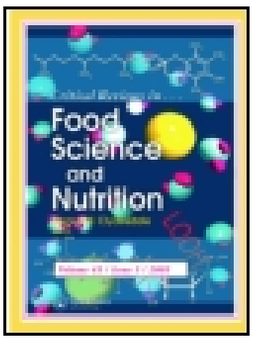

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Milk fat globule membrane glycoproteins: valuable ingredients for lactic acid bacteria encapsulation?

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Abstract

The membrane (Milk Fat Globule Membrane – MFGM) surrounding the milk fat globule is becoming increasingly studied for its use in food applications due to proven nutritional and technological properties. This review focuses first on current researches which have been led on the MFGM structure and composition and also on laboratory and industrial purification and isolation methods developed in the last few years. The nutritional, health benefits and techno-functional properties of the MFGM are then discussed. Finally, new techno-functional opportunities of MFGM glycoproteins as a possible ingredient for Lactic Acid Bacteria (LAB) encapsulation are detailed. The ability of MFGM to form liposomes entrapping bioactive compounds has been already demonstrated. One drawback is that liposomes are too small to be used for bacteria encapsulation. For the first time, this review points out the numerous advantages to use MFGM glycoproteins as a protecting, encapsulating matrix for bacteria and especially for LAB.

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Keywords

Milk fat globule membrane; lactic acid bacteria; encapsulation; interaction; techno-functionality; nutritional properties

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1. Introduction

The milk fat globule membrane (MFGM) *i.e.* the thin membrane surrounding the milk fat globule reveals to be especially interesting due to its specific composition in proteins and polar lipids which makes it an excellent candidate to develop a wide range of new innovative applications such as emulsifiers, stabilizers, and health promoters in food and non-food outlets (Dewettinck et al., 2008; Jiménez-Flores & Brisson, 2008; Singh, 2006). Buttermilk and butter serum are two dairy products particularly rich in MFGM. Both are low-cost and available in large quantities but have been considered invaluable by-products of the milk-fat industry for years. However over the last two decades they have gained considerable attention due to their high MFGM content (Vanderghem et al., 2010). Numerous works were developed to isolate and purify MFGM from milk fat globule, making it easier to study the MFGM properties (Corredig & Dalgleish, 1997; Dewettinck et al., 2008; Fong et al., 2007; Holzmüller et al., 2016a; Sachdeva & Buchheim, 1997; Singh, 2006; Ye et al., 2002).

Recently, the MFGM fraction was used for encapsulating bioactive compounds. The properties of MFGM-derived phospholipids to form liposomes have been optimized to encapsulate bioactive compounds such as lactoferrin, vitamin C, tea polyphenol or β-carotene (Farhang et al., 2012; Gülseren et al., 2012; Jin et al., 2016; Liu et al., 2013; Thompson et al., 2009). However, to the best knowledge of the authors, the potential interest of MFGM glycoproteins in the encapsulation field and especially for Lactic Acid Bacteria (LAB) has never been investigated. Most LAB are likely to benefit human health and therefore present a probiotic potential (Gilliland, 1990; Naidu et al., 1999). Due to their positive health effects

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LAB are incorporated into many food products. LAB encapsulation is a very widespread method used to entrap bacteria and protect their viability and functionality during food storage and digestion (Cook et al., 2012; Tripathi & Giri, 2014; de Vos et al., 2010). The scientific community constantly seeks to improve the encapsulation efficiency of LAB with more efficient encapsulation materials. Due to the innovative properties of MFGM, its health benefits (Dewettinck et al., 2008; Singh, 2006), its ability to bind with LAB (Brisson et al., 2010; Laloy et al., 1996; Lopez et al., 2006; Oberg et al., 1993; Tunick et al., 1993), and to recent improvements of the purification methods used for their extraction (Corredig et al., 2003; Holzmüller et al., 2016a; Le et al., 2009; Rombaut et al., 2007; Sachdeva & Buchheim, 1997), MFGM could be potentially used as a new component for LAB encapsulation. This review focuses on the potential interest of MFGM glycoproteins in LAB encapsulation. The structure, composition, health benefits, binding properties and techno-functional properties of the MFGM are successively approached in this paper. The last part of the paper presents arguments in favor of using MFGM as a new material for LAB encapsulation.

2. Milk fat globule membrane

a. Structure

The milk fat globule is composed of a triacylglyceride core surrounded by the MFGM. The MFGM is organized into a trilayer structure with a thickness of 10 – 50 nm. The monolayer inner membrane contains proteins and polar lipids derived from the endoplasmic reticulum. The outer membrane is a double layer also containing proteins and polar lipids, both coming from the apical plasma membrane of the mammary epithelial cells which surrounds fat globules during

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their secretion (Heid & Keenan, 2005). The MFGM structure is schematically represented on **Figure 1 – insert 1**. Recently, microscopy techniques such as Confocal Laser Scanning Microscopy (CLSM) improved knowledge of the MFGM structure and organization (Evers et al., 2008; Lopez et al., 2010). Evers et al. (2008) used lipophilic probes and lectins respectively to identify MFGM composition and structure heterogeneities. Lopez et al. (2010) also used specific probes for triacylglycerols, glycolipids and glycoproteins to identify some MFGM structural features such as the presence of a rigid domain composed of sphingomyelin and cholesterol, as well as the existence of a heterogeneous area with glycolipids and glycoproteins.

b. Composition

The MFGM consists in phospholipids, sphingolipids, and membrane-specific proteins, mainly glycoproteins. Many papers dealing with the MFGM composition have already been published (Dewettinck et al., 2008; Elías-Argote et al., 2013; El-Loly, 2011; Mather, 2000; Singh, 2006; Smoczyński et al., 2012). The MFGM composition can differ a lot from one study to another. The reasons for these changes in composition include: cow's age, milk microbiological quality, lactation stage and/or the season. Also, the literature attests that the choice of the MFGM isolation method can introduce many drawbacks and therefore variations (Evers, 2004). The major components of the bovine MFGM are described in the following paragraphs.

i. Composition of the lipid fraction

Bovine MFGM lipids are mainly composed of polar lipids such as phospholipids. Triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acids, and sterols are also present in the MFGM lipid fraction (**Table 1**). Many reviews mentioned a high level of triacylglycerols (56

– 62%) in MFGM although this must be attributed to the isolation methods. During MFGM isolation, triacylglycerols from the core of the milk fat globule can easily contaminate the MFGM fraction (Walstra, 1985).

Polar lipids are minor amphiphilic milk fat components. They stand for 0.2 – 1% of the total milk lipids (Lopez, 2011) but for 26 – 40% of the MFGM lipids (Fong et al., 2007; Sánchez-Juanes et al., 2009; Singh, 2006; Smoczyński et al., 2012). They are constituted of a hydrophobic tail combined with a hydrophilic head group and are mainly found in the MFGM. MFGM polar lipids are rich in phospholipids and sphingolipids. The major polar lipids found in all mammal species are: phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), lysophosphatidylcholine (LPC), and sphingomyelin (SM) (**Table 1**).

ii. Composition of the protein fraction

Bovine MFGM proteins stand for only 1 – 2% of the total bovine milk proteins but for 25 – 70% of the MFGM composition (Elías-Argote et al., 2013; Riccio, 2004). Their concentrations depend on the purification method used to obtain the MFGM (Holzmüller et al., 2016a, 2016b). The MFGM is composed of about 40 proteins ranging from 15 to 240 kDa (Dewettinck et al., 2008; Mather, 2000; Ye et al., 2002). The major proteins of the bovine MFGM are the mucin MUC1, the redox enzyme xanthine dehydrogenase/oxidase (XDH/XO), butyrophilin (BTN), cluster of differentiation (CD36), Periodic Acid Schiff 6/7 (PAS 6/7), Periodic Acid Schiff III (PAS III, also called MUC15), adipophilin (ADPH) and fatty-acid binding proteins (FABP). All proteins are glycosylated except the latter two which are also smaller (Dewettinck et al., 2008; Mather, 2000; Ye et al., 2002) (**Table 1**).

