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Microstructural and lipid composition changes in milk fat globules during milk powder manufacture

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Running title: Changes of MFG during milk powder processing

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Abstract

The purpose of this study was to investigate the effects of milk powder processing conditions (pasteurisation, homogenisation and spray-drying) on the microstructure and composition of fat globules in cow milk. In general, the process of pasteurisation results in fewer changes in the microstructure and lipid composition than do other processing methods. The phospholipid, sterol and the fatty acid composition of the phospholipids, with the exception of the fatty acids of total lipids, underwent significant changes under different processing conditions compared with the untreated milk fat globules. The contents and distribution of the phospholipids in pasteurised milk indicated a high level of stability, but the amounts of phosphatidylethanolamine, phosphatidylserine and sphingomyelin were significantly affected by homogenisation. A reduction in the cholesterol content was observed after pasteurisation and homogenisation. The results indicate that processing had a significant effect on the composition and structure of the membrane of the phospholipids in milk fat globules.

Keywords: milk fat globule, microstructure, lipids composition, pasteurisation, homogenisation, spray-drying

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Introduction

Milk, which is 87% water, has been recognised as an excellent source of nutrition for mammal newborns. Fat is a major component of milk, which exists as small droplets between 0.2 and 10 μm that are called milk fat globules (MFGs). Native MFGs have a special physical structure with a triacylglycerol (TAG) core inside and a tri-layer membrane outside that is derived from a unique secretion mechanism. The membrane is composed mainly of proteins, phospholipids, cholesterol, enzymes and other minor components.[1] Scientific evidence supports breastfeeding as one of the most cost-effective interventions for the improvement of health and the prevention of illness in early childhood.[2, 3] Therefore, the milk of other mammals has been used to make breast-milk substitutes, such as infant formula and other milk products and complementary foods.[4] Although the dairy industry is working to duplicate human milk,[5] it is difficult to replicate the native biological structure and composition of the MFGs. Because milk powder is processed by heat treatment, homogenisation and spray-drying may modify the supramolecular structure and composition of the MFGs.

Many studies have reported changes in the protein components during heat treatment[6, 7] and homogenisation.[8, 9] The phospholipid content was simultaneously changed by heat treatment and homogenisation.[10, 11] Some researchers have used confocal laser scanning microscopy (CLSM) to investigate the surface structure of MFGs with different types of processing to determine the phospholipid profiles.[12, 13] However, these studies did not directly compare batches of milk with the same composition, and the information regarding the lipid composition of the MFGs included only phospholipids. In addition, information is scarce about the effects of the sterol composition of the MFGs during a series of processing conditions. Our knowledge of the changes in the lipid composition of milk powder manufacture is

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therefore fragmented and incomplete. In general, data are lacking on the changes in the lipid composition of MFGs subjected to different treatments to produce milk powder.

A number of studies have used scanning electron microscopy to study the changes in the microstructure of MFGs after heat treatment[14] and the effects of processing of buttermilk.[15] Balasuriya et al.[16] used atomic force microscopy to study the changes in the morphological and nanomechanical properties of the MFG membrane (MFGM) during processing. More recently, CLSM has become a highly successful technique for study of the microstructure of MFG in its native environment with the use of fluorescent dyes.[17-20]

The purpose of our study was to determine the effects of various unit operations during processing on the microstructure and lipid components of MFGs that have the same composition. The results of this study could be useful for dairy manufacturers because it highlights the influence of the physicochemical properties of MFGs during a succession of technological treatments.

Materials and Methods

Samples and Reagents

Raw milk (RM): Raw whole milk was purchased from a local dairy farm (Tianzi Cow Milk Farm, Wuxi, China). One fourth of the milk was stored at 4°C and characterised within 24 h after collection.

Pasteurised milk (PM): The remainder was kept in a double-walled vessel at 63°C ± 0.5°C in a heating bath maintained at 65°C. The temperature was monitored with a thermocouple. After 30 min of treatment, the samples were transferred to disposable 15-mL polyethylene terephthalate conical, sterile test tubes.

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Pasteurised and homogenised milk (PHM): The two-thirds of the PM was homogenised in a two-stage homogeniser (nmGEN 7400H, Stansted, Essex, UK). The homogenisation pressure was 150 bars in the first stage and 100 bars in the second.

Spray-drying milk (SDM): One half of the homogenised milk was processed in a laboratory-scale spray-dryer LabPlant SD-05 (Huddersfield, England). The milk was fed into the main chamber by a peristaltic pump, and the feed flow rate was controlled by the pump rotation speed. The inlet air temperature was 160°C, and the outlet air temperature was 80°C. The spray-dried powder (1 g) was dispersed in 10 mL of deionised water for analysis.

A lipid-soluble Nile red fluorescent dye (9-diethylamino-5H-benzoalpha-phenoxazine-5-one, Sigma-Aldrich, St. Louis, MO; 42 μg/mL in acetone) was used to stain the triacylglycerol core of the MFGs. The fluorescent dye N-(lissamine rhodamine B sulfonyl) dioleoyl-phosphatidylethanolamine (Rd-DOPE; Avanti Polar Lipids, Inc. Alabaster, AL; 1 mg/mL in chloroform) was used to label the phospholipids. The fatty acids, 5a-cholestane and phospholipid standards used to study the composition of the fatty acids, sterols and phospholipids were purchased from Sigma. All other reagents were of analytical grade (Shanghai Chemical Reagents Co., Shanghai, China).

Particle Size Measurements

The MFG size distributions were measured by laser light scattering using a Mastersizer 2000 (Malvern Instruments, Malvern, UK) equipped with a He/Ne laser (λ = 633 nm) and an electroluminescent diode (λ = 466 nm). The refractive index of the milk fat was taken to be 1.460 at 466 nm and 1.458 at 633 nm. The samples were diluted in 100 ml of water directly in the measurement cell of the apparatus to reach

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10% obscuration. The casein micelles were dissociated by addition of 1 ml of buffer (35 mM ethylenediaminetetraacetic acid/NaOH, pH 7.0) to the milk in the apparatus. The size distributions of the MFGs were characterised by the volume-weighted mean diameter $D_{4,3}$ defined as $\sum n_i d_i^4 / \sum n_i d_i^3$, the volume/surface mean diameter $D_{3,2}$ defined as $\sum n_i d_i^3 / \sum n_i d_i^2$, and the number-weighted mean diameter $D_{1,0}$ defined as $\sum n_i d_i^1 / \sum n_i$, where n_i is the number of fat globules of diameter d_i. The $D_{3,2}$ values were selected for analysis because this parameter is very sensitive to the presence of smaller particles, and the $D_{4,3}$ values were also selected because this parameter is very sensitive to the largest particles.

