0.02(^{a})</td>
<td>11.23 ± 0.20(^{e})</td>
<td>0.989</td>
</tr>
<tr>
<td>0</td>
<td>2.3</td>
<td>0.194 ± 0.008(^{f})</td>
<td>0.44 ± 0.01(^{a})</td>
<td>15.85 ± 0.17(^{g})</td>
<td>0.980</td>
</tr>
<tr>
<td>0.3</td>
<td>0.3</td>
<td>0.008 ± 0.001(^{ab})</td>
<td>0.94 ± 0.03(^{de})</td>
<td>6.37 ± 0.53(^{a})</td>
<td>0.998</td>
</tr>
<tr>
<td>1.3</td>
<td>0.3</td>
<td>0.008 ± 0.000(^{ab})</td>
<td>0.93 ± 0.02(^{de})</td>
<td>6.68 ± 0.02(^{ab})</td>
<td>0.999</td>
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<tr>
<td>2.3</td>
<td>0.3</td>
<td>0.016 ± 0.002(^{abc})</td>
<td>0.84 ± 0.00(^{ed})</td>
<td>9.07 ± 0.69(^{cd})</td>
<td>0.998</td>
</tr>
<tr>
<td>0.3</td>
<td>1.3</td>
<td>0.006 ± 0.000(^{a})</td>
<td>0.97 ± 0.00(^{e})</td>
<td>5.71 ± 0.07(^{a})</td>
<td>0.996</td>
</tr>
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<td>1.3</td>
<td>1.3</td>
<td>0.013 ± 0.001(^{abc})</td>
<td>0.88 ± 0.02(^{cd})</td>
<td>8.10 ± 0.51(^{bc})</td>
<td>0.996</td>
</tr>
<tr>
<td>2.3</td>
<td>1.3</td>
<td>0.023 ± 0.006(^{bc})</td>
<td>0.80 ± 0.02(^{c})</td>
<td>10.34 ± 0.66(^{de})</td>
<td>0.999</td>
</tr>
<tr>
<td>0.3</td>
<td>2.3</td>
<td>0.011 ± 0.002(^{ab})</td>
<td>0.92 ± 0.03(^{de})</td>
<td>8.43 ± 0.69(^{c})</td>
<td>0.999</td>
</tr>
<tr>
<td>1.3</td>
<td>2.3</td>
<td>0.017 ± 0.002(^{abc})</td>
<td>0.88 ± 0.01(^{cd})</td>
<td>10.28 ± 0.87(^{de})</td>
<td>0.999</td>
</tr>
</tbody>
</table>

\(^{a-f}\) Values within a column followed by different superscripts are significantly different (Tukey’s test, \(P \leq 0.05\)).

\(^{1}\) Mean values ± SD of three replicates

The flow data fitted well to the power law model, as indicated by \(R^2 > 0.97\) for all emulsions. Consequently, the consistency index \(K\) and flow behavior index \(n\) can be used to describe the flow behavior of these emulsions (Table 4-2). All emulsions prepared with proteins (0.3-2.3%) whether or not in combination with PLs (0.3-2.3%) showed a flow behavior close (\(n\) approaching 1) to that of Newtonian liquids and a relatively low consistency index \(K\) (Figure 4-4A and B, Table 4-2). In contrast, emulsions prepared with PLs (0.3-2.3%) showed a pronounced shear-thinning behavior. Since the extracted PLs concentrate still contained a high lipid content, the lipid phase could be incorporated into the oil phase by dilution. This could be a reason for the observed high consistency index \((K)\) in emulsion prepared with PLs when the concentration of PLs used was high (2.3%). Scott et al. (2003a) reported that the higher PLs and unsaturated fatty acid content (oleic, and linoleic acid) of MFGM fragments resulted in an increase in the fluidity of the emulsions. The increasing trend of the consistency
index $K$ caused by the concentration of proteins and PLs was consistent with the changing trend in the apparent viscosity.

Figure 4-5. Interaction effect between proteins and PLs on $d_{3,2}$ after dilution in SDS solution (A), shear stress at 100 $s^{-1}$ (B), consistency index $K$ (C) and flow behavior index $n$ (D).

4.4 Conclusions

Results from this work evidenced that MFGM protein and PLs and their concentration could affect the physicochemical properties of oil-in-water emulsions. At a low emulsifier concentration (< 2.3% in total), there was an interacting effect between proteins and PLs on
the droplet size of emulsions. At 2.3% proteins, irrespective of the presence of PLs in emulsions, showed a narrow droplet size distribution with small average diameter size. Visible phase separation was similar that of emulsions stabilized with 0.3% of both proteins alone and a combination of proteins and PLs. The PLs in this system did not retard the instability in emulsions prepared with a combination of proteins and PLs. The rheograms of shear stress versus shear rate were best fitted with the power law model and the emulsions showed pseudoplastic flow behavior. In conclusion, the use of MFGM protein concentrate separated from BM-MFGM in the preparation of emulsions had better emulsifying/stabilizing properties compared to those of the PLs concentrate.
Chapter 5. Adsorption Behavior of Combined MFGM Proteins and PLs at the Interface of Oil-in-Water Emulsions

This chapter is redrafted after:

Abstract
BM-MFGM material used in Chapter 2 and Chapter 3 contains a bulk of MFGM specific components and a number of other components derived from milk serum. The interactions between these components can alter the physicochemical properties of O/W emulsions. This study is a continuation of the work in Chapter 4. MFGM protein and PLs concentrate were separated from BM-MFGM material and used in the preparation of emulsions, individually or in combination, at various concentrations as described in Chapter 4. The surface characteristics and adsorption behavior at the interface of O/W emulsions were examined. The results showed that proteins were more preferentially adsorbed at the emulsion droplet surface compared to PLs. The adsorbed proteins were not replaced by the addition of polar lipids. At 2.3% proteins, irrespective of the presence and concentrations of polar lipids in emulsions, the surface protein load of droplets seemed to have reached saturation. In conclusion, MFGM protein concentrate had superior emulsifying ability compared to polar lipids concentrate.

5.1 Introduction
Milk proteins and low molecular weight surfactants are key components for emulsion stability in many food systems (Cornec et al., 1998). The stability of O/W emulsions depends to a large extent on the properties of the surface-active components in the system. Low molecular weight surfactants such as PLs contain ionogenic groups, exerting a repulsive force which prevents the coalescence of FGs (Shimizu et al., 1980). Furthermore, proteins stabilize emulsions against coalescence by forming a viscoelastic adsorbed layer around the droplets (Murray and Dickinson, 1996). This protective film plays an important role in stabilizing emulsions by generating a steric/electrostatic repulsion, making the droplets resistant against rupture (Bos and van Vliet, 2001, Tcholakova et al., 2002, Wilde et al., 2004). In a system
that contains both small molecular surfactants (e.g. PLs) and proteins, the former may bind to the latter through hydrophobic and electrostatic interactions for ionic surfactants, and hydrophobic interactions for non-ionic surfactants (Coke et al., 1990, McClements, 2004). Proteins are reversibly adsorbed at the fluid-fluid interface and can be replaced by PLs and PLs can interact with proteins and change their functionality (Dickinson, 1999). The combination of both PLs and proteins can alter emulsion stability (Dickinson and Ritzoulis, 2000, Kelley and McClements, 2003, Gunning et al., 2004, Pugnaloni et al., 2004, Wilde et al., 2004). The capability of proteins to interact with PLs determines the mechanism of protein removal from the interface. The interaction may increase or decrease the surface activity of proteins as well as that of PLs. Moreover, the formation of a surfactant/protein complex is involved in the competition between protein and PLs (Clark et al., 1991, Nylander et al., 1997, Bos and van Vliet, 2001, McClements, 2004).

As presented in Chapter 1, the emulsifying properties of MFGM isolated from reconstituted buttermilk have been studied over the past years. Most of the studies focused on the effect of MFGM and fat content, processing treatment of MFGM before and after isolation, emulsion preparation conditions (such as emulsifying time, homogenization pressure, temperature, pH) on creaming stability, fat droplet sizes, rheological characteristics and surface protein coverage of O/W emulsions (Corredig and Dalgleish, 1997b, Wong and Kitts, 2003, Roesch et al., 2004, Sodini et al., 2006). However, MFGM material isolated from reconstituted buttermilk by MF still contains a bulk of MFGM specific components (e.g. MFGM-specific proteins and PLs) and, to some extent, other contaminating components derived from milk serum (e.g. caseins, β-LG and α-LA) (Malin et al., 1994). Thus far, the influence of MFGM-specific proteins and PLs, separately, or the alteration in interactions of PLs with other components of the membrane (e.g. whey proteins, caseins and MFGM-specific proteins) at the oil droplet surface during emulsification have not been elucidated (except for Chapter 4).

Also, no information is available on the possible effect of PLs and MFGM proteins on the surface active properties as well as on the competitive adsorption behavior between the two component groups at the interface of O/W emulsions. To get a better understanding of the role of MFGM proteins and PLs or on possible competitive adsorption at the interface surface of oil-in-water emulsions, the effect of various concentrations of MFGM proteins and PLs, obtained from MFGM material isolated from reconstituted buttermilk, on the surface characteristics and adsorption behavior at the interface emulsions was investigated. Moreover,
the optimum balance of PLs and MFGM proteins content as well as the minimum proteins and/or PLs coverage required for preparing stable emulsions would be explored.

5.2 Materials and methods

5.2.1 Materials and chemicals

Chemicals for analysis and materials for emulsion preparation were described in 2.2.1. MFGM protein concentrate and PLs concentrate used in this study were the obtained products as described in Chapter 4.

5.2.2 Emulsion preparation

The experimental set-up and the procedure of emulsion preparation were explained in 4.2.2 and 3.2.4.

5.2.3 Determination of adsorption behavior of proteins and PLs at the oil-water interface and their surface load

The adsorbed proportion of proteins at the interface, the surface protein and PLs load of emulsions prepared with various concentrations of proteins and PLs were determined as described in 3.2.5.4. The specific surface area (SSA) (m$^2$ g$^{-1}$ emulsion) was calculated via equation [5.1]

$$SSA = \frac{6 \times C_{oil}}{d_{3,2} \times \rho_{emulsion}}$$

Where $C_{oil}$ is the concentration of soybean oil in emulsion (35% w/w), $d_{3,2}$ is the Sauter mean diameter of the oil droplets and $\rho_{emulsion}$ is the emulsion density (970kg/m$^3$).

Similar to the determination of adsorbed proteins, the adsorption of a specific PL at the surface of the oil droplets was determined. The cream and serum phase were separated by centrifugation of the emulsions as described in 3.2.5.4. The PL content in the cream phase and in the emulsion was analyzed, after extraction, using HPLC as described in 2.2.3. The proportion of an individual PL component (GluCer, LacCer, PI, PE, PS, PC, and SM) in the cream phase compared to that of the PL component in the emulsion was calculated according to the following equation [5.2]:

$$\text{Surface proportion of a specific PL component (\%)} = \frac{\text{concentration of a PL component in cream phase}}{\text{concentration of the PL in emulsion}} \times 100 \quad [5.2]$$
Chapter 5. Adsorption behavior of MFGM proteins and PLs at O/W interface

5.2.4 Cryo-scanning electron microscopy (Cryo-SEM)

The microstructure of the emulsions prepared with various concentrations of proteins and PLs was visualized using a JSM-7100 F TTLS LV TFEG-SEM (Jeol Europe BV, Zaventem, Belgium) under high vacuum and at an accelerated voltage of 15 keV. Prior to electron beam targeting, the samples were vitrified in liquid nitrogen and transferred to a PP3000T cryo-transfer system (Quorum Technologies Ltd., East Sussex, UK) at -140°C. The samples were fractured, using a knife, followed by sublimation for 30 min at 90°C in order to reveal a more detailed microstructure. Prior to the transfer from the cryo preparation room to the SEM chamber, a thin layer of a conductive metal (Pt) was deposited on the samples. This coating process prevents charging of specimens by an electron beam.

