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Dairy Sci. & Technol. (2014) 94:439–453
DOI 10.1007/s13594-014-0171-8

ORIGINAL PAPER

The antiproliferative properties of the milk fat globule membrane are affected by extensive heating

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Received: 3 November 2013 / Revised: 28 March 2014 / Accepted: 28 April 2014 /
Published online: 23 May 2014

Abstract The milk fat globule membrane (MFGM), the material surrounding milk fat globules, is not only interesting from a technological standpoint but it also shows great potential as a health ingredient, as it exerts cytotoxic and apoptotic effects against colon cancer cells. Although the effects of milk processing on the MFGM composition and functionality are well documented, less is understood on how processing may affect its bioactivity. This study aimed to determine if heating can affect the antiproliferative capacity of the MFGM. To do so, MFGM was extracted from milk heated at 80 °C for 10 min, as this temperature/time regime is known to cause extensive protein-protein interactions with changes in the processing functionality of milk. Two cell lines, whose morphological features are representative of two different stages of colon carcinogenesis (HT-29 and Caco-2), were used to test the antiproliferative capacity of MFGM isolates obtained either from untreated or heated milk. Cell proliferation analysis showed a similar dose-dependent decrease of DNA synthesis in both cell lines exposed to 6.25–200 μg of MFGM protein.mL^{-1}, isolated from unheated milk. The heat treatment diminished the efficacy of the MFGM isolates, as only the highest concentrations of MFGM tested following heating showed a significant effect on cell proliferation. The decreased ability of MFGM isolates to affect the carcinoma cell proliferation was attributed to changes in composition, mainly phospholipid losses. Changes to the supramolecular structure of the MFGM caused by heating may also have played a role. This work demonstrates the importance of processing history in assessing the biological functionality of MFGM.

Keywords Antiproliferative · Bioactivity · Bioefficacy · Milk heating · MFGM

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1 Introduction

The milk fat globule membrane (MFGM) represents a very small fraction of the total mass of fat globules (2–6%) (McPherson 1984), but despite this, its commercial and nutritional value should not be underestimated. In nature, the MFGM protects the lipid fraction from enzymatic degradation, oxidation, and physical instability (Ye et al. 2002). Common dairy practices such as cooling, heating, homogenization, and shear markedly affect the physico-chemical properties of the fat globule membrane (Michalski et al. 2002; Sharma and Dalgleish 1994). Among them, heat and homogenization are the most important factors affecting the composition and structure of MFGM, as they induce protein denaturation, structural rearrangements, and aggregation (Kim and Jimenez Flores 1995; Michalski et al. 2002; Morin et al. 2007). Heating affects the composition of the MFGM as adsorption of proteins on the MFGM surface or desorption of membrane material may occur. In addition to protein aggregation, it has been reported that heating also causes changes in the iron content, solubility, and the emulsifying properties of MFGM isolates (Corredig 1998).

During heating, partial displacement of membrane material by skim milk proteins occurs, due to breakdown or competitive adsorption (Kim and Jimenez Flores 1995; Houlihan et al. 1992a, b). Serum proteins (especially β-lactoglobulin) deposit on the MFGM, filling in gaps in the membrane surface (Houlihan et al. 1992a, b). β-lactoglobulin (β-lg) molecules bind to the MFGM directly through sulfhydryl-disulfide interchange reactions as well as non-covalent interactions (Dalgleish and Banks 1991; Kim and Jimenez Flores 1995). Regardless of the mechanism by which these interactions occur, it is recognized that the level of structural and compositional rearrangements on the MFGM are dependent on the extent of the heat treatment and shear applied to the fat globules (Michalski et al. 2002; Michalski and Januel 2006).

Starting at 60–65 °C, milk proteins undergo structural changes, and associations occur between β-lg and the MFGM (Kim and Jimenez Flores 1995; Sharma and Dalgleish 1994). It has been reported that the cysteine residues present in the MFGM can be highly reactive, and temperatures as low as 60 °C can generate aggregates and disulfide-stabilized complexes, like those occurring between butyrophilin (BTN) and xanthine oxidase (XO), which form intermolecular disulfide bonds. Temperatures above 70 °C result in the exposure of reactive thiol groups, allowing thiol disulfide interchange reactions and the formation of disulfide-linked aggregates as well as partial or total loss of PAS6/7 from the MFGM (Lee and Sherbon 2002; Evers 2004). However, at lower temperatures (60–70 °C), the MFGM proteins may initiate reactions by providing reactive thiol groups, before the actual full exposure of the β-lg structure occurs (Ye et al. 2004a). Heating also causes modifications to the phospholipid composition (Evers 2004; Houlihan et al. 1992a). The changes in lipid composition are dependent on processing conditions as well as temperature regimes. A 20% lipid loss has been reported after heating at 80 °C for 3–18 min (Lee and Sherbon 2002). However, the details of compositional changes have yet to be reported.

Although the compositional changes occurring to the MFGM after processing have been widely studied, much less is known of their effects on biological functionality. An understanding of the effect of heating on the bioefficacy of MFGM is necessary to be able to clearly understand how to process foods with enhanced functionality. A better

understanding of how heat treatment can affect MFGM functional properties may lead to new processing practices to obtain dairy products with improved nutritional properties.

