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Bovine Milk Fat Globule Membrane Proteins Are Affected By Centrifugal Washing Processes

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ABSTRACT: The impact of washing on the release of proteins from the milk fat globule membrane (MFGM) was examined by applying washing procedures with different degrees of stringency to milk fat globule (MFG) surfaces in simulated milk ultrafiltrate buffer solution. Three washing methods, M1 (3000*g*, 5 min, three washes), M2 (3750*g*, 15 min, one wash), and M3 (15000*g*, 20 min, three washes) were chosen. MFG ζ-potential increased after M3 washing ($P < 0.05$), suggesting surface damage. For M1, in which the native MFG surface was least damaged, cluster of differentiation 36 (CD 36) and periodic acid schiff 6/7 proteins were more strongly bound to the MFGM compared with other major membrane proteins. For M3, CD 36 together with fatty acid-binding protein was more strongly bound to the MFGM. Washing by centrifugation and redispersal of the fat globules damaged the MFGM, with release into the aqueous phase of some membrane-associated proteins. The current results show the impact of washing processes on retention of functional MFGM proteins.

KEYWORDS: *milk fat globule membrane, membrane proteins, membrane structure, confocal laser scanning microscopy*

INTRODUCTION

Bovine milk contains around 3.5−4.7% (w/v) fat in the form of an oil-in-water milk fat globule (MFG) emulsion.[1−4] MFGs are stabilized by the milk fat globule membrane (MFGM) layer, a biofunctional multilayer system encapsulating the triacylglycerol inner core.[2,5] Although the MFGM is a thin layer system of around 10−20 nm thickness[6−8] and accounts for about 2−6% of the mass of native MFGs[9] (about 0.07−0.3% of the mass of bovine milk), it has a complicated structural organization integrating numerous enzymes, other proteins, and lipids. The MFGM has considerable nutritional and technical value as a food ingredient,[4,10] and the structural arrangement is likely to have an impact on the efficacy of the functional components.

Proteomics and lipidomics have been carried out to study the composition of the MFGM,[11,12] sourced milk from human[13,14] or bovine milk.[15−19] Methods such as laser diffraction, dynamic light scattering, microscopy, spectroscopy, and chromatography have been applied to understand the composition and structure of the MFGM.[20−23] Reviews had been published on the structure and functionality of MFG and MFGM;[3,4,24] however, the structure of the native MFGM is still unclear because of its fragile and treatment-sensitive nature.[9] Different types of experimental approaches are needed to provide information to develop the current MFGM models.[7,25,26]

In light of recent developments of the understanding on nutrient bioavailability, the importance of the macrostructure of food matrixes in regulating the rate of digestion and absorption have been emphasized. Such work has focused on the biofunctional nature of the MFGM structure in physiological processes.[24] Several models of the MFGM have been deduced to explain experimental results, featuring a trilayer structure, transmembrane and inner-core proteins, and liquid-ordered regions (or lipid rafts) rich in cholesterol and sphingomyelin.[3,16,25,27] McPherson and Kitchen[28] discussed the existence of a protein inner layer of the MFGM. Murray et al.[29] found glycoproteins in the isolated inner coat material of the MFGM, in contrast to the more widely held belief that glycoproteins are exposed at the outer MFGM surface. In a more recent review, Keenan and Mather[5] emphasized the possible existence of a protein dense layer from the perspective of MFG origin and secretion. Direct evidence for the existence of an inner protein dense layer from studies of postsecretion milk is still scarce.

Protein structural organization in the MFGM has been reviewed by Mather.[7,30] It is known that key MFGM proteins are not bound to the membrane structure with equal binding force. For instance, mucin 1 (MUC 1), xanthine dehydrogenase/oxidase (XDH/XO), mucin 15 (MUC 15), periodic acid Schiff III (PAS III), and PAS 6/7 are recognized to be bound more loosely compared with cluster of differentiation (CD 36), butyrophilin (BTN), and adipophilin (ADPH). The distribution of these key MFGM proteins between the water-insoluble MFGM pellet and the MFGM supernatant during MFGM isolation has been reviewed;[30,31] however, there is little information on the binding strength of major MFGM proteins. It is widely accepted that both MFG and MFGM isolation processes may induce MFGM material loss, especially the loosely bound peripheral proteins,[30] but the relative loss of specific membrane proteins during MFG isolation from unpasteurized milk is not well-known.

Fractionation of MFGM, which is essential for studying the structure, composition and biofunctionality, is achieved by three major steps: (1) isolation of washed MFGs (in physiological buffer) from unpasteurized milk; (2) release of MFGM from washed MFGs via physical or chemical means (or both); and (3) fractionation of MFGM materials via physical or

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chemical methods (or both).[28,30] Patton and Huston[32] developed a washing method based on convenience of operation, less structural change to the native MFGM, and acceptable level of protein retention, but other washing procedures employing different centrifugal forces, time of centrifugation, and washing buffers are still used.[33,34] Washing procedures may induce damage to the MFG surface, resulting in contamination with milk serum proteins, and will consequently alter the composition of the extracted MFGM and lead to conclusions about structure based on spurious results.

The impact of three washing procedures on the composition of the MFGM from unpasteurized bovine milk was examined to provide evidence for protein structural organization in the bovine MFGM. Different centrifugal washing forces (from mild to intensive) and duration were employed to gain insight into the competitive binding strengths of key MFGM proteins. The compositional changes of the MFGM were studied on the surface of stable MFGs without the need to destabilize MFGs and remove the MFGM from the surface.