5.2.5 Statistical analysis

Statistical analysis was performed using the SPSS version 21 (IBM Inc., Chicago, IL., USA). One-way analysis of variance (ANOVA) and Tukey’s test were used for multiple comparisons of means. A two-way ANOVA was performed to compare the interaction of the effects of various levels of two factors in a factorial experiment. The differences were considered statistically significant at $P \leq 0.05$. Multivariate principal component analysis (PCA) is a data reduction technique that compresses the number of correlated variables into a smaller number of uncorrelated variables (i.e., principal components). The average value of the relative proportion of absorbed proteins (XO, BTN, caseins, $\beta$-LG, and $\alpha$-LA) and adsorbed PLs (GluCer, LacCer, PI, PE, PS, PC, and SM) at the surface of emulsion prepared with different concentration combinations of MFGM protein and PLs concentrate were evaluated using PCA with Varimax rotation.

5.3 Results and discussion

5.3.1 Adsorption behavior of proteins and PLs at the oil-water interface

The adsorbed proteins at the surface of emulsions prepared with various concentrations of proteins and PLs were studied by comparing the SDS-PAGE pattern of the cream phase with that of the serum phase (Figure 5-1).

SDS-PAGE of the cream and serum phase of the emulsions following centrifugation showed that with the increase in the protein concentration the high band intensities of proteins were observed in the serum phase. At constant protein concentration, the PL concentration in the system does not seem to affect the $\beta$-LG and $\alpha$-LA band intensities in the serum phase as well.
Chapter 5. Adsorption behavior of MFGM proteins and PLs at O/W interface

as in the cream phase. At 0.3% proteins combined with various PLs concentrations (0.3-2.3%), almost all XO and BTN were adsorbed at the oil droplet surface, i.e. the serum phase contained only traces of these proteins (Lane 4’-6’). Contrary to XO and BTN, a low band intensity of caseins was observed in the cream phase (lane 4-6). When increasing the protein concentrations and regardless of the concentrations of PLs, the serum phase contained a considerable amount of proteins. In such samples, XO, BTN, caseins, β-LG, and α-LA were absent in the serum phase at 0.3% proteins, but at higher concentrations (≥ 1.3%) not only the bands of XO and BTN but also the bands of β-LG, and α-LA appeared in the serum phase.

**Figure 5-1.** SDS-PAGE patterns of different samples. Lanes 1-3: cream phase of emulsions prepared with 0.3, 1.3 and 2.3% proteins, respectively; Lanes 1’-3’: serum phase of emulsions prepared with 0.3, 1.3 and 2.3% proteins, respectively; Lanes 4 and 4’: cream and serum phase of emulsion prepared with 0.3% proteins and 0.3% PLs; Lanes 5 and 5’: cream and serum phase of emulsions prepared with 0.3% proteins and 1.3% PLs; Lanes 6 and 6’: cream and serum phase of 0.3% proteins and 2.3% PLs; Lanes 7 and 7’: cream and serum phase of 1.3% proteins and 0.3% PLs; Lanes 8 and 8’: cream and serum phase of 1.3% proteins and 1.3% PLs; Lanes 9 and 9’: cream and serum phase of 1.3% proteins and 2.3% PLs; Lanes 10 and 10’: cream and serum phase of 2.3% proteins and 0.3% PLs; Lanes 11 and 11’: cream and serum phase of 2.3% proteins and 1.3% PLs; Lane 12: molecular mass (kDa) standards. The load on each lane was 13µg total proteins.
The relative proportion of individual adsorbed protein in the cream phase and in the original emulsion (i.e. the sum of that protein in the cream phase and in the serum phase) was estimated by using color intensity analysis and is shown in Figure 5-2. The adsorption behavior of individual PLs at the surface of oil-in-water emulsions prepared with various concentrations of proteins and PLs is shown in Figure 5-3.

**Figure 5-2.** Sodium dodecyl sulfate PAGE band intensity of (A) MFGM-specific proteins (XO, BTN) and (B) non-MFGM proteins (caseins, β-LG, α-LA) in cream phase relative to the sum of those in cream and serum phase of emulsions prepared with various concentrations of proteins and PLs. CP = cream phase; SP = serum phase.
The result clearly shows that the adsorbed proportion of XO and BTN at the surface of emulsions prepared with both proteins and PLs was higher than that of emulsions prepared with solely proteins (Figure 5-2A). This may indicate that the presence of PLs enhanced the adsorption of XO and BTN at the droplet surface, because XO and BTN are capable of being acylated with fatty acids and BTN can also be acylated with phosphate (Keenan et al., 1982, Spitsberg and Gorewit, 1997, Mather, 2000). In another study of Singh (2006) it was prospected that oleic, palmitic and stearic acid are the main fatty acids bound with both XO and BTN. Therefore, they may be incorporated in the formation of a protein-PLs complex which could be the reason for this behavior. An opposite trend was observed with caseins; the adsorbed caseins at the surface strongly decreased with increasing the concentrations of PLs in the system from 0 to 2.3% (Figure 5-2B). Fang and Dalgleish (1993b) reported that the presence of phospholipids decreased the amount of protein necessary to cover the oil-in-water interface. In those studies, a synergistic effect of proteins and phospholipids was described whereby phospholipids adsorbed in the gaps present at the interface, caused by the presence of an insufficient amount of proteins. Depending on their molecular nature, PLs can displace proteins from the interface and/or bind to proteins inducing the formation of new surface-active molecules (Nylander et al., 1997). The result in the present study showed that SM was more effective in removing the casein from the interface (Figure 5-2B and Figure 5-3A). This result was in agreement with the finding of Krisdhasima et al. (1993) that reported casein had higher affinity to be adsorbed at the hydrophilic surfaces. Since SM is a water soluble polar lipid, it may displace casein more effectively. The lowest adsorbed proportion of caseins at the surface of emulsions prepared with a combination of 0.3% proteins and 2.3% PLs could result from a displacement of caseins by certain PLs. The results in Figure 5-2 show that, at a low protein concentration (0.3%), the presence of PLs in emulsions affects the competitive adsorption between caseins and MFGM-specific proteins at the interface. The incorporation of XO and BTN at the interface was increased, while the incorporation of caseins was decreased.

The adsorption of all polar lipid components at the surface increased with increasing concentrations of PLs from 0.3 to 2.3%, when no proteins were present or when used at 0.3%. At 2.3% proteins, the adsorption of polar lipids was not affected by their concentrations in the emulsion. In addition, the proportion of polar lipids adsorbed at the surface in emulsions containing 2.3% proteins was lower than that of emulsions prepared with 1.3% proteins.
Figure 5-3. Adsorption of (A) sphingolipids (GluCer, LacCer, SM) and (B) glycerophospholipids (PI, PE, PS and PC) at the droplet surface of emulsions prepared with various concentrations of proteins and PLs. CP = cream phase

Identifying the overall competitive adsorption behavior of proteins and PLs at the oil-water interface was difficult because of the changes in the proportion of individual adsorbed proteins and PLs when emulsions were prepared with different concentrations of proteins and PLs. To identify the relationships between the adsorption behavior of proteins (e.g. XO, BTN,
caseins, β-LG and α-LA) and PLs (e.g. GluCer, LacCer, PI, PE, PS, PC and SM) at the droplet surface, multivariate principle components analysis (PCA) was applied and the results are shown in Figure 5-4.

**Figure 5-4.** Principal component analysis (PCA) loadings for (A) the proportion of adsorption of proteins and PLs at the interface of oil-in-water emulsions and scores for (B) emulsion samples.

The first principal component (PC1) explained 60.0% of the variation across the samples, whereas the second principal component (PC2) explained 30.1% of the variance. In the Figure 4A, it can be seen that the surface-adsorbed proportion of GluCer, LacCer, PI, PE, PS, PC and SM is positively correlated with PC1, whereas the adsorbed caseins are negatively correlated.
Chapter 5. Adsorption behavior of MFGM proteins and PLs at O/W interface

with PC1. In addition, the adsorbed XO, BTN, caseins, β-LG and α-LA positively correlated with PC2. Consequently, these response factors can be divided into three groups based on their distribution in Figure 5-4A. In Figure 5-4B, the sample points were plotted increasingly toward the positive side of the PC2 axis with the decrease in the protein concentrations (2.3% to 0.3%) in the emulsions. This indicated that the surface-adsorbed proportion of MFGM-specific proteins (i.e. XO and BTN) and whey proteins (i.e. β-LG and α-LA) was decreased with increasing protein concentrations from 0.3 to 2.3%, regardless of the concentrations of polar lipids. In contrast, the surface-adsorbed proportion of polar lipid components and caseins were affected by both the proteins and polar lipids concentrations. By increasing the PLs concentrations (0.3 – 2.3%) and decreasing the protein concentrations (2.3 – 0.3%), the sample points were plotted increasingly toward the positive side of the PC1 axis. Among 14 emulsions, emulsions prepared with either 2.3% PLs alone or in combination with 0.3% proteins, were strongly positively correlated with PC1. The difference in the type of proteins (i.e. MFGM-specific proteins, caseins and whey proteins), the HLB of PLs, the total proteins and PL concentrations in the emulsion could play some role in this contrast. Depending on their molecular nature, PLs can displace proteins from the interface or/and bind to proteins inducing the formation of new surface-active molecules (Nylander et al., 1997).

In conclusion, the PCA result of this study shows that the adsorption of species of PLs (i.e. GluCer, LacCer, PI, PE, PS, PC and SM) at the interface revealed a strongly negative correlation to the amount of adsorbed caseins at the surface. This means that a higher amount of adsorbed GluCer, LacCer, PI, PE, PS, PC and SM at the surface resulted in a lower amount of adsorbed caseins. The amount of XO, BTN, β-LG and α-LA adsorbed at the surface was mainly affected by the protein concentrations present in the emulsions.

In this work, only the emulsifying properties of MFGM group components or fractions (i.e. MFGM proteins and PLs) were studied. More work should be conducted on the emulsifying properties of individual MFGM-specific proteins (e.g. MUC1, PASIII, PAS6/7, …) in oil-in-water emulsion, as no data on this topic is available in literature. For a complete view on the role of all specific protein components of MFGM adsorbed at the interface, standards are essential, which could possibly be obtained by the purification of each individual protein using different techniques.

5.3.2 The SSA, the surface protein load (Γ_{Pro}) and PLs load (Γ_{PL})
The SSA, $\Gamma_{\text{Pro}}$ and $\Gamma_{\text{PL}}$ of emulsions prepared with different combinations of MFGM protein and PLs concentrate are given in Table 5-1. The effect of the interaction between the concentration of proteins and polar lipids on the $\Gamma_{\text{Pro}}$ and $\Gamma_{\text{PL}}$ of emulsions is shown in Figure 5-5.

The emulsifying ability of surface-active components can be quantified by measuring the specific surface area of oil droplets. The larger the specific surface area generated (smaller droplets), the better the emulsifying ability of the material (i.e. proteins or/and PLs). In Table 5-1, emulsions prepared with MFGM protein concentrate had a far higher emulsifying ability than the PLs concentrate. At the same concentration (2.3%), the SSA of emulsions prepared with MFGM protein concentrate was 4.0 times higher than that of emulsions prepared with the PLs concentrate (Table 5-1).