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In addition to the major MFGM proteins other proteins are present in smaller amounts within the bovine MFGM. These proteins include enzymes, immunoglobulins, proteins derived from the cytoplasm of secretory-epithelial cells, skim milk constituents, and proteins from milk leucocytes (Fong et al., 2007; Mather, 2000) which are exhaustively listed by Mather (2000).

c. Isolation and purification

MFGM can be isolated from raw milk (Le et al., 2009), buttermilk, butter serum, or whey (Corredig et al., 2003; Holzmüller & Kulozik, 2016; Rombaut et al., 2007; Sachdeva & Buchheim, 1997). The MFGM isolation in laboratory consists in a four-step procedure: fat globule separation, cream washing, MFGM release from the globules, and MFGM material collection (Dewettinck et al., 2008; Singh, 2006) (**Figure 2**). Isolation of fat globules from milk may be performed using either centrifugation or a top-cream separator (Dewettinck et al., 2008). Once isolated, the cream is washed several times with a buffer: two (Ye et al., 2002), three (Fong et al., 2007) or more times (Sánchez-Juanes et al., 2009) depending on the authors. Dewettinck et al. (2008) proposed an exhaustive list of buffers used to wash the cream. Distilled or deionized water, sucrose saline solution with or without pH buffering, isotonic phosphate buffer solution, phosphate-saline buffer, or simulated milk ultrafiltrate are all examples of available buffers. The MFGM is then released from the triacylglyceride fat core using various methods including churning, stirring, cycles of freezing and thawing, treatment with detergents, suspension in polar and aprotic or bile salts solvents (Elías-Argote et al., 2013; Singh, 2006). The final step consists in the release of MFGM material. The membrane can be collected by ultracentrifugation *i.e.* typically 90,000 to 100,000 × g for 60 min (Singh, 2006), proteins precipitation at low pH,

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induction of membrane aggregation with ammonium sulfate followed by ultracentrifugation, freeze drying or microfiltration (Dewettinck et al., 2008; Elías-Argote et al., 2013).

At an industrial scale, buttermilk and butter serum are used as a suitable source of MFGM (**Figure 3**). They are the main by-product of the butter-making process and their current commercial value is somewhat limited. Buttermilk and butter serum have gained a lot of attention thanks to the many potential applications of MFGM as an ingredient used to design healthy products. Many studies focused on the isolation of MFGM from the buttermilk. Filtration methods were developed to isolate MFGM with a high protein content. Residual lactose and whey proteins can be removed easily from buttermilk via microfiltration due to their small size (Holzmüller & Kulozik, 2016) but some difficulties can occur with residual casein micelles due to the similarity in size between MFGM fragments and micellar caseins (Sachdeva & Buchheim, 1997). Casein micelles should consequently be removed from buttermilk before filtration. Sachdeva & Buchheim (1997) used rennetting or acid coagulation and subsequent microfiltration with a membrane of 0.2 µm to eliminate casein micelles from buttermilk. Another approach consists in dissociating casein micelles in smaller particles using sodium citrate and then proceed to high speed centrifugation (Corredig & Dalgleish, 1997). Microfiltration with a 0.1 µm pore size membrane instead of a high speed centrifugation may improve caseins removal with a reduction of casein micelles contamination to only 6% final (Corredig et al., 2003). Recently, Holzmüller & Kulozik (2016) followed a two-step approach to isolate MFGM from milk proteins. Native casein micelles were removed from buttermilk by rennet-induced coagulation and then residual whey proteins were eliminated using diafiltration in order to obtain a purified MFGM fraction. With this technique, 70% of peripheral membrane proteins were recovered.

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Either at laboratory and industrial scale, isolation techniques are always responsible of material losses such as protein or phospholipids. For example, cream washing impacts MFGM proteins at laboratory scale. After three washing steps, only 30% of PAS 6/7 and 10% of BTN or XO/XDH were found in washed cream compared to non-washed cream (Holzmüller et al., 2016b) (**Figure 2**). At industrial scale, the MFGM proteins yield is influenced by the pH and the temperature of the rennet-induced coagulation of casein micelles before microfiltration. A high pH and a low temperature improve the MFGM proteins retention yield in buttermilk whey (Holzmüller et al., 2016a). Sachdeva & Buchheim, (1997) showed that processing (coagulation and microfiltration) affects the phospholipids recovery: only 70 – 77% phospholipids from the MFGM were recovered.

d. Nutritional and health benefits of MFGM

MFGM has gained considerable attention due to its nutritional and health benefits. Many studies show the important role of some individual MFGM components when isolated, such as its proteins and polar lipids. Others also feature health benefits associated with MFGM diet supplementation (**Table 2, 3 and 4**). Due to their positive health effects MFGM are expected to be more and more used in functional foods.

i. Health benefits brought by MFGM proteins

Some MFGM proteins have been found to feature anticancer effects (**Table 2**). Fatty acid binding proteins (FABP) are able to inhibit the growth of breast cancer cell lines at low concentrations (Spitsberg & Gorewit, 2002). The inhibitory action of FABP has been demonstrated through their interaction with the cluster of differentiation CD36 (Gorewit &

Spitsberg, 1998). Vissac et al. (2002) evaluated the presence of breast cancer susceptibility proteins (BRCA1 and BRCA2) in milk. These proteins also inhibit the growth of various cell types including breast cancer-inducing cells. They are also implicated in DNA repair process and BRCA2 was one of the direct regulators of cytokinesis (Daniels et al., 2004; Vissac et al., 2002).

MFGM proteins possess antimicrobial, antiviral, and antiadhesive effects (**Table 2**). XDH is involved in the production of reactive oxygen species including superoxide, hydrogen peroxide, nitric oxide, and peroxynitrite involved in inflammatory responses and antimicrobial activities (Harrison, 2006; Martin et al., 2004). Both proteins MUC1 and PAS6/7 present protective effect against rotavirus infection (Kvistgaard et al., 2004; Newburg et al., 1998; Yolken et al., 1992). In addition, MUC1 prevents pathogenic bacteria from binding to Caco-2 cells such as *E. coli*, *S. thyphimurium*, *S. aureus* and *B. subtilis* thanks to its antiadhesive properties (Parker et al., 2009).