MATERIALS AND METHODS

Materials. Fluorescent headgroup-labeled phospholipid analogue 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rd-DOPE) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), dissolved in chloroform (1 mg/mL), and sealed with a Teflon lid under N_2 and kept at −20 °C until used. Fast Green FCF (disodium 3-[*N*-ethyl-*N*-[4-[[4-[*N*-ethyl-*N*-(3-sulfonatobenzyl)amino]phenyl](4-hydroxy-2-sulfonatophenyl)-methylene]-2,5-cyclohexadien-1-ylidene]ammoniomethyl] benzenesulfonate) (1 mg/mL in deionized water) was kept at ambient temperature before use. Simulated milk ultrafiltrate (SMUF) at pH 6.5 was prepared[35] and used as a buffer and washing solution. All other chemicals were of analytical grade (Merck, Darmstadt, Germany).

Isolation of Milk Fat Globules. Bovine milk was obtained from a single Jersey cow, to limit biovariation, at midlactation from a local farm (Port Chalmers, Otago, New Zealand); all milk samples were collected in the morning between 8 and 9 a.m. directly after milking. The cow was pasture-fed and milked in the same manner (in-line vacuum milking machine) for six consecutive sampling days. The milk was not pasteurized postmilking. Milk from a Jersey cow was chosen for its relatively high fat content and larger volume-based MFG size compared with Friesian cows' milk.[36] Incorporation of air through beating or stirring, temperature history, and aging during milking, transportation, and storage are known to cause compositional and structural changes to the MFGM.[2] To minimize these effects, unpasteurized milk was cooled to ambient temperature (20−22 °C) immediately after milking without any mechanical treatment to minimize damage to the MFGs and ensure minimal variation in replicate samples.

Three methods were chosen to isolate MFGs from fresh unpasteurized milk; namely, M1 (3000*g*, 5 min, cream + three washes),[15] M2 (3750*g*, 15 min, one wash),[32] and M3 (15000*g*, 20 min, cream + three washes).[34] The M1 and M3 procedures[15,34] were randomly carried out in triplicate over the six sampling days, and M2 as a control method was carried out six times over the sampling days. The M1 and M3 washes are considered to be conventional MFG isolation procedures. In all cases, cream was initially collected from milk under the M1, M2, or M3 centrifugation condition (designated as M1-cream, M2-cream, and M3-cream) and then resuspended into 10 volumes of SMUF. After 1 h at ambient temperature with gentle agitation, the dispersion was centrifuged under the same conditions (M1, M2, or M3) again to wash off the non-MFGM proteins from the MFG surface, and the top MFG cream layer was collected. This procedure was designated as the first washing step (M1-1, M2-1, and M3-1). The centrifugation and washing process was repeated twice more for M1 and M3. A sucrose density gradient single wash separation was applied in M2 with slight modification.[32] Briefly, in a 50 mL centrifuge tube (Nalgene Centrifuge Ware, New York, NY), 15 mL of sucrose-conditioned milk (5% w/v) was layered under 30 mL of SMUF buffer using a syringe coupled with a ∼100 mm length and ∼1 mm diameter needle. The prepared sample was centrifuged (3750*g*, 15 min), and the top layer containing washed MFGs was collected. The temperature of the SMUF buffer was kept at 30 °C. The centrifugation temperature was 25 °C for all experiments. Washing of milk took place on the same day as milking as MFGs from uncooled milk samples were more readily dispersed into the SMUF washing solution than MFGs isolated from milk held overnight at 4 °C (results not shown). Milk and cream fractions collected after each centrifugation process were kept at −80 °C until further analysis.

Fat Globule Size and Specific Surface Area. Laser diffraction equipped with a red wavelength diode laser at 655.0 nm for larger particles (>500 nm) and a blue light emitting diode at 405.0 nm for smaller particles (<500 nm) was used to measure particle size distribution and specific surface area on a volume and surface area basis (model LA-950, Horiba, Irvine, CA). Optical parameters were adopted from previous reports,[37] with modifications. The refractive index of MFGs was set to 1.460 for the diode laser and 1.470 for the light-emitting diode. Samples of resuspended MFGs were first diluted with SMUF to give the same fat content of the original milk. The MFG dispersions and milk were diluted 2-fold (v/v) with 35 mM ethylene diamine tetraacetic acid (EDTA) containing 2% (w/v) sodium dodecyl sulfate (SDS) buffer solution at pH 7.0. The EDTA was used to dissociate casein micelles, and SDS, to disperse aggregated MFGs. Deionized water was used as the continuous medium. Mode size (peak of frequency distribution), volume mean diameter (d_{43}), surface mean diameter (d_{32}), and specific surface area (S.S.A.) were calculated[9] using the Horiba diffraction software. Measurements were carried out in triplicate.

Determination of ζ-Potential. The ζ-potential of milk fat globules was measured using a Zetasizer nano ZS90 (Malvern Instruments Ltd., Worcestershire, U.K.) using the Smoluchowski approximation because the thickness of MFGM is widely recognized to be less than 20 nm, which is smaller than the diameter of MFGs (0.1−10 μm). Milk and cream dispersions were diluted 8×10^{-3} in SMUF. Each sample was measured as five replicates.

Total Protein and Fat Content. The total protein content of milk and SMUF-washed MFGs was determined by a Coomassie blue-staining protein assay.[38] Total fat content was determined gravimetrically after a Folch total lipid extraction.[39] Milk and dispersed MFG fractions were mixed in a chloroform/methanol (2:1 v/v) solvent at a ratio of 1:8 (v/v) and 1:20 (v/v), respectively. NaCl solution (0.2 volumes of 0.9% w/v) was used to wash the sample-solvent mixture to enhance the recovery of lipids from extraction. The chloroform phase, containing lipids, was dried under a gentle N_2 gas flow.