**Table 5-1. The specific surface area (SSA), surface protein load ($\Gamma_{\text{Pro}}$) and PLs load ($\Gamma_{\text{PL}}$) of emulsions as affected by concentrations of proteins and PLs**

<table>
<thead>
<tr>
<th>Proteins (wt%)</th>
<th>PLs (wt%)</th>
<th>SSA (m$^2$ g$^{-1}$)</th>
<th>$\Gamma_{\text{Pro}}$ (mg m$^{-2}$)</th>
<th>$\Gamma_{\text{PL}}$ (mg m$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.3</td>
<td>0.22 ± 0.01$^a$</td>
<td>ND</td>
<td>3.04 ± 0.12$^e$</td>
</tr>
<tr>
<td>0</td>
<td>1.3</td>
<td>0.91 ± 0.05$^{bc}$</td>
<td>ND</td>
<td>3.90 ± 0.18$^f$</td>
</tr>
<tr>
<td>0</td>
<td>2.3</td>
<td>1.01 ± 0.04$^{bc}$</td>
<td>ND</td>
<td>9.68 ± 0.55$^h$</td>
</tr>
<tr>
<td>0.3</td>
<td>0</td>
<td>0.54 ± 0.05$^{ab}$</td>
<td>2.28 ± 0.11$^d$</td>
<td>ND</td>
</tr>
<tr>
<td>0.3</td>
<td>0.3</td>
<td>0.84 ± 0.02$^{bc}$</td>
<td>1.50 ± 0.04$^{ab}$</td>
<td>0.79 ± 0.03$^{bc}$</td>
</tr>
<tr>
<td>0.3</td>
<td>1.3</td>
<td>0.91 ± 0.07$^{bc}$</td>
<td>1.52 ± 0.08$^{ab}$</td>
<td>3.62 ± 0.35$^{ef}$</td>
</tr>
<tr>
<td>0.3</td>
<td>2.3</td>
<td>1.28 ± 0.02$^c$</td>
<td>1.25 ± 0.09$^a$</td>
<td>7.31 ± 0.08$^g$</td>
</tr>
<tr>
<td>1.3</td>
<td>0</td>
<td>1.83 ± 0.13$^d$</td>
<td>3.41 ± 0.20$^e$</td>
<td>ND</td>
</tr>
<tr>
<td>1.3</td>
<td>0.3</td>
<td>2.46 ± 0.20$^e$</td>
<td>2.07 ± 0.12$^{cd}$</td>
<td>0.22 ± 0.02$^{ab}$</td>
</tr>
<tr>
<td>1.3</td>
<td>1.3</td>
<td>3.48 ± 0.10$^{fg}$</td>
<td>1.84 ± 0.05$^{bc}$</td>
<td>0.91 ± 0.02$^e$</td>
</tr>
<tr>
<td>1.3</td>
<td>2.3</td>
<td>3.01 ± 0.31$^f$</td>
<td>2.10 ± 0.23$^{cd}$</td>
<td>1.93 ± 0.21$^d$</td>
</tr>
<tr>
<td>2.3</td>
<td>0</td>
<td>5.36 ± 0.35$^h$</td>
<td>1.99 ± 0.02$^{cd}$</td>
<td>ND</td>
</tr>
<tr>
<td>2.3</td>
<td>0.3</td>
<td>3.58 ± 0.11$^g$</td>
<td>2.29 ± 0.11$^d$</td>
<td>0.09 ± 0.01$^a$</td>
</tr>
<tr>
<td>2.3</td>
<td>1.3</td>
<td>3.33 ± 0.25$^{fg}$</td>
<td>2.28 ± 0.22$^{d}$</td>
<td>0.46 ± 0.05$^{abc}$</td>
</tr>
</tbody>
</table>

$^{a-g}$ Values within a column followed by different superscript letters are significantly different (Tukey test, $P \leq 0.05$).

$^i$Mean values ± SD of three replicates (ND, not determined)
Chapter 5. Adsorption behavior of MFGM proteins and PLs at O/W interface

Two-way ANOVA analysis of the data in Table 5-1 indicated that the surface polar lipids load in emulsions prepared with both proteins and polar lipids strongly depended on both the concentrations of proteins and polar lipids \((P < 0.001)\) (Figure 5-5B). In contrast, the interaction between the concentrations of proteins and PLs did not influence significantly the surface protein load \((P = 0.09)\) (Figure 5-5A). Between the two emulsifiers, proteins showed a stronger effect on the surface protein load \((P < 0.001)\) than polar lipids \((P = 0.049)\).

In emulsions prepared with proteins alone as emulsifier, the surface protein load was significantly \((P < 0.05)\) increased from 2.28 to 3.41 mg m\(^{-2}\) with an increase in protein concentrations from 0.3\% to 1.3\%, respectively. Further increase in the protein concentrations to 2.3\% resulted in a decrease in the surface protein load (i.e. 2.0 mg m\(^{-2}\)) (Table 5-1). At protein concentrations of 1.3\% and below, it seemed that the system contained insufficient surface active agents to cover the entire freshly created oil surface and the formation of large-sized droplets in emulsions, probably, due to coalescence or/and flocculation (Figure 5-6). Therefore, the SSA of the emulsions was low due to the large droplet sizes and the high surface protein load. The surface protein load in present study was in line with the values of 1.5-3.4 mg m\(^{-2}\) reported for whey proteins and sodium caseinate (Oortwijn and Walstra, 1979, Courthaudon et al., 1991b, Dalgleish et al., 1995, Dickinson, 1998).

![Figure 5-5](image-url)  
*Figure 5-5. Effect of interaction between proteins and PLs on the (A) \(\Gamma_{Pro}\) and (B) \(\Gamma_{PL}\) of emulsions.*
In emulsions prepared with solely PLs concentrate, the data demonstrated that the SSA increased slightly, while the adsorption of all PL components increased markedly with an increase in PLs concentrations from 1.3% to 2.3% (Table 5-1 and Figure 5-3). According to Equation [3.2] (section 3.2.5.4), the surface PLs load depends on both the total surface PLs content and the SSA. Due to the more pronounced increase in the total surface PLs content than the increase in the SSA, the surface PLs load increased (9.68 mg m\(^{-2}\) at 2.3% PLs).

Elwell et al. (2004) reported that, when homogenized, the larger droplets of pre-mixed emulsions were crushed to smaller droplets. Depending on the concentrations and firmness of the emulsifier, the newly formed (small) droplets rapidly re-coalesce. Higher surface emulsifier content weakens the droplet re-coalescence. In a study by van Aken and van Vliet (2002) it was demonstrated that the formation of droplet coalescence can be avoided if the system contains sufficient amount of emulsifier (e.g. proteins and PLs) to cover all droplets. Emulsions prepared with 2.3% PLs still contained droplets with a large average droplet size after dilution in SDS 1% compared to that of emulsions prepared with proteins (as discussed in 4.3.1). This could be due to absence of a sufficient amount of emulsifier to cover the interface and to prevent coalescence. Bos and van Vliet (2001) also reported that emulsions stabilized with PLs mostly show low stability against coalescence. Our results showed that emulsions prepared with polar lipids had less emulsifying ability than emulsions prepared with proteins when used at the same concentration. More specifically, the surface PLs load of emulsions prepared with PLs was 4.8 times higher than the surface protein load of emulsions prepared with proteins at the same concentration (2.3%).

In the case of emulsions prepared with both proteins and PLs, at a constant protein concentration (0.3; 1.3 or 2.3%), the surface PLs load increased markedly with increasing the concentration of PLs from 0.3 to 2.3%. No significant difference in the surface protein load was observed with the different concentrations of PLs in the emulsions (0.3; 1.3 and 2.3%) (Table 5-1). In contrast, at a constant PLs concentrations (0.3; 1.3 or 2.3%), the surface PLs load strongly decreased with an increase in the concentration of proteins, especially at high PLs concentrations (1.3 and 2.3%), while the surface protein load increased to a lesser extent. Presumably, when the total emulsifier concentration is increased above 2.3%, the emulsifier molecules do not adsorb to the surface, and are to be found free in the bulk phase or loosely attached to the interface as multilayers. This is an indication that there was an excess of emulsifiers present in the emulsions. This result was confirmed by the data on the proportion of adsorbed protein and PLs at the droplet surfaces of the emulsions, as presented in section
5.3.1. Dickinson and Iveson (1993a) reported that exceeding a certain amount of emulsifier caused a decrease in the SSA. The SSA of emulsions prepared with a combination of 2.3% proteins and 0.3% PLs was lower than that of emulsions prepared with 2.3% proteins alone (Table 5-1). In general, it can be concluded that under the experimental conditions used in work package, the optimum emulsifier concentration for coverage and stabilization of droplets is 2.3% proteins or a combination of 1.3% proteins with a varying concentration of PLs from 0.3 to 2.3.

5.3.3 Cryo-SEM

The differences in the oil droplets surface of emulsion prepared with either MFGM protein or PLs concentrate or in combination of both at various concentrations are shown in Figure 5-6.

Cryo-SEM has been widely used to determine the microstructure of emulsions. John et al. (2004) have shown that cryo-SEM is a valuable tool which enables the elucidation of the oil-surfactant-water interaction in emulsions.

![Cryo-SEM images at 4000x magnification of emulsions prepared with varying concentrations of proteins and PLs A: 2.3% PLs; B: 2.3% proteins; C: 1.3% proteins and 1.3% PLs; D: 0.3% PLs; E: 0.3% proteins; F: 0.3% proteins and 0.3% PLs. Examples of water and oil domains are indicated by solid and dot arrows, respectively.](image)

Figure 5-6. Cryo-SEM images at 4000x magnification of emulsions prepared with varying concentrations of proteins and PLs A: 2.3% PLs; B: 2.3% proteins; C: 1.3% proteins and 1.3% PLs; D: 0.3% PLs; E: 0.3% proteins; F: 0.3% proteins and 0.3% PLs. Examples of water and oil domains are indicated by solid and dot arrows, respectively.
The cryo-SEM micrographs shown in Figure 5-6 present the differences in microstructure of emulsions with varying concentrations of proteins and PLs. Coalescence or/and bridging flocculation due to incomplete coverage of the oil droplets surface, which occurred at low concentrations of emulsifiers (i.e. proteins and PLs), is evidenced by the large droplets in Figure 5-6D – F. While the oil droplets are seen to join with adjacent oil droplets, sharing the thin interfacial film, leading to the loss in oil droplet identity with emulsion prepared with either 0.3% proteins or in combination with 0.3% PLs (Figure 5-6E and F), coalescence of small droplets during homogenization may occur in emulsions prepared with 0.3% PLs, resulting in an increase in droplet sizes of the emulsion. The thin interfacial layer possesses a low repulsive barrier and therefore in these emulsions rapid phase separation occurs. In addition, extensive water domains are evident (Figure 5-6D – F). This result was confirmed by the data on the stability of emulsions. Emulsions prepared with 0.3% of either proteins or PLs or in combination of both, showed high phase separation after 2 days of storage at 4°C (as discussed in 4.3.3).

In contrast, emulsion prepared with 2.3% proteins showed a microstructure consisting of individual oil droplet with well-resolved interfaces in the aqueous proteins network, having a pore structure below 0.5 µm (Figure 5-6B). This could be due to the fact that sufficient emulsifier was available for covering the oil droplets. The droplets were largely preserved with good coverage of the proteins at the oil droplet surfaces, resulting in a high repulsive barrier due to steric and electrostatic forces and an increased emulsion stability. For emulsions prepared with either 2.3% PLs or a combination of 1.3% PLs and 1.3% proteins, the oil droplets were connected to each other but in a different way compared to emulsion containing 2.3% proteins.