BTN and PAS6/7 impact positively the behavior as well as the central nervous system disorders (**Table 2**). BTN is able to suppress multiple sclerosis (Berer et al., 2005) and to modulate positively experimental autoimmune encephalomyelitis, a pathogenic autoimmune response to myelin oligodendrocyte glycoprotein (Stefferl et al., 2000). Beside, BTN influences pathogenesis of autistic behavior (Vojdani et al., 2002). PAS6/7 is involved in epithelization, cell polarization, cell movement and rearrangement, neurite outgrowth, and synaptic activity in the central nervous system (Riccio, 2004). It was also demonstrated that the administration of exosomes encapsulated with MFG-E8 may be an interesting therapeutic approach in sepsis. MFG-E8 is a glycoprotein that was originally identified as a component of milk fat globules budding from the mammary epithelia during lactation. MFG-E8 is able to accelerate the clearance of accumulating

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apoptotic cells like B cells, CD4 T cells, DCs, vascular endothelial cells, and enteric epithelial cells in sepsis (Wu et al., 2017).

ii. Health benefits brought by MFGM polar lipids

Sphingomyelin (SM) is the major sphingolipid membrane component and features many demonstrated health benefits (Dewettinck et al., 2008) (**Table 3**). SM influences colorectal cancer by inhibiting both early and late stages of colon carcinogenesis in mice (Dillehay et al., 1994). A dietary SM supplementation decreases colonic inflammation and inflammation-drive colorectal cancer (Mazzei et al., 2011). Zhang et al. (2008) explained this positive effect on colon carcinogenesis with an upregulation of colonic alkaline sphingomyelinase, which is a key enzyme involved in SM digestion in the gut. SM digestion generates biologically active products regulating cell growth, differentiation, and apoptosis involved in colorectal cancer (Hannun & Obeid, 2008). SM diet is also known to attenuate the negative effects of high-fat diet which include high serum and hepatic lipids contents, gut dysbiosis, intestinal barrier dysfunction, etc. For example, SM decreases hepatic steatosis development with lower hepatic triacylglyceride and cholesterol rates (Norris et al., 2017). After 4 weeks of SM diet, high-fat-fed mice had gained less weight and had reduced serum cholesterol compare to high-fat-fed mice without SM diet (Norris et al., 2016). Other work also demonstrated that SM diet presents lower hepatic lipid-lowering properties and lower cholesterol intestinal absorption (Chung et al., 2013; Eckhardt et al., 2002). SM also features many other acknowledged health effects and plays an important role during neonatal gut maturation (Mutsumi et al., 2003), myelination of the developing central nervous system (Oshida et al., 2003), and in the resistance to certain types of

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food-born gastrointestinal infections induced by *Listeria monocytogenes*, *Campylobacter jejuni* or *Salmonella enteritidis* (Sprong et al., 2002).

Other phospholipids of the MFGM present health benefits (**Table 3**). PS seems to be able to reduce many neuronal ageing-induced disorders in animals. PS has shown to restore normal memory on a variety of tasks (McDaniel et al., 2003) and to improve memory for people with Alzheimer (Zhang et al., 2015). PS also enhances the exercise capacity of exercising humans (Kingsley, 2012). PC, which is one of the most abundant MFGM phospholipids, can support clinical liver recovery after a toxic chemical attack, or acute or viral damage (Kidd, 2002), reduce necrotizing enterocolitis in hospitalized preterm infants (Carlson et al., 1998) and protect human gastrointestinal mucosa against toxic attack by decreasing acute aspirin-induced damage on gastric mucosa (Anand et al., 1999). Other phospholipids such as PI or LPC can respectively promote plasma cholesterol transport and metabolism or display a strong gastro-protective role in the gastric mucosa against irritants (Kivinen et al., 1995; Stamler et al., 2000).

iii. Health benefits brought by a MFGM-supplemented diet

In addition to the health effects featured by its individual components, MFGM can be used without further purification as a food supplement for *in vivo* and clinical studies (**Table 4**).

The MFGM possesses protective effect against colon carcinogenesis. The anticarcinogenic capacity of MFGM was demonstrated over the colorectal carcinoma cell lines HT-29. The presence of MFGM decreased the cell proliferation and activated one of the major markers of programmed cell death (Zanabria et al., 2013). A dietary MFGM conferred prevention of colon cancer in Fisher-344 rats by decreasing the incidence of aberrant crypt Foci (Snow et al., 2010).

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Defatted bovine MFGM, a fraction rich in proteins and glycoproteins, has been found to prevent enterohemorrhagic *E. coli* adhesion on HT-29 human cells (Ross et al., 2016). A novel infant milk formula (Nuturis®) has been developed to mimic the human milk fat globule structure with large fat droplets recovered by phospholipids, milk proteins, and cholesterol (Gallier et al., 2015). Indeed, the milk fat structure of human milk and infant milk formula are totally different. Human milk contains dispersed fat droplets with large fat droplets enveloped by a phospholipid membrane whereas infant milk formula contains small fat droplets primarily coated by proteins. The novel infant milk formula proposed by Gallier et al. (2015) is supposed to feature infant health benefits by mimicking more closely the structure of human milk fat globules. A recent study led on mice proved that mice fed with this formula present a healthier growth and a decrease of body fat accumulation during adulthood (Baars et al., 2016).

Many clinical studies also explored the effects of MFGM supplementation on infant and children diets using double-blinded randomized controlled trials (Hernell et al., 2016). Zavaleta et al. (2011) supplemented infant food with a MFGM-enriched protein fraction (Lacprodan MFGM-10; Arla Foods Ingredients, Viby, Denmark). Children (from 6- to 11-month-old) supplemented with MFGM presented lower longitudinal prevalence of diarrhea and lower incidence of bloody diarrhea than children with no MFGM supplementation. Veereman-Wauters et al. (2012) evaluated the effect of MFGM-enriched phospholipidic fraction (INPULSE; Büllinger SA, Büllingen, Belgium) on young children (around 4 years old). Fever remained fewer days amongst children under supplementation; these children also feature lower parental scoring of internal, external, and total behavior problems. Another team tested the supplementation of infant food with an MFGM-enriched protein fraction amongst 2- to 6-month old children

(Lacprodan MFGM-10; Arla Foods Ingredients, Viby, Denmark). Children presented higher cognitive scores (Timby et al., 2014a), a reduction of the risk of acute otitis (Timby et al., 2015), immunomodulatory effects on humoral response against *Pneumococcus* vaccine (Timby et al., 2015), and a higher total serum cholesterol (Timby et al., 2014b). Another clinical study using double-blinded randomized controlled trials tested the dietary supplementation with MFGM on adult diets. This supplementation associated with habitual exercise improved the physical performance of middle-aged Japanese adults (Ota et al., 2015). MFGM supplementation also improved endurance capacity and muscle function in these middle-aged adults; this was probably due to stimulation of the neuromuscular development (Haramizu et al., 2014; Soga et al., 2005). Demmer et al. (2016) compared the influence of high-fat meals composed of palm oil vs. palm oil with MFGM on obese adults. MFGM supplementation reduced the negative effects induced by high-saturated fatty acids and reduced postprandial cholesterol, inflammatory markers, and insulin response.

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3. Interaction between bacteria and MFGM-glycoproteins

a. Lactic acid bacteria-MFGM interaction

In raw milk, caseins and whey proteins, lactose, and fat globules structure the matrix. LAB are also present and the study of their location can reveal their adhesive preferences for the different milk components.