Characterization and Quantitation of MFG-Associated Protein Components. Protein characterization and relative quantification was carried out by SDS−polyacrylamide gel electrophoresis (PAGE) under reducing conditions. The precast gel (bis-Tris 4−12% polyacrylamide gel), NuPAGE LDS (lithium dodecyl sulfate) pH of 8.4 sample buffer (4×), NuPAGE reducing agent (dithiothreitol), and SimplyBlue Safe Stain (Coomassie Brilliant Blue) staining agent were obtained from Life Technologies Corporation (Carlsbad, CA). The dispersed MFG fractions were initially ultrasonicated at 50 °C to achieve a fine and homogeneous suspension system. Milk was diluted four times in SMUF. Dispersed MFG fractions were diluted in SMUF to yield a protein content of 5 mg/mL to ensure accurate comparison of band densities in stained gels. Samples (6.5 μL) were mixed with LDS sample buffer (2.5 μL) and reducing agent (1 μL) and heated at 90 °C for 10 min before loading onto precast polyacrylamide gels. Electrophoresis was run at constant voltage (160 V) for 1 h. Stained gels were scanned at 300 dpi (ImageScanner III, GE Healthcare, Uppsala, Sweden), and band density was measured using image analysis software (ImageQuant TL, GE Healthcare). Molecular weight (MW) markers (3.5−260 kDa Novex sharp prestained protein markers (Life Technologies Corporation) were used to determine the MW of MFGM proteins.

Table 1. Milk Fat Globule Diameters, Specific Surface Area and ζ-potential from Different Sample Fractions[a]

	modal diameter (μm)	d_{43} (μm)	d_{32} (μm)	S.S.A.[b] (m^2/g fat)	ζ-potential (mV)
unpasteurized milk	3.58 ± 0.03 g	3.57 ± 0.04 g	3.53 ± 0.03 e	1.70 ± 0.01 a	−12.24 ± 0.26 bcd
M1-cream	4.62 ± 0.26 cd	4.77 ± 0.07 cde	3.82 ± 0.17 ef	1.57 ± 0.07 bc	−12.38 ± 0.32 cd
M1-1 wash	4.79 ± 0.01 bc	5.11 ± 0.03 bc	4.47 ± 0.08 c	1.34 ± 0.02 e	−11.97 ± 0.16 b
M1-2 wash	5.02 ± 0.36 b	5.44 ± 0.25 b	4.65 ± 0.06 b	1.29 ± 0.02 e	−12.02 ± 0.27 bc
M1-3 wash	5.48 ± 0.01 a	7.69 ± 0.73 a	5.14 ± 0.03 a	1.17 ± 0.01 f	−11.38 ± 0.36 a
M2-cream	4.15 ± 0.01 ef	4.32 ± 0.09 ef	3.81 ± 0.07 ef	1.57 ± 0.03 bc	−12.50 ± 0.29 d
M2-1 wash	4.13 ± 0.01 f	4.18 ± 0.04 f	3.86 ± 0.19 e	1.55 ± 0.07 c	−12.11 ± 0.35 bc
M3-cream	4.15 ± 0.02 ef	4.30 ± 0.15 ef	3.67 ± 0.07 fg	1.64 ± 0.03 ab	−12.49 ± 0.39 d
M3-1 wash	4.20 ± 0.02 ef	4.68 ± 0.13 cde	3.82 ± 0.05 ef	1.57 ± 0.02 bc	−12.38 ± 0.19 bcd
M3-2 wash	4.18 ± 0.02 ef	4.66 ± 0.27 de	3.71 ± 0.07 ef	1.62 ± 0.03 bc	−12.93 ± 0.77 e
M3-3 wash	4.39 ± 0.30 de	4.90 ± 0.43 cd	4.14 ± 0.11 d	1.45 ± 0.04 d	−13.04 ± 0.65 e

[a]Means within a column with different letters of significance differ ($P < 0.05$); $n = 3$ for size and specific surface area data, and $n = 5$ for ζ-potential data. [b]Specific surface area.

Table 2. Total Protein Change and Main Nonmilk Fat Globule Membrane (MFGM) Proteins on the Milk Fat Globule Surface after Washing[a]

	total protein (%)[b]		main non-MFGM proteins[b]	
	fat basis	S.S.A.[c] basis	(mg/g of fat)	(mg/m^2 of S.S.A.)
M1-cream	100	100	n.a.[d]	n.a.
M1-1 wash	66.57 ± 3.11 d	78.00 ± 3.65 b	0.28 ± 0.04 b	0.21 ± 0.03 b
M1-2 wash	50.78 ± 1.96 f	61.81 ± 2.39 d	0.27 ± 0.03 b	0.19 ± 0.04 b
M1-3 wash	42.56 ± 3.78 g	59.20 ± 2.34 d	0.15 ± 0.01 e	0.13 ± 0.01 c
M2-cream	100	100	n.a.	n.a.
M2-1 wash	77.22 ± 3.46 b	78.28 ± 2.94 b	0.90 ± 0.07 a	0.58 ± 0.04 a
M3-cream	100	100	n.a.	n.a.
M3-1 wash	84.88 ± 0.98 a	87.95 ± 0.32 a	0.23 ± 0.02 bc	0.14 ± 0.01 c
M3-2 wash	72.20 ± 3.74 c	71.50 ± 1.47 c	0.21 ± 0.02 cd	0.14 ± 0.01 c
M3-3 wash	61.91 ± 1.76 e	70.02 ± 1.99 c	0.17 ± 0.01 de	0.11 ± 0.00 c

[a]Means within a column with different letters differ ($P < 0.05$); $n = 3$ for specific surface area data, and $n = 3$ for fat compositional data. [b]Calculated on a total fat content and S.S.A. basis. [c]Specific surface area. [d]Not available; gel band density of non-MFGM proteins were too high and beyond the range of the quantification method.

Identification of protein bands was carried out by comparison with published MW data[7,11,30,34] using standard curves from major milk proteins.[33] The relative densities of protein bands on different gels were calibrated using the 110 and 160 kDa bands of the MW markers, allowing a comparison of protein band densities across different gels. Samples were run in duplicate on SDS−PAGE gels.