Furthermore, in the cryo-SEM micrographs a mesh-like structure with small holes was observed, caused by the disappearance of the amorphous water when the samples were subjected to sublimation. The surface structure of emulsions prepared with either PLs alone or in combination with proteins at high concentrations, showed a distinct difference in surface structure between emulsions with 2.3% proteins and the other emulsions (i.e. 2.3% PLs or a combination of 1.3% proteins and 1.3% PLs) (Figure 5-6A – C). The emulsions containing 2.3% proteins displayed smooth droplet surfaces with only the imprint from the structure created by the vitrified water phase (Figure 5-6B). In contrast, emulsions containing 2.3% PLs or a combination of 1.3% proteins and 1.3% PLs had a more rough appearance with bumps and holes on the surface of the oil droplets and it seemed that there were several layers
of emulsifier covering the droplets. Almost all droplets in emulsions containing 2.3% proteins were broken across the interfacial layer after freeze-fracture while the droplets in emulsion containing PLs were broken along the interfacial layer and the droplets were almost always intact. The mechanism of this phenomenon needs to be further investigated. However, the results that can be drawn from the micrographs are in good agreement with data on \(d_{3,2}\) and emulsion stability (see 4.3.1 and 4.3.3).

5.4 Conclusions

The surface characteristics and adsorption behavior at the surface of oil-in-water emulsions were affected by the concentration and the type of emulsifiers (i.e., MFGM protein and PLs concentrate) used. By increasing the concentrations of MFGM protein concentrate, the surface protein load increased slightly, whereas the surface polar lipids load markedly decreased with increasing the concentrations of PLs concentrate. The presence of PLs affected the competition between caseins and MFGM-specific proteins (i.e., XO and BTN) for being adsorbed at the oil droplet surface. However, no competitive displacement between proteins and PLs was observed at the surface of these emulsions. Differences in microstructure of the interfacial domain between emulsions prepared with proteins and PLs were also found. At an identical concentration, MFGM protein concentrate had a better emulsifying ability than PLs concentrate. The optimum emulsifier concentration for coverage and stabilization of droplets is 2.3% proteins or a combination of 1.3% proteins with concentrations of PLs from 0.3 to 2.3%. In conclusion, the use of MFGM protein concentrate from reconstituted buttermilk in the preparation of emulsions had a greater impact at the oil-in-water interface compared to the PLs concentrate.
Chapter 6. Potential of MFGM Materials to Improve the Whipping Properties of Recombined Cream

This chapter is redrafted after:

Abstract

This work package is an application of the work in Chapter 3. All MFGM materials of Chapter 3 (BM-MFGM, Whey-MFGM, Lacprodan®PL-20) were used to make RC. The effect of MFGM on the fat crystallization behavior, shear-induced partial coalescence and on the whipping properties of RC was investigated and compared to those of NC and RC made with BMP. RC was prepared by homogenizing 35% (w/w) anhydrous milk fat into an aqueous phase containing individual BM-MFGM, Whey-MFGM, Lacprodan®PL20, or BMP or a mixture of MFGM materials and BMP (4:6, w/w). Amongst the four emulsifier materials used in this study, Whey-MFGM revealed a similar crystallization behavior to NC. After 5 days of storage, the solid fat content (SFC) of RC Whey-MFGM40, BM-MFGM40, Lacprodan40 and Lacprodan100 was similar to that of NC, whereas the SFC of BM-MFGM100 and BMP100 was higher than that of NC. RC Whey-MFGM40 showed improved whipping properties, namely a high overrun, no serum loss and reasonable firmness. Shear-induced partial coalescence of Whey-MFGM40 was slower compared to that of NC. Our data indicated that a mixture of Whey-MFGM and BMP can improve the whipping properties of the RC.

6.1 Introduction

Natural cream is a dairy product, rich in fat, directly obtained from fresh cows’ milk by separation of the FGs from the skimmed milk. RC is produced by recombining milk ingredients with or without the addition of potable water. The advantages of RC over NC are the cost reduction for storage of the raw materials, the possibility to modify and standardize the composition and the desired properties and the independence of the composition towards the milking seasons.
Chapter 6. Potential of MFGM materials to improve the whipping properties of RC

Whipping of cream is a destabilization mechanism, whereby milk FGs partially coalesce. The susceptibility towards partial coalescence is highly related to the strength of the interfacial layer. The use of RC for whipping is hardly new. However, whipped RC has divergent physicochemical and sensory properties compared to whipped NC. A controlled destabilization or partial coalescence during whipping of RC is needed to develop a network of agglomerated fat, retaining the air bubbles. This leads to the typical texture and physical appearance of the product. Several studies have been performed on improving the whipping properties of RC. For example, Fredrick (2011) reported that improved whipping properties of RC can be achieved by using a binary mixture of monoacylglycerols rich in oleic acid and monoacylglycerols rich in stearic acid. When low molecular weight surfactants (e.g., monoacylglycerols, diacylglycerols, and polysorbates) replace protein on the interface, due their high affinity to the surface and their capability to reduce surface tension, weaker spots on the interface and partial coalescence are promoted (Goff, 1997a). Emam-Djome et al. (2008) reported that the addition of whey protein concentrate (WPC) decreased the maximum overrun and serum loss and increased the whipping time. An increase in the fat to protein ratio in emulsions stabilized by WPC and sodium caseinate resulted in a decreased whipping time and overrun and in an increased stability and firmness (Van Camp et al., 1996).

In view of its technological properties, MFGM materials isolated from dairy by-products can be used as a novel ingredient for the development of emulsified products. From an economic point of view, by-products in dairy processing still have a lower price compared to mainstream products. The utilization of these sources to isolate the functional MFGM materials, their use as emulsifiers in oil-water emulsions (Chapter 3), and their application in the production/development of new products, may bring great benefit. However, to date no studies have been published evaluating the specific applications of these MFGM materials in whipping cream.

The objective of this study was to evaluate the potential of different MFGM materials as emulsifier to improve the whipping properties of RC. The different MFGM materials used in Chapter 3 (BM-MFGM, Whey-MFGM, Lacprodan®PL-20), were used individually or mixed with BMP (4:6, w/w) in the preparation of RC. The physicochemical properties (i.e. the fat crystallization behavior, the solid fat content, the interfacial protein load and shear-induced partial coalescence) and the whipping properties of these RCs were investigated and compared to NC and RC prepared with only BMP.
6.2 Materials and methods

6.2.1 Chemicals and materials

All the materials (BMP, BM-MFGM, Whey-MFGM and Lacprodan®PL20) as well as chemicals for analysis used in this work package were described in Chapter 3 (see 3.2.1). Anhydrous milk fat was obtained from FrieslandCampina (Lummen, Belgium). NC with 35% (w/w) fat was purchased from a local supermarket.

6.2.2 Determination of the composition of the emulsifying materials, the PLs and the protein profile in the aqueous phase of creams

The content of total protein, total lipids, ash, PLs and dry matter as well as the protein profile of the emulsifying materials in this work package were determined and discussed in section 3.3.1.

Table 6-1. The experimental design and composition of RCs.

<table>
<thead>
<tr>
<th>Type of cream</th>
<th>MFGM materials /BMP ratio</th>
<th>MFGM materials (% dry matter)</th>
<th>BMP (% dry matter)</th>
<th>Water (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey-MFGM40</td>
<td>4 6</td>
<td>3.44</td>
<td>3.82</td>
<td>54.74</td>
</tr>
<tr>
<td>BM-MFGM40</td>
<td>4 6</td>
<td>1.32</td>
<td>3.82</td>
<td>59.86</td>
</tr>
<tr>
<td>Lacprodan40</td>
<td>4 6</td>
<td>1.76</td>
<td>3.82</td>
<td>59.42</td>
</tr>
<tr>
<td>Whey-MFGM100</td>
<td>10 0</td>
<td>8.52</td>
<td>0</td>
<td>56.48</td>
</tr>
<tr>
<td>BM-MFGM100</td>
<td>10 0</td>
<td>3.30</td>
<td>0</td>
<td>61.70</td>
</tr>
<tr>
<td>Lacprodan100</td>
<td>10 0</td>
<td>4.41</td>
<td>0</td>
<td>60.59</td>
</tr>
<tr>
<td>BMP100</td>
<td>0 10</td>
<td>6.37</td>
<td>58.63</td>
<td></td>
</tr>
</tbody>
</table>

All creams contained 35% w/w of anhydrous milk fat and a total protein content of 2.3 g 100 g⁻¹ cream. Abbreviation: Whey-MFGM40 = cream with mixture of Whey-MFGM and BMP (4/6 w/w); BM-MFGM40 = cream with mixture of BM-MFGM and BMP (4/6 w/w); Lacprodan40 = cream with mixture of Lacprodan®PL20 and BMP (4/6 w/w); Whey-MFGM100 = cream with individual Whey-MFGM; BM-MFGM100 = cream with individual BM-MFGM; Lacprodan100 = cream with individual Lacprodan®PL20; BMP100 = cream with individual buttermilk powder.
The aqueous phase of RCs was prepared as given in Table 6-1, without adding AMF. For NC, the aqueous phase was obtained by churning the NC with a Hobart N50 mixer (Kampenhout, Belgium) and separating the buttermilk from the milk fat by filtration, using paper filters.

The total PL content, the PLs composition and the protein profile of the aqueous phase of cream was determined using methods as described in section 2.2.3.

### 6.2.3 Cream preparation

RCs made with emulsifiers, different in composition, were prepared to compare the effect of different MFGM materials with varying PLs content and protein composition, on the whipping properties. The MFGM materials used are: BM-MFGM, Whey-MFGM, Lacprodan®PL20, BMP or mixtures of MFGM and BMP. A constant protein content (2.3 g 100 g⁻¹ of RC) was retained for all the samples as used for the preparation of the O/W emulsion in Chapter 2 and Chapter 3. For RC prepared with a mixture of the MFGM material and BMP in a 4:6 ratio (on dry weigh basis) was used due to practical considerations. Results of screening tests showed no significant difference between the ratio 4:6 and 5:5. The composition of the RCs is shown in Table 6-1.

The emulsifying materials were dissolved in deionized water using a magnetic stirrer and stored overnight at 4°C for complete hydration. Then, 35% AMF at 50°C and 0.02% κ-carrageenan were added to the aqueous phase. This mixture was heated up to 60°C and pre-homogenized with an ULTRA-Turrax T25 basic (see 2.2.4) at 13,000 rpm for 6 min. The homogenization was performed in a two-step laboratory-scale high-pressure homogenizer (see 2.2.4) at 2/0 MPa. After homogenization, the RCs were cooled rapidly to 5°C and aged at this temperature for 7 days. The physicochemical and whipping properties of the RC were determined and compared with those of NC. All the experiments were carried out in triplicate.

### 6.2.4 Determination of physicochemical properties

#### 6.2.4.1 Isothermal crystallization behavior of cream

Differential scanning calorimetry (DSC; TA Q1000 DSC (TA Instruments, New castle, Delaware, USA)) was used to study the isothermal crystallization behavior of cream at 5°C according to Fredrick et al. (2011). Prior to analysis, the DSC was calibrated with indium (TA Instruments, New castle, Delaware, USA), azobenzene (Sigma-Aldrich, Bornem, Belgium) and undecane (Acros Organics, Geel, Belgium). The system was purged with nitrogen gas.
Cream samples (5-15 mg) were weighed on an analytical balance (Sartorius, CP225D, Germany). Hermetic alodined aluminium pans (TA instruments) were used to seal the samples and an empty pan was used as reference. The time-temperature program (t-T) was applied as follows: (i) holding the sample at 65°C for 10 min, (ii) cooling at 25°C min⁻¹ to 5°C and (iii) holding at 5°C for \(x\) min (\(x\) may vary from 0 to 120 min) and (iv) finally heating at 20°C min⁻¹ until 65°C. The t-T program was repeated for several defined isothermal periods. The recorded melting curves were integrated using a horizontal linear baseline with a fixed end point at 55°C. The melting heat (J g⁻¹) was plotted as function of the holding time at 5°C.

