JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 2157–2163 2157
DOI:10.1021/jf903617q

Dietary Milk Fat Globule Membrane Reduces the Incidence of Aberrant Crypt Foci in Fischer-344 Rats

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Milk fat globule membrane (MFGM) is a biopolymer composed primarily of membrane proteins and lipids that surround the fat globules in milk. Although it is considered to have potential as a bioactive ingredient, few feeding studies have been conducted to measure its potential benefits. The aim of this investigation was to determine if dietary MFGM confers protection against colon carcinogenesis compared to diets containing corn oil (CO) or anhydrous milk fat (AMF). Male, weanling Fischer-344 rats were randomly assigned to one of three dietary treatments that differed only in the fat source: (1) AIN-76A diet, corn oil; (2) AIN-76A diet, AMF; and (3) AIN-76A diet, 50% MFGM, 50% AMF. Each diet contained 50 g/kg diet of fat. With the exception of the fat source, diets were formulated to be identical in macro and micro nutrient content. Animals were injected with 1,2-dimethylhydrazine once per week at weeks 3 and 4, and fed experimental diets for a total of 13 weeks. Over the course of the study dietary treatment did not affect food consumption, weight gain or body composition. After 13 weeks animals were sacrificed, colons were removed and aberrant crypt foci (ACF) were counted by microscopy. Rats fed the MFGM diet (n = 16) had significantly fewer ACF (20.9 $\pm$ 5.7) compared to rats fed corn oil (n = 17) or AMF (n = 16) diets (31.3 $\pm$ 9.5 and 29.8 $\pm$ 11.4 respectively; $P < 0.05$). Gene expression analysis of colonic mucosa did not reveal differential expression of candidate colon cancer genes, and the sphingolipid profile of the colonic mucosa was not affected by diet. While there were notable and significant differences in plasma and red blood cell lipids, there was no relationship to the cancer protection. These results support previous findings that dietary sphingolipids are protective against colon carcinogenesis yet extend this finding to MFGM, a milk fat fraction available as a food ingredient.

KEYWORDS: Milk fat globular membrane (MFGM); anhydrous milk fat (AMF); colon cancer; sphingolipid; sphingomyelin; aberrant crypt foci (ACF)

INTRODUCTION

Colon cancer is the third most commonly diagnosed cancer in the United States and the second most common cause of cancer-related deaths (*1*). Diet is a well-recognized contributing factor to the etiology of cancer and may be associated with 35−70% of the incidence of colon