Microstructural Imaging by Confocal Laser Scanning Microscopy. Confocal laser scanning microscopy (CLSM; Zeiss 710 upright microscope, Jena, Germany) was used to assess protein and phospholipid changes on the MFG surface in situ. MFG-associated proteins were labeled with fluorescent Fast Green FCF, and MFGM phospholipids (PLs), with fluorescent Rd-DOPE.[13,15,16] Prior to staining, the suspended MFGs and milk were diluted 5-fold with SMUF at pH 6.5. The staining method was adopted from previous work in our research group[15] with slight modifications. Briefly, prepared samples (1 mL) were mixed with Fast Green FCF at a ratio of 100:6 (v/v) in Eppendorf tubes for 10 min. Rd-DOPE solution (1 mL of 1 mg/mL) was placed on a concave microscope slide, and chloroform solvent was dried in a vacuum desiccator before mixing with the protein-stained MFG sample because chloroform may induce structure changes in the MFGM. Protein-stained MFG suspension samples (25 μL) were pipetted onto the Rd-DOPE deposited slide surface with gentle mixing using a pipet tip. An incubation time of 20 min at ambient temperature was required to allow Rd-DOPE fluorescent dye to stain the MFGM. All staining procedures were carried out in a dark room to minimize loss of fluorescence signal (photobleaching). Agarose (low melting point 37 °C) temperature-controlled gel solution (50 μL, 0.5% w/v in deionized water) was mixed with stained samples to fix the MFGs onto the microscope slide.[15,16] A coverslip was gently applied to the top of the sample.

Three channels—Rd-DOPE channel 1, Fast Green FCF channel 2, and transmitted light channel 3—were used for CLSM observation. Channel 3 was used to locate the physical position of MFGs in the agarose gel.[16] Rd-DOPE-labeled MFGM PLs and Fast Green FCF-labeled MFG-associated proteins were excited using a green HeNe laser at 543 nm and red HeNe laser at 633 nm, respectively. The emitted light was collected between 570 and 625 nm for Rd-DOPE and between 632 and 639 nm for Fast Green FCF. Experiments were carried out in the absence of fluorescent stains to confirm that MFGs do not display intrinsic fluorescence. The configuration of the confocal microscope was kept the same across all samples because alteration would be likely to affect the level of the fluorescent signals. This enabled relative quantitative changes of lipids and proteins on the surface of MFGs to be determined.

Statistical Analysis. One-way analysis of variance tests were carried out using Minitab 16 (Minitab Inc., State College, PA). Significant differences ($P < 0.05$) were determined using Fisher's test in paired comparisons between sample means.

RESULTS

Milk Fat Globule Characterization. The MFG diameters, S.S.A., and ζ-potential of milk and washed fractions are presented in Table 1. MFG modal diameters, d_{43} and d_{32}, were larger after M1 processing with a corresponding decrease in S.S.A. M2 and M3 did not induce significant change in the particle size distribution after washing compared with their corresponding unpasteurized cream fractions, with the exception of the third wash for M3. The ζ-potential was significantly reduced only after the third M1 wash compared

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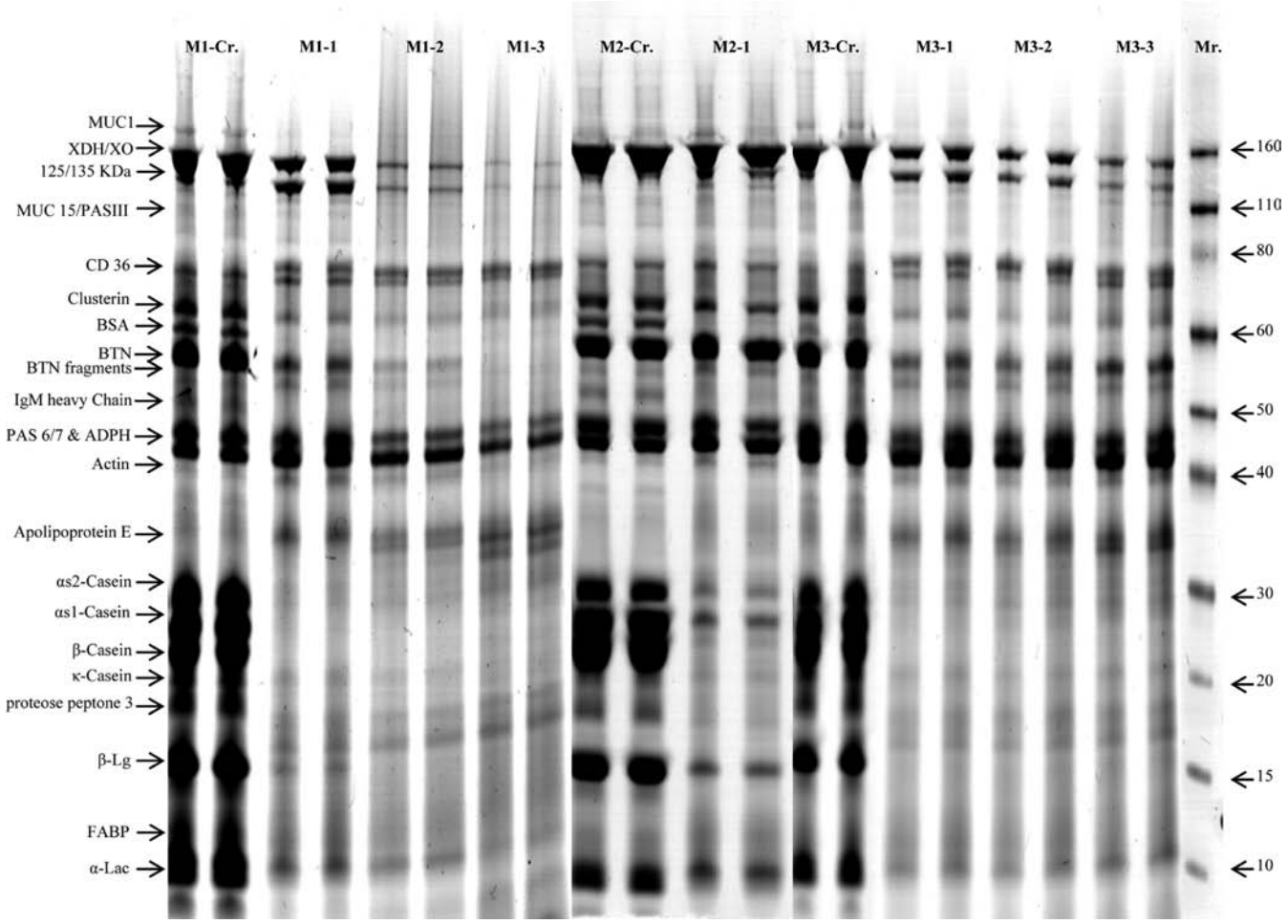