### 6.2.4.2 Solid fat content (SFC)

SFC was measured with a Maran Ultra 23 MHz pulsed-field-gradient nuclear magnetic resonance (NMR) (Oxford Instruments, Abingdon, UK). An indirect SFC method as described by Fredrick et al. (2011) was applied to determine the SFC content of cream. Since the aqueous phase of the cream contributes to the FID-signal (free induction decay), the measured FID-signal (at 70\(\mu\)s) of the cream per gram (\(L_{\text{cr}}\)) was corrected for the aqueous phase. Cream sample (0.5 mL) was filled in the NMR-tubes and the mass was determined on an analytical balance (Sartorius, CP225D, Germany). All samples were placed in a water bath at 45°C and after 10 minutes their FID-signal was recorded at 45°C. Then, the samples were transferred to the water bath at 5°C and the FID-signal was recorded after holding the samples for 2 h, 1 day, 3 days and 5 days of storage at 5°C. To avoid interruption of the isothermal period the NMR-probe was thermally controlled at 5°C. For each sample the NMR measurements were done in triplicate. All calculations of the SFC content of cream were according to Fredrick et al. (2011).

### 6.2.4.3 Shear-induced partial coalescence

Shear-induced partial coalescence measurements were performed after 7 days of storage at 5°C using rotational viscosimetry in an AR2000 controlled stress rheometer (TA instruments, Brussels, Belgium) equipped with a starch pasting cell geometry composed of a jacket, removable cup and an impeller (diameter 32 mm, height 122 mm). Each time, 30 g of sample was weighed into the cup and after an equilibration period at 20°C, a constant shear rate of 150 s⁻¹ was applied to induce partial coalescence (Fredrick et al., 2013). The change in the apparent viscosity of the cream as a function of time was recorded until a maximum was reached. This maximum corresponds to the occurrence of phase inversion. All measurements were performed in duplicate.
6.2.4.4 Droplet size

The droplet size of the creams was measured using a long bench Malvern Mastersizer S (Malvern Instruments, Malvern, UK) with an MS17-automated sample dispersion unit as described in section 2.2.5.1. The presentation code 1.492, 0.01, 1.330 corresponding respectively to the particle refractive index, particle absorption, and dispersant refractive index, was used. Prior to the measurements, 1% of the cream was heated (50°C) to avoid scattering of fat crystals present in the milk FGs and then dissolved in 1% SDS solution. For each composition, three creams were prepared independently and measured at least in duplicate.

6.2.4.5 Surface protein load

The surface protein load at the O/W interface of the RCs and of NC was determined according to the method described by Fredrick et al. (2013) with some modifications. The simulated milk ultrafiltrate (SMUF) was prepared according to the procedure of Jenness and Koops (1962). All samples were centrifuged at 4500 g for 45 min at 20°C in a Sigma 4K15 centrifuge (Sartorius AG, Göttingen, Germany). The protein content of the upper fat layer was determined by the Kjeldahl method as described in section 2.2.3. The total fat surface area (m²) was calculated from the fat volume fraction (mL) and the specific surface area (SSA) (m² mL⁻¹), where SSA was derived from the d₃,₂. The surface protein load (Γₚṟo, mg m⁻²) was calculated from equation [3.1] as described in section 3.2.5.4.

6.2.4.6 Whipping properties

a. Whipping time

After seven days of storage, approximately 1000 g of RC was whipped in a Hobart mixer equipped with a ‘D’ wire agitator at a speed of 240 rpm. For each treatment, three independently prepared RCs were whipped until a desired quality of whipped cream was obtained as described by Fredrick et al. (2013). The whipping time (tₘₗₚ) was noted. Whipping properties were determined based on the measurement of overrun, serum loss and firmness and compared to those of the NC.

b. Overrun

The overrun is a measure for the volume of air trapped in the whipped cream. It can be calculated by comparing the weight of equal volumes of the unwhipped and whipped creams (Fredrick et al., 2013). For each whipped cream, seven plastic cups of 200 mL were used for
the overrun measurements. Initially, the weight of the empty cups was determined. Then, the unwhipped cream was completely filled in one out of the seven cups to know the mass of the cream before whipping ($M_1$). After whipping, the six remaining cups were filled with whipped cream and weighted and averaged ($M_2$). The overrun of the whipped cream was calculated using following equation [6.1]:

$$\text{% overrun} = \frac{M_1 - M_2}{M_2} \times 100$$

\[6.1\]

c. Serum loss

The serum loss was used as a measure for the stability of whipped cream. A defined mass of whipped cream (30 g) was transferred into a funnel placed on top of a flask. The amount of serum drained from the whipped cream was weighed after storing the cream for 1 hour at 20°C and also for 24 hours at 5°C. The percentage serum loss was calculated using following equation [6.2]:

$$\text{% serum loss} = \frac{M_2}{M_1} \times 100$$

\[6.2\]

with, $M_1$ is the initial mass of the whipped cream and $M_2$ the mass of the released serum.

d. Firmness

The cups with the whipped cream for the overrun measurements were used afterwards for the firmness measurements by a penetration test using an Instron 5942 texture analyser (Instron, Norwood, US). An acrylic cylindrical probe ($\Phi 25$ mm) attached to a 500N load cell was used for these measurements. The probe penetrated into the sample to a depth of 20 mm at a rate of $1 \text{ mm s}^{-1}$ with a trigger value of 0.1 N. The firmness is defined as the force required to reach this penetration depth. The firmness of the whipped cream was measured after 1 hour and 24 hours of storage at 5°C.

6.2.5 Statistical analysis

Statistical analysis was performed using the SPSS version 21 (IBM Inc., Chicago, IL., USA). One-way analysis of variance (ANOVA) and Tukey’s test were used for multiple comparison of means. The differences were considered statistically significant at $P \leq 0.05$. The average value of the composition in the aqueous phase of creams (total PLs, the ratio of casein to whey proteins) and the whipping properties of cream ($t_{ch}$, $t_{wh}$, overrun, serum loss and
firmness) in different creams were evaluated using principal component analysis (PCA) with maximum variation rotation.

6.3 Results

6.3.1 Composition of the experimental materials and the aqueous phase of creams

The composition of the experimental materials was presented and discussed in section 3.3.1. The total concentration and composition of PLs were quantified in the aqueous phase of RCs and in NC and are shown in Table 6-2.

There are two groups of phospholipids: the glycerophospholipids (PC, PE, PS and PI) and the sphingolipids (SM, GluCer and LacCer) (Contarini and Povolo, 2013). Content and composition of the aqueous phases of the creams were different because the emulsifying materials used for the experiments differed in their PLs content and composition (Table 6-2).

It is shown that the PLs content in the aqueous phase of NC and in the RC of BMP100, BM-MFGM40 and BM-MFGM100 was significantly different from that in the aqueous phase of the RCs of Whey-MFGM40, Whey-MFGM100, Lacprodan40 and Lacprodan100. This is logical because Whey-MFGM and Lacprodan® PL20 contained a higher level of PLs compared to BMP and BM-MFGM material (Table 6-2). Although the PLs content of Lacprodan® PL20 was 1.91 times higher than that of Whey-MFGM (section 3.3.1), the total PLs content in the aqueous phase of Whey-MFGM100 and Whey-MFGM40 was not significantly different from that of Lacprodan100 and Lacprodan40, respectively. This is explained by the difference in concentration of the emulsifying materials used to prepare the RCs since the protein content of all RCs was standardized at 2.3 g 100 g−1 dry matter (Table 6-1).

In general, the aqueous phase of creams contained a high proportion of PC (26.6-32.9%), PE (22.0-29.9%), SM (14.5-23.8%) and a low proportion of GluCer (1.7-5.7%), LacCer (6.3-9.6%) and PS (1.2-4.0%). This is in accordance with what has been reported for raw milk (Rombaut and Dewettinck, 2006c, Contarini and Povolo, 2013). As compared to the PLs composition of RCs, the aqueous phase of NC contains only a small amount of SM (9.27%). The proportion of GluCer and LacCer was higher in NC than in RCs.

Comparison of the protein profile of the aqueous phase of the RCs and that of NC is shown in Figure 6-1.
The major MFGM proteins XO and BTN were present in a high intensity in the SDS-PAGE patterns of the aqueous phases of all creams except for the aqueous phase of Whey-MFGM100 (lane 5). Based on densitometry, band intensities of XO of the aqueous phase of Lacprodan40 (lane 4) were found to be 2.1 and 1.4 times higher than those of NC (lane 1) and Whey-MFGM40 (lane 2). There was no difference with the other aqueous phases. Comparing the band intensities of BTN, the band intensity of the aqueous phase of BM-MFGM100 (lane 6) was found to be 2.5 and 2.0 times higher than that of NC and Whey-MFGM40, respectively. Besides the MFGM specific proteins, the aqueous phase of different creams contained different ratios of casein to whey proteins (Figure 6-1).
### Table 6-2. Total concentration and composition of PLs in the aqueous phase of NC and RC prepared with MFGM materials

<table>
<thead>
<tr>
<th>Aqueous phase of cream</th>
<th>Composition (% of total PLs)</th>
<th>Total PLs (% w/w of sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GluCer</td>
<td>LacCer</td>
</tr>
<tr>
<td>Whey-MFGM40</td>
<td>2.66 ± 0.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.26 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-MFGM40</td>
<td>5.54 ± 0.06&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>9.63 ± 0.71&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lacprodan40</td>
<td>3.94 ± 0.82&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.75 ± 0.08&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMP100</td>
<td>2.82 ± 0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.91 ± 0.15&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Whey-MFGM100</td>
<td>1.24 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.31± 0.78&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BM-MFGM 100</td>
<td>5.70 ± 0.09&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>8.78 ± 0.17&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lacprodan100</td>
<td>1.71 ± 0.43&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.31 ± 0.18&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>NC</td>
<td>6.89 ± 0.73&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.88± 1.26&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> Data are expressed as mean values ± SD of three replicates; different superscript letters within a column refer to statistically significant differences (Tukey’s test, \( P \leq 0.05 \)).
6.3.2 Physical properties of creams

The isothermal crystallization at 5°C for different type of creams was studied by the indirect DSC-method. Figure 6-2A and B show the crystallization profiles of NC versus RCs prepared with individual MFGM material and with a mixture of MFGM material and BMP (4:6, w/w), respectively.

Table 6-3. Crystallization temperature ($T_{cr}$) of different type of creams

<table>
<thead>
<tr>
<th>Type of cream</th>
<th>$T_{cr}$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey-MFGM40</td>
<td>$17.72 \pm 0.61^{cd}$</td>
</tr>
<tr>
<td>BM-MFGM40</td>
<td>$17.34 \pm 0.28^{bcd}$</td>
</tr>
<tr>
<td>Lacprodan40</td>
<td>$16.54 \pm 0.15^{bc}$</td>
</tr>
<tr>
<td>Whey-MFGM100</td>
<td>$17.42 \pm 0.48^{bcd}$</td>
</tr>
<tr>
<td>BM-MFGM100</td>
<td>$18.07 \pm 0.63^{d}$</td>
</tr>
<tr>
<td>Lacprodan100</td>
<td>$16.38 \pm 0.33^{ab}$</td>
</tr>
<tr>
<td>BMP100</td>
<td>$18.17 \pm 0.44^{d}$</td>
</tr>
<tr>
<td>NC</td>
<td>$15.21 \pm 0.45^{a}$</td>
</tr>
</tbody>
</table>

$^{1}$Data are expressed as mean values ± SD of three replicates; superscript letters a-d refer to statistically significant differences (Tukey’s test, $P \leq 0.05$).