Recently, it was shown that MFGM isolated from untreated milk induces apoptosis in the adenocarcinoma HT-29 cell line, as shown by an increase in caspase-3 production and the reduction of lactate dehydrogenase enzyme production by the remaining viable cells (Zanabria et al. 2013). The present work aimed to evaluate if heat treatment affects the antiproliferative capacity of the MFGM isolate. A high heat treatment (80 °C for 10 min) was chosen, as this treatment is known to cause extensive changes to the structure of whey proteins and the formation of whey protein aggregates and to modify the processing functionality of milk (Guyomarc'h et al. 2003; Ye et al. 2004b). For this study, the colon cancer cell lines Caco-2 and HT-29 were used, because they express high and lower levels of COX-2, respectively, and so may represent different stages of colorectal carcinogenesis (Dommels et al. 2003).

2 Materials and methods

2.1 Isolation of MFGM

MFGM isolates were prepared from fresh raw milk samples collected at the Elora Research Station (Guelph, ON, Canada) under sterile conditions, as previously described (Zanabria et al. 2013). Also, MFGM isolates were obtained from milk heated at 80 °C for 10 min. For this, raw milk was distributed into 500-mL bottles (400 mL per bottle) and placed into a 90 °C water bath, with gentle swirling until the desired temperature (80 °C) was attained (about 4 min). The bottles were then transferred to a temperature-controlled water bath at 80 °C for 10 min, with gentle agitation. After treatment was completed, samples were cooled, and MFGM was isolated as previously described (Zanabria et al. 2013).

Cream from heated or unheated milk, obtained by centrifugation (4,800×g for 30 min at 4 °C) (Beckman Coulter, Mississauga, ON, Canada), was washed three times with endotoxin-free water (Hyclone, Fisher Sci., Mississauga, ON, Canada) (1:10 cream/water ratio). Two slow freezing-thawing cycles were carried out (−20 °C/37 °C) to disrupt the fat globules, and afterwards, 1% sodium citrate was added to minimize casein contamination. After 30 min of incubation at room temperature, the MFGM was collected by ultracentrifugation at 60,000×g. for 1 h at 4 °C (Optima LE-80 K, Beckman Coulter, Mississauga, ON, Canada). The MFGM pellets were homogeneously resuspended in 3–5 mL of endotoxin-free water and freeze-dried. The MFGM preparations were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (20% acrylamide gel) and transmission electron microscopy (Zanabria et al. 2013).

For cell stimulation, the freeze-dried material was reconstituted in endotoxin-free water (2:1 $w \cdot v^{-1}$), and the protein content was determined by the Lowry assay (DC Protein Assay, BioRad Laboratories, Mississauga, ON, Canada) using BSA as standard (Biorad). The preparations had less than 15 EU of LPS.mg^{-1} protein (1.5 ng of LPS.mg^{-1} protein) as determined using the Limulus Amebocyte Lysate Pyrochrome® kit (Associates of Cape Cod Inc., Cape Cod, MA, USA).

2.2 Phospholipid determination

A modified Folch procedure (Folch et al. 1957) was used to extract the phospholipid fraction from MFGM samples. Briefly, the freeze-dried MFGM isolates (40–50 mg) were suspended in 1 mL of 0.1-mol.L^{-1} NaCl solution and mixed for 1 min. Subsequently, 4 mL of chloroform/methanol solution (2:1 $v \cdot v^{-1}$) was added, and the mixture was agitated vigorously for 1–2 min. The homogenate was then centrifuged at 400×g for 15 min at room temperature (Heraeus Multifuge X1R centrifuge, Thermo Scientific, Langenselbold, Germany), and the lower organic phase containing lipids was harvested. This operation was repeated three times, and the remaining lower phase evaporated under a stream of nitrogen. The dried lipid samples were either used immediately or stored at −20 °C.

Total phosphorous content was measured using plasma-optical emission spectrometry (ICP-OES) at the Laboratory Services (University of Guelph, ON, Canada), following the guidelines of ISO 17025 and the AOAC Official Method 2011.14. Lipid phosphorus contents were then multiplied by a factor of 25 to give the PL content (Sanchez-Juanes et al. 2009).

Phospholipid composition was also measured using nuclear magnetic resonance (^{31}P NMR) spectroscopy. For this analysis, extracted MFGM lipids were dissolved in a ternary solvent (Larijani et al. 2000), consisting of a mixture of $CDCl_3$/CD_3OD/K_4EDTA (100:40:20 by vol.). The K_4EDTA solution was prepared by dissolving the chemical reagent in D_2O (0.2 mol.L^{-1}). The lipid sample and the solvent system were then gently mixed in a glass vial, and the emulsion was stored overnight at room temperature until phase separation was complete. Care was taken to ensure that there was a clear separation of the organic and aqueous phase. From the two liquid phases, an aliquot (600 μL) of the lower phase (mainly chloroform-based) was transferred into a 5-mm NMR tube, and the internal calibration standard (2 μL of triethyl phosphate) was added. ^{31}P NMR analysis was performed on a Bruker AVANCE III 600 MHz spectrometer operating at 243 MHz and equipped with deuterium field-frequency stabilization and automatic field-homogeneity adjustment capability. All measurements were carried out at 297°K using a 30° pulse angle, 50-μs pulse width, 10-s relaxation delay, and 2-s acquisition time. Five pure PL classes, including L-α-phosphatidyl choline (PC), L-α-phosphatidyl ethanolamine (PE), L-α-phosphatidyl serine (PS) and sphingomyelin (SM) (Avanti Polar Lipids, Alabaster, AL, USA), and L-α-phosphatidyl inositol (PI) (Sigma-Aldrich, Oakville, ON, Canada) were analyzed by NMR either individually or in combination, to test the possible interference or interaction among PL species. The number of scans varied: 16 and 600 scans for the standards and MFGM samples, respectively. The chemical shifts (δ) and line-widths were measured digitally and referenced to triethyl phosphate, using orthophosphoric acid (85% $v\ v^{-1}$) as external standard (δ=−1.0±0.01 ppm) (Helmerich and Koehler 2003). Owing to the presence of three deuterated solvents, the field was adjusted by systematically locking on the $CDCl_3$ signal for a better reproducibility (Larijani et al. 2000). Calculation of the relative PL concentrations in the samples was based on integrals, and the quantitative analysis was carried out through an interactive fitting (deconvolution) of the spectra (assuming a Lorentzian shape for the ^{31}P NMR signals) to get the peak areas by the use of the program TopSpin™ 2.1 (Bruker BioSpin GmbH, Bruker Corporation, Germany).