Figure 1. SDS−PAGE gel of milk fat globule-associated proteins after different washing processes. M1-Cr.: original cream. centrifugation conditions M1, 3000*g*; 5 min; M2, 3750*g*; 15 min; M3, 15000*g*; 20 min. The final number after the dash indicates the washing step. Mr., molecular weight markers (kDa); MUC1, mucin 1; XDH/XO, xanthine dehydrogenase/oxidase; MUC15, mucin 15; CD 36, cluster of differentiation 36; BTN, butyrophilin; IgM, immunoglobulin M; PAS 6/7, periodic acid Schiff 6/7; FABP, fatty acid-binding protein.

with unpasteurized milk and was significantly increased after the second M3 wash. M2 processing did not alter the ζ-potential significantly from unpasteurized milk to the M2−1 fraction.

Gross Protein Profile in Washed MFG Fractions. Table 2 shows the percentage of initial total protein retained on the surface after washing and amounts of non-MFGM proteins (including whey and casein proteins) in the washed MFG fractions. Results were calculated on both a fat content and S.S.A. basis. The protein content of the initial cream was considered as the reference and designated as a 100% protein load. The absolute value of non-MFGM protein content in the initial cream fractions could not be estimated from SDS−PAGE because the protein load exceeded the upper limit of the standard curve. This was necessary to give clear band images of the MFGM proteins. Generally, all washing processes resulted in protein loss. M1 induced a lower ($P < 0.05$) total protein retention, 42.56% (fat basis) and 59.20% (S.S.A. basis); compared with M2, 77.22% (fat basis) and 78.28% (S.S.A. basis); and M3, 61.91% (fat basis) and 70.02% (S.S.A. basis), after the final washing steps. However, the retention of non-MFGM proteins on MFGs was higher ($P < 0.05$) in M1 (0.27 mg/g fat basis and 0.19 mg/m^2 S.S.A. basis) than in M3 (0.21 mg/g fat basis and 0.14 mg/m^2 S.S.A. basis) after the second-step washing, and was not significantly different after the third washing step in M1 and M3. Although M2 showed relatively high total protein recovery after washing, it resulted in a higher ($P < 0.05$) retention of non-MFGM protein compared with M1 and M3.

Identification of Proteins. The SDS−PAGE patterns of MFG-associated proteins in different MFG fractions from the initial cream to the final washing step are shown in Figure 1. Non-MFGM protein bands were the most dense in the initial cream samples for M1, M2, and M3 and were largely removed after one washing step. The M2 process was least effective at removing caseins and whey proteins.

The quantification of the partial removal of MFGM proteins on MFG surfaces during the M1 and M3 washing procedures is shown in Figure 2. The relative changes in density from the SDS−PAGE gels of seven key MFGM protein bands (MUC 1, XO, MUC 15/PAS III, CD 36, BTN, PAS 6/7, and FABP) after three washing steps were calculated. Results are shown as a percentage change from the amount of a particular MFGM protein present in the initial cream fraction. Because the total protein loading volumes were the same for all samples, the relative compositional changes of specific MFGM proteins after washing may be presented as enrichment or depletion, on a total protein basis. Enrichment, which has a positive percentage