Food structuration as well as bacteria spatial distribution have often been investigated and especially during cheese manufacture and ripening. Bacteria location in the matrix was found to play an essential role during the ripening and the flavors development steps in cheeses (Hickey et

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al., 2015a). Various microscopy methods were used in order to be able to quickly detect, count, and visualize bacteria in cheese matrices (Hickey et al., 2015a, 2015b). For example, Laloy et al. (1996) visualized bacteria location in Cheddar cheese with Transmission Electron Microscopy (TEM). Microstructure and fat concentration were found to influence bacteria location and retention in cheese. Comparison between free, reduced, and full fat Cheddar highlighted the importance of fat to improve bacteria retention in the matrix. Also, the preferential location of bacteria was observed to be directly in contact with the MFGM or at the casein-fat interface. Besides, after 1--2 months of ripening, bacteria were entrapped in the MFGM. The organization of fat, protein, and bacteria was observed using CLSM in Emmental cheeses during ripening. Bacteria location was again systematically close to the whey proteins/fat interface (Lopez et al., 2006). Others studies used Scanning Electron Microscopy (SEM) to study the microstructure of Mozzarella cheese; bacteria were located respectively either at the whey proteins-fat interface or at the fat globule surface (Oberg et al., 1993; Tunick et al., 1993). Infant formula containing LAB with MFGM components was explored using electron microscopy and the association of these two entities has recently been patented (Benyacoub et al., 2011). All these microscopic observations revealed the preferential location of bacteria at the protein-fat interface or directly in contact with the MFGM. However, some precautions should be taken when interpreting microscopy images. Even if microscopy methods are now well mastered, the observation may be affected by the presence of artifacts and results may then be misinterpreted (Burgain et al., 2017). With SEM, the native morphology of the sample may be affected in the chamber vacuum. Artifacts may be the result of unfavorable specimen-beam interaction in SEM. The deleterious preparation procedures required for TEM may also create artifacts during the observation.

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Concerning CLSM, the method requires the addition of suitable fluorescent probes and the protocol involves the use of solvents or water which can affect the sample integrity (Burgain et al., 2017). Taking into account the important part of artifacts in the microscopic image interpretations, microscopic observations may only allow to roughly observe the bacterial location in the cheese matrices but these results need to be confirmed using other methods.

In the literature, other techniques were used to investigate the interactions between LAB and food matrices. Brisson et al. (2010) used sucrose density gradient, bacterial DNA quantification, binding rate, and force measurements with optical tweezers or CLSM to visualize and characterize interactions between *Lactobacillus reuteri* and MFGM. Interactions between LABs and MFGM depend on the environment and the bacterial surface composition. Brisson et al. (2010) demonstrated that the bacterial surface hydrophobicity played a key role in the adhesion with MFGM. A microbial adhesion to hydrocarbon test (MATH) was used to correlate the hydrophobic cell surface properties of the bacteria to their affinity to MFGM. Bacteria with hydrophobic surface presented a high affinity toward hydrophobic compounds such as MFGM. The hydrophobic surface properties of bacteria depended on their surface composition. The presence of many proteins (Brisson et al., 2010) on their surface but also of other compounds such as peptidoglycans, exopolysaccharides, or (lipo)teichoic acids improved bacterial hydrophobicity. Therefore, bacterial surface hydrophobicity could be a good indicator to predict bacterial adhesion toward MFGM in dairy products.

Atomic force microscopy (AFM) in force mode was also used to better characterize bacteria and food matrices interactions (Burgain et al., 2014). Personal author's works used AFM to study the

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interaction between *L. rhamnosus* GG and MFGM (unpublished data of our team). For this, the bacteria and MFGM were adsorbed on a functionalized mica and an AFM probe, respectively. The experimental procedure requires bringing the AFM probe coated with MFGM into contact with the bacterial surface in order to form a molecule-molecule complex governed by intermolecular bonds. When the AFM probe is retracted from the substrate surface, a retraction curve is recorded and the analysis of the retraction curve allowed to detect the presence and to determine the nature of the interaction between bacteria and MFGM. When the probe was nude (**Figure 4A**), the retraction curve was linear, without rupture peaks. This profile revealed the absence of interactions between the probe and the bacteria. On the contrary, when the AFM probe was coated with MFGM (**Figure 4B**), numerous rupture peaks were observed that demonstrated the existing interactions between *L. rhamnosus* GG and the MFGM.

Quantitative affinity measurements between LAB and various milk lipids were performed by Bacherio et al. (2007) using immunoblotting techniques. No interactions with triacylglycerol were found; interactions with phospholipids were however revealed. Since the MFGM is essentially composed of polar lipids, LAB may interact with the MFGM through their direct binding with polar lipids. A dairy matrix was hypothesized in **Figure 1** which summarizes the information found in the literature about bacteria location preferences. The dairy matrix could represents cheese, yoghurt or a milk product. All these matrices are composed of fat globules surrounded by the MFGM, milk proteins as caseins or whey proteins, and lactose. The different compounds are represented in **Figure 1**. According to the literature, bacteria seem to be preferentially located at the surface of the MFGM or at the proteins/fat globules interface. Bacteria may also be found in whey pockets (Lopez et al., 2006).

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b. MUC1: an important MFGM component involved in the interaction with LAB

The adhesion properties of LAB and especially of *Lactobacillus* strains on intestinal mucin have already been described (Nishiyama et al., 2016). Many cell wall-anchored proteins of the *Lactobacillus* surface have been reported to act as mucin adhesion factors: the mucus binding protein family of *L. acidophilus* and *L. reuteri* strains (Buck et al., 2005; Roos & Jonsson, 2002), the mannose-specific adhesin of *L. plantarum* (Pretzer et al., 2005), the mucus-binding factor of *L. rhamnosus GG* (von Ossowski et al., 2011), the mucus-binding protein A of *L. reuteri* strains (Etzold et al., 2014; Jensen et al., 2014), and the spaC subunits of spaCBA pili of *L. rhamnosus* GG (Etzold et al., 2014; Kankainen et al., 2009; von Ossowski et al., 2010; Reunanen et al., 2012). Other surface proteins such as the elongation factor Tu, the glyceraldehyde 3-phosphate dehydrogenase, and the chaperonin GroEL are present on *Lactobacillus* surface and expressed mucin-binding properties (Bergonzelli et al., 2006; Granato et al., 2004; Kinoshita et al., 2008). The proteins of the ABC transporters such as the protein Lam29 or the mucus adhesion-promoting protein (Macías-Rodríguez et al., 2009; Miyoshi et al., 2006) also feature mucin-adhesive factors. The large amount of mucin-adhesive factors found on LAB strains demonstrates the high affinity between LAB and the intestinal mucin.

There are many similarities between the MUC glycoproteins found on the MFGM and human mucin found on intestinal cells that need to be taken into account when exploring the role of MUC1 in the interaction with LAB. The intestinal human mucin and the MFGM MUC1 are both large proteins which are highly glycosylated (mainly O-glycosylated). They are both composed

of N-acetylgalactosamine, N-acetylglucosamine, fucose, galactose, sialic acid, mannose, and sulfate in different ratios (Bansil & Turner, 2006; Patton et al., 1995). Because these two glycosylated proteins have similarities, it can be hypothesized that the glycoprotein MUC1 found in the bovine MFGM interacts with LAB and govern their location in the food matrix.