Apparent Zeta-potential

The MFG electrophoretic mobility was measured by electrophoretic light scattering using a Malvern Zetasizer 2000 (Malvern Instruments, Worcestershire, UK). The apparent zeta-potential of the MFGs was calculated from their electrophoretic mobility μ, according to Henry's equation: $\zeta - \text{potential} = (\mu \cdot 6\pi\eta/\varepsilon)/f(ka)$, where η and ε are the viscosity and the dielectric constant of the solution, respectively, at the temperature of measurement. "1/κ" is the Debye length and "a" is the radius of the particle. The Smoluchowski approximation, assuming f(κa) = 1.5, was used. The samples were prepared by suspending 10 μL of MFG in 10 mL of buffer (20 mM imidazole, 50 mM NaCl and 5 mM $CaCl_2$, pH 7.0; η = 0.89 cp, ε = 79), and the zeta-potential was measured at 25°C. The average of three measurements was reported as the zeta-potential.

Surface Tension

The surface tension of the different types of processed milk was determined with a

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surface tension meter (Dataphysics, DCAT21, Germany). The surface temperature of both native and processed milk was kept constant at 25°C.

Confocal Laser Scanning Microscopy

The microstructure of the MFGs was analysed with a Zeiss LSM 710 Meta confocal microscope. A 63 × 1.4 oil-immersion objective was used for all images. The neutral lipids were stained with Nile red. The phospholipids of the MFGM were labelled with Rd-DOPE. Confocal experiments were performed with an argon laser operating at a 488-nm excitation wavelength (emissions were detected from 500 to 530 nm) and a He-Ne laser operating at a 543-nm excitation wavelength (emissions were detected from 565 to 615 nm). The milk sample used for CLSM observation was prepared as previously reported.[21] About 100 μL of the Nile red solution was added to 1 mL of milk. About 20 μL of the Rd-DOPE solution was added to 0.5 mL of milk. After labelling, the samples were kept at room temperature for 30 min; 100 μL of the stained milk was then slowly mixed with 100 μL of the agarose (5 g/L) and stored at 45°C. One drop of the mixture was deposited on a slide and quickly covered with a coverslip.

Analysis of Lipid Composition

Extraction of total lipids

Extraction of the total lipids of milk was carried out using the method described by Folch et al.[22] with some modifications. A 10-mL milk sample was homogenised with 200 mL 2:1 chloroform-methanol (v/v), exposed to an ultrasonic wave for 10 min and centrifuged for 10 min at 4500 rpm. The organic phase that contained the milk lipids was collected and equilibrated with one-fourth volume of a saline solution (NaCl

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0.86%, w/w). The extract was transferred to a separation funnel for 20 min, and the liquid phase was filtered and evaporated under a vacuum. The total lipids extracted were stored at –20°C until gas chromatography (GC) and high-performance liquid chromatography were performed.

Analysis of Fatty Acid Composition

We accurately weighed 20 mg of milk fat into a sealable tube and added 2 mL of hexane and 500 μL of 2-mol/L KOH-CH_3OH. For extraction, the mixture was vortexed for 5 min and 5 mL of deionised water was added. After shaking, the upper layer was collected and dried over anhydrous sodium sulfate, and 1 μL of the resulting fatty acid methyl ester solution was analysed with GC.

GC was performed with a capillary column TRACE TR-FAME (60 m × 0.25 mm × 0.25 μm, Thermo Fisher, USA) mounted in an Agilent 7820 gas chromatograph (Agilent Corp., USA) equipped with a flame ionisation detector, and the injector and detector temperatures were set at 230°C and 250°C, respectively. Nitrogen carrier gas at 1 mL min^{-1} was split in a 1:100 ratio. The oven temperature was maintained at 60°C for 3 min, programmed to increase to 175°C at 5°C min^{-1} and held for 15 min, and then programmed to increase to 220°C at 2°C min^{-1} and held for 10 min.

Analysis of phospholipids

A 100-mg sample of milk fat was dissolved with 1 mL chloroform/methanol (88:12, v/v) and transferred into capped test tubes for high-performance liquid chromatography. Phospholipids were analysed with high performance liquid chromatography with an evaporative light scattering detector as described by Rombaut et al.[23] A silica column (4.6 mm × 250 mm, 5-μm particle size) conjugated

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with a precolumn was used in this study. Nitrogen was used as the nebulising gas at a flow rate of 1 L/min, and the evaporating temperature was 85°C. The elution program was isocratic with 87.5:12:0.5 (v/v/v) chloroform/methanol/triethylamine buffer (pH 3, 1 M formic acid) from 0 to 10 min and then a linear gradient with 87.5:12:0.5 (v/v/v) at 11 min to 28:60:12 (v/v/v) at 45 min. The mobile phase was returned to the initial conditions at 47 min, and the column was allowed to equilibrate until the next injection at 55 min. The flow rate was maintained at 0.5 mL min^{-1}, the injection volume was 10 μL and the samples and the column were equilibrated at 40°C.

Analysis the Fatty Acids of Phospholipids

The phospholipid classes were separated by one-dimensional double development high-performance thin-layer chromatography using hexane/diethyl ether/acetic acid (80:20:1, v/v/v). The bands of absorbent containing the phospholipid fraction were scraped off the plates into test tubes. The phospholipids were then extracted three times with chloroform (1 mL each). The fatty acid methyl esters of the phospholipids were prepared with a method adapted from Lopez et al.[24], and the procedure was then continued as described above for analysis of total fatty acids.

Analysis of Sterols

The sterol content was measured according to the method described by Changmo Li et al.[25] A 100-mg sample of milk fat was placed in a sealable tube; 200 μL of internal standard (0.597 mg/mL 5α-cholestane in hexane) and 2 mL of 2-M KOH in ethanol were added, sealed, heated at 85°C for 1 h and cooled in cold water, and then 2 mL of distilled water and 5 mL of hexane were added. The unsaponifiable matter was extracted three times with hexane. The hexane phase was then dried and silylated with

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215 400-µL (BSTFA + TMCS) at 70°C for 30 min, the residue was dissolved in 1 mL of
216 hexane, and 1 µL of the product was analysed by GC–mass spectrometry.

217 The sterol samples were analysed with a Thermo Scientific France DSQ GC–
218 mass spectrometer equipped with a DB-5 capillary column (30 m; 0.25 mmi.d.,
219 0.52-µm film thickness; Agilent Corp.). Helium was used as the carrier gas with a
220 flow rate of 1 mL min^{-1}, and the split ratio was 1:50. The oven temperature was
221 maintained at 150°C for 1 min, increased to 300°C at a rate of 10°C min^{-1} and held for
222 15 min at 300°C. The scan time was 1 s, and the mass range was 50 to 500 (m/z). The
223 sterols were identified by comparison of their mass spectra with those of the
224 corresponding standards.

225

226 **Statistical Analysis**

227 The peaks were separated and identified by comparing the retention times with those
228 of the standards. All sample results were expressed as mean ± SD. The experiments
229 were run in triplicate. Statistical analysis software (version 9.0, SAS Institute, Inc.,
230 Cary, NC) was used for data treatment. The results were considered statistically
231 significant for p values of less than 0.05.