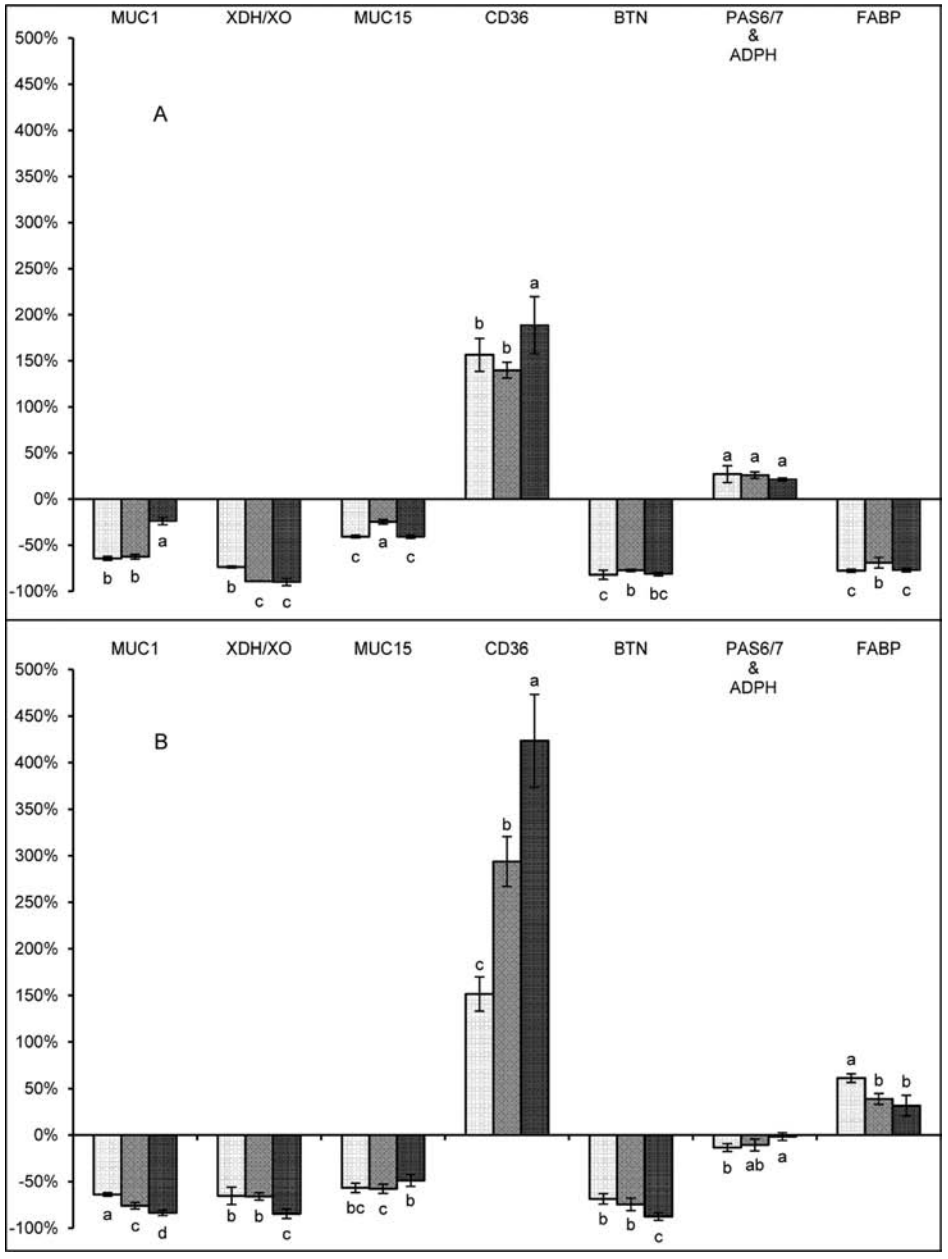

Figure 2. Relative change to the amounts of major milk fat globule proteins after different washing processes compared with the original cream (n = 2). Light to dark shading refers to one to three washes. A, M1 washes; B, M3 washes. Columns for each MFGM protein within each subfigure with different superscript letters do not differ significantly ($P < 0.05$). Abbreviations for proteins are given in Figure 1.

change, represents relatively strong MFGM-binding proteins, such as CD 36 and PAS 6/7 in M1 and CD 36 and FABP in M3. CD 36 was the strongest ($P < 0.05$) MFGM-binding protein and was enriched on the MFG surface by ∼150%, even after the first washing step for both M1 and M3, and was further enriched by around 200−400% after the final M1 and M3 washing steps, respectively. PAS 6/7 and FABP were the second strongest MFGM-binding proteins after M1 and M3 processing, respectively. The rest of the major MFGM proteins were more loosely bound to the MFGM.

Microstructural Imaging by CLSM. Only MFGs within the fluorescent z-stack (confocal focus) with clear and sharp boundary interfaces were used for characterizing structural and compositional changes. The microstructural comparison of unwashed initial cream and the corresponding final washed fraction for the three washing methods (M1, M2, and M3) are shown in Figure 3. Most of the MFGs were well-covered by PLs, and very little fluorescent stained protein was evident on the surface of MFGs after a single M1 centrifugation (Figure 3A−C), but there were some structural changes after the third M1 wash, as shown by the evident loss of PLs (white arrows, Figure 3F). These irregularly shaped MFGs (Figure 3F, white arrows) could be due to the MFGs being compressed by the agarose and coverslip or artifacts from the microscopic imaging. For the M2 wash (Figure 3G−L), some surfaces were apparently bare without fluorescence from Fast Green FCF or Rd-DOPE (white arrows, Figure 3L), as well as rough surfaces coated with PLs (red arrows, Figure 3I). Less Rd-DOPE and Fast Green FCF fluorescent signals were observed in the M3 cream fraction (Figure 3M−O); however, the Fast Green FCF-labeled protein layer became more pronounced after three M3 washings (Figure 3P−R).