To our knowledge, the binding capacity of MUC1 on LAB was never investigated but has already been demonstrated on pathogenic bacteria. A recent review reported the anti-adhesive properties of MFGM-associated glycoproteins, particularly the MFGM, against bacteria food enteropathogens (Douëllou et al., 2017). Parker et al. (2009) demonstrated that bovine MUC1 is able to bind enteric pathogen bacteria such as *E. coli*, *S. Thyphimurium*, *S. aureus*, or *B. subtilis*. The interaction Bovine MUC1 can also interact with *E. coli* and inhibit its binding to bovine mammary epithelial cells grown *in vitro* (Sando et al., 2009). Bovine MUC1 may thus be able to prevent the adhesion of bacteria onto epithelial surfaces (Parker et al., 2009; Sando et al., 2009) and consequently possesses anti-invasive characteristics. This effect was more pronounced for Gram negative vs. Gram positive bacteria. These two types of bacteria differ by the structure of their cell wall. Contrary to Gram positive bacteria, Gram negative bacteria possess an outer membrane with high lipid and phospholipids content. The interaction between phospholipids and intestinal mucin was recently highlighted (Li et al., 2017). Li et al. (2017) suggest that the presence of phospholipids on the cell walls of Gram negative bacteria may promote interaction with the bovine protein MUC1.

Results obtained on interactions between pathogenic bacteria and bovine MUC1 are promising but more work has to be done to confirm the few data published on the subject. Inhibition of

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pathogens adhesion through their binding with MFGM is an interesting way of research but it should be keep in mind that MFGM binding with pathogens may also have negative impacts. Indeed, it may increase the antibiotic resistance or the formation of biofilm (Ali-Vehmas et al., 1997; Pasvolsky et al., 2014). Also, pathogens binding in cell cultures is not obvious. It depends on many factors: cells growth, method used to follow the attachment of pathogenic bacteria to the cells. A standardized protocol needs to be created to compare and reproduce these findings.

By taking into account these studies one can wonder: what could happen if MFGM MUC1 can also bind LAB in a dairy matrix?

4. Techno-functional properties of MFGM

a. Properties which are already identified

i. Emulsion properties

MFGM is a natural emulsifier present in milk. Membrane components and especially phospholipids contribute largely to the MFGM emulsifying properties due to their amphiphilic properties. The MFGM emulsifying properties stabilize fat globules in milk. Shimizu et al. (1980) demonstrated the important role of MFGM proteins and glycoproteins on fat stabilization by interrupting the clustering of fat globules. They also highlighted the importance of MFGM phospholipids on fat globules stability since phospholipids interrupt the coalescence of fat globules thanks to repulsive forces. Kanno et al. (1991) studied the stability of a milk fat emulsion made with various amounts of MFGM fraction (20 to 80 mg MFGM material/g fat). MFGM fractions increase the stability of the fat globule emulsion. For emulsions containing the

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highest level of MFGM material, the stability of milk fat globules was similar to those of natural fat globules. Corredig and Dalgleish (1998) characterized the interface of emulsions prepared with a fraction of MFGM isolated from fresh raw milk. MFGM components were adsorbed onto the surface of oil-in-water emulsions; this confirms the good emulsifying capacity of MFGM. Roesch et al. (2004) separated MFGM fractions using microfiltration in the presence of citrate. MFGM in oil-in-water emulsions reduced the oil droplet size and improved creaming stability.

ii. Use of MFGM-derived phospholipids to encapsulate bioactive compounds

A liposome is a spherical vesicle formed through the self-assembly of amphiphilic molecules such as phospholipids with a diameter ranging from 20 nm to several microns. Liposomes are constituted of a bilayer with an aqueous core inside and a hydrophobic layer on the surface. Industrial applications concern cosmetic and pharmaceutic industries for the protection and release of nutraceuticals and drugs (Maherani et al., 2011; Singh, 2006). In food industry, liposomes can be used within many potential applications for the encapsulation of bioactive compounds (Kim & Baianu, 1991; Reineccius, 1995). Lecithin from soybean or egg yolk which is typically used in liposomes is very expensive (Mozafari et al., 2008; Thompson et al., 2006). MFGM has recently been proposed as an alternative to traditional materials (Thompson et al., 2006). Its rich phospholipid content combined with its low cost make it an ideal material to replace traditional lecithins. Thompson & Singh (2006) prepared for the first time liposomes using MFGM phospholipids. Their stability was significantly different from those made using soy phospholipids (Thompson et al., 2006). MFGM liposomes possess a higher phase transition

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temperature, a thicker membrane, and a lower membrane permeability. In addition, dispersions obtained from these liposomes were more stable for all studied pH during heat treatments and storage (4 – 35 °C) compared to soy lecithin-made liposomes (Thompson et al., 2006). It is suggested that MFGM-made liposomes are more resistant to environmental stresses due to their thicker membranes and to their different phospholipids organization within the membrane layer (Thompson & Singh, 2006).

Liposomes prepared with MFGM-derived phospholipids have been also studied to encapsulate bioactive compounds (**Table 5**). MFGM-made liposomes are better for the entrapment of hydrophobic (β-carotene *e.g.*) and hydrophilic (potassium chloride *e.g.*) compounds (Thompson et al., 2009), and of tea polyphenols (Gülseren et al., 2012). Jin et al. (2016) encapsulated curcumin (an antitumor, antioxidant and anti-inflammatory molecule) in various liposomes. MFGM liposomes presented a higher encapsulation efficiency, a higher zeta-potential, and a smaller particle size range; they also slow down *in vitro* release compared to lecithin liposomes. The dispersion and the stability of MFGM liposomes are also better than those of lecithin liposomes. Lactoferrin was also stabilized in MFGM-derived phospholipids liposomes (Liu et al., 2013). During *in vitro* gastric digestion, the entrapment efficiency of lactoferrin remained unchanged over time and was not function of pepsin concentration, whereas during the intestinal digestion the MFGM phospholipids were hydrolyzed by pancreatic lipase. MFGM liposomes were stable and able to protect proteins during the gastric phase with a final release in the intestine. Nanoliposomes prepared with MFGM-derived phospholipids were also used to stabilize acid ascorbic as a vitamin supplement or an antioxidant molecule (Farhang et al., 2012). During storage at 4 °C and after 7 weeks, 70% of vitamins remained in the liposomes.

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Considering all these studies MFGM-derived phospholipids liposomes seem to be very promising carriers for both hydrophobic and hydrophilic molecules.

b. A new potential techno-functional opportunity for the MFGM: the use of its glycoprotein fraction in encapsulation

This section highlights the potential role of the MFGM glycoproteins fraction in LAB encapsulation. Even if the ability of MFGM-derived fractions to form liposomes has already been exploited to encapsulate bioactive compounds, it has not been developed as a fraction for LAB encapsulation (Burgain et al., 2011). Many encapsulating materials are able to protect bacteria including alginate, κ-carrageenan, starch, gelatin, chitosan, or milk proteins (Burgain et al., 2011); but the scientific community constantly seeks to improve the encapsulation efficiency of LAB and therefore is always looking for new encapsulation materials. MFGM glycoproteins possess many advantages for LAB encapsulation. First, several arguments revealed the health benefits brought by both MFGM proteins and a MFGM diet supplementation (see section 2d of the review). The addition of MFGM glycoproteins in the matrix will be an advantage for improving consumer health. The second argument is the role of MFGM in the preferential location of bacteria within the food matrix (see section 3 of the review). A lot of studies dealing with the location of bacteria within cheeses matrices demonstrated the existing interaction between MFGM and LAB. It is thus suggested that this interaction may be mediated by a glycoprotein which could be MUC1 in the MFGM and a mucin-binding factor on the surface of bacteria. Burgain et al. (2014) recently demonstrated the role of interactions between bacteria and milk matrix compounds to increase the bacteria encapsulation efficiency and to improve its

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location inside the matrix in order to be more efficient for bacterial release within the intestine. A patent described interaction between LAB and MFGM (Benyacoub et al., 2011). Authors also believed that MFGM incorporation in microparticles may facilitate the transport of bacteria during the gastrointestinal digestion and ensure their delivery to the appropriate site (Benyacoub et al., 2011). By interacting with LAB, the MFGM glycoproteins may improve the bacterial location inside the microparticle. Bacteria are therefore more protected against stomach conditions (low pH, presence of pepsin) and can thus reach their target site (the intestine) while remaining in a viable and functional state.