2.3 Cell culture

Two human adenocarcinoma cell lines (HT-29 and Caco-2) were employed to test the antiproliferative capacity of the native MFGM isolates and to determine the half maximal dose (EC50) required to inhibit their proliferation by 50%. The cell lines were obtained from the Canadian Research Institute for Food Safety (CRIFS, ON, Canada) culture collection. Both cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 2-mmol.L^{-1} glutamine, 1% penicillin-streptomycin (dual antibiotic solution 10,000 U.mL^{-1}), and 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen Canada Inc., Burlington, ON, Canada). Medium for Caco-2 cells was further enriched with 0.025-mol.L^{-1} HEPES buffer solution and 0.1-mmol.L^{-1} MEM non-essential amino acid solution (Life Technologies Inc., Burlington, ON, Canada). Cells (20–30 passage number) were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C and grown as monolayers in 25- or 75-cm^2 T-flasks (Corning Inc., NY, USA). Cell culture medium was changed every 48 h and cells passed weekly (80–90% confluence) using 0.25% trypsin containing 0.38 g.L^{-1} of EDTA-4Na (Life Technologies Inc.). Prior to analysis, cells were confirmed to be at exponential growth phase by the sulforhodamine B (SRB) assay before stimulation, with cell density estimated at 4-h intervals for a 48-h total period. Medium concentration was kept constant throughout all analyses, eliminating possible diluting effects due to reconstitution of the MFGM samples in LPS-free water.

2.4 Cell proliferation and determination of the EC50 value

Cell proliferation was determined using the Cell Proliferation ELISA BrdU (colorimetric) kit (Roche Diagnostics, QC, Canada). Ten dose-points (0–200 μg of MFGM.mL^{-1} based on protein) were applied for the determination of the half maximal amount required to cause a 50% reduction in cell proliferation (EC50). To assess the effect of heating on MFGM bioactivity, only six dose-points were investigated, along with the commercial compounds melphalan and *N*-acetyl-D-sphingosine (Sigma), included as positive controls at 0.05 mmol.L^{-1} and 20 μmol.L^{-1}, respectively. Cell stimulation and analysis were carried out as previously described (Zanabria et al. 2013). The labeled molecules were detected by substrate reaction and the final product quantified by measuring the absorbance at 450 nm using a Multilabel Counter (Wallac 1420 Victor Perkin-Elmer Life Sciences, Peterborough, ON, Canada). Results were expressed as percentage with respect to control cells (set as 100%) grown under regular conditions.

2.5 Statistical analysis

All experiments were performed at least three times unless otherwise detailed in the graphs or tables. Results are expressed as mean±SD. Differences among means were determined by one-way ANOVA and subsequent Tukey's comparison test using the IBM SPSS Statistics v.20 software. The four parametric equation model was applied for the EC50 determination using Sigma Plot v. 10. Differences at $P<0.05$ were considered to be statistically significant.

3 Results

3.1 Characterization of the MFGM samples

Figure 1 shows the heterogeneous microstructures of the MFGM isolates derived from unheated and heated milk, as observed by transmission electron microscopy. Samples derived from heated milk showed a more diffuse structure and more material surrounding the MFGM fragments, compared to the samples obtained from unheated milk. The long fragments of MFGM were still visible in both samples. Heat-treated MFGM showed the presence of casein micelles coexisting with fragments of membrane material.

Compositional changes in the MFGM protein profiles were observed by SDS-PAGE as shown in Fig. 2. A larger proportion of the sample remained unresolved on top of the gel for MFGM isolates from heated milk. The isolates from heated milk also seemed to contain both quantitative and qualitative changes in protein content. Traces of caseins were present in the unheated sample (Fig. 2, lane 2), possibly as a result of mechanical treatments (Dalgleish and Banks 1991). When the MFGM was obtained from heated milk, there was a considerable amount of skim milk-derived proteins in the MFGM isolates. Increasing amounts of β-lactoglobulin, α-lactalbumin, and κ-casein were recovered, as expected, after heating milk at temperatures above 75 °C (Ye et al. 2004b). In the case of α-la, previous findings are ambiguous as to whether this whey protein is present or absent in MFGM isolates when milk is heated (Corredig 1998; Dalgleish and Banks 1991). However, Fig. 2 shows the presence of some α-la in the MFGM isolate.