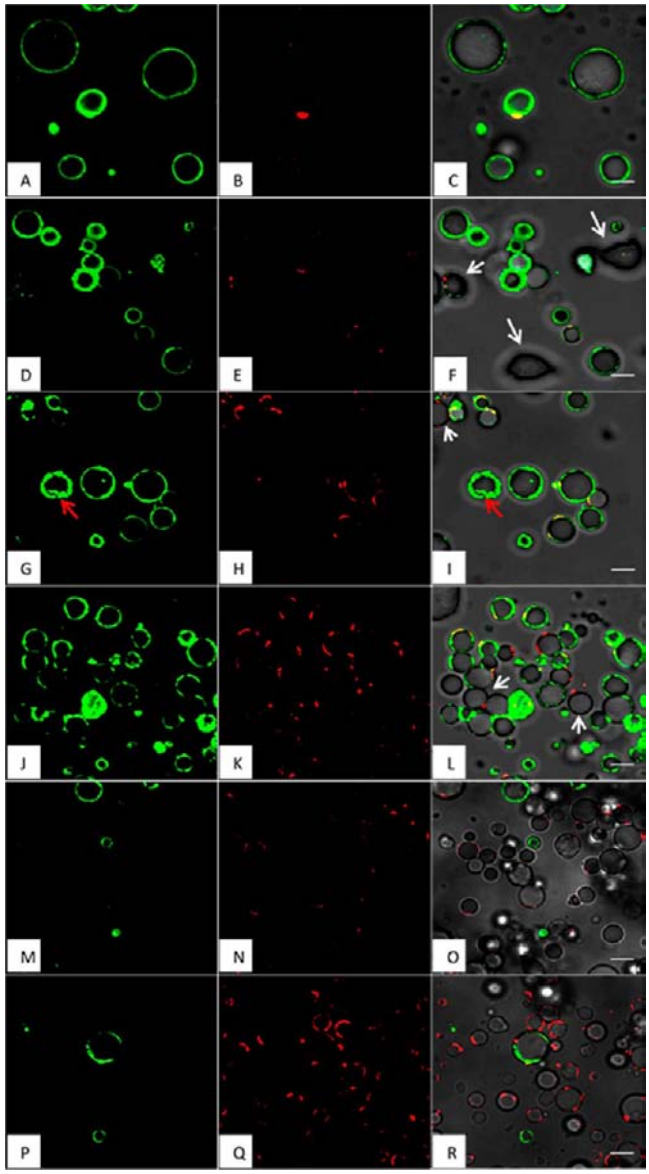

Figure 3. Confocal micrographs of double-stained milk fat globules after washing using three different methods. A–C, M1-cream; D–F, M1 after three washes; row G–I, M2-cream; J–L, M2 after one wash; M–O, M3-cream; P–R, M3 after three washes. Conditions for M1, M2, and M3 are given in the text. Column 1, PLs channel (Rd-DOPE staining); column 2, protein channel (Fast Green FCF staining); and column 3, merged channels of PLs, protein, and T-MPT (transmitted light channel). White arrows, surfaces without evident protein or phospholipids; red arrows, rough Rd-DOPE-labeled phospholipid layer. Scale bar is 5 μm.

DISCUSSION

Unpasteurized milk was sourced from a single Jersey cow to minimize variables, such as breed and age of the cow, feed, and stage of lactation, which could affect the composition and structure of the MFGM and the mean size of MFGs. A single source of milk was also deemed necessary to avoid the problem of structural changes that might occur in pooled milk that is refrigerated and agitated on the farm.

The ζ-potential at the surface of shear of MFGs was used as one of the criteria to estimate the stringency of the washing processes and damage to the MFGM.[20] The mean ζ-potential of MFGs in unpasteurized milk was −12.24 mV, in agreement with published data in the range of −10 to −14 mV.[9,26] The ζ-potential of MFGs increased for the M3 procedure (in absolute values) after the second and third washing steps. This increase could be considered to be due to exposure of an electron-dense proteinaceous coat (inner protein monolayer of the MFGM)[7] during mechanical treatments. This exposure can be attributed to damage to MFG surfaces (the outer phospholipid bilayer) because the total protein and non-MFGM protein load onto MFG surfaces did not increase with the number of wash steps in M3 (Table 2).

The increase in the ζ-potential and greater protein content after the intensive M3 washing procedure, compared with M1 and M2, could be related to the existence of a protein coat under the outer leaflet phospholipid bilayer in the MFGM, which has been reported in other model systems.[7,25,26] This is supported by the observed increase in protein content after M3 washing compared with M1 and M2 (Figure 3), which may be facilitated by damage to the phospholipid outer bilayer. Although M3 is the most intensive washing procedure used in this study, the retention of total protein on MFG surfaces in each of the isolated fractions was higher than that in the corresponding fractions in M1 and M2 (Table 2). Previous results from our group showed the absence of a detectable triglyceride inner core and a greater proportion of MFGM in vesicle-like structures smaller than 2 μm.[21] MFGM vesicles and milk microsomes have been found in the skimmed phase of milk, and these small "MFGM liposomes" are thought to be formed from MFGM material released from native MFGs during centrifugation.[40] However, other research groups found that these "MFGM liposomes" did not originated from shedding of the MFGM during centrifugation processes.[41,42] The fat contents of M3 samples were significantly higher ($P < 0.05$) than the specific paired samples in M1 and M2 (i.e., M3-cream is compared with M1-cream and M2-cream; results not shown). It can therefore be assumed that a larger number of smaller MFGs collected during the more stringent centrifugation process of M3 will result in more recovered MFGM, and specifically, more MFGM proteins.

Non-MFGM proteins, bovine serum albumin and β-casein (CN), were removed after the first washing step in M1, M2, and M3 (Figure 1). For the caseins, α-CN (combined α_{s1}-CN and α_{s2}-CN) were removed in the first washing for M1 and M3 but were still evident in the M2-1 fraction, whereas κ-CN could not be washed off by M2 and M3 but was still found after the third M1 wash (Figure 1). Bands representing β-lactoglobulin and α-lactalbumin were observed after the final washing in all three washing procedures, suggesting that whey proteins were relatively more resistant to the washing processes in all three methods, especially for M2. In practice, it is almost impossible to remove all of the non-MFGM proteins from the MFG suspensions, as evident by residual nonmembrane proteins remaining on the surface of MFGs[33,34] and after the final washing in the current results (Figure 1). It should also be noted that nonmembrane proteins (such as whey proteins) in the cream fractions could be present both on the surface of MFGs and in the aqueous phase surrounding the milk fat globules.

Results of changes in key MFGM proteins (Figure 2 A,B) suggest that CD 36 is the strongest protein bound to the MFGM in both M1 and M3, in agreement with a recent review of the MFGM.[7] The CD 36 protein contains contiguous stretches of hydrophobic amino acid residues close to both the N- and C-termini which face the cytoplasm (thus, are

cytoplasmic-oriented) and function as transmembrane anchors; the hydrophobic pockets in folded polypeptides are also formed by exoplasmic domains.[30,43] Therefore, the two hydrophobic anchors and the hydrophobic exoplasmic pocket serve as unique structural elements of CD 36 that may contribute to the strong affinity to MFGM.