The opportunity to use MFGM glycoproteins to encapsulate LAB requires the purification of the MFGM glycoprotein fraction. Up to now in this review, the purification and the isolation methods allowed to collect a MFGM fraction composed of a mixture of phospholipids and glycoproteins. However some studies demonstrated that the purification of MFGM glycoproteins is possible. One patent has already been issued on the isolation of MUC1 from bovine milk and whey (Yang et al., 2001) and another one was issued on the isolation of xanthine oxidase from raw whole milk (Zikakis, 1979). The PAS-6 and PAS-7 glycoproteins were also purified from the MFGM in another study (Kim et al., 1992).

The very good knowledge of the health benefits brought by MFGM glycoproteins, the role of MFGM in interaction with LAB and the promising researches on MFGM glycoproteins purification tend to show the great potential of MFGM glycoproteins for the formulation of new encapsulating matrices to entrap and protect bacteria until they can reach their target.

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Future trends

For a long time, buttermilk and butter serum were poorly valorized as they were considered by-products of the food industry. However, thanks to their high MFGM content and the growing interest of the scientific community and industry to this fraction, this position is changing. The demonstrated nutritional properties and the attractive physico-chemical properties (emulsion capacity, liposome formation, interaction with LAB, etc.) of the MFGM will promote the use of buttermilk and butter serum. This review proposes to develop the use of MFGM as new bioactive component for LAB encapsulation.

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Table 1: Lipids and proteins fractions of the MFGM (adapted from (Dewettinck et al., 2008; Fong et al., 2007; Jiménez-Flores & Brisson, 2008; Sánchez-Juanes et al., 2009; Singh, 2006; Smoczyński et al., 2012).

Group of compounds	**Percentage (%)**	**Molecular Weight (kDa)**
Triacylglycerols	56.0 – 62.0	-
Diacylglycerols	2.1 – 9.0	-
Monoacylglycerols	0.4	-
Free fatty acids	0.6 – 6.0	-
Sterols	0.2 – 2.0	-
Phospholipids	26.0 – 40.6	-
Sphingomyelin	18.8 – 22.0	-
Phosphatidylcholine	27.4 – 36.0	-
Phosphatidylethanolamine	27.0 – 33.0	-
Phosphatidylinositol	11.0	-
Phosphatidylserine	4.0	-
Lysophosphatidylcholine	2.0	-
Proteins	0.2 – 2.0	
BRCA1 and BRCA2	-	210
Mucin I (MUC1)	-	160 – 200
Xanthine oxidase (XO)	-	146 – 155
PAS III	-	94 – 100
CD36	-	76 – 78
Butyrophilin (BTN)	-	66 – 67
Adidophilin	-	52

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Periodic acid Schiff 6/7 (PAS 6/7) (lactadherin)	-	47 – 59
Proteose peptone 3 (PP3)	-	18 – 34
Fatty acid binding protein (FABP)	-	13 – 15

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Table 2: Health aspect of major milk proteins of the milk fat globule membrane.

Component	Health aspects	References
Mucin I (Muc1)	Antiadhesive effect	(Parker et al., 2009)
	Protective effect against rotavirus infection	(Kvistgaard et al., 2004; Yolken et al., 1992)
Xanthine dehydrogenase/oxidase (XDH/XO)	Antimicrobial agent	(Martin et al., 2004)
	Source of ROS/anti-inflammatory properties	(Harrison, 2006)
Butyrophilin (BTN)	Suppression of multiple sclerosis	(Berer et al., 2005)
	Development of experimental autoimmune encephalomyelitis	(Stefferl et al., 2000)
	Influence on pathogenesis of autistic behavior	(Vojdani et al., 2002)
	Regulation of immunity	(Rhodes et al., 2016)
Periodic acid Schiff 6/7 (PAS 6/7)	Protection from viral infections in the gut	(Kvistgaard et al., 2004; Newburg et al., 1998)
(lactadherin)	Epithelialization, cell polarization, cell movement and rearrangement, neurite outgrowth, synaptic activity in the central nervous system	(Riccio, 2004)
Fatty acid binding protein (FABP)	Breast cancer cells lines inhibition	(Gorewit & Spitsberg, 1998)
Cluster of differentiation (CD36)	Anticancer properties by interacting with FABP	(Gorewit & Spitsberg, 1998)
Breast cancer susceptibility proteins (BRCA1 and BRCA2)	Breast cancer DNA repair process inhibition	(Daniels et al., 2004; Vissac et al., 2002)

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Table 3: Health aspect of MFGM-derived phospholipids.

Components	Heath aspects	References
Sphingomyelin (SM)	Modulator of carcinogenesis	(Dillehay et al., 1994)
	Suppression of cholesterol absorption	(Chung et al., 2013; Eckhardt et al., 2002)
	Colon tumors inhibition	(Zhang et al., 2008)
	Neonatal gut maturation	(Mutsumi et al., 2003)
	Hepatic steatosis and adipose tissue inflammation attenuation	(Norris et al., 2017)
	Myelination of the developing central nervous system	(Oshida et al., 2003)
	Suppression of colonic inflammation and inflammation-driven colorectal cancer	(Mazzei et al., 2011)
	Protection against gastrointestinal infections	(Sprong et al., 2002)
	Lipids metabolism improvement	(Norris et al., 2016)
Phosphatidylcholine (PC)	Necrotizing enterocolitis reduction	(Carlson et al., 1998)
	Support liver recovery from toxic chemical attack or viral damage	(Kidd, 2002)
	Protection of the human gastric intestinal mucosa against toxic attack	(Anand et al., 1999)
Phosphatidylinositol (PI)	Promotion of plasma cholesterol transport and metabolism	(Stamler et al., 2000)
Phosphatidylserine (PS)	Restoration of normal memory on a variety of tasks	(McDaniel et al., 2003)
	Improvement of memory on Alzheimer patients	(Zhang et al., 2015)
	Improve exercise capacity of exercising humans	(Kingsley, 2012)
Lysophosphatidylcholine (LPC)	Strong gastro-protective role in the duodenal mucosa	(Kivinen et al., 1995)

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Table 4: Effect of MFGM supplementation to diet in health.