232

233 **Results and Discussion**

234 **Size Distribution, Zeta-Potential and Surface Tension of MFGs**

235 The size distributions, apparent zeta-potentials and surface tension of the MFGs were
236 determined to characterise the physicochemical properties of the MFGs dispersed in
237 different processing conditions. The parameters of the particle size distribution,
238 zeta-potential and surface tension of the MFGs are presented in Table 1. The mean
239 $D_{3,2}$ values of the different processes showed that homogenisation and spray-drying

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produced lower values than pasteurisation, and the $D_{4,3}$ values were higher than the corresponding $D_{3,2}$ values in all treatments. The size distributions of MFG in RM, PM, HM and SDM as determined by laser light scattering are also presented in Figure 1. The laser light scattering measurements showed that raw MFGs have a unimodal size distribution ranging from about 1 to 10 μm, with a mean volume-weighted diameter $D_{4,3}$ of 3.70 ± 0.31 μm, which is similar to that seen in previous studies.[26] Pasteurisation caused the MFG size to increase slightly from a $D_{4,3}$ of 3.70 ± 0.31 μm to a $D_{4,3}$ of 3.97 ± 0.21 μm. The results demonstrate that heat treatment, when not associated with homogenisation, does not induce significant changes in the MFG size.[27] Upon homogenisation, the MFGs were reduced to around 1 μm, resulting in a greater droplet surface area. The sample of PHM had a narrower fat globule volume distribution than the other milk preparations. These results reflect those of previous studies on the effects of processing conditions on the size of fat globules using laser light scattering.[28] The MFG size distributions of the spray-dried powders showed a uniform distribution of MFGs, with a majority of globules between 0.1 and 4.0 μm, but with a small proportion of globules between 10 and 15 μm. There was a possible disruption of the fat globules that caused smaller fat globules to form and a small proportion of the fat globules to coalesce into large globules during spray-drying. The drying process causes a range of structural and physicochemical modifications, which in turn influence the reconstitution and absorption of milk proteins and causes the $D_{4,3}$ of the MFGs to increase slightly to 2.48 ± 0.97 μm.

The zeta-potentials calculated from electrophoretic mobility are suitable for determination of the effects of a process and are capable of detecting changes in the MFGM when no significant change in the MFG size distribution has occurred.[29] The zeta-potentials for RM, PM, PHM and SDM were –9.44 ± 0.66, –10.04 ± 0.68, –12.33

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± 0.40 and –15.3 ± 0.42 mV, respectively, indicating a tendency to increase during milk powder processing methods (Table 1). This result reveals that the changes in the zeta-potentials were related to their particle size ($D_{3,2}$). The gradual increase in the zeta-potentials with PHM was due to the fact that homogenisation led to the disruption of MFGM and decreased the size of the MFGs (from 3.31 ± 0.11 to 0.917 ± 0.69 μm). As the interfacial area increased, the native MFGM could no longer cover the globule entirely. Thus the fraction of the MFGM surface covered by plasma proteins increased. The absolute value of the zeta-potential significantly increased with the spray-drying process, from about –12.33 ± 0.40 mV for PHM to –15.3 ± 0.42 mV for SDM. The changes in the zeta-potentials were largely due to the surface components of the MFGs and the minerals in the aqueous environment. The final zeta-potential value of –15.3 ± 0.42 mV was interpreted as the surface layer of fat globules that was almost totally composed of proteins and was no longer covered by the native MFGM after spray-drying.

The rupture of the MFGs that occurs during homogenisation forms a new membrane that is mostly composed of protein and other surface-active components, which creates a new interface. Casein micelles are the major protein fraction adsorbed, even if a portion of the native membrane remains associated to the fat droplets. Thus the surface tension of the milk also increases with changes in the interface of the MFGM.

Microstructure of MFGs With Various Kinds of Processing

The microstructures of different kinds of processed MFGs were observed with CLSM (Figure 2). Nile red fluorescent probe was used to label the TAG, which was exclusively located in the core of the MFGs. The results of image analysis of the

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CLSM micrographs were in agreement with the size distributions determined by laser light scattering, and the MFGs from RM and PM did not reveal any significant differences (Figure 2A and B). Figure 2C shows that homogenisation reduced the size of MFGs and that these globules were all similar in size, unlike the MFGs from RM, which were of varying sizes. Figure 2D shows that a small proportion of large globules were dispersed in the system. It is possible that during processing methods such as pasteurisation, homogenisation and spray-drying, the MFG structure was completely damaged and the TAG of the MFG coalesced and was then entrapped by some fragments of the MFGM and proteins. In the spray-drying process, the smaller particles dried and became solid more quickly, so they were able to collide with larger ones that were still viscous and remain trapped on their surface. If the larger particles were no longer sticky, the collisions with small particles created depressions on their surface.[30]

The Rh-DOPE fluorescent probe–labelled MFGs from different processing conditions were observed by CLSM and are shown in Figure 3. Because Rh-DOPE is a polar headgroup–labelled phospholipid probe, it can be incorporated with minimal perturbation into the bilayer of the MFGM that contains about 25% to 65% phospholipids, which makes it a suitable choice for CLSM imaging.[12] Figure 3A shows the emission fluorescence of Rh-DOPE in the form of red rings at the periphery of the MFGs of RM. Dark areas absent of the extrinsic fluorescence probe were seen on the surface of the globules, which are marked by white arrows. These domains were interpreted as lipid rafts according to the research of Lopez.[19] The interior of the MFGs appeared as nonfluorescent black areas that were mainly composed of TAG. As already observed in Figure 3B, the MFGs of the PM looked similar to those of the RM. However, the red emission fluorescence rings were much looser and the circular

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areas were incomplete, perhaps because the treatment temperatures reached the phase transition temperature of the outer phospholipid bilayer membrane, resulting in polar lipids from the gel to liquid crystalline phase transition. More dark areas existed on the surfaces of globules because small amounts of MFGM were lost during pasteurisation.

The widely different appearances of the microstructure of homogenised and spray-drying MFGs as compared with those of the RM and PM are shown in Figure 3C and D. The fluorescence probe in Figure 3C and D was spotted, perhaps because the rupture of the native MFG during homogenisation and spray-drying results in a newly formed membrane that is mostly composed of adsorbed caseins. Therefore, the Rh-DOPE fluorescent probe could not be inserted into the membrane composed of proteins.