PAS 6/7 (and ADPH) was shown to be less easily removed after M1 washing (Figure 2A); however, it has been reported that PAS 6/7 can be displaced from washed fat globules into the skim milk phase using a concentrated salt washing solution.[44,45] The molecular weight of PAS 6/7 is similar to that of ADPH, the latter of which is associated with the lipid droplet in the protein inner layer underneath the phospholipid bilayer in the MFGM[7] and considered as a stable membrane protein on MFG, and should be taken into account. The quantification of PAS 6/7 from the SDS−PAGE gel may have been overestimated by inclusion of ADPH; therefore, the apparent enrichment of the PAS 6/7 and ADPH band (Figure 2A) could be due to the presence of the more stable ADPH. Unlike other key MFGM proteins, PAS 6/7 are not transmembrane proteins but, instead, are exoplasmic, peripheral proteins bound to anionic phospholipids in the membrane via the C-terminus.[30,46] Therefore, it is reasonable to consider that the affinity of PAS 6/7 to MFGM depends on the amount of retained phospholipids rather than the internal structure and morphology of the phospholipid bilayer backbone of MFGM. M1 is considered as a mild washing treatment and did not induce much structural or physical damage to the MFGM phospholipid backbone on the surface of MFGs (Figure 3A−F), and the ζ-potential did not increase with the number of washing steps; however, rearrangement of the lipid components in the MFGM during washing might allow the release of transmembrane proteins into the washing buffer, resulting in enrichment of the PAS 6/7 and ADPH band.

The MUC 1, MUC 15, XDH/XO, and BTN proteins were depleted with washing in both the M1 and M3 procedures and are therefore considered to be proteins relatively weakly bound to the MFGM. The structural feature of MUC 15, which lacks a membrane anchor and contains an extensive exoplasmic domain,[7] may explain the lower binding to the MFGM. It is speculated that during the washing procedures, the structural organization of MFGM is altered and the soluble XDH/XO, which is located in the protein coat (underneath the phospholipid bilayer) in the MFGM,[7] is released into the washing buffer (SMUF in this case). The BTN protein is released with XDH/XO into the washing buffer as XDH/XO is bound to the cytoplasmic tail of BTN.[47] The formation of a supramolecular complex between XDH/XO and BTN is thought to be an essential step in the assembly of the MFGM, and XDH/XO is considered as a linker between proteins in the MFGM bilayer and proteins on the surface of the lipid inner core of MFGs.[30,48] The majority of MFGM-associated XDH/XO can be washed off using a concentrated salt solution.[31] Although the washing procedures used in these studies were different from the current study, it is still implied that XDH/XO is not a firmly bound membrane protein. Surprisingly, BTN has been found to be firmly bound, resisting extraction with chaotropic agents and detergents;[49,50] however, the MFGM was removed from the surface of destabilized MFGs in the aforementioned research, whereas this current study focused on the effects occurring during the washing processes on the surface of native MFGs.

In the M3 washing procedure, when the MFGM was damaged (as assessed by the ζ-potential), CD 36 and FABP showed enrichment, and all other key membrane proteins were depleted after the M3 process. The coenrichment of CD 36 and FABP is probably due to CD 36 being a strong membrane-binding protein, with interprotein binding between CD 36 and FABP.[7] Although protein−protein interactions (CD 36 with FABP, and XDH/OX with BTN) in the MFGM are known,[7] the depletion of FABP after the M1 washing process (Figure 2A) suggests that CD 36 and FABP proteins are partially present in an uncomplexed form due to FABP being depleted as CD 36 was enriched.

The CLSM images (Figure 3) may present quantitative information based on color (fluorescent intensities) changes because the parameters of the CLSM were strictly controlled for all measurements. In CLSM images (Figure 3O, for example), it must be noted that the apparently bare MFG surfaces in the fluorescent focus plane of the confocal *z*-stack do not mean that membrane phospholipids and proteins were fully washed off from the surfaces of these MFGs. This phenomenon was due to the CLSM's being set at a power level that was not sufficient to excite the lesser amount of fluorescent probes on the surfaces of these MFGs. However, these apparently bare MFG surfaces indicate that some depletion of phospholipids has occurred in situ; therefore, some structural changes to the MFGM have taken place. The fluorescently labeled phospholipid layer on the apparently bare surfaces can be made visible by increasing the power of the CLSM (results not shown).

The increased extent of the protein-stained surfaces after three M3 washings (Figure 3Q,R) could indicate that MFGM proteins have been exposed, rather than being due to adsorption of milk serum proteins, which are largely absent after more than one washing (Figure 1). The observation of a protein-based membrane after three M3 washings is supported by the higher percentage of total proteins remaining on the surface, and these are mainly MFGM proteins due to the lower amounts of non-MFGM proteins (Table 2). Evidently, the M1 washing procedure was not sufficiently stringent to expose membrane proteins to such a large extent or cause the adsorption of milk serum proteins (where the outer phospholipid layer was not damaged). The observed increase in the protein stained surface after one M2 washing (Figure 3K,L) is likely due to a larger amount of adsorbed non-MGFM serum proteins (Table 2). From the ζ-potential, confocal, and protein measurement results, there is evidence to suggest that intensive washing procedures damaged the outer leaflet phospholipid bilayer of MFGM. The exposure of membrane proteins after the more stringent washing processes supports the notion of a protein monolayer underneath the phospholipid bilayer, as deduced in previous models.[7,25]

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Notes

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■ ABBREVIATIONS USED

MFGM, milk fat globule membrane; MFG, milk fat globule; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CD 36, cluster of differentiation 36; MUC, mucin; XDH/XO, xanthine dehydrogenase/oxidase; BTN, butyrophilin; ADPH, adipophilin; SMUF, simulated milk ultrafiltrate; LDS, lithium dodecyl sulfate; MW, molecular weight; CLSM, confocal laser scanning microscopy; PL, phospholipid; S.S.A., specific surface area; PAS, periodic acid Schiff; FABP, fatty acid binding protein; CN, casein

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