For all samples melting heat are already detected from the moment the temperature reaches 5°C ($t = 0$ min) indicating that milk fat in cream already started to crystallize during the preceded cooling. Table 6-3 shows the crystallization temperatures ($T_{ch}$) of the NC and the RCs prepared with different MFGM materials and a mixture of MFGM material and BMP. The $T_{cr}$ of NC was significantly different from that of RCs, except for Lacprodan100. The $T_{cr}$ of RC BMP100, i.e. $18.17 \pm 0.44^{o}C$, was higher than the value of $11.1 \pm 0.6^{o}C$ reported by Fredrick et al. (2011), while the $T_{cr}$ of NC obtained in this study is in line with the result of the Fredrick study ($14.4 \pm 0.2^{o}C$). This can probably be explained by the difference in t-T history during processing (e.g. the production of cream on the lab scale conditions and of a typical industrial RC), of the starting materials and the storage conditions.
Figure 6-2. Isothermal crystallization curves at 5°C of NC and RC prepared with individual MFGM material (A) and with a mixture of MFGM material and BMP (4:6, w/w) (B) constructed by plotting the melting heat per g cream sample as function of the holding time.

Figure 6-2A and B show the isothermal crystallization curves of NC and RCs prepared with different MFGM materials at 5°C after fast cooling (25°C min⁻¹). The crystallization profiles all show a two-step crystallization. In the first step, α-crystals are formed during the cooling and the beginning of the isothermal period. Secondly, β' -crystals are shaped at the expense of α-crystals (second step) while still some α-crystals remain present. However, the latter have a different composition than the initially created α-crystals during cooling. In RCs of BM-MFGM40, BM-MFGM100 and BMP100, some differences in the crystallization kinetics can clearly be detected compared to that of NC and other RCs. Concretely, interfacial heterogeneous nucleation took place during cooling. The slope of the second step of RC of BM-MFGM40, BM-MFGM100 and BMP100 is also steeper, hence the α-β’ polymorphic transition seems to be faster than that of the NC and other RCs. As a consequence, the crystallization of these RCs started at a higher temperature and the crystal growth and the α-β’ polymorphic transition was accelerated. Wiking et al. (2009) and Vanhoutte et al. (2002) reported that adding soybean PLs in a mixture of milk fat and water retarded nucleation and crystal growth of the milk fat. Miura et al. (2006) investigated the effect of bovine and soybean PLs on the milk fat crystallization and concluded that soybean PLs decreased the crystallization temperature during cooling and consequently lowered the solid fat content compared to RCs containing bovine PLs. The PLs content in the aqueous phase of RC of BM-MFGM100, BM-MFGM40 and BMP100 was significantly lower.
than that in the aqueous phase of the RCs of Whey-MFGM40, Whey-MFGM100, Lacprodan40 and Lacprodan100 (Table 6-2). Besides, the melting heat required to melt the crystals, created during each isothermal period of the RCs was higher than that of NC. The difference in nucleation mechanism and crystallization kinetics may result in a different number, size, arrangement and morphology of the crystals in the fat globule. However, microscopic analysis, to visualize fat crystals in the emulsion droplets, still needs to be performed.

To induce partial coalescence, a continuous fat crystal network is needed (Boode et al., 1993). This means that the SFC has a strong influence on the rate of partial coalescence. Figure 6-3 shows the SFC of NC and of RC prepared with individual MFGM material or with a mixture of MFGM material and BMP (4:6, w/w) after 2 h, 1 day, 3 days and 5 days storage at 5°C.

![Figure 6-3](image)

**Figure 6-3.** Solid fat content (SFC) of NC and RC prepared with individual MFGM material and with a mixture of MFGM material and BMP (4:6, w/w) after storage for 2h, 1 day, 3days and 5 days at 5°C.

From the DSC-data shown in Figure 6-2, after 120 min, it seems that an equilibrium in milk fat crystallization was reached in all RCs and NC. However, SFC measurements show some extra crystallization during the first day. The polymorphic transition probably continues after 120 min of crystallization at 5°C most likely combined with some additional crystallization of the triacylglycerols (TAGs) which did not crystallize immediately. The equilibrium SFC of RCs and
NC is reached after 3 days of storage, except for the RC of BM-MFGM100. Among all types of cream, RC of Whey-MFGM100 showed the lowest amount of SFC during 5 days of storage. The SFC of RC of Whey-MFGM40, BM-MFGM40, Lacprodan40 and Lacprodan100 was similar to that of NC, whereas the SFC of BM-MFGM100 and BMP100 was higher than that of NC.

Table 6-4. The $t_{ch}$, $d_{3.2}$ and the $\Gamma_{pro}$ at the oil-water interface of different types of creams.

<table>
<thead>
<tr>
<th>Type of cream</th>
<th>$t_{ch}$ (min)</th>
<th>$d_{3.2}$ ($\mu m$)</th>
<th>$\Gamma$ (mg m$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>($\gamma=150$ s$^{-1}$ at 20°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whey-MFGM40</td>
<td>45.9 ± 0.82$^g$</td>
<td>2.63 ± 0.13$^a$</td>
<td>9.60 ± 0.22$^{a,b}$</td>
</tr>
<tr>
<td>BM-MFGM40</td>
<td>12.2 ± 1.05$^c$</td>
<td>2.66 ± 0.06$^a$</td>
<td>10.77 ± 0.44$^b$</td>
</tr>
<tr>
<td>Lacprodan40</td>
<td>5.0 ± 0.21$^b$</td>
<td>2.56 ± 0.22$^a$</td>
<td>9.25 ± 0.91$^{a,b}$</td>
</tr>
<tr>
<td>BMP100</td>
<td>2.3 ± 0.26$^a$</td>
<td>2.78 ± 0.10$^{ab}$</td>
<td>10.38 ± 1.02$^b$</td>
</tr>
<tr>
<td>Whey-MFGM100</td>
<td>67.0 ± 0.80$^h$</td>
<td>2.62 ± 0.13$^a$</td>
<td>8.29 ± 0.23$^a$</td>
</tr>
<tr>
<td>BM-MFGM 100</td>
<td>20.8 ± 0.15$^d$</td>
<td>2.69 ± 0.08$^a$</td>
<td>10.42 ± 0.20$^b$</td>
</tr>
<tr>
<td>Lacprodan100</td>
<td>27.8 ± 0.26$^f$</td>
<td>2.58 ± 0.09$^a$</td>
<td>10.11 ± 0.47$^b$</td>
</tr>
<tr>
<td>NC</td>
<td>24.6 ± 0.46$^e$</td>
<td>3.01 ± 0.05$^b$</td>
<td>10.40 ± 0.84$^{b}$</td>
</tr>
</tbody>
</table>

$^1$Data are expressed as mean values ± SD of three replicates; different superscript letters within a column refer to statistically significant differences (Tukey’s test, $P \leq 0.05$).

After storing the RC at 5°C for 7 days, the shear-induced partial coalescence was measured as a function of time by applying a constant shear rate of 150 s$^{-1}$ at 20°C. The results are expressed as the churning time ($t_{ch}$). Table 6-4 shows that the $t_{ch}$ of NC and of RC was significantly different from each other. The longest $t_{ch}$ was observed for the RC of Whey-MFGM100 ($t_{ch} = 67$ min), while the churning time of RC of BMP100 was the shortest ($t_{ch} = 2.3$ min). The $t_{ch}$ of NC was 24.6 min (Table 6-4). FGs in raw milk have a volume surface average diameter of about 3.4 µm. The average particle size of NC is similar to that of raw milk. The average particle size of all RCs was not significantly different (Table 6-4).

The surface protein load at the interface of different types of cream was analyzed and is shown in Table 6-4. RC of Whey-MFGM100 is characterized by a significantly lower protein load as compared to other RCs and NC, except for Whey-MFGM40 and Lacprodan40. However, the values obtained in this study are within the ranges suggested by Melsen (1987). According to Melsen (1987) the surface protein load varies depending on the ratio of whey proteins to the intact casein micelles and to the casein sub-micelles located at the interface. The surface protein
load of an interface fully covered with whey proteins, intact casein micelles and casein submicelles, amounts to 2-3 mg m\(^{-2}\), 40 mg m\(^{-2}\) and 5 mg m\(^{-2}\), respectively. In addition, protein adsorption can be influenced by heat treatment of the protein dispersion, the homogenization temperature, the protein to fat ratio, the fat globule size and the presence of small-molecules surfactants (Oortwijn and Walstra, 1979). In the present study, the interfacial layer of RCs and NC probably consisted of a mixture of serum proteins, caseins, MFGM-specific proteins and PLs and was different in NC and in each RC. It should be noted that in the emulsifying materials many different substances are present, such as whey proteins, caseins, MFGM proteins, PLs and minerals, which may influence the surface protein composition. The competitive adsorption on the surface of FGs of these substances still needs to be further investigated.

**6.3.3 Whipping properties**

The whipping properties, namely, whipping time, overrun (Figure 6-4), serum loss (Figure 6-5) and firmness (Figure 6-6) were determined for NC and RCs.

**Figure 6-4.** Whipping time (A) and overrun (B) of NC and RC prepared with a mixture of MFGM material and BMP, individual MFGM material or BMP.
Chapter 6. Potential of MFGM materials to improve the whipping properties of RC

Figure 6-5. Serum loss of RC BM-MFGM40, Lacprodan40, Whey-MFGM100, BM-MFGM100, Lacprodan100, BMP100 after 1 h at 20°C and 24 h at 5°C. No serum loss with NC and RC Whey-MFGM40 (data not shown).

Figure 6-6. The firmness of NC and RC Whey-MFGM40, BM-MFGM40, Lacprodan40, Whey-MFGM100, BM-MFGM100, Lacprodan100, BMP100 after 1 h at 5°C and 24 h at 5°C.
Chapter 6. Potential of MFGM materials to improve the whipping properties of RC

The results showed that whipped creams stabilized with Whey-MFGM40 had, as it was also the case for NC, no serum loss neither after 1 h at 20°C or after 24 h at 5°C (data not shown). Particularly, a high overrun and good firmness of these RCs after whipping were observed, being comparable to that of NC (Figure 6-4B and Figure 6-6, respectively). Compared to RC, Whey-MFGM40, Whey-MFGM100 showed the longest whipping time, a low overrun and firmness, no serum loss after 1 h at 20°C and only 0.65% serum loss after 24 h at 5°C (Figure 6-4A, B, Figure 6-6 and Figure 6-5, respectively).

Among the eight creams, RC of BM-MFGM40, Lacprodan40, BM-MFGM100, Lacprodan100 or BMP100 showed considerable serum loss after 1 h at 20°C and after 24 h at 5°C. The largest serum loss was observed for RC of BM-MFGM100. RC of BM-MFGM40 had different characteristics compared to that of BM-MFGM100. RC of BM-MFGM40 showed less serum loss and a higher firmness compared to the RC of BM-MFGM100 (Figure 6-5, Figure 6-4 and Figure 6-6, respectively).

6.4 Discussion

To obtain the desired product properties in whipped cream, partial coalescence is necessary. It influences the macroscopic product properties like overrun, firmness and stability. The increase in crystal size at rest and in Couette flow, affects the emulsion stability. Consequently, the emulsion will be more susceptible to partial coalescence. The rate and extent of partial coalescence initially depends on the t-T history during processing. It is mainly influenced by the crystallization of milk fat and its kinetics. The differences in the concentration and composition of components (e.g. PLs, lactose and minerals) in the aqueous phase of RCs and NC may influence the fat crystallization behavior (nucleation, crystal growth rate and polymorphic evolutions) and, therefore, the shear-induced partial coalescence and the whipping behavior of creams.