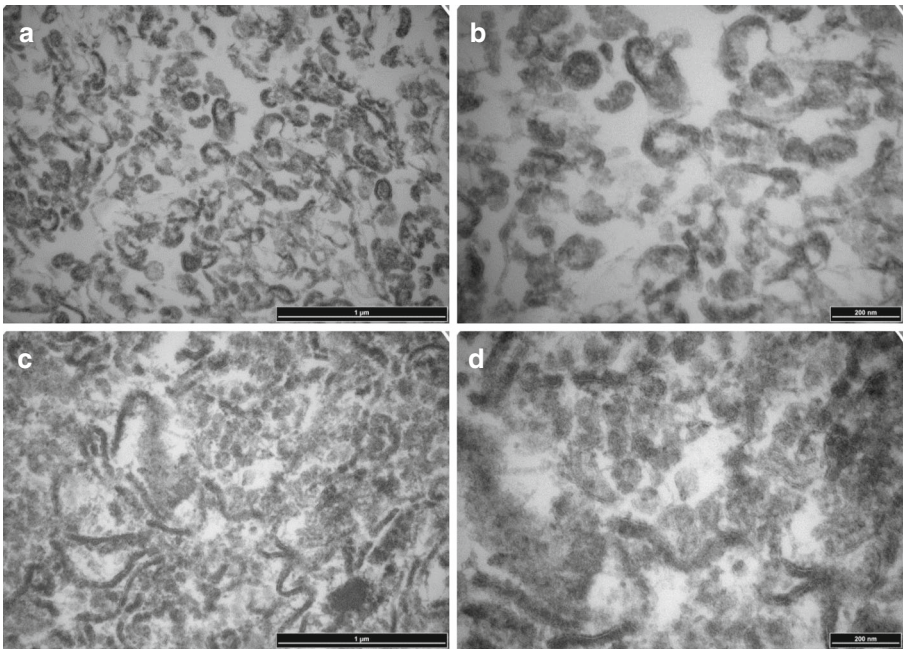

Fig. 1 Transmission electron micrographs of MFGM isolates obtained from unheated (**a**, **b**) and heated milk (**c**, **d**). *Bar size* 1 μm (**a**, **c**); 200 nm (**b**, **d**)

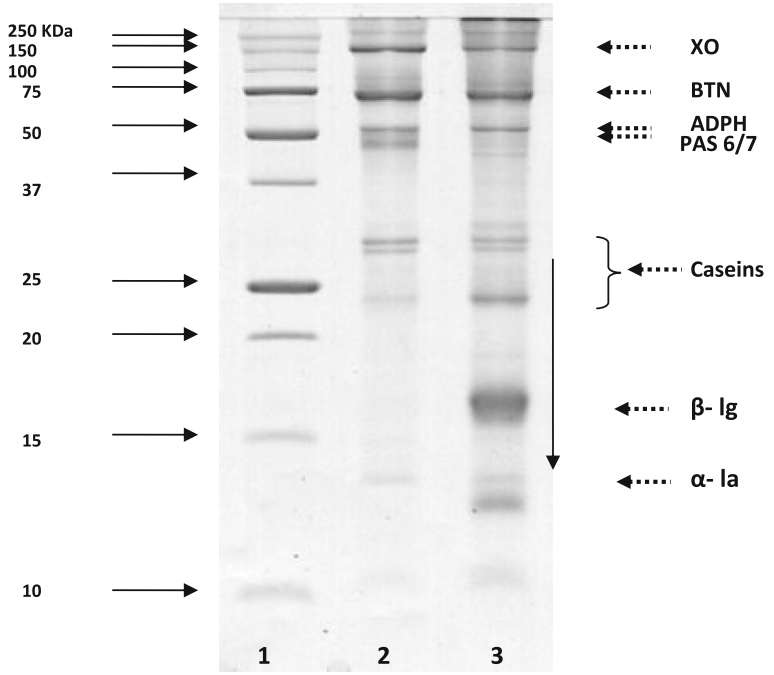

Fig. 2 SDS-PAGE of MFGM isolates from raw milk (*lane 2*) and heat-treated milk (*lane 3*). *Lane 1* molecular mass standard. *Arrow* indicates the direction of migration. Each *lane* was loaded with 15 μg of MFGM isolate (based on total solids)

With regard to the MFGM-derived proteins, ten major polypeptide bands were observed by SDS-PAGE of the raw isolates, including XO (with a reported molecular mass of 145 $kg.mol^{-1}$), PAS III (94 $kg.mol^{-1}$), BTN (67 $kg.mol^{-1}$), and PAS 6 and PAS7 (50 and 47 $kg.mol^{-1}$, respectively) (Mather 2000). There were differences in the distribution of the band intensities between the unheated and the heated sample. PAS 6 and 7 were markedly decreased, probably because of their loose association to the MFGM (Lee and Sherbon 2002; Houlihan et al. 1992a). Previous studies relate the loss of these proteins to the presence of serum components in the sample (Houlihan et al. 1992a, b) and to the fact that PAS 7 seems to be heat-labile while PAS 6 is more thermo-resistant (Ye et al. 2004).