Fatty Acid Composition of Various Processing Milk

The fatty acid profiles in RM, PM, PHM and SDM are presented in Table 2. The predominant fatty acids in cow milk are palmitic acid (C16:0), oleic acid (C18:1), myristic acid (C14:0) and stearic acid (C18:0), which together account for roughly 83.08% of the total fatty acids. The experimental fatty acid contents obtained here were in good agreement with those reported by Zou et al.[31] The saturated fatty acids accounted for 71.46%. The relative amount of SFA, some of which is supposed to be detrimental to human health, was not found to be statistically significantly different ($P > 0.05$) between the different processing conditions. Monounsaturated fatty acids and polyunsaturated fatty acids accounted for 27.33% and 1.21%, respectively. Linolenic acid (C18:3n-3) was the major polyunsaturated fatty acid, with a relative concentration of 0.3%. The values for polyunsaturated fatty acids were lower than

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those in other reports by Talpur (4.34% to 5.76%).[32] The differences in the percentages of monounsaturated and polyunsaturated fatty acids observed in this study could be caused by the breed, the feeding system, seasonal changes, or other factors.[33]

The results in Table 2 show that the fatty acid composition of milk fat varied slightly amongst the various fatty acids fractions in this study, but the differences were not statistically significant. Therefore, the fatty acid profiles were not related to processing by pasteurisation, homogenisation and spray-drying. This means that the TAG in the treated samples will not be hydrolysed by the action of enzymes and will therefore be less susceptible to further oxidation. The results were in accordance with those of a study by Rodríguez-Alcalá, which showed that thermal and high-pressure processing did not produce significant changes to the fatty acid composition of milk fat.[34, 35]

Phospholipid Profiles and Fatty Acid Composition of the Phospholipid Fraction

Phospholipids with both lipophilic and hydrophilic properties are located on the MFGM and therefore contribute significantly to the emulsification of the membrane. The distribution of phospholipids is asymmetric; phosphatidylcholine (PC) and sphingomyelin (SM) are largely located on the outside of the membrane, and phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) are concentrated on the inside.[36] The phospholipid contents of RM, PM, PHM and SDM are shown in Figure 4. According to the data in the literature, the major phospholipids in milk are PE (19.8% to 42.0%, w/w), PC (19.2% to 37.3%, w/w), PS (1.9% to 10.5%, w/w) and PI (0.6% to 11.8%, w/w) and the major sphingolipid was SM (18.0% to 34.1%, w/w).[37-39] These differences were probably a result of the

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methods, breed, or feeding regimen.[24]

The content and distribution of the phospholipids in the PM samples in this study indicate a high level of stability. After homogenisation and spray-drying, the phospholipid content and composition showed significant changes compared with PM. The amounts of PE, PS and SM were significantly affected by homogenisation (Figure 4). During pasteurisation and homogenisation, a part of the MFGM was rearranged at the surface of the globule with the absorbed whey proteins and caseins.[40] Homogenisation led to a loss of PC and SM that in the outer layer and resulted in a higher proportion of PE and PS. However, the proportion of PE decreased from 28% to 22% after spray-drying. PS and PI also decreased to a much smaller extent, but the relative content of PC and SM increased. The decrease in the PE content seemed to be attributable mainly to these phospholipids, which are mostly polyunsaturated phospholipids and are more easily oxidised and denatured during high spray-drying temperatures.

To obtain the phospholipid fraction from the total lipids, thin-layer chromatography separation was carried out. The band relative to phospholipids was collected and extracted. The phospholipid extracts were methylated, and the fatty acids were analysed by GC. The fatty acid contents of the total phospholipids from each processing method are shown in Table 3.

The major fatty acids associated with the phospholipids were C14:0, C16:0, C18:0, C18:1 and C18:2. These results are consistent with the reports by Fong[41]. The fatty acids of the phospholipids in SDM were significantly less saturated than the fatty acids from RM, PM and PHM. It is likely that the decrease in saturated fatty acids (C16:0 and C18:0) was mainly due to a lower proportion of SM in SDM. SM is a highly saturated phospholipid that is associated with cholesterol format the lipid raft

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390 in MFGM. Gallier reported that the main fatty acids in SM were C16:0, C22:0 and
391 C24:0 and that the SM content was constant for RM, processed milk and buttermilk
392 powder.[10] This might be because the samples had been obtained from different
393 sources or had undergone different treatments. The changes in the unsaturated fatty
394 acid (C18:1 and C18:2) composition of the phospholipids were consistent with the
395 changes in the phospholipid (PE and PC) content during each processing method
396 (Figure 4).
397
398 **Minor Sterol Profile**
399 Sterols comprise a minor fraction of MFG, and the main sterols are cholesterols. The
400 cholesterols present in the MFGM may be located in the SM-rich domains and may
401 play a role in the formation of the microdomains.[19] The sterol fraction of milk is of
402 nutritional interest because high levels of cholesterol in plasma (modulated by the
403 cholesterol ingested) are associated with a greater risk of cardiovascular disease.
404 Squalene and small quantities of other sterols (lathosterol, lanosterol) and two
405 phytosterols (stigmasterol, β-sitosterol) were detected in this study (Figure 5). One
406 small peak had a relative retention time similar to that of campesterol in the
407 chromatograms, but mass spectra could not be assigned to this sterol.

408 The cholesterol content of cow MFG was similar to that in previous reports on
409 cow milk, but the contents of other minor sterols were different than these reports.[42]
410 The cholesterol concentration in milk fat was shown to be influenced by an
411 interaction between the time of year of sampling and the breed.[43] As shown in Figure
412 4, the cholesterol level kept decreased as processing continued. After pasteurisation
413 and homogenisation, the cholesterol content decreased from 220.08 to 172.46 μg/g,

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414 and the other minor sterols also showed downward trends. These results indicate that
415 the cholesterol content decreased with the damage of the MFG by processing.
416 Whether the observed increase in the sterol and squalene content after spray-drying is
417 caused by extraction of the equivalent milk lipid or the MFG was completely
418 destroyed during spray-drying, resulting in the complete extraction of the sterols in
419 MFGM, requires further investigation.
420

421 **Conclusions**

422 This study indicates that different processing methods resulted both in significant
423 changes in the structure and lipid composition of MFGs. Pasteurisation of MFGs did
424 not show any obvious effects compared to the other treatments. Homogenisation of
425 MFGs resulted in a dramatic decrease in the size and protein absorption of the newly
426 formed interface. After spray-drying, the microstructure and lipid composition of
427 MFGM showed the most profound changes. The nutritional and health properties of
428 MFG result from its lipid composition but also depend on its microstructure. The
429 possible effects of milk powder processing methods on its health and nutritional
430 properties should be further explored.
431