Our data demonstrated that the fat crystallization behavior, the shear-induced partial coalescence and the whipping properties of creams were affected by the composition of the aqueous phase in the reconstituted creams. Among the RCs and NC, the rate of partial coalescence of the FGs in NC and RC was found to be very different (Table 6-4). The difference in the $t_{ch}$ of NC and all RCs can be due to the difference in the fat crystallization behavior, the composition of the aqueous phases and the nature of the newly formed fat globule membrane (Table 6-2 and Figure 6-1). In emulsions the crystallization behavior, the emulsion stability and whipping properties can be affected to a large extent by the particle size distribution. The smaller the droplet, the
higher the supercooling needed to induce crystallization (Walstra and Vanberesteyn, 1975, Lopez et al., 2002). The latter counts until the supercooling for homogeneous nucleation is reached. In the present study, the average particle size of all RCs was not significantly different so the effect of this factor was expected to be small. The $d_{3,2}$ of NC was significantly different from that of RCs (Table 6-4). In NC, the droplet size is a consequence of a natural process, while the droplet size of RC depends on the energy-input during processing as well as on the availability of surface active materials (Ward et al., 2006). In RC, the PLs and proteins (caseins, whey proteins and MFGM-specific proteins) are present in the membrane and were found to have a strong effect on the whipping properties of RCs. The shear-induced partial coalescence rate was also affected by the fat crystallization behavior and by the SFC of NC and RC (Figure 6-2 and Figure 6-3). The increased nucleation rate in the RCs was demonstrated by the increased crystallization temperature observed upon cooling, (Table 6-3) implying a higher number of nucleation sites, and hence, more primary crystals within one droplet are shaped at the interface. After primary nucleation has been established during cooling, the crystallization will readily spread throughout the whole volume via secondary nucleation. The latter is favored in milk fat because of their complex TAG-composition (Walstra, 1998). The difference in crystallization behavior induced by different MFGM materials, are considered to affect the $t_{ch}$ and the whipping properties of creams.

RC stabilized by Whey-MFGM has a long $t_{ch}$, a high overrun and a reduced serum loss as compared to RCs made with BM-MFGM and Lacprodan®PL20 (Table 6-4, Figure 6-4B and Figure 6-5, respectively). Goff (1997a) reported that emulsions stabilized by whey proteins were more susceptible to partial coalescence than when they were stabilized with caseins. MFGM materials used in this study were isolated from the by-products of dairy industrial processing. The denatured whey proteins can affect the stability of the RCs. The presence of pre-denatured whey proteins in these RCs contribute to the higher stability against fat droplet aggregation and reduce the sensitivity to partial coalescence. The rate of partial coalescence is lower for FGs stabilized by proteins, because they form a thicker and more viscous elastic membrane at the oil-water interface. Particularly these FGs were stabilized by whey proteins and it took a long time before they started to fuse and aggregate to initialize the partial coalescence and to show an increase in viscosity. Besides, the high amounts of PLs in MFGM enriched materials can also provide stability to whipped cream. In our study the FGs of RC of Whey-MFGM100 were stabilized mostly by whey proteins and PLs. The whey proteins β-LG and α-LA form elastic interfaces and increase the stability of emulsions against partial coalescence. In addition, the SFC
and the surface protein load of RC of Whey-MFGM100 was lowest compared to that of other creams (Figure 6-3 and Figure 6-4). These could be the reason for the observed longer $t_{ch}$ and $t_{wh}$ of RC Whey-MFGM100 (Figure 6-4). However, the whipping properties of cream, amongst others, were not dependent on the concentration of PLs in this RC. On the other hand, the effect of the chemical structure of the PLs on the emulsifying properties may also be important. In fact, the size, molecular weight and relative polarity of each polar head contributes to the emulsifying properties and should be taken into account (Birkett, 1983). The differences in the content of PLs, the polarity, the molecular weight of the phosphate head group, and the composition of the fatty acid tail may influence the fat crystallization behavior as well as the SFC and consequently also the partial coalescence and the whipping properties of cream. Besides, the high amount of ash content in MFGM enriched materials can also provide stability to whipped cream. The Whey-MFGM material used in this study has the highest ash content (minerals e.g., Ca$^{2+}$) (Table 3-2) which probably also contributes to the modified whipping properties. Calcium (Ca$^{2+}$) being a divalent cation, can form calcium bridges between adjacent FGs in the last stage of whipping resulting in a whipped cream in which the air bubbles are stabilized by aggregated FGs rather than partially coalesced FGs. The calcium content contributes further to this aggregation mechanism of FGs and, hence, improves the whipping properties (Kieseker and Zadow, 1973, Besner and Kessler, 1998). Among the seven RCs, RC of Whey-MFGM100 and of Whey-MFGM40 had a higher stability resulting in no serum loss compared to NC. It was observed that by whipping with RC of BM-MFGM100 more air was introduced and a less dense structure was built-up resulting in a high overrun, high serum loss and low firmness (Figure 6-4B, Figure 6-5 and Figure 6-6, respectively). RC of BM-MFGM40 had different characteristics compared to the RC of BM-MFGM100. RC of BM-MFGM40 showed less serum loss and a higher firmness compared to BM-MFGM100. In both cases, a high overrun was observed. Even though the BM-MFGM was isolated from the BMP, the composition of the two materials was different. Consequently, the mechanism behind nucleation and behind crystal growth was found to be different between RC of BM-MFGM100, BM-MFGM40 and BMP100 (Figure 6-2) and the SFC, after prolong storage 5°C, was slightly different (Figure 6-3), although similarities in crystallization kinetics of RC of BM-MFGM100, BM-MFGM40 and BMP100 were observed (Figure 6-2). In addition, the protein composition of the two materials was different. The ratio of caseins to whey proteins of BMP was higher compared to that of BM-MFGM (Table 3-2). As a result, BM-MFGM40 had higher ratio of caseins to whey proteins compared to BM-MFGM100. It is known that these milk proteins have a high water holding capacity (Kneifel et al., 1991) and
may reduce the serum loss as compared to BM-MFGM100. As already indicated above, in RC of BM-MFGM100 more air was introduced during whipping and a less dense structure was built-up, resulting in a higher overrun and a lower firmness. In our view this could, to a certain extent, be due to the presence of a high amount of MFGM-specific proteins such as XO and BTN in BM-MFGM. In the case of RC of BMP100, the amount of caseins was high. However, casein micelles were present when BMP was dissolved in water. Those micelles are large and thus could stabilize neighboring fat droplets by positioning themselves at the interface of the FGs (forming globule clusters). This is in agreement with microscopic observation (data not shown). Consequently, the clusters of globules, being bigger in size compared to the separate globules, can collide more easily during churning. Vanapallli et al. (2002) reported that the long lasting contact between globules in clusters favored the occurrence of partial coalescence. Consequently, a lower whipping time was observed (Figure 6-4A).

Principal component analysis (PCA) is an effective mathematical method to reduce the dimensionality of multivariate data. To investigate the correlations between the composition of the aqueous phase (PLs content and the ratio of casein to whey proteins) and the whipping properties ($t_{ch}$, $t_{wh}$, overrun, serum loss and firmness), these variables were subjected to PCA, and the results are shown in Figure 6-7.

The first principal component (PC1) explained 52.7% of the variation across the samples, whereas the second principal component (PC2) explained 24.1% of the variance. The cumulative variance contribution of PC1 and PC2 was 76.8%. In the PCA factor loading plot (Figure 6-7A), it can be seen that $t_{ch}$, $t_{wh}$ and PLs content positively correlated with PC1, whereas the ratio of casein to whey proteins and firmness negatively correlated with PC1. In addition, on PC2, overrun and serum loss exhibited heavier factor loadings. The PLs content and the caseins and whey proteins ratio in the aqueous phase showed a slightly negative correlation to the overrun and the serum loss. Consequently, we could divide these response factors into three groups based on their distribution in Figure 6-7A. The PCA scores of different cream samples are shown in Figure 6-7B. The center point corresponds to the RC of Whey-MFGM40. While the NC and the RC of Whey-MFGM100 are strongly negatively and positively correlated with PC1, respectively, the RC of BM-MFGM100 is positioned into the positive side of the PC2 axis. The difference in the total PLs content, the ratio of casein to whey protein and the band intensity of the MFGM specific proteins (XO and BTN) in the aqueous phase of creams (Table 6-2 and Figure 6-1) were responsible for this contrast.
Chapter 6. Potential of MFGM materials to improve the whipping properties of RC

In conclusion, PCA results show that there was a strongly contrasting correlation between the total PLs content and the caseins and whey proteins ratio in creams. The PLs in creams showed a strong positive correlation to the $t_{ch}$ and $t_{wh}$. This means that the higher the PLs content in the aqueous phase of creams within the range of our study, the longer the $t_{ch}$ and $t_{wh}$. This finding is

**Figure 6-7.** Principal component analysis (PCA) loadings for (A) the composition in the aqueous phases and the whipping properties of creams and scores for (B) cream samples. The first two principal components, PC1 and PC2, explained 52.7 and 24.1% of the variance, respectively.
in agreement with some findings of Ihara et al. (2011). Whereas, the ratio of casein to whey protein in the creams was strongly positively correlated with the firmness of the creams, RC of BM-MFGM100 showed a high overrun and serum loss. This can be due to the presence of a higher amount of MFGM-specific proteins such as XO and BTN compared to other creams. However, thus far, no information concerning the effect of MFGM-specific proteins on the physical properties and textural characteristics of whipped cream is available. The detailed effect of the MFGM-specific proteins in whipped cream still needs to be further investigated.

6.5 Conclusions

MFGM affected the physicochemical and whipping properties of RC. The present results demonstrated that the differences in composition of the MFGM sources have a very important influence on the characteristics of the whipped RCs. When using BM-MFGM as an emulsifier, the crystallization started at higher temperature and the crystals growth and the α-β’ polymorphic transition was accelerated compared to the NC. The overrun of whipped cream of BM-MFGM100 increased. RC prepared with Whey-MFGM as emulsifier (Whey-MFGM100) led to a long $t_{ch}$ due to the presence of a high amount of whey proteins, PLs, minerals and low amount of SFC. The use of 60% BMP in RC next to the 40% of MFGM-enriched material was also found to have an impact on the physicochemical and whipping properties, especially the serum loss reduction. Amongst the four emulsifier materials used in this study, Whey-MFGM had a high potential to improve the whipping properties of RC. The RC prepared with a mixture of Whey-MFGM and BMP resulted in a product with no serum loss, a high overrun and a reasonable firmness. These characteristics are comparable with the whipping properties of NC and compared well with RC without the addition of extra emulsifying material (i.e., only with BMP).
Chapter 7. General Conclusions and Perspectives

MFGM is believed to be a good emulsifying agent, given its natural presence at the interface between milk fat and the serum phase and its function in the stabilization of fat globules against coalescence. MFGM material isolated from dairy by-products contains various proteins and PLs which are efficient natural surface-active agents for emulsion preparation. The composition and technological functionalities of MFGM material isolated from dairy by-products depend on the type of dairy sources and on the treatments during milk processing. In addition, in an emulsion system that contains both PLs and proteins, the interaction of PLs and proteins may increase or decrease the surface activity of proteins as well as that of PLs.

7.1 Summary of major findings

Previous works reported in the literature have suggested that the utilization of MFGM, which contains both MFGM proteins and PLs, isolated from dairy by-products such as buttermilk, butter serum and buttermilk whey can improve the emulsifying/stabilizing stability of oil in water emulsions because of the presence of high content in polar lipids. However, the role of individual MFGM proteins and PLs moiety as well as the optimum balance between the PLs and MFGM proteins content, on the stabilization of O/W emulsions is lacking. In addition, the effect of PLs and MFGM proteins on the surface active properties as well as on the competitive adsorption behavior between the two component groups at the interface of O/W emulsions had not been addressed in literature.