Supplementation	Type of study	Health effects	References
***IN VIVO* STUDIES**			
MFGM	Study in Fisher-344 rats	- Prevention of colon cancer	(Snow et al., 2010)
MFGM	Human colon cancer cells HT-29	- Anticarcinogenic capacity	(Zanabria et al., 2013)
Defatted-MFGM	Human cells HT-29	- Prevented association of enterohemorrhagic *E. coli*	(Ross et al., 2016)
Novel infant milk formula (Nuturis®) composed of MFGM fragment	Study in mices	- Healthier growth - Decrease of body fat accumulation	(Baars et al., 2016)
CLINICAL STUDIES (double-blinded randomized controlled trials)			
MFGM-enriched proteins fraction (Lacprodan MFGM-10; Arla Foods Ingredients, Viby, Denmark)	With 6- to 11-month-old children	- Lower longitudinal prevalence of diarrhea - Lower incidence of bloody diarrhea	(Zavaleta et al., 2011)
MFGM-enriched phospholipids fraction (INPULSE; Büllinger SA, Büllingen, Belgium)	With young children (≈ 4.4 years old)	- Fewer day with fever - Lower parental scoring of internal, external and total behavior problems	(Veereman-Wauters et al., 2012)
MFGM-enriched proteins fraction (Lacprodan MFGM-10; Arla Foods Ingredients, Viby, Denmark)	With 2- to 6-month-old children	- Risk of acute otitis reduced - Immunomodulatory effects on humoral response against pneumococcus vaccine - Higher total serum cholesterol	(Timby et al., 2014b, 2014a, 2015)
MFGM supplementation	With middle-aged	- Improve exercise capacity	(Ota et al.,

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associated with habitual exercise	adults	- Improve muscle function	2015)
MFGM plus palm oil	With obese adults	- Decrease negative effects of high-saturated fatty acid meals	(Demmer et al., 2016)
		- Decrease postprandial cholesterol, inflammatory markers and insulin response	

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Table 5: Encapsulation of bioactive compounds in MFGM-derived phospholipids liposomes.

Matrices	Encapsulated compounds	References
Chitosan-coated liposomes prepared with MFGM-derived phospholipids	Antidiabetic peptides	(Li et al., 2015)
MFGM-derived phospholipids	Curcumin	(Jin et al., 2016)
	Lactoferrin	(Liu et al., 2013)
	Tea polyphenol	(Gülseren & Corredig, 2013)
	β-carotene	(Thompson et al., 2009)
	Potassium chromate	(Thompson et al., 2009)
	Ascorbic acid	(Farhang et al., 2012)

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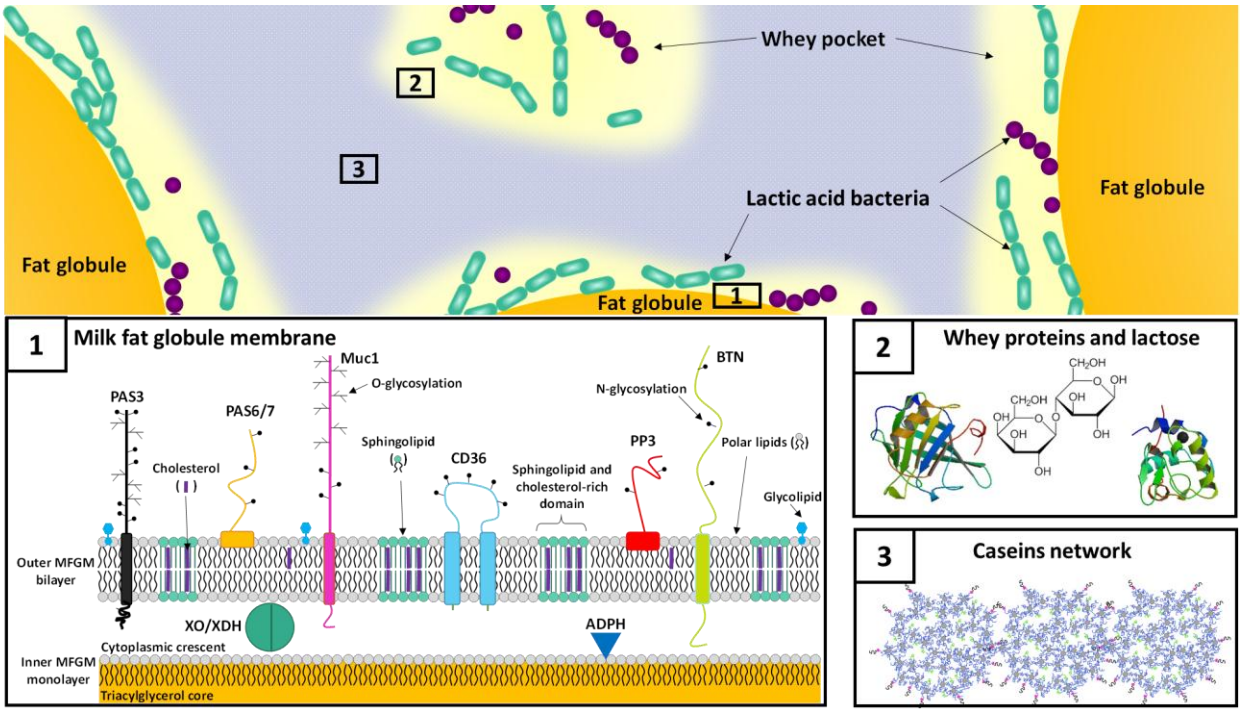

Figure 1: Milk components and bacterial organization in a cheese matrix.

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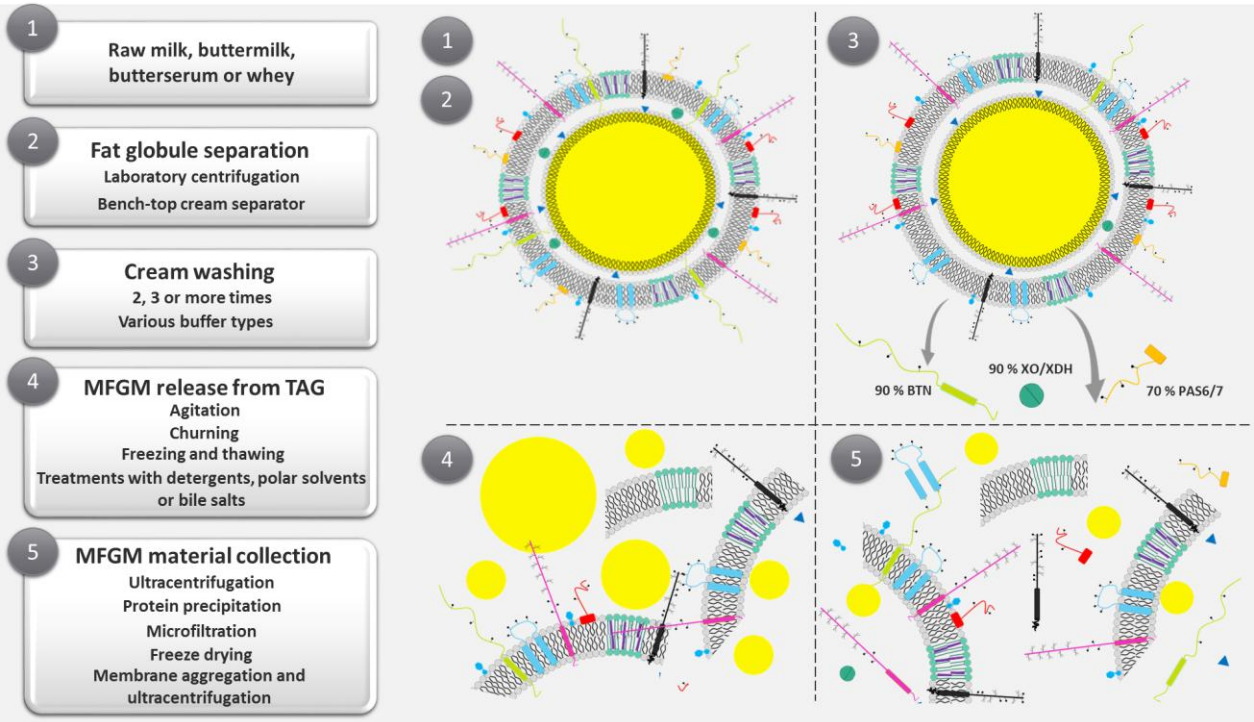

Figure 2: Isolation of the MFGM at laboratory scale.