432 **Conflict of interest**

433 The authors declare that there are no conflicts of interest.
434

435 **Acknowledgments**

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References

1. B. J. Kitchen, *J. Dairy Res.*, 1977, **44**, 469-482.
2. S. Arifeen, R. E. Black, G. Antelman, A. Baqui, L. Caulfield and S. Becker, *Pediatrics*, 2001, **108**, e67-e67.
3. C.-H. Hsia, M.-C. Shen, J.-S. Lin, Y.-K. Wen, K.-L. Hwang, T.-M. Cham and N.-C. Yang, *Nutrition Research*, 2009, **29**, 190-196.
4. P. J. Aggett, C. Agostini, O. Goulet, O. Hernell, B. Koletzko, H. L. Lafeber, K. F. Michaelsen, J. Rigo and L. T. Weaver, *J Pediatr Gastr Nutr*, 2001, **32**, 256-258.
5. A. L. Lock and D. E. Bauman, *Lipids*, 2004, **39**, 1197-1206.
6. A. Ye, H. Singh, M. W. Taylor and S. Anema, *Le Lait*, 2004, **84**, 269-283.
7. A. Ye, H. Singh, M. W. Taylor and S. Anema, *International Dairy Journal*, 2002, **12**, 393-402.
8. A. Zamora, V. Ferragut, B. Guamis and A. J. Trujillo, *Food Hydrocolloids*, 2012, **29**, 135-143.
9. M. Cano-Ruiz and R. Richter, *J. Dairy Sci.*, 1997, **80**, 2732-2739.
10. S. Gallier, D. Gragson, C. Cabral, R. Jimenez-Flores and D. W. Everett, *J Agric Food Chem*, 2010, **58**, 10503-10511.

RSC Advances Accepted Manuscript

11. S. J. E. Lee and J. W. Sherbon, *J. Dairy Res.*, 2002, **69**, 555-567.

12. S. Gallier, D. Gragson, R. Jime?nez-Flores and D. Everett, *Journal of Agricultural and Food Chemistry*, 2010, **58**, 4250-4257.

13. L. Ong, R. R. Dagastine, S. E. Kentish and S. L. Gras, *J Food Sci*, 2010, **75**, E135-145.

14. D. Bermúdez-Aguirre, R. Mawson and G. Barbosa-Cánovas, *Journal of Food Science*, 2008, **73**, E325-E332.

15. P. Morin, R. Jiménez-Flores and Y. Pouliot, *International Dairy Journal*, 2007, **17**, 1179-1187.

16. T. S. Balasuriya, L. Ong, S. L. Gras and R. R. Dagastine, *RSC Advances*, 2012, **2**, 2384-2394.

17. H. T. H. Nguyen, L. Ong, E. Beaucher, M.-N. Madec, S. E. Kentish, S. L. Gras and C. Lopez, *Food Research International*, 2015, **67**, 35-43.

18. C. Lopez, *Current Opinion in Colloid & Interface Science*, 2011, **16**, 391-404.

19. C. Lopez, M. N. Madec and R. Jimenez-Flores, *Food Chem.*, 2010, **120**, 22-33.

20. J. M. Evers, R. G. Haverkamp, S. E. Holroyd, G. B. Jameson, D. D. S. Mackenzie and O. J. McCarthy, *International Dairy Journal*, 2008, **18**, 1081-1089.

21. C. Lopez and O. Menard, *Colloids and surfaces. B, Biointerfaces*, 2011, **83**, 29-41.

22. J. Folch, M. Lees and G. Sloane-Stanley, *J. biol. Chem*, 1957, **226**, 497-509.

RSC Advances Accepted Manuscript

23. R. Rombaut, J. Camp and K. Dewettinck, *J Dairy Sci*, 2005, **88**, 482-488.

24. C. Lopez, V. Briard-Bion, O. Menard, F. Rousseau, P. Pradel and J.-M. Besle, *Journal of Agricultural and Food Chemistry*, 2008, **56**, 5226-5236.

25. C. Li, Y. Yao, G. Zhao, W. Cheng, H. Liu, C. Liu, Z. Shi, Y. Chen and S. Wang, *J Agric Food Chem*, 2011, **59**, 12493-12498.

26. C. Lopez, B. Camier and J.-Y. Gassi, *International Dairy Journal*, 2007, **17**, 235-247.

27. M.-C. Michalski and C. Januel, *Trends in Food Science & Technology*, 2006, **17**, 423-437.

28. M. C. Michalski, R. Cariou, F. Michel and C. Garnier, *J. Dairy Sci.*, 2002, **85**, 2451-2461.

29. M. C. Michalski, F. Michel, D. Sainmont and V. Briard, *Colloids and Surfaces B: Biointerfaces*, 2002, **23**, 23-30.

30. M.-L. Vignolles, R. Jeantet, C. Lopez and P. Schuck, *Le Lait*, 2007, **87**, 187-236.

31. X. Zou, J. Huang, Q. Jin, Z. Guo, Y. Liu, L. Cheong, X. Xu and X. Wang, *J Agric Food Chem*, 2013, **61**, 7070-7080.

32. F. N. Talpur, M. I. Bhanger and M. Y. Khuhawar, *Journal of Food Composition and Analysis*, 2006, **19**, 698-703.

33. R. G. Jensen, *J. Dairy Sci.*, 2002, **85**, 295-350.

34. L. M. Rodríguez-Alcalá, F. Harte and J. Fontecha, *Innovative Food Science & Emerging Technologies*, 2009, **10**, 32-36.

RSC Advances Accepted Manuscript

35. L. Rodríguez-Alcalá, P. Castro-Gómez, X. Felipe, L. Noriega and J. Fontecha, *LWT-Food Science and Technology*, 2015, **1**, 265-270.

36. H. Deeth, *Australian Journal of Dairy Technology*, 1997, **52**, 44-46.

37. J. Bitman and D. L. Wood, *J. Dairy Sci.*, 1990, **73**, 1208-1216.

38. R. Rombaut, J. V. Camp and K. Dewettinck, *J. Dairy Sci.*, 2005, **88**, 482-488.

39. R. Rombaut and K. Dewettinck, *International Dairy Journal*, 2006, **16**, 1362-1373.

40. M.-C. Michalski, F. Michel and C. Geneste, *Lait*, 2002, **82**, 193-208.

41. B. Y. Fong, C. S. Norris and A. K. H. MacGibbon, *International Dairy Journal*, 2007, **17**, 275-288.

42. M. P. Giovanna Contarini*, Eliana Bonfitto, Silvia Berardi, *International Dairy Journal*, 2002, **12**, 573-578.

43. H. Goudjil, S. Torrado, J. Fontecha, I. Martínez-Castro, M. J. Fraga and M. Juárez, *Le Lait*, 2003, **83**, 153-160.

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518 **Figure Captions**

519 **Figure 1.** Size distributions of milk fat globules for different processing methods
520 determined using laser light scattering.

521

522 **Figure 2.** Confocal laser scanning two-dimensional micrographs of milk fat globules
523 for different processing methods stained with Nile red fluorescent probe. (A) RM, (B)
524 PM, (C) PHM, (D) SDM.

525

526 **Figure 3.** Confocal laser scanning two-dimensional micrographs of milk fat globules
527 for different processing methods stained with Rh-DOPE fluorescent probe. (A) RM,
528 (B) PM, (C) PHM, (D) SDM.

529

530 **Figure 4.** Comparison of the percentages of phospholipids (PE, PI, PS, PC and SM)
531 in MFG after different processing methods.