The major accomplishments of this doctoral research are:

- Giving a greater insight into the possibility of using MF for isolating MFGM materials from dairy by-products and the efficiency of these MFGM materials as emulsifiers for improving the stability of emulsions.
- Understanding of the separated roles of MFGM protein fraction and PL fraction and the possible interaction between PLs and membrane-specific proteins and their potential emulsifying properties.
- Preliminary application of the MFGM materials isolated from dairy by-products as a novel ingredient for improving the whipping properties of RC

The side streams of dairy processing contain valuable substances that are of interest for being isolated and purified. Proper utilization of these cheap by-products as sources for the isolation of MFGM material and for the use of its functional properties in the development of new products.
Chapter 7. General conclusions and perspectives

has great economical and technological value. MF was used to isolate MFGM materials from reconstituted buttermilk and buttermilk whey. The concentration of PLs increased about 2.8 times after MF, while the presence of MFGM-specific proteins in the MFGM isolate from reconstituted buttermilk (BM-MFGM) also increased significantly. However, a high concentration of serum milk proteins (caseins and whey proteins) was found still to be present in the isolates. The presence of these protein components in the material can interfere with the effects of MFGM components, making it difficult to evaluate the functional properties of MFGM components. To further decrease the concentration of non-MFGM proteins, more DF steps can be considered (Corredig et al., 2003). However, it is noted that even with more extensive DF, the purification of the filtered products, in term of MFGM proteins, would not be comparable with that of MFGM material isolated from raw milk, because heat treatment during BMP production may induce an interaction between β–LG, α–LA, κ–casein and the surface of FGs. With MF of buttermilk whey, the isolated MFGM material (Whey-MFGM) had a high concentration of PLs (12.1g 100 g⁻¹ Whey-MFGM, on dry basis). However, the MFGM material still had a high mineral content, due to the addition of calcium chloride as process additive during the coagulation step in cheese making. In addition, the MFGM-specific proteins were lost together with the coagulated caseins upon acidification. The functionality of MFGM material isolated from buttermilk whey could be affected by the contaminating Ca²⁺ content and the absence of MFGM-specific protein components as discussed in Chapter 3 and Chapter 6. Modification of the MF parameters is still needed in order to obtain MFGM material from buttermilk whey with a lower mineral content.

The use of BM-MFGM, BMP, SMP and SC in which the concentration of PLs and the composition of proteins were different, was shown to have a significantly different effects on the properties of emulsions such as particle size distribution, microstructure, rheology and stability. Emulsions prepared with BM-MFGM had characteristics similar to SC emulsions, with a narrow particle size distributions, a very low viscosity and a Newtonian flow behaviour. In addition, emulsions prepared with BM-MFGM had a good stability compared to those of SC. Thus, BM-MFGM material has superior emulsifying properties as compared to BMP, SMP and SC and may present an alternative ingredient in food products. This can be caused by the difference in composition of the four materials of which BM-MFGM contained the highest concentration in PLs, exhibited the highest band intensities of MFGM-specific proteins and the lowest casein-to-whey proteins ratio and ash content. To get a better understanding of the participation of MFGM components, different MFGM materials and a mixture of MFGM material and BMP, different in
composition, were investigated. It was found that the compositions of the MFGM materials isolated from different sources were significantly different from each other. The emulsifying properties of MFGM materials were strongly influenced by their compositions (i.e. PLs, MFGM-specific proteins, whey proteins, caseins, and minerals) and especially the levels at which these substances were present. The use of BM-MFGM or commercial Lacprodan®PL20 (individually or mixed with BMP as emulsifier) in the preparation of emulsions resulted in similar particle size distribution patterns, microstructures and rheological properties, despite the fact that the PLs content in BM-MFGM material was 2.5 times lower compared to that of Lacprodan®PL20. Moreover, emulsions prepared with Whey-MFGM contained the highest amount of PLs together with the presence of many large aggregates of droplets and showed shear-thinning behavior. The absence of MFGM-specific proteins and the presence of high amount of mineral in Whey-MFGM material could influence the interfacial layer of emulsions. BM-MFGM contains both MFGM-specific proteins and PLs, therefore it was chosen as MFGM material for further investigated influence of individual concentration of PLs and MFGM proteins on the emulsifying properties of oil in water emulsions.

Solvent extraction was applied to fractionate BM-MFGM into two materials: MFGM protein and polar lipid concentrate. The influence of the concentrations of PLs and proteins, separately or in combination, on the physicochemical properties of oil-in-water emulsions and possible interaction between them at the interface during emulsification was elucidated. It was found that at a low emulsifier concentration (< 2.3% in total), the combination of both proteins and PLs in oil-water emulsion led to a positive interaction between proteins and PLs resulting in an enhancement in their emulsifying properties, especially in the formation of an emulsion exhibiting good stability and low apparent viscosity. Proteins had a greater impact on the stabilization of oil droplet emulsions than PLs. The surface characteristics and adsorption behavior at the surface of oil-in-water emulsions were dependent on the concentrations and the type of emulsifier (i.e. MFGM proteins or PLs) used. The distributed-to-surface proportion of proteins decreased with increasing protein concentrations added, whereas the surface adsorbed proportion of PLs increased with increasing the used concentrations of PLs. At identical concentration (2.3%), more material (about 4.8 times more) was needed to cover the oil/water interface for PLs (i.e. 9.68 mg m\(^{-2}\) of PLs load) than for MFGM proteins emulsions (i.e. 2 mg m\(^{-2}\) of protein load). No competitive displacement between proteins and PLs was observed at the surface of these emulsions. Between the two MFGM fractions, MFGM protein concentrate had greater emulsifying/stabilizing properties compared to the PLs concentrate. Differences in
microstructure of the interfacial domain between emulsions prepared with MFGM proteins and those prepared with PLs were also found. While, after freeze-fracture, almost all droplets in emulsions prepared with MFGM protein concentrate were broken across the interfacial layer, the droplets in emulsion prepared polar lipid concentrate were broken along the interfacial layer and the droplets were almost always intact. The reason behind this phenomenon still needs to be investigated.

Overall, the conclusions and the possible mechanisms based on the results of this dissertation for emulsifying ability of MFGM components in oil-in-water emulsions can be summarized in Figure 7-1.

**Figure 7-1.** Schematic presentation of possible mechanisms (not to scale) for the stabilization of oil-in-water emulsions with milk protein (A), MFGM PLs (B), MFGM proteins (C) and a mixture of MFGM proteins and PLs (D).
MFGM was also found to affect the physicochemical and whipping properties of RC. Preliminary experiments were carried out to evaluate the possibility of using MFGM materials to improve the whipping properties of RC. It was found that the physicochemical and whipping properties of RC were strongly affected by the concentrations and composition of MFGM components in the aqueous phase. When using BM-MFGM as an emulsifier, the crystallization started at higher temperature and the crystals growth and the $\alpha-\beta'$ polymorphic transition was accelerated compared to the natural cream. The overrun of whipped RC prepared with BM-MFGM as emulsifier (BM-MFGM100) increased. RC prepared with Whey-MFGM as emulsifier (Whey-MFGM100) led to a long churning time due to the presence of a high amount of whey proteins, PLs, minerals and a low amount of SFC. Amongst the four emulsifier materials, namely BM-MFGM, Whey-MFGM, Lacprodan®PL20 and BMP, Whey-MFGM had the highest potential to improve the whipping properties of RC. In contrast, MFGM material had inferior emulsifying/stabilizing properties in oil-in-water emulsions containing 35% soybean oil compared to BM-MFGM and Lacprodan®PL20. This indicated that the emulsifying properties of MFGM material isolated from dairy by-products depended on the sources for isolating the material and the types of applied products.

7.2 Future perspectives in MFGM research

Due to the health-promoting properties and beneficial technological functionalities of MFGM material, it is an interesting and novel ingredient for the development of emulsified products. Based on the findings of this work further work could be undertaken:

- An attempt was made to investigate individual and interacting effects of MFGM protein and polar lipid concentrate on the surface of oil-in-water emulsions. However, the present study still did not evaluate the effect of contaminating proteins (caseins and whey proteins) and MFGM-specific proteins separately. Fractionation and purification procedures for the production of MFGM-specific proteins should be further investigated. In addition, the MFGM protein and polar lipid were separated by solvent extraction which is considered as an unfriendly method to human health and environment. To be more applicable to food use, another extraction method should be developed.

- The differences in the types and chemical structures of PLs or alternative interactions between individual PLs or interactions with other membrane components, could be of importance for the emulsification and stabilization of oil-in-water emulsions. This study overviewed the interaction between PLs and MFGM proteins at the surface of O/W emulsion regardless their molecular
structure and their composition in fatty acid, which may affect the emulsifying properties in different ways. Future research should be done, investigating the impact of different polar lipid species on their emulsifying properties.

- The influence of the functional components of the MFGM on the microstructural properties of emulsified products and the difference in compositions need to be examined. Confocal laser scanning microscopy (CLSM) could be a useful mean to visualize heterogeneities in the distribution of PLs and MFGM proteins relating to the treatment during milk processing. The effect of the isolation procedures on the composition of the MFGM isolate should be investigated. Then the effect of different composition of these MFGM isolates on the characteristics of emulsions could be investigated by double-staining the surface of the milk fat globules, followed by CLSM observation.

- Although the possibility of using MFGM material to improve the whipping properties of RC was noticed, the relation between the physical properties of individual MFGM components and their whipping properties is still left to be unraveled. This aspect could be another interesting topic for future research.

While milk protein isolates, caseinate and whey protein concentrates are commonly used as functional ingredients in a wide variety of food products, less attention has been given to the protein fraction derived from the milk fat globule membrane. Only a few reports are available on the preparation of MFGM from commercially available sources and the opportunities to exploit fully the utilization of MFGM as a functional material are so far limited by the lack of available products and commercially feasible preparation methods. The development of methods for the extraction of MFGM from buttermilk, a low value by-product from the dairy industry, through microfiltration may increase the opportunity to produce this ingredient on a commercial scale. The big challenge lies in a high cost and a low yield of the isolates. In addition, the use of processed MFGM material may result in different effects compared with native MFGM. This has to be taken into account when developing products with desired and controlled characteristics. Therefore, before the economics of such processes can be appreciated, the unique functionality of MFGM isolates needs to be understood better.
References


References


References


References


References


Tadros, F. T. 2013. Emulsion formation, stability and rheology Wiley-VCH Verlag GmbH & Co.KGaA.


References


PHAN Thi Thanh Que was born on July 23rd, 1974 in Long An, Viet Nam. She obtained a Bachelor in Food Technology in 1997 from Can Tho University, Viet Nam. Since then she has been a lecturer at Department of Food Technology, Can Tho University, Viet Nam. In 2000, she was granted a scholarship by the Flemish Interuniversity Council (V.L.I.R) to follow a two-years master study in Postharvest and Food Preservation Engineering at the Katholieke Universiteit Leuven in Belgium. After Msc. graduation, she continued teaching at Department of Food Technology, Can Tho University.

In June 2010, she started her PhD research with financial support of the Ministry of Education and Training, Viet Nam (MOET, project 322) in the Laboratory of Food Technology and Engineering, Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University. Her research led to a PhD dissertation entitled “Functionality of milk fat globule membrane material” under supervisions of Prof. dr. ir. Koen Dewettinck and Dr. Thien Trung Le (from Faculty of Food Science and Technology, Nong Lam University, Viet Nam). During her research, she supervised several master thesis students, and joined in other scientific as well as social activities in the lab. She is author of several publications in peer-reviewed scientific journals and has attended several international and national scientific conferences.

Publications in international peer-reviewed articles


Submitted manuscripts


Chapter in book


Contributions at international conferences and PhD symposium

Poster presentations

Phan, T. T. Q., Xu, H. and Dewettinck, K. Effect of milk fat globule membrane enriched materials on partial coalescence and whipping properties of recombined cream. Poster presentation at 18th PhD symposium on Agricultural and Applied Biological Sciences, 8th February 2013, Ghent University, Gent, Belgium.


Conference proceeding


Oral presentation