The amount of phospholipids (PL) in the MFGM isolates from raw and heated milk was estimated using the total phosphorous content and was 161±5.9 and 137±2.1 μg of $PL.mg^{-1}$ of protein, respectively, values that are within the range previously reported (Keenan and Patton 1995; Sanchez-Juanes et al. 2009). The results from this study confirmed previous reports that heating milk causes a loss of total PL from the MFGM (Lee and Sherbon 2002). However, this is still somewhat controversial, as Houlihan et al. (1992b) reported that heating milk at 80 °C for 20 min resulted in significant losses of triacylglycerols but not of PL from the MFGM. Discrepancies may be due to differences in the treatment as well as the methodologies used to obtain the MFGM isolates, as it is known that processing may modify the structure of the membrane and result in decreased solubility in the extraction solvents (Morin et al. 2007). It is important to note at this point that PAS6/7 present in human milk has been identified

as a lactadherin capable of phospholipid binding (Morin et al. 2007). The loss of PAS6/7 in the heated sample (Fig. 2, lane 3) may explain the decrease in MFGM phospholipids during the heating or washing steps.

The ^{31}P NMR spectroscopy profiles for the MFGM isolates of raw and heated milk are shown in Fig. 3 (a and b in scale). Peaks were identified based on the chemical shifts obtained from individual standards (Table 1). The trend in chemical shifts of the phospholipid classes obtained in the mixture of standards and the MFGM samples agreed with previously reported data (Estrada et al. 2008; Garcia et al. 2012; Helmerich and Koehler 2003). Less peak resolution was noted for the signal from PL of heated MFGM, and it may be due to lipid-lipid interactions (i.e., micellization) or changes in the spatial distribution of the P-groups during heating. The common irreproducibility of the exact resonance of PL is due to the sensitivity of the chemical shift of a phosphate group to very slight changes in its conformation, its environment, or the acyl chain composition (Larijani et al. 2000). As observed in Table 1, more than one PL standard resulted in two or more peaks, and some even shared similar chemical shift ranges, obstructing their identification when in a mixture. Nonetheless, the ^{31}P NMR confirmed that heated samples showed a smaller amount of total phospholipids than raw MFGM isolates, and it also allowed qualitative evaluation of PL distribution within the samples.

The initial composition of the MFGM isolate originating from raw milk was similar to previously reported data for milk, highlighting the presence of the major PL classes, including PC (21–36%), SM (18–35%), PE (20–46%), and, in relative minor proportion, PI (7–18%) and PS (4–9%) (Sanchez-Juanes et al. 2009; Murgia et al. 2003). The heat-treated sample showed fewer PL species along with a peak at 3.42 ppm, which may represent a different assembly of the PL. The PI signal was almost absent from the heat-treated sample, and many other peaks were significantly reduced (SM + PS, PE, PC). Few studies have focused on processing changes occurring in individual PL. Milk heating caused a significant reduction of the major PL species as more than 50% of PC, SM, and PE were lost, supporting the hypothesis of the partial dislocation or interaction of serum components with the exposed PL species mainly located in the bilayer outer leaflet (Danthine et al. 2000). The peak corresponding to SM + PS was the most affected (~75% of the original area), followed by PC and PE (with an area decrease of approximately 65 and 50%, respectively).

3.2 Effect of native MFGM isolates on colon cancer cell proliferation

Figure 4a, b shows similar trends to those previously reported for HT-29 cells (Zanabria et al. 2013). Caco-2 and HT-29 cell lines were negatively affected when treated with the MFGM native isolates, although Caco-2 cells seemed to be more resistant (a significant higher amount of MFGM isolate was required to achieve the same effect upon cell proliferation). The EC50 values for Caco-2 and HT-29 cells were 102 and 93 μg of protein MFGM.mL^{-1}, respectively.

3.3 Effect of milk heating on the bioactivity of the MFGM isolates

Heating of milk at 80 °C for 10 min caused interactions between serum and MFGM proteins and resulted in the formation of complexes between proteins and the partial

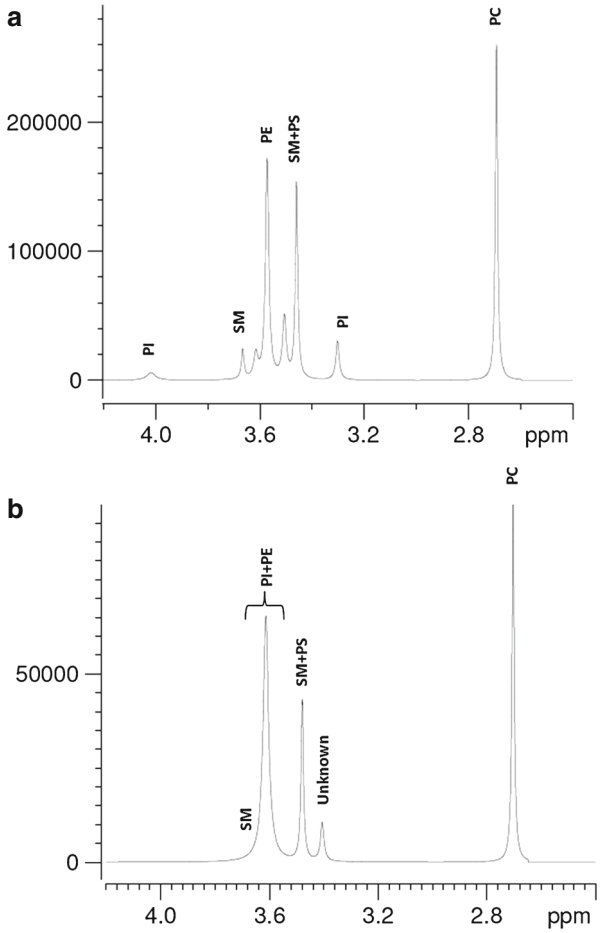

Fig. 3 Phospholipid profile of the MFGM isolates obtained from raw (**a**) and heated (**b**) milk obtained by ^{31}P NMR spectroscopy analysis. *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PS* phosphatidylserine, *SM* sphingomyelin. *Figures* are representative of duplicate experiments

loss of individual components. Cell proliferation tests revealed that the bioactivity was significantly reduced as a result of the heat treatment applied. It is important to point out that the samples were compared based on total protein. In HT-29 cells, heated MFGM induced a similar dose-response to that obtained with the native isolate, yet lower concentrations (<50 μg of protein.mL^{-1}) did not induce any significant antiproliferative effect (Fig. 5a). While 50% of antiproliferative capacity was achieved when 100 μg of protein raw MFGM.mL^{-1} was used, the same amount of heat-treated sample only achieved 20%. Furthermore, in agreement with previous results reported for the native isolate (Fig. 4b), Caco-2 cells seemed to be more resistant to the heated MFGM antiproliferative activity (Fig. 5b), being only affected by the highest concentration (200 μg.mL^{-1}, $P<0.05$).

The highest dose of the heated MFGM isolate tested produced about a 40% reduction in Caco-2 cell proliferation. Conversely, the lowest concentrations caused an increment in cell proliferation, increasing cell DNA synthesis up to 35%. Table 2

Table 1 Chemical shifts (δ) of phospholipid classes and internal reference standard analyzed by ^{31}P NMR spectroscopy

Lipid Class	δ^{31}P NMR (ppm)
PC	+ 2.682 to+2.712
PE	+ 3.526 to+3.613
PI (1st peak)	+ 3.213 to+3.320
PI (2nd peak)	+ 3.516 to+3.560
PI (3rd peak)	+ 4.165 to+4.416
PS	+ 3.429 to+3.470
SM (1st peak)	+ 3.453 to+3.476
SM (2nd peak)	+ 3.662 to+3.684
Triethyl phosphate	+ 2.051 to+2.073

Results represent the ranges obtained from the three samples (dilutions) analyzed for each compound

Data is a representative of three experiments

PC phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PS* phosphatidylserine, *SM* sphingomyelin

summarizes the data related to the MFGM antiproliferative capacity obtained from the study on the two different cell lines and compares the effect of the MFGM isolates with those obtained using commercially available anticarcinogenic drugs (C2-ceramide and melphalan).

4 Discussion

In the dairy industry, heating (e.g., pasteurization) of milk is employed to improve safety, increase shelf-life, and influence the properties of processed milk products. To date, few studies have focused on the effect of processing (i.e., heating) on the biofunctionality of MFGM components. As previously reported (Kim and Jimenez Flores 1995; Ye et al. 2004a), heating at 80 °C for 10 min ensured the formation of protein aggregates, by causing significant changes in the MFGM composition and its supramolecular structure. The composition of the MFGM isolated from unheated milk was similar to that previously reported in the literature, with no bands corresponding to whey proteins (Ye et al. 2002), indicating that these proteins, along with most of the caseins, were efficiently removed by the washing procedure. By heating under those conditions, it was then possible to test the hypothesis that composition and supramolecular structure affect the efficacy of MFGM on cancer cells.

The antiproliferative capacity of the native MFGM was tested with two different cell lines (HT-29 and Caco-2). A dose-dependant effect was observed in both cases, although to a different extent depending on the cell type. Differences in metabolism and absorption between the cells (e.g., nutrient absorption, cell division rate) as well as innate cell characteristics (i.e., protein expression, which reflects different stages of colorectal carcinogenesis) may be the reason for their different responses. A recent

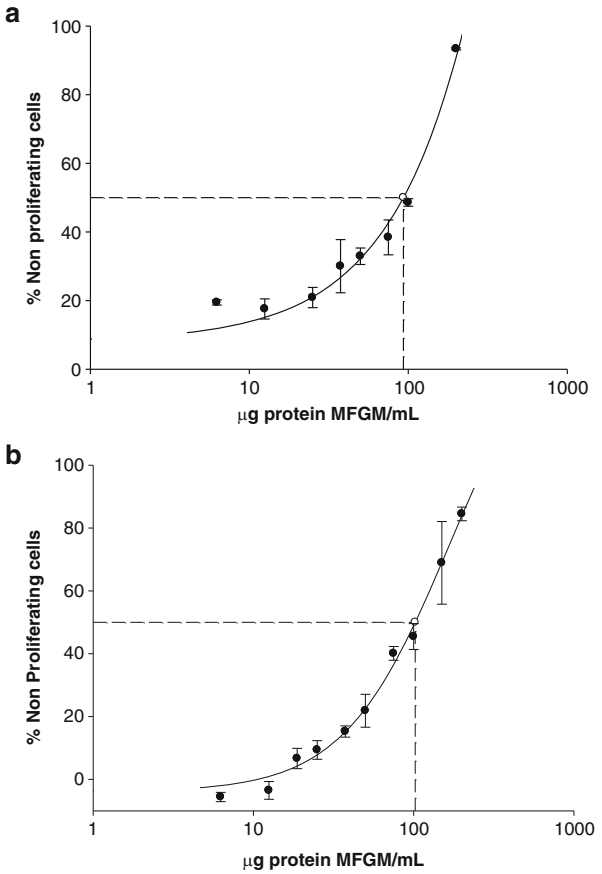

Fig. 4 Dose-response curve for **a** HT-29 and **b** Caco-2 cells when stimulated with native MFGM isolate. Cell proliferation was assessed by quantification of the pyrimidine analog BrdU incorporation into newly synthesized cellular DNA. Results represent the means±SD of three independent replicate experiments. EC50 value is shown (*empty symbol*). For statistical analysis see Table 2

study demonstrated that sphingomyelin treatment did not significantly alter mRNA levels but had a significant effect on protein levels encoded by genes critical to the early stages of colon cancer, such as beta-catenin, connexin-43, and Bcl-2 (Simon et al. 2009). Different levels of these proteins in cells may cause different responses to MFGM.

Cytotoxic as well as apoptotic effects have been shown to be responsible for the anticarcinogenic activity of the native MFGM (Zanabria et al. 2013). Additionally, Hintze et al. (2011) reported changes in gene expression in Caco-2 cells after treatment with a MFGM isolate obtained after cream churning, with the majority of the genes affected being identified as metallothioneins (MT), which code for small molecular weight, cysteine-rich proteins with redox, and metal-binding activities. Their work suggested that the toxicity caused by the MFGM on these cells could have been induced by oxidative stress (mediated through XO) or caused by the high concentration of zinc present in the untreated MFGM extract. The present investigation was not designed to identify the agents responsible for such differences; rather, it aimed to

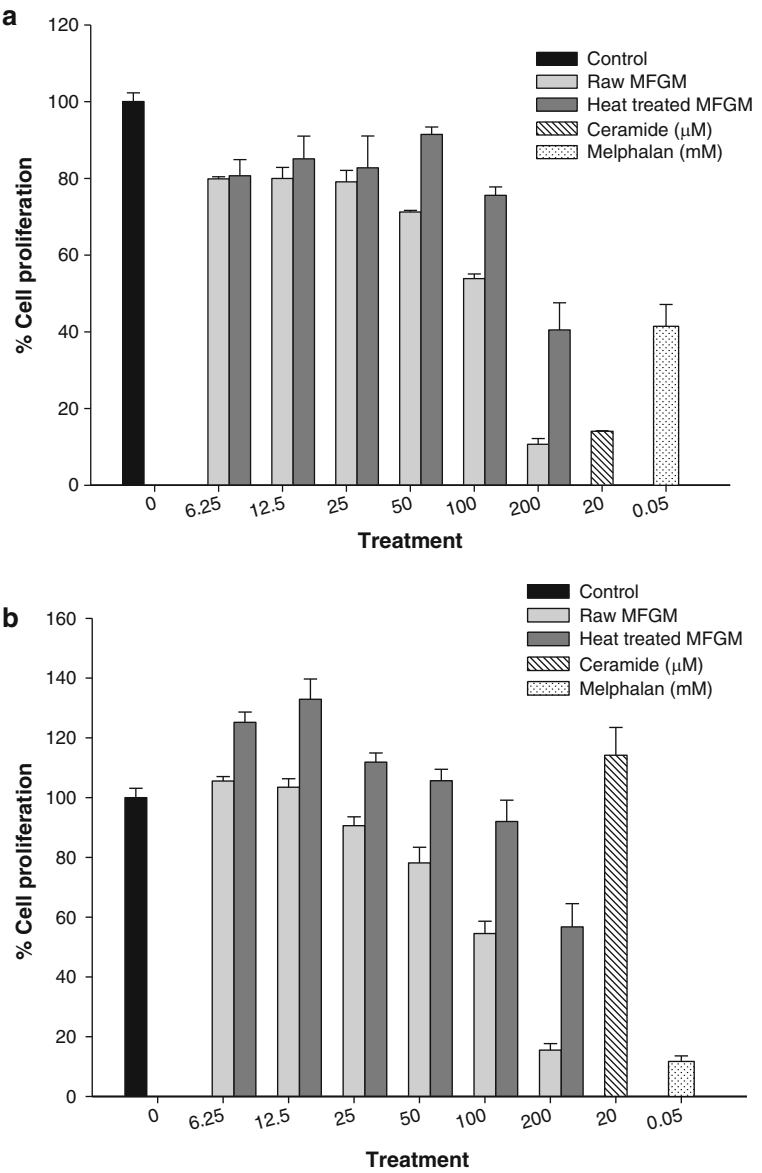

Fig. 5 Effect of heat treatment on the bioactivity of MFGM on **a** HT-29 and **b** Caco-2 cells. Cells were treated with MFGM isolates (μg protein.mL^{-1}) derived from raw (native) or heat-treated (80 °C, 10 min) milk for 24 h. Cell proliferation was assessed by the BrdU colorimetric method. Results represent the means±SD of three independent replicate experiments. For statistical analysis see Table 2

evaluate the simultaneous action of all the biocomponents present in the MFGM (proteins, PL, enzymes, glycoproteins) either individually or in combination.

Heating (at 80 °C for 10 min) had a great impact on the bioactivity of the MFGM isolates. It has already been shown that processing has a major impact on the MFGM technological functionality (Morin et al. 2007; Corredig 1998). Also in this study, it was confirmed that such extent of heating caused a decrease in total protein and

Table 2 Effect of different stimulants on the proliferation (%) of two human adenocarcinoma cell lines (HT-29 and Caco-2)

Stimulant	HT-29		Caco-2	
	Native	Heat-treated	Native	Heat-treated
0-MFGM	100.0±2.3a	100.0±2.3a	100.0±3.1a	100±3.1c,d
6.25 - MFGM	80.1±0.6b	80.7±4.2b,c	105.6±1.5a	125.2±3.4a,b
12.5 - MFGM	81.2±2.9b	85.1±5.9b,c	103.5±2.8a	132.9±6.7 a
25 - MFGM	79.1±3.0b	82.8±8.3b,c	90.6±3.0b	111.9±3.1b,c
50 - MFGM	71.2±0.4c	91.5±1.9a,b	78.1±5.2c	105.6±3.8c,d
100 - MFGM	53.9±1.2d	75.6±2.2c	54.5±4.1d	92.0±7.1d
200 - MFGM	10.7±1.5e	40.5±7.1d	15.5±2.2e	56.7±7.8e
Ceramide	14.1±0.1a	–	114.2±9.3a	–
Melphalan	41.4±5.7b	–	11.7±1.8b	–

Cells were treated with either the commercial drugs ceramide (20 $\mu mol.L^{-1}$) or melphalan (0.05 $mmol.L^{-1}$) (positive controls) or with MFGM isolates (μg $protein.mL^{-1}$) derived from raw (native) or heat-treated (80 °C, 10 min) milk for 24 h

Cell proliferation was assessed by the BrdU colorimetric method

Results represent the means±SD of three independent replicate experiments

Values within the same column that have different letters are significantly different ($P<0.05$)

phospholipid content as well as the formation of complexes with skim milk-derived proteins. This work demonstrated for the first time that extensive heating caused a significant reduction in the antiproliferative capacity of the MFGM isolates. Further research is needed to identify critical time-temperature combinations that affect the bioefficacy of the MFGM isolates.

Interestingly, the results obtained using ceramide (20 $\mu mol.L^{-1}$) and melphalan (0.05 $mmol.L^{-1}$) as positive controls confirmed the hypothesis that sphingolipids play a key role in the MFGM bioactivity. As shown in Table 2, C2 ceramide, a synthetic analog of natural ceramide arising from sphingolipid metabolism (Hintze et al. 2011), shows a greater effect on HT-29 cells compared to that observed on Caco-2 cells. The results obtained for the native MFGM isolate (Fig. 4) are fully in line with these findings. In contrast, melphalan caused a greater impact on Caco-2 cells compared to ceramide. Ceramide was effective only at higher concentrations (data not shown). Once the milk was heated, a reduction in PL content (including SM) was observed in the MFGM fractions, and the reduction in bioactivity observed in these samples would further support the role of PL in the bioactivity of the native MFGM.

It is also important to note that individual proteins, such as XO, known to cause oxidative stress by generating reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) (Hintze et al. 2011), along with the formation of disulfide-linked aggregates, or even the presence of certain minerals (i.e., zinc, copper) in the native MFGM isolate but absent in the heated sample, may have contributed to the loss of its bioactivity. As reported previously (Ye et al. 2002; Mather 2000; Corredig 1998), heating milk causes not only the association between XO and BTN, which form a supramolecular complex, but also the loss of mineral components. The formation of

supramolecular structures with skim milk-derived proteins may also play a role in the reduction of antiproliferative capacity.

Though the specific compounds that are directly responsible for the changes in the antiproliferative capacity of the MFGM have not been yet defined, these results suggest that protein-protein interactions, protein denaturation, and phospholipid reduction, along with the structural changes known to occur during heating in the MFGM isolates, directly influence bioefficacy. These results raise new questions related to which active components play a role in the antiproliferative effect observed with the colon cancer cell lines. After heating, the antiproliferative activity was substantially reduced but not inhibited, and it is important to point out that the proliferation tests were carried out based on total protein content. Hence, it may be speculated that a change in composition, more than structure, may be responsible for the decrease in bioactivity.

5 Conclusions

Compositional and structural changes occurring to the MFGM as a result of milk heating (80 °C, 10 min) greatly reduced the in vitro antiproliferative capacity of the native isolates. Bioactivity of heated MFGM was only observed at the highest concentrations used in this study (200 μg protein.mL^{-1}). The results suggested that a change in composition (protein and phospholipids) and the formation of heat-induced supramolecular aggregates may play a role in the decrease of bioactivity. Further investigation is needed to better understand the specific components responsible for the reduction in the bioactivity of heated MFGM fractions and the critical time-temperature combinations that would better preserve bioactivity. This research emphasizes the need for further investigation into the effects of milk heating on MFGM bioactivity, with the ultimate goal of obtaining a fundamental understanding of the phenomena involved, so improvements in process design can be done to protect MFGM properties. Knowing the history of the source material utilized for MFGM isolation would be critical for defining its nutritional properties and may shed some light on the debate related to conflicting in vivo and in vitro data.

Acknowledgments This study was supported by the Canadian Dairy Commission (CDC), Ontario Dairy Council, Dairy Farmers of Canada, and Natural Sciences and Engineering Research Council of Canada (NSERC). The authors would like to thank Ms. Laura Wright for her help with the milk collection procedure at the experimental farm.

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