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Food emulsifiers based on Milk Fat Globule Membranes and their Interactions with calcium and casein phosphoproteins

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Abstract

Natural food surfactants were produced from membrane-derived fragments of milk fat globules (MFGM) obtained from cream and buttermilk by microfiltration with diafiltration followed by churning. The obtained MFGM fragments were demonstrated for their emulsifying and functional properties and as substitute of commercial phospholipids (lecithin). Fine emulsions (droplet size ~1.2 μm) were obtained by stabilization of the oil/water interface after low energy sonication. Stable emulsions were obtained with lecithin in the pH range between 5.8 and 7.8, whereas MFGM components enabled better stability above pH 6. Coalescence took place in the presence of calcium, owing to electrostatic screening. The stability of the emulsions increased

with the addition of casein, which formed droplet flocs. The calcium-binding ability of MFGM is proposed to inhibit protein (casein) flocculation, leading to a highly interacting network that prevents phase separation and stable MFGM-based food emulsions.

Keywords: MFGM, food emulsion, casein, phospholipid, emulsifier, sonication

1 Introduction

The milk fat globule membrane (MFGM) is a fascinating multilamellar structure comprising polar lipids and proteins that act as natural emulsifiers in milk. The unique composition and structure of the MFGM endows technological, nutritional and biological functions. Its amphiphilicity and inherent role as stabilizer of fat globules make MFGM a suitable option as food emulsifier. Moreover, MFGM can bring antimicrobial and antiviral activities and contribute with other functions such as those already identified in gut physiology and development of the central nervous system in newborns (Milard et al., 2018; Dewettinck, et al., 2008; Contarini and Povolo, 2013; Gallier, et al., 2015). These are some of the many reasons for the current interest in MFGM isolation and its application as ingredient of functional foods (Singh, 2006; Gallier, et al., 2017) and, especially, as emulsifier (Livney, et al., 2017; Lopez, et al., 2017; Phan, et al., 2014; Phan, et al., 2016). For instance, Gallier et al. (2015) and Lopez et al. (2015) used MFGM in the formulation of an infant milk for enhanced metabolic and digestive properties and to mimic the properties of human milk, respectively. In these applications, the biological and technological functionality of MFGM have been found to depend on its surface composition (MFGM *vs.* serum proteins) (Garcia, et al., 2014; Berton-Carabin, et al., 2013).

Despite its potential benefits, MFGM adoption in dairy products is still far from realization. This is mainly because the typical process used in the dairy industry leads to MFGM

47 disruption, undermining its functionality (Holzmüller et al., 2016; Corredig and Dalgleish, 1998).
48 Furthermore, the methods used so far for MFGM isolation have been ineffective, mainly because
49 of low yields and purity (serum proteins). Recently we proposed a chemical-free, fully integrated
50 method to separate proteins and MFGM (*ideal butter making*) by using microfiltration of raw
51 bovine cream prior to butter making (Jukkola et al., 2018). Up to 90% of the serum proteins were
52 separated by microfiltration before cream churning. Hence, the MFGM results into the (*ideal*)
53 buttermilk, with no need for further purification steps.

54 Although polar lipid emulsions have been investigated extensively, the interactions
55 between MFGM and major milk proteins and their effect on emulsion behavior are subjects that
56 remain unexplored, limiting the formulation of MFGM in food systems. Owing to their negative
57 charges, polar lipid emulsifiers are known to provide good stability against aggregation and
58 coalescence (McClements and Gumus, 2016) and their combination with other surfactants can
59 tailor emulsions properties via electrostatic and colloidal interactions. The synergistic effects of
60 proteins and phospholipids as emulsifiers have been reported (McClements and Jafari, 2018; Xue
61 and Zhong, 2014). For instance, stable emulsions were produced by combining lecithin and
62 casein (Garcia-Moreno et al., 2014) while phospholipids can displace adsorbed proteins by
63 competitive effects at the interface (Livney et al., 2017; Fang and Dalgleish, 1996). MFGM is
64 rich in polar lipids and proteins, which in the right conditions are surface active, eliminating the
65 need for additional surfactants for emulsification. However, MFGM interactions and/or
66 complexation with other milk proteins can be destabilizing and the formation of secondary
67 adsorption layers or competitive displacement are factors that require consideration.

68 The interactions with casein involve association with the polar lipid domains of MFGM
69 (Gallier et al., 2012). Furthermore, Obeid et al. (2019) demonstrated that casein interaction

favors polar lipids in the liquid-disordered phase where a wide intermolecular distance between the lipids allows protein adsorption. Moreover, the presence of anionic polar lipids prevents interactions by electrostatic repulsion and the emulsifying properties of MFGM decrease with the incorporation of whey proteins (Corredig and Dalgleish, 1998). Furthermore, compared to the lipid fraction, the proteins from MFGM were demonstrated to contribute to emulsion stabilization (Phan et al., 2016). Overall, membrane proteins appear to have important roles in stabilizing milk fat globules and MFGM. Hence, understanding MFGM and protein interactions is critical for designing food emulsions based on MFGM, the main subject of this study.