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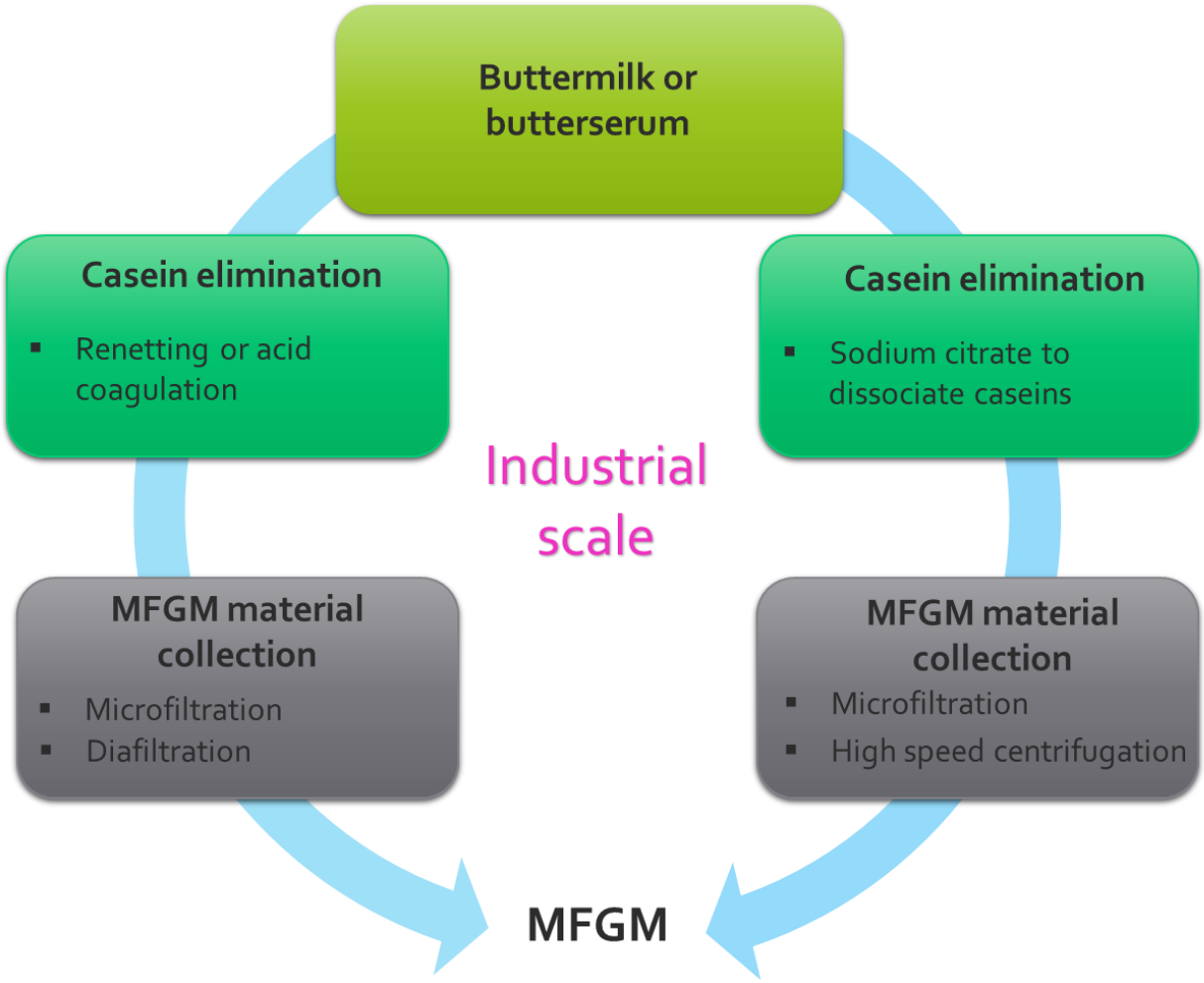

Figure 3: Isolation of the MFGM at industrial scale.

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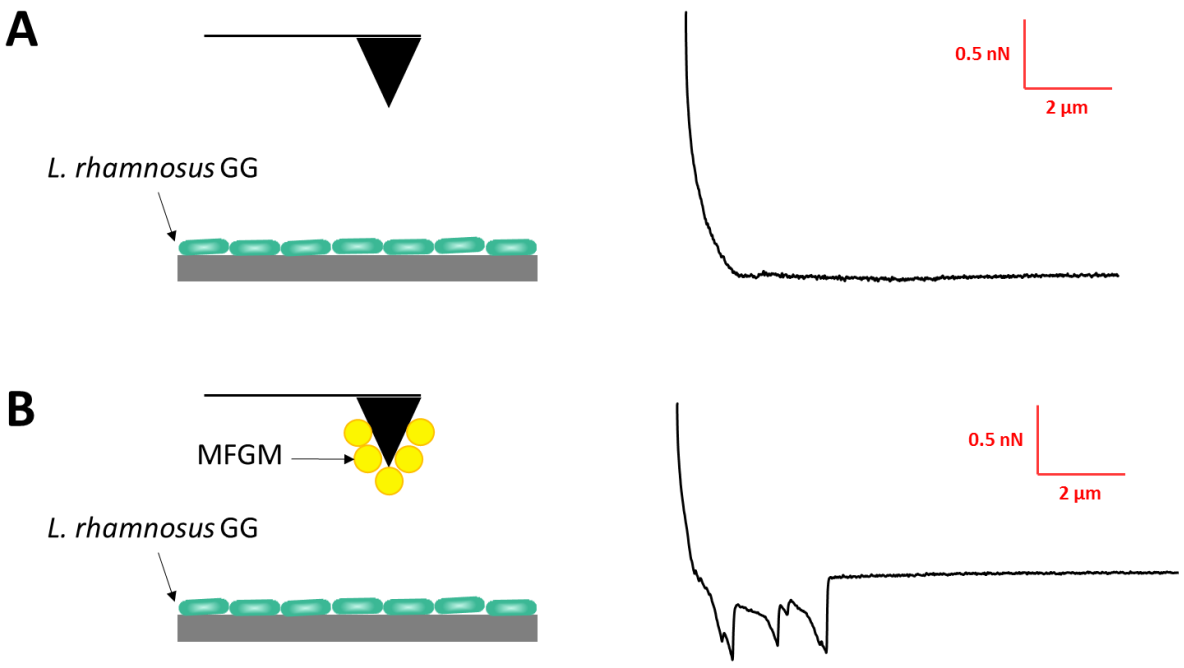

Figure 4: Interaction between *L. rhamnosus* GG and MFGM observed by AFM (author unpublished personal data). Retraction curve without adhesive events between the nude AFM probe and bacteria (A). Retraction curve with adhesive events between MFGM and bacteria (B).

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