532

533 **Figure 5.** Contents of squalene and sterols (cholesterol, lathosterol, lanosterol,
534 stigmasterol and β-sitosterol) in MFG after different processing methods.

535

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536 **Table 1.** Size distribution, zeta-potentials and surface tension of MFG after different
537 processing methods.

Size parameters	RM	PM	PHM	SDM
$D_{3,2}$(μm)	3.31±0.11	3.57±0.47	0.917±0.69	0.847±0.56
$D_{1,0}$(μm)	2.63±0.27	2.878±0.32	0.532±0.08	0.348±0.25
$D_{4,3}$(μm)	3.70±0.31	3.97±0.21	1.358±0.46	2.480±0.97
zeta-potentials(mV)	-9.44±0.66	-10.04±0.68	-12.33±0.40	-15.3±0.42
surface tension(mN/m)	41.207±0.023	40.773±0.019	42.817±0.013	44.646±0.022

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Table 2. Fatty acid composition of MFG after different processing methods [a].

Fatty acid	RM	PM	PHM	SDM
C4:0	1.00± 0.02 c	1.25± 0.04 b	1.23± 0.01 b	1.50± 0.05 a
C8:0	0.99± 0.03 c	1.06± 0.01 b	1.17± 0.03 a	1.16± 0.01 a
C10:0	2.85± 0.04 c	2.91± 0.03 c	3.27± 0.05 a	3.16± 0.05 b
C11:0	0.08±0.01 a	0.08±0.02 a	0.10±0.02 a	0.09±0.02 a
C12:0	3.87±0.05 b	3.89±0.07 b	4.21±0.07 a	4.21±0.11 a
C13:0	0.14±0.02 a	0.15±0.03 a	0.16±0.03 a	0.15±0.03 a
C14:0	12.84±0.46 a	12.63±0.28 a	12.99±0.35 a	13.16±0.14 a
C14:1	1.44±0.08 a	1.44±0.05 a	1.49±0.10 a	1.50±0.25 a
C15:0	1.44±0.05 a	1.43±0.08 a	1.43±0.03 a	1.45±0.07 a
C15:1	0.02±0.003 a	0.01±0.002 b	0.01±0.003 b	0.02±0.001 a
C16:0	35.50±1.30 a	35.11±0.52 a	34.70±1.46 a	35.06±1.12 a
C16:1	1.94±0.30 a	1.89±0.25 a	1.83±0.37 a	1.83±0.46 a
C17:0	0.79±0.24 a	0.80±0.16 a	0.79±0.18 a	0.79±0.05 a
C17:1	0.29±0.11 a	0.30±0.05 a	0.29±0.02 a	0.30±0.03 a
C18:0	11.66±0.72 a	11.70±1.13 a	11.47±0.91 a	11.29±0.06 a
C18:1t	0.42±0.04 a	0.45±0.04 a	0.45±0.01 a	0.45±0.06 a
C18:1	23.08±1.14 a	23.31±1.17 a	22.74±1.18 a	22.34±1.37 a
C18:2t	0.27±0.02 a	0.28±0.04 a	0.27±0.06 a	0.28±0.02 a
C18:3n-6	0.06±0.004 a	0.06±0.005 a	0.07±0.007 a	0.07±0.004 a
C18:3n-3	0.30±0.05 a	0.30±0.01 a	0.31±0.02 a	0.31±0.06 a
C20:0	0.22±0.008 a	0.22±0.04 a	0.21±0.02 a	0.21±0.03 a
C20:1	0.12±0.02 a	0.10±0.03 a	0.11±0.02 a	0.08±0.02 a
C21:0	0.04±0.006 a	0.04±0.002 a	0.05±0.009 a	0.05±0.01 a
C20:2	0.06±0.01 a	0.05±0.01 ab	0.05±0.004 ab	0.04±0.007 a
C20:3n-6	0.15±0.02 a	0.15±0.01 a	0.17±0.02 a	0.15±0.02 a
C20:4n-6(ARA)	0.22±0.01 a	0.23±0.04 a	0.23±0.03 a	0.22±0.04 a
C22:1	0.01±0.003 b	0.02±0.001 a	0.02±0.004 a	0.01±0.003 b
C20:5n-3(EPA)	0.14±0.02 a	0.09±0.01 b	0.12±0.01 ab	0.09±0.02 b
C23:0	0.05±0.006 b	0.04±0.006 b	0.09±0.01 a	0.04±0.009 b
C22:2	0.01±0.002 a	0.01±0.003 a	0.01±0.001 a	nd
SFA	71.47±2.96 a	71.31±2.418a	71.85±3.179a	72.31±1.759 a
MUFA	27.32±1.696a	27.52±1.593a	26.94±1.707a	26.53±2.194a
PUFA	1.21±0.134 a	1.17±0.128a	1.23±0.152 a	1.16±0.171 a

[a] Means with different superscript letters are significantly different ($P < 0.05$). RM, raw milk; PM, pasteurized milk; PHM, pasteurized and homogenized milk; SDM, spray drying milk; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

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547 **Table 3.** Fatty acid composition of phospholipids of MFG after different processing
548 methods [a].

Fatty acids	RM	PM	PHM	SDM
C4:0	0.14± 0.02 b	0.21± 0.02 a	0.23± 0.03 a	0.20 ± 0.03a
C6:0	0.16± 0.01 d	0.38± 0.03 b	0.58± 0.03 a	0.22± 0.02 c
C8:0	0.19± 0.02 c	1.38± 0.13 a	0.38± 0.04 b	0.46± 0.01 b
C10:0	0.59± 0.03 c	1.92± 0.02 a	1.93± 0.02 a	0.65± 0.04 b
C11:0	0.11± 0.02 d	0.87± 0.02 a	0.77± 0.03 b	0.26± 0.02 c
C12:0	1.21± 0.04b	2.83± 0.27a	3.22± 0.12a	1.50± 0.33b
C13:0	0.16± 0.02d	0.53± 0.04a	0.43± 0.03b	0.27± 0.02c
C14:0	6.95± 0.04a	6.32± 0.14b	4.77± 0.20d	5.42± 0.23c
C14:1	0.47± 0.13b	0.88± 0.12a	0.23± 0.03c	0.35± 0.04bc
C15:0	1.13± 0.04b	1.74± 0.13a	0.75± 0.11c	0.85± 0.16c
C15:1	0.20± 0.05c	0.53± 0.03b	0.73± 0.09a	0.22± 0.04c
C16:0	28.03± 1.49a	22.61± 1.12b	23.25± 1.46b	21.24± 0.78b
C16:1	1.82± 0.15a	1.88± 0.09a	1.24± 0.09b	1.77± 0.1a
C17:0	0.83± 0.02b	1.00± 0.09a	1.00± 0.11a	1.02± 0.07a
C17:1	0.31± 0.02b	0.25± 0.02c	0.44± 0.03a	0.43± 0.02a
C18:0	16.59± 0.82b	13.00± 0.50d	18.56± 0.62a	14.53± 0.69c
C18:1	27.79± 1.03b	24.98±1.13c	23.31±0.75c	30.15±0.69a
C18:2	9.92±0.27c	17.61±0.83ab	16.74±0.75b	18.40±0.84a
C20:0	0.62±0.09ab	0.54±0.03b	0.68±0.04a	0.61±0.07ab
C21:0	0.15±0.01b	0.05±0.01c	0.05±0.02c	0.19±0.04a
C20:3n-6	0.65±0.04b	0.34±0.03d	0.43±0.03c	0.93±0.04a
C22:1	0.27± 0.02b	0.15± 0.02c	0.28± 0.03b	0.33± 0.02a
C20:5n-3(EPA)	0.65± 0.04	nd	nd	nd
C23:0	0.99± 0.11	nd	nd	nd
C22:2	0.09± 0.02	nd	nd	nd
SFA	57.85±2.68a	53.38±2.55 a	56.60±2.86 a	47.42±2.49 b
MUFA	30.86±1.40 b	28.67±1.41 b	26.23±1.02 c	33.25±0.91 a
PUFA	11.31±0.31c	17.95±0.86ab	17.17±0.78 b	19.33±0.88 a