We prepared and characterized natural surface-active material derived from MFGM, as that from *ideal buttermilk* powder and compared the emulsifying properties against a commercially available lecithin phospholipid. The emulsions were formulated as model food systems at three values of pH and in the presence or absence of casein and calcium. Furthermore, the interactions between MFGM, lecithin and casein were studied for their cooperative emulsifying potential.

2 Materials and methods

2.1 Materials

Raw bovine cream (fat 40.2%, protein 2.0%, dry matter 50.2%) was used for MFGM material production. The MFGM material (fat 73.5%, protein 16.5%, ash 1.08%) was produced by microfiltration with diafiltration and fat globule separation (Tetra Alcross®, Tetra Pak, Denmark) according to Jukkola et al. (2018), followed by churning and freeze-drying the buttermilk. Briefly, 10 kg of the raw cream was heated to 50 °C and diafiltered with 90 kg of deionized water. A 1.4 μm cut-off membrane (Membralox®, Pall Corporation, France) was used

to separate fat globules from the cream non-fat solids. Then, the cream was heated to 75 °C in a water bath for pasteurization, followed by cooling to 5 °C to allow fat crystallization. On the following day, the cream was ripened at 19 °C for 2 h to partially melt the fat crystals, followed by cooling to 12 °C for churning. The cream was churned (ELBA 30, Elecrem, France) and MFGM-rich buttermilk (fat 0.8%, protein 0.27%, ash <0.15%) was collected. Finally, the buttermilk was freeze dried (Alpha 1-4 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) and the obtained MFGM material was stored at -20 °C to be used in the experiments.

Sunflower oil was purchased from the local supermarket. Bovine non-micellar casein and sodium azide were purchased from Sigma-Aldrich (Merck KGaA, Germany). Commercially available sunflower lecithin (Unilec SF-DP, UAE) was used for the emulsion studies (>96% phospholipids). All the chemicals used were of analytical grade.

2.2 Surfactant solutions and emulsion preparation

Aqueous solutions were prepared with ultrapure water and 1.2 g of the respective emulsifier added for a final concentration of 2% (based on 60 mL of aqueous phase). Such concentration (2%) was chosen in the emulsion experiments to ensure effective emulsification and for comparison purposes. MFGM was dissolved using a magnetic stirrer and heated to 50-60 °C to melt the fats. Then, the solution was gently homogenized using a sonicator (5x20s, 40%, Ultrasonic Sonifier S-450, Branson Ultrasonics Corporation, USA). Lecithin did not require heating nor sonication for dissolution. Casein was dissolved by mixing 1.2 g casein powder in ultrapure water. The solution was heated to 80-90 °C and 0.1M NaOH was added until the casein was dissolved for a final volume of 20 mL. Calcium solution was prepared by mixing 3.53 g $CaCl_2$ in 1L of ultrapure water.

Emulsions were formulated using 2.0% (w/v) MFGM or lecithin. The emulsions were optionally supplemented with casein (0.1% concentration), calcium (30 mM) or both. In such cases, 1 mL of either casein or calcium solution was added before emulsification. The volume of water needed to achieve the target water-to-oil ratio (90:10) was added. The pH of each solution was adjusted to 5.8, 6.8 or 7.8 using 0.1 M NaOH or 0.1 M HCl. Finally, 0.05% of sodium azide was added to the aqueous phase of each emulsion to prevent microbial spoilage.

The emulsions (62.22 mL total volume each) were formulated with 10% of the oil phase (90% aqueous phase) using sunflower oil (2.22 mL). The respective system was sonicated 5x40s (40% amplitude) on ice bath to form the emulsions. The emulsions were stored at ambient temperature, protected from light and the headspace of the vials were filled with nitrogen gas to minimize oxidation. Three replicates were prepared for center points (pH 6.8).

2.3 Protein profiling of the MFGM

Proteins from MFGM were analyzed by reducing tricine-sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) according to Jukkola et al. (2018) and Hinz et al. (2007). Briefly, the MFGM was diluted and mixed with tricine buffer (0.1 M Tris–HCl buffer pH 6.8, 20% glycerol, 4% SDS, 10% beta-mercaptoethanol, 0.02% bromophenol blue). The proteins were denatured at 100 °C for 5 min and loaded in a Criterion 8–16% TGX gel (Bio-Rad Laboratories Inc., US). A running buffer comprising trizma base (3.03 g/L), glycine (14.42 g/L) and SDS (1.0 g/L) was used and the electrophoretic migration for the gel was performed at 200 V and 100 mA. The proteins were stained with Coomassie blue and identified by using molecular weight standards.

2.4 Surface activity and emulsifier properties

2.4.1 Surface tension

The dynamic surface tension of the MFGM and lecithin solutions was measured by a KSV BPA-800P bubble pressure tensiometer (KSV Instruments, Finland) with a capillary radius of 0.13 mm. The measurements were performed in triplicate at 22 °C for 30 minutes. The average surface tension was plotted as a function of bubble life time with 13 s resolution.

2.4.1 Emulsion Droplet size

Droplet size distribution was measured for each freshly made emulsion and after 1, 2, 3 and 7 days of storage using a Mastersizer 2000 (Malvern Instruments, Malvern, UK) based on light scattering (Ye et al., 2011; Jukkola et al., 2016). A refractive index for the dispersed phase, e.g., sunflower oil was assumed (1.473). The samples were analysed in triplicate.

2.4.2 Emulsion Stability

A scanning light scattering instrument (Turbiscan MA 2000, Formulaction, Toulouse, France) was used for measuring the stability of the emulsions according to Juliano et al. (2011). Six milliliters of each emulsion were pipetted into the Turbiscan scanning tube. The headspace was flushed with a stream of nitrogen gas to reduce the effects of oil oxidation. Backscattered light was measured in freshly prepared samples, after 1, 2, 3 and 7 days of storage and the stability was plotted in terms of emulsion clarification layer thickness versus time. The capped test tubes were stored at ambient temperature and the measurements were performed in duplicate.

2.4.3 Microstructural analysis

Confocal laser scanning microscopy, CLSM (Leica TCS SP2, Leica Microsystems, Germany) was used to examine the microstructure of the emulsions according to Jukkola et al. (2018). Briefly, the emulsion samples were stained (10 µL/1mL) with Nile red (0.05% in acetone) in order to visualize the oil droplets (Gallier et al., 2010; Garcia, et al., 2014). At least 20 min staining time was allowed. Then, the sample was mixed (50:50) with 0.5% agarose (Top Vision LM, Thermo-Fisher Scientific, Spain) and approximately 10 µl of the mixture was

pipetted on microscopy glass and allowed to set for at least 20 min under sealed cover glass. The samples were excited using Argon laser at 488 nm and objected with a 63X oil-immersion lens (Leica, Germany). Emission wavelengths were collected between 550–650 nm. The imaging was done after emulsification and after 7 days of storage. Approximately 10 individual images were recorded for each sample.

Visual observation of the emulsions was performed after storing each emulsion in a glass vial. The headspace of the vials was flushed with nitrogen and the emulsions were stored in a dark room. The emulsions were observed weekly for 4 weeks, after which they were observed monthly for 4 months.

3 Results and discussion

3.1 Protein profiling

SDS-PAGE electrophoretic separation was used to confirm enrichment of the membrane material in *ideal buttermilk* and to identify the membrane-specific proteins present in MFGM. They were proteins not released from the MFGM upon processing and included MUC1/Myocin, XO (Xanthine Oxidase), adipophilin, butyrophilin (BTN) and periodic acid and Shiff (PAS) 6/7 (Figure 1). Some remains of casein were present in the MFGM sample, whereas α-La was absent (~14kD), as a result of the fact that unpasteurized cream was used for obtaining the MFGM-material (Hansen et al., 2018). Moreover, the band intensity of the MFGM proteins compared to those for casein and whey proteins indicated the enrichment of MFGM components and effective separation of milk serum proteins. Similar MFGM protein profile was obtained previously by Hansen et al. (2018) and Jukkola et al. (2018).

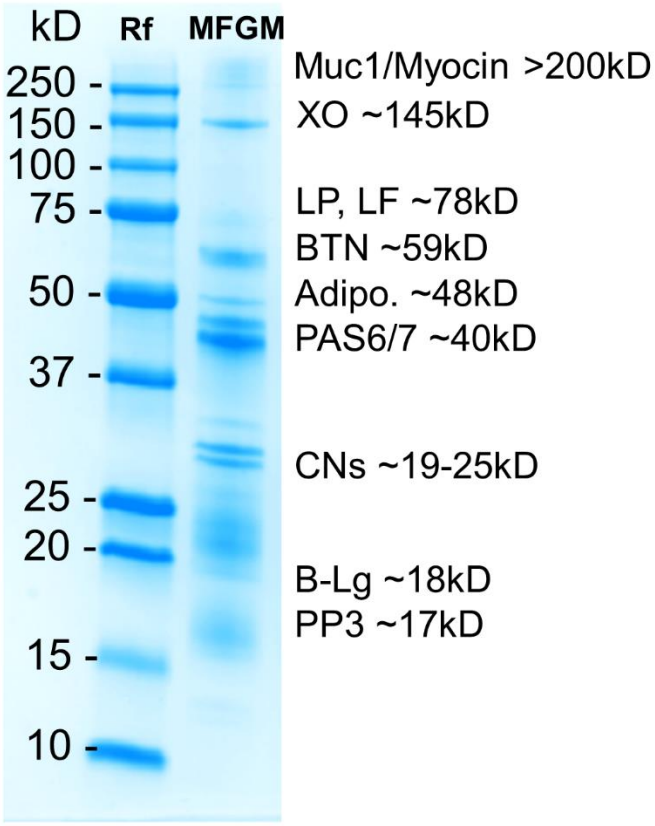

Figure 1. SDS-PAGE protein profiling of milk fat globule membrane material. Rf = reference molecular weight marker, MFGM= milk fat globule membrane. Muc1 = mucin 1, XO = xanthine oxidase, LP = lactoperoxidase, LF = lactoferrin, BTN = butyrophilin, Adipo. = adipophilin, PAS6/7 = periodic acid and shiff 6/7, CNs = caseins, B-Lg = betalactoglobulin, PP3 = proteose peptone 3.

3.2 Surface activity

Surface tension values were taken as indicative of the surface activity and the ability of the system to stabilize the increased interfacial area formed upon shearing and therefore to reduce the initial size of the emulsion droplets (McClements and Gumus, 2016). Furthermore, the emulsifier adsorption rate needs to be fast to avoid droplet coalescence upon emulsification. As expected, the surface tension of both MFGM and lecithin solutions were reduced with time

196 (Fig. 2). Moreover, both solutions reached the corresponding surface tension equilibrium at
197 similar bubble life times. Lecithin lowered the surface tension to about 57 Nm/m at equilibrium.
198 MFGM lowered the surface tension to a similar value (55 Nm/m) but faster, possibly due to
199 small monomeric proteins present in the MFGM material. Similar values were previously
200 recorded for MFGM by Malik et al., (2015). Overall, the surface tension measurement indicates
201 MFGM to be surface active and to have a potential as a replacement for lecithin emulsifiers.
202 The saturation of oil droplet surfaces with surfactant is important to achieve small droplet
203 sizes (McClements and Gumus, 2016) since otherwise droplets collide upon multi-step
204 sonication, leading to coalesce. The sufficient inventory of the emulsifier (concentration) was
205 confirmed in emulsification tests (data not shown). We note that even though the same
206 concentration was adopted for both surfactant solutions, the MFGM fragments may consist of a
207 relatively larger quantity of non-surface-active material, making the effective surfactant
208 concentration lower. In particular, the MFGM-material was isolated from microfiltered cream by
209 freeze-drying of the buttermilk. Hence, neutral fat (triacylglycerols), ever present in buttermilk,
210 are expected to be present.

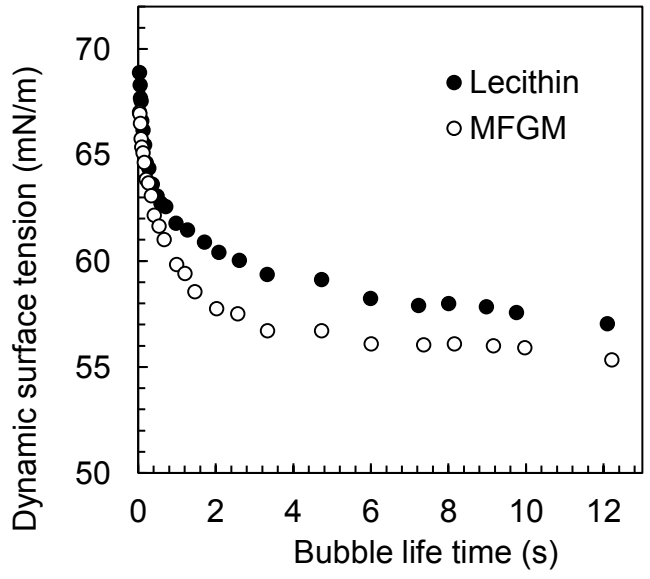

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Figure 2. Dynamic surface tension of aqueous solutions (2% m/v) of lecithin and MFGM fragments as a function of bubble life time.

3.3 Emulsion behavior

Lecithin and MFGM produced oil (sunflower oil)/water emulsions with a mean droplet size of 0.6 μm and 1.2 μm, respectively. The larger droplet size observed in the MFGM-based emulsion was partly anticipated due to its heterogenous droplet composition. Moreover, despite the overpowered effect of some MFGM components to lower the surface tension, the MFGM most likely contained lesser amount of fast adsorbing surface active material compared to lecithin. Hence, producing larger droplet size distribution. The type of emulsifying method affects significantly to the droplet size distribution (Heffernan et al., 2011). Moreover, the droplet size distribution for both lecithin and MFGM emulsions show tailing of large droplets (Fig. 3), indicating the inefficiency of the sonication method and the instability of the systems against creaming. Furthermore, the mean droplet size for lecithin emulsion increased by 0.47 μm after 7-day storage (from 0.61±0.01 μm to 1.08±0.34 μm), whereas for MFGM emulsions the droplet size increased by 0.86 μm during the same time period (Fig. 3(A) and (B), respectively).

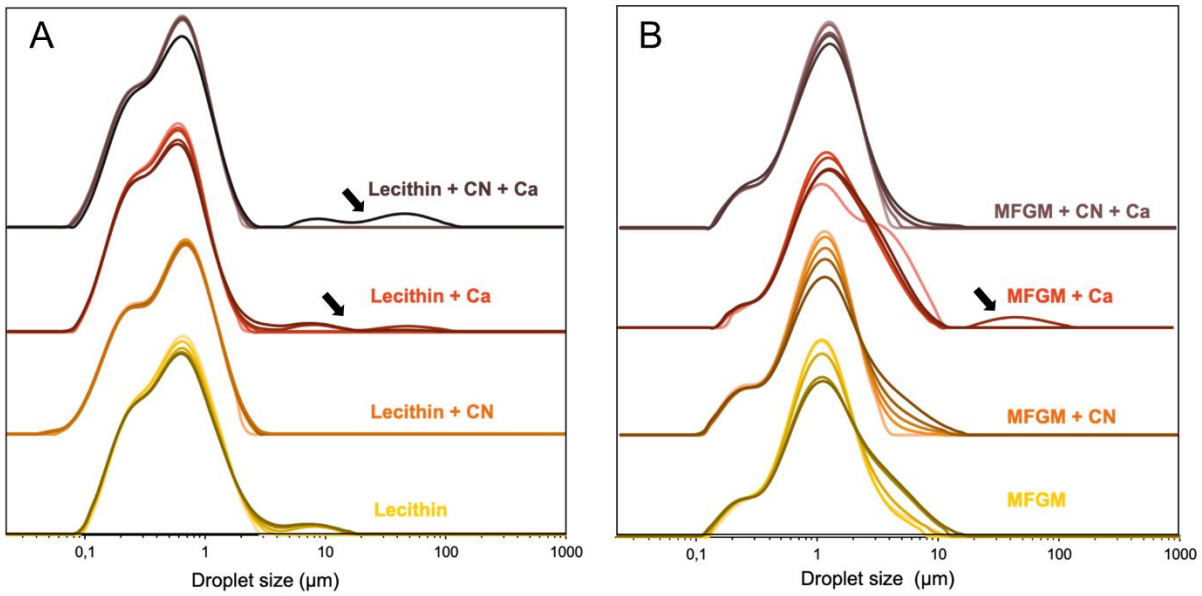

Figure 3. Oil droplet size distribution in emulsions stabilized with (A) Lecithin and (B) MFGM (yellow). The histograms for the same systems in the presence of casein (CN, orange), calcium (Ca, red) or both (purple) are also shown after 7-day storage time. The increased storage time is indicated by the darker contrast of the colors used in the respective profiles. The arrows point to formation of large droplets or aggregates (for interpretation of the references to color, the reader is referred to the web version of this article).

3.3.1 Effect of pH. The droplet size measured for emulsions stabilized with lecithin was found not to depend on the pH of the aqueous phase (Fig 4). This was not expected since the deprotonation of lecithin and phospholipids depend on pH. In particular, Damodaran et al.(, 2010) reported pK_1 ~4 for phospholipids and Lopez et al.(, 2017) demonstrated destabilization of phospholipid-based emulsions at pH 4. In contrast, pH was found to affect MFGM emulsion droplet size, which varied from 1.2 µm to 7 µm (5.8 – 7.8 pH range). At pH 5.8 the mean droplet size of MFGM emulsion increased dramatically whereas at pH 6.8 and 7.8 the mean droplet size

remained around 1.3 μm and 1.2 μm, respectively. Some MFGM proteins have a pI of 4-6 (Kanno and Kim, 1990; Ye et al., 2011) and therefore lowering the pH below 6 increases the MFGM emulsion droplet size due to more limited repulsive surface charges.

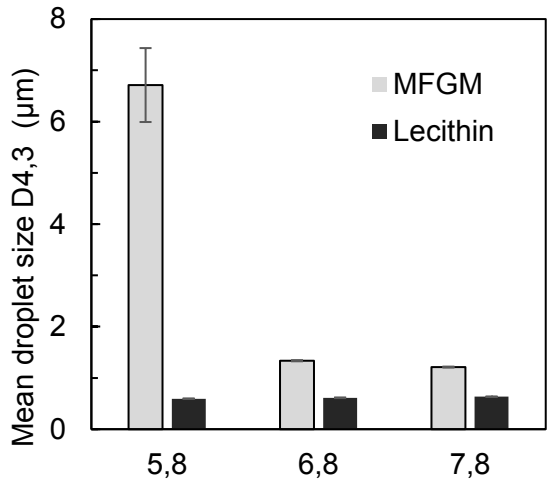

Figure 4. Mean droplet size of O/W emulsions prepared with MFGM and Lecithin at the given pH of the aqueous phase

3.3.2 Effect of casein and calcium. In the presence of casein, the lecithin-stabilized emulsions showed a slightly reduced mean droplet size (0.56±0.01 μm) that increased marginally after 7 days, by 0.03 μm (Fig. 3(a)). As discussed previously, several authors have reported a synergistic effect with polar lipids when using proteins as co-emulsifiers (Xue and Zhong, 2014;Garcia-Moreno, et al., 2014; McClements and Jafari, 2018). This may lead to different functional properties, for instance, one of the components may form the emulsion while the other improve its stability or physiological functionality (McClements and Jafari, 2018). The use of proteins as co-surfactant together with lecithin was previously reported to decrease emulsion droplet size and increase stability (Xue et al., 2015; Xue and Zhong, 2014). The presence of calcium in the aqueous phase of the emulsions stabilized with lecithin increased the mean droplet

size; moreover, the emulsions became less stable (after 3-days a 0.67 μm increase in the mean droplet size was recorded, Fig. 3(A)). This is possibly due to screening of the electrostatic forces and the reduced inter-droplet repulsion. The combination of casein and calcium led to larger droplets upon storage (2.02 μm increase), possibly due to calcium-induced protein flocculation and electrostatic shielding. Similar observation was made by McCarthy et al. (2014) who showed an increase in casein emulsion droplet size due to calcium-induced self-aggregation.

The presence of casein improved the emulsification with MFGM slightly, lowering the mean droplet size from 1.34±0.11 μm to 1.03±0.02 μm in freshly prepared samples. However, the mean droplet size of such emulsions increased by 0.70 μm upon storage. Overall, compared to the case of lecithin-stabilized emulsions, the presence of casein in MFGM-based emulsions had a relatively small impact on stability. The interaction of casein is more pronounced with lecithin where the lipids express less repulsion and their phase state is expected to be more favourable for interaction compared to that of MFGM (Obeid et al., 2019), thus allowing casein adsorption. This is supported by results from experiments with Langmuir monolayers (MFGM and pure phospholipids) formed at the air/water interface to allow control of the lateral packing of the molecules and to study, *via* the surface pressure isotherms, the interactions with proteins (casein) injected within the sub-phase (see *supplementary material).* Casein interacts with MFGM in the liquid-disordered phase (Obeid et al., 2019), consisting mainly of phosphatidylcholine (PC), where the wide intermolecular distance between the lipids favours higher protein adsorption. Furthermore, the complex structure of MFGM may partly induce repulsive electrostatic forces, which limit casein interaction. Overall, the interaction between casein and MFGM is expected to be limited and very specific (Lopez, et al., 2010).

283 The presence of calcium was found to increase the MFGM emulsion droplet size and
284 lower the stability, similar to what was observed for lecithin-based emulsions (Fig. 3(b)).
285 Interestingly, the stability of MFGM-based emulsions increased in the presence of both, casein
286 and calcium, leading to a relatively minor increase in droplet size, by 0.43-μm (the smallest
287 increase among MFGM emulsions). Previously, calcium was found to complex with MFGM
288 fragments and to prevent precipitation (Damodaran, 2010). Hence, calcium has a critical effect
289 on MFGM structure and stability since it may inhibit hydrophobic interactions between the
290 membrane-bound proteins. On the other hand, it is well known that the presence of calcium may
291 create calcium bridges between protein molecules leading to flocculation, as was observed in the
292 case of lecithin emulsions (Ye and Singh, 2001). However, the observed calcium-induced
293 flocculation of MFGM-based emulsions is most likely limited due to several factors. Firstly, the
294 calcium concentration may have been too low to induce protein bridging (Müller-Buschbaum et
295 al., 2007). Secondly, MFGM glycoproteins may have inhibited the calcium-induced protein
296 flocculation due to their calcium binding ability and by blocking the calcium binding sites on
297 casein molecules (McCarthy et al., 2014; Müller-Buschbaum et al., 2007; Ye et al., 2012).
298 Lastly, the calcium-induced protein complexes may have increased steric repulsion on MFGM-
299 coated droplets. Moreover, addition of calcium prior to emulsification was reported to increase
300 surface coverage due to adsorption of calcium-casein aggregates on the surface of the droplets
301 (Dickinson, 2010). While the native MFGM bound to the surface of the fat globules consists of
302 three polar lipid layers and protruding proteins, that of the system considered in this study is
303 derived from the churning process, which is disruptive and results in fragments, polar lipid
304 vesicles and individual components in buttermilk. Therefore, we propose that the MFGM does
305 not return to its native form after high shear emulsification; instead, it is most likely to form a

surfactant monolayer with combination of adsorbed fragments. Moreover, some parts of the newly formed surface might contain three layers of the MFGM and secondary adsorption layer of casein-calcium complexes.

3.4 Emulsion stability and microstructure

3.4.1 Stability. The stability of the emulsions was observed by clarification of the emulsion, detected by decreased backscattering on the lower layer of the emulsion. Furthermore, CLSM images of the emulsion microstructure and photo images of the emulsion vials were recorded. The emulsions were found to be unstable against creaming due to large droplet fraction (>1 μm) present in the freshly prepared emulsions. The stability measurements showed little or no difference between the lecithin emulsions (Fig. 5A). The strongest clarification was found for emulsions that used lecithin alone but the differences between the samples were insignificant.

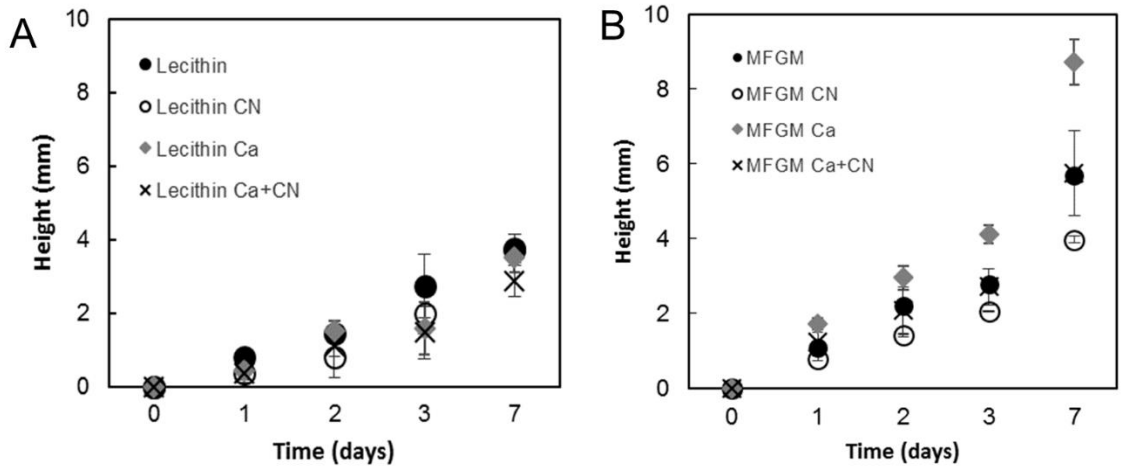

Figure 5. Height of the emulsion clarification layer as measured by backscattered light upon 7 days of storage of A) lecithin and B) MFGM emulsions supplemented with casein (CN), calcium (Ca) or both. A threshold of -15.0% and -22.5% was adopted for calculation of the clarification kinetics from the backscattered data for lecithin and MFGM, respectively.

322 The MFGM emulsions were found unstable against creaming due to their droplet size, >1
323 μm. The addition of calcium promoted destabilization of MFGM-based emulsions (Fig. 5B). The
324 strongest clarification was recorded for calcium-supplemented MFGM emulsions. No significant
325 difference between the MFGM and casein-supplemented MFGM emulsions was observed after
326 3-day storage. The presence of both casein and calcium in MFGM-stabilized emulsions led to
327 similar results as for those obtained in their absence. Hence, the destabilizing effect of calcium
328 was prevented by addition of casein, as explained previously in section 3.3.
329 Point of notice is that the volume of the clarification layer between the lecithin and
330 MFGM samples are not fully comparable, since different thresholds were used for calculation of
331 the separation kinetics to ensure fitting of the backscattering data. Hence, the data should be used
332 individually for evaluating the emulsion stability. The oil-in-water emulsion may separate into
333 the clarified and cream layers through numerous mechanisms, including aggregation and
334 coalescence. According to the Stokes' law the droplets move due to gravitational forces
335 depending on their diameter, density and shear viscosity. Thus, smaller droplets are more stable
336 compared to the larger ones, whereas coalescence or aggregation increases the rate of instability.
337 Hence, lecithin emulsions are expected to be more stable compared to MFGM because of their
338 smaller droplet size.
339 ***3.4.2 Visual and microstructural observations.*** The emulsions remained visually stable
340 upon 4 weeks, except MFGM emulsion at pH 5.8 with added calcium, which showed
341 clarification after 2 weeks of storage. After 4 months of storage, the MFGM, lecithin and their
342 casein versions showed some creaming or clarification (Fig. 6). Furthermore, the calcium
343 supplemented samples were strongly clarified (MFGM) or showed some oiling off (lecithin) and
344 the MFGM emulsion with added casein and calcium showed clarification at pH 5.8. The stability

of the lecithin emulsions was not affected by pH (5.8-7.8; *data not shown*) and overall the differences were smaller compared to MFGM.

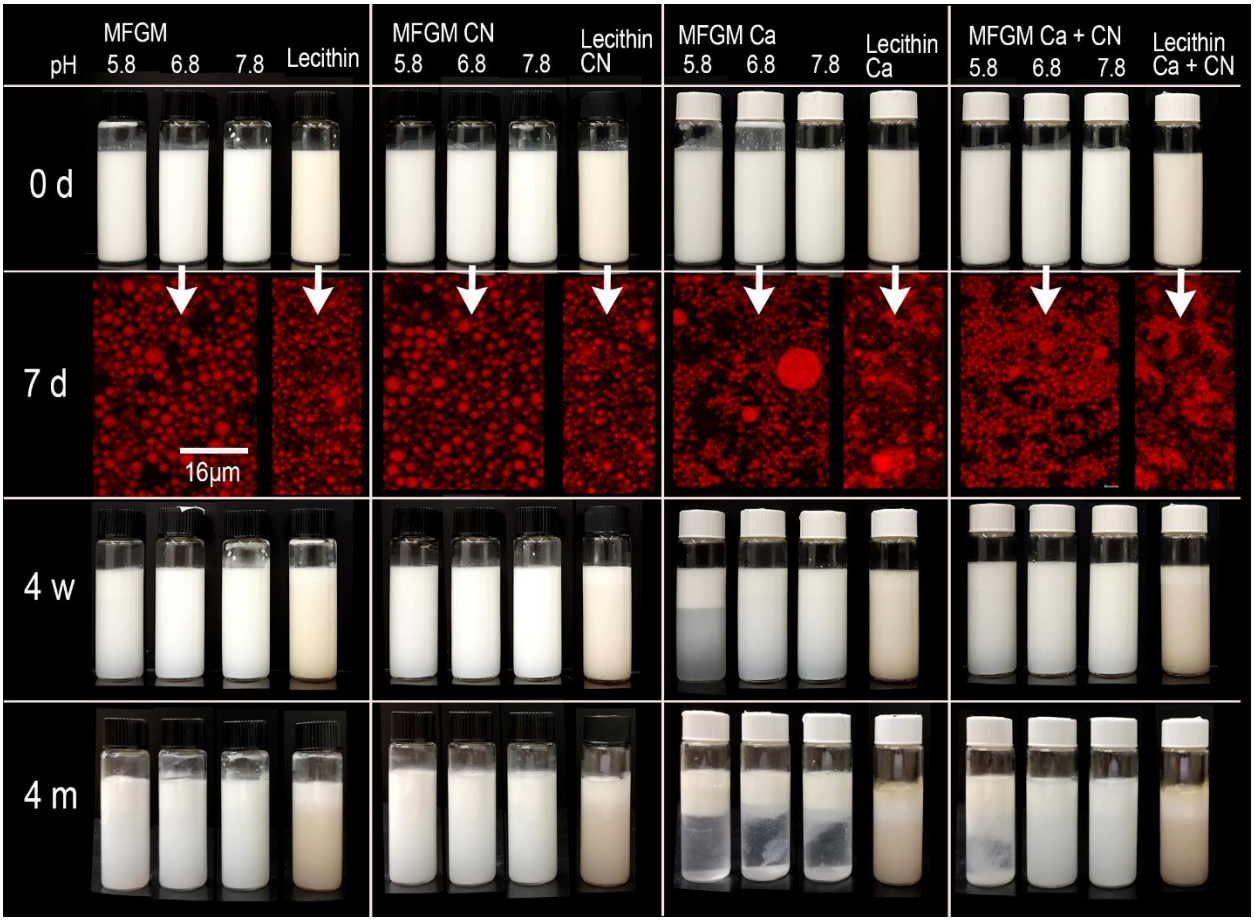

Figure 6. Visual observation of MFGM emulsions at pH 5.8, 6.8 and 7.8 and lecithin emulsions at pH 6.8 (6 mL each) upon 4-week and 4-month storage and confocal laser scanning microscopy images of MFGM and lecithin emulsions at pH 6.8 after 7 days of storage. The emulsions were supplemented with casein (CN), calcium (Ca) or both.

The microstructural images of the lecithin emulsions revealed very fine droplets, which increased in size in the presence or absence of casein upon 7 days of storage (Fig. 6). Hence, the increase in droplet size was most likely caused by coalescence. The microstructure of calcium-supplemented lecithin emulsions showed heterogenous droplet size distribution, including small flocs and larger droplets, suggesting that calcium reduced droplet repulsion and favored

flocculation and coalescence. Lastly, the casein and calcium supplemented lecithin emulsion showed little to no difference in the microstructure upon 7 days of storage compared to that of freshly prepared emulsion: the droplets were flocculated but visually their size remained very small (Fig. 6). In contrast, the droplet size measurements showed an increase in casein and calcium supplemented emulsion droplet size upon storage. Hence, it can be argued whether the flocs disturbed the droplet size measurement, or if the largest droplets were absent.

No significant difference between the microstructure of MFGM and casein-supplemented MFGM emulsions were recorded (Fig. 6). In contrast, the addition of calcium increased the number of very large emulsions droplets, especially after 7 days of storage, hence making the emulsion less stable. The instability of calcium-supplemented emulsion was attributed to the decrease of electrostatic repulsion and droplet coalescence. However, adding calcium together with casein produced an emulsion with a more homogenous microstructure and relatively small droplet size. In contrast to lecithin emulsions, the MFGM emulsions showed no or limited flocculation (microscopy images, Fig. 6), possibly due to heterogeneity of the interface, allowing only partial interaction with casein. We note, however, the limitations of microscopy for evaluation of droplet flocculation, especially since the sample was mixed with agarose upon the preparation. However, the microstructural images show flocs in each individual lecithin image, except in pure lecithin and casein-supplemented emulsions after 7 days of storage where no flocculation was recorded. Overall, the evaluation of the emulsion microstructure corroborates our previous findings for MFGM-stabilized emulsion and their interactions with casein and calcium.

Emulsions based on pure lecithin and MFGM as well as those supplemented with casein are most stable with creaming observed only after long-term storage. Calcium addition leads to

380 droplet coalescence and separation and destabilizes the emulsions. Emulsions in the presence of
381 both, calcium and casein, are more stable compared to those produced in the presence of calcium
382 only. However, creaming of such emulsions is stronger compared to those based on pure lecithin
383 or MFGM due to formation of flocs, leading to complete clarification at low pH (MFGM).
384 Hence, the addition of calcium and casein together promote flocculation, as observed in CLSM
385 images and by the measured increase in mean droplet size.
386
387 **Conclusions**
388 Components of the milk fat globule membrane, a natural emulsifier in milk, were
389 collected from the *ideal butter* process and used to stabilize food emulsions. The results were
390 compared with those after using a commercial lecithin. The MFGM fragments are surface active
391 and lowered the surface tension to values similar to those achieved by pure lecithin. MFGM-
392 material formed emulsions by sonication with characteristic droplet sizes of ~1.2 μm. That from
393 lecithin corresponded to 0.6 μm. The emulsifying effect of MFGM may be hindered by the
394 presence of non-surface-active components present in the buttermilk. Furthermore, the MFGM
395 emulsions were found to be sensitive to pH <6, most likely due to the protein fraction present in
396 MFGM. Both MFGM and lecithin emulsions were destabilized by the addition of calcium
397 whereas casein had a stabilizing effect. The interaction of casein was stronger at the lecithin
398 interface compared to that of MFGM due to packing and the composition of the latter interface,
399 as also shown by the results in emulsion stability. Moreover, the calcium-induced droplet
400 coalescence and destabilization was inhibited in the presence of casein due to formation of
401 emulsion flocs. MFGM is expected to bind calcium and inhibit its destabilizing effect.

Overall, this study sets the basis for the formulation of natural food emulsion with MFGM fragments, especially in the field of dairy products, which contain calcium and caseins. MFGM may be considered as a natural, nutritionally valuable, emulsifier in future food applications. Detailed information about the MFGM-casein interaction and the effect of different emulsifying processes (e.g. high-pressure homogenization) on the MFGM emulsion stability remains a subject for further elucidation.

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