549 [a] Means with different superscript letters are significantly different ($P < 0.05$). For
550 abbreviation, see Table 2.
551

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552 **Figure 1**

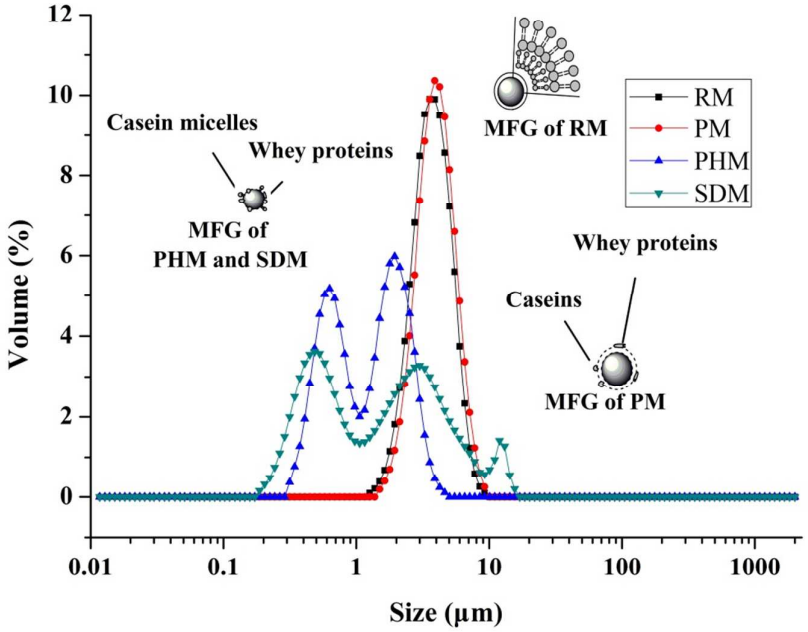

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Figure 2

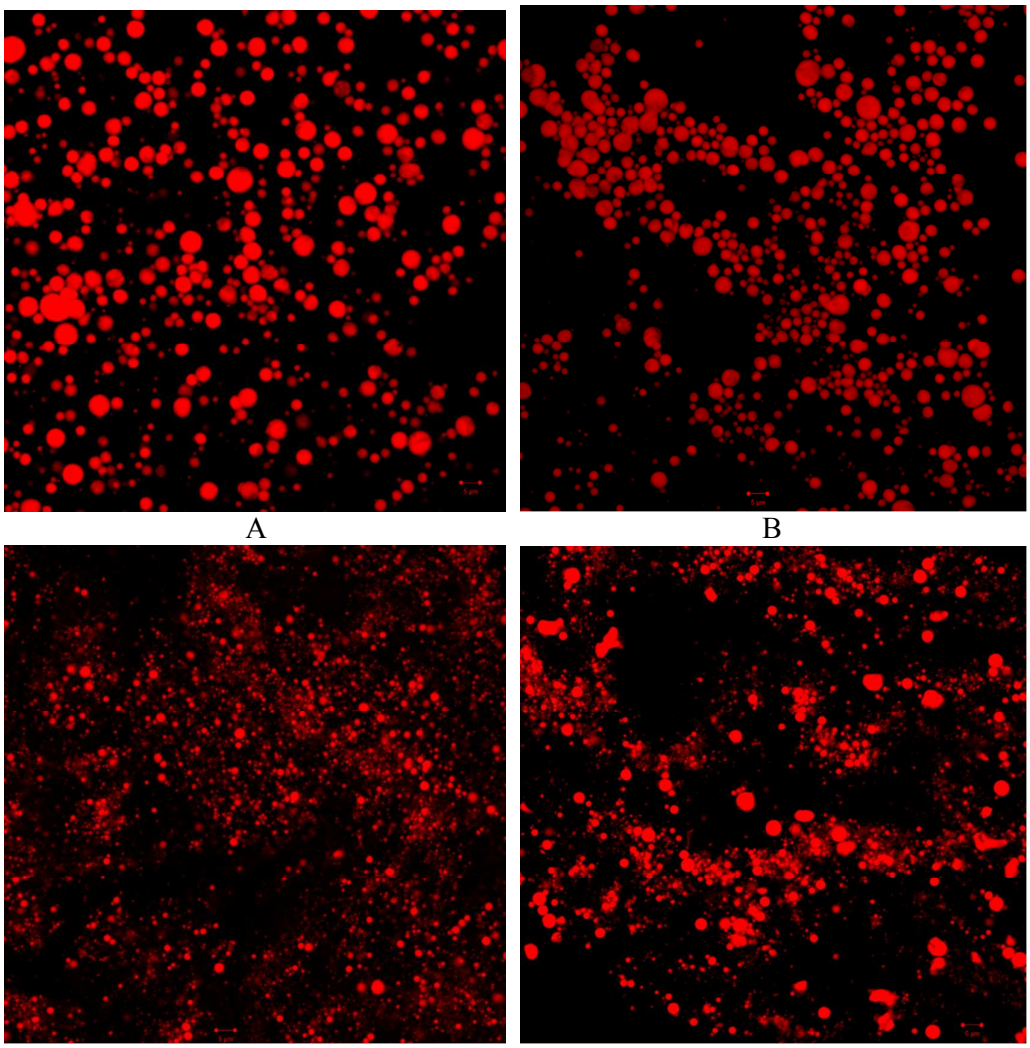

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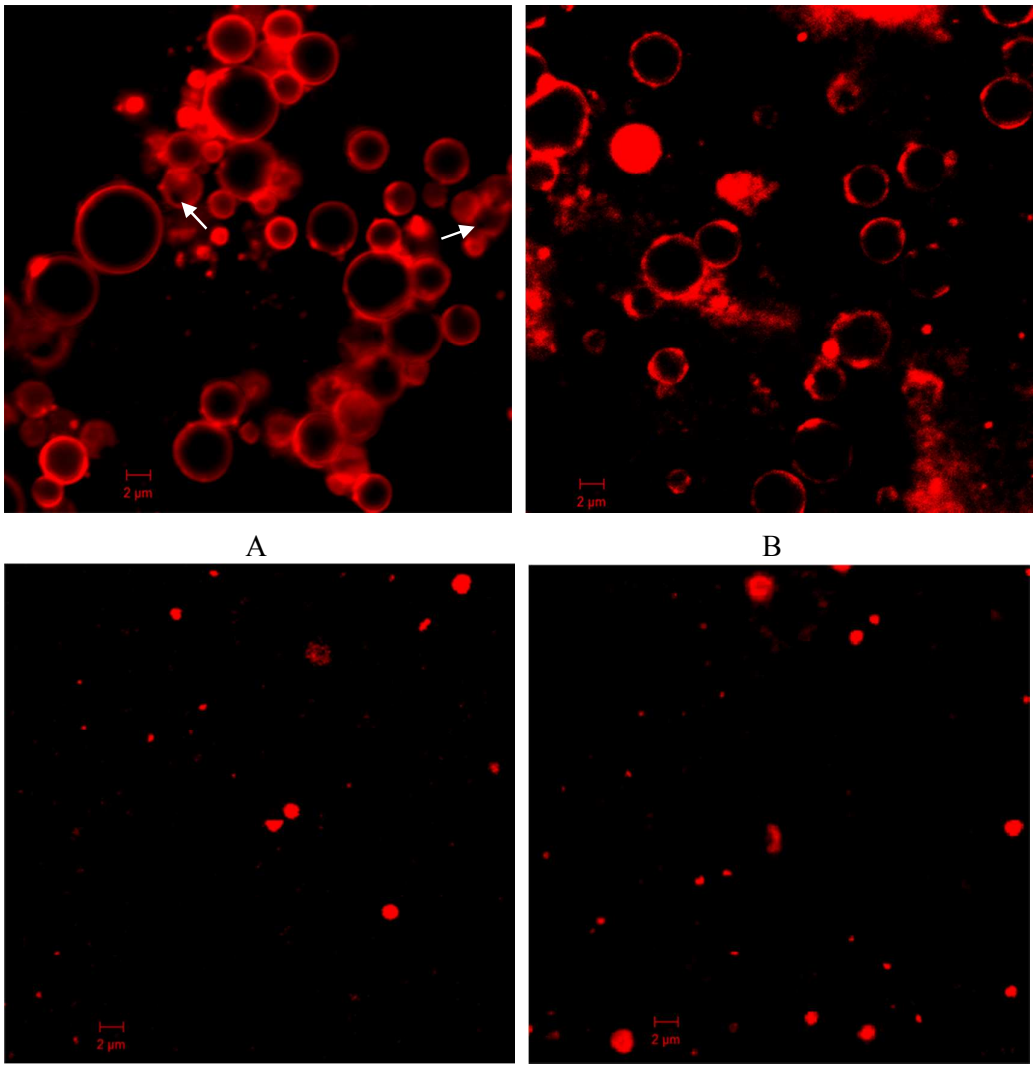
Figure 3



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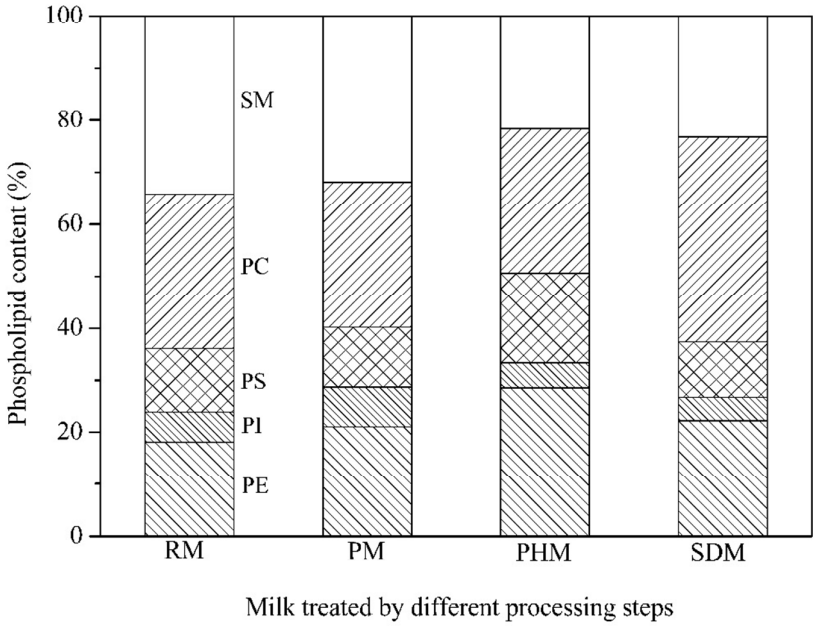
569 **Figure 4**



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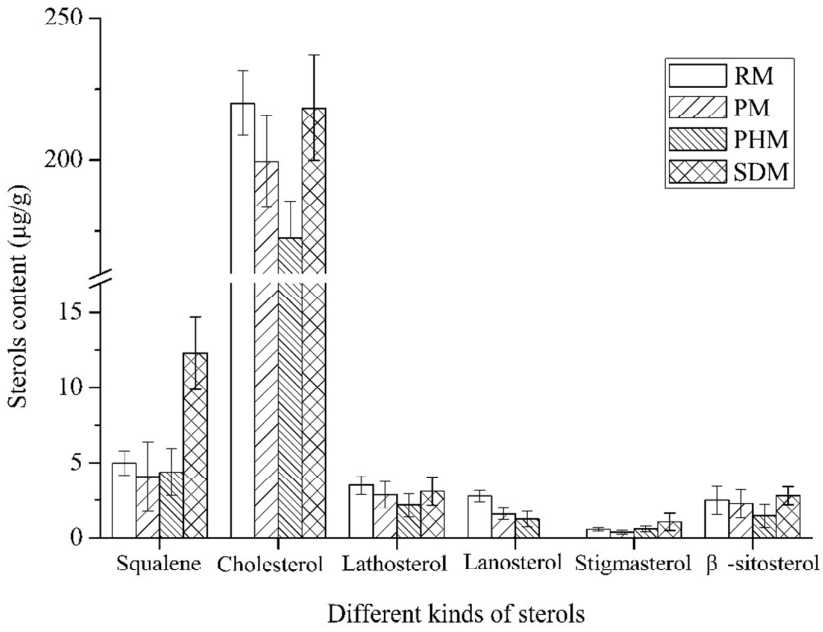
572 **Figure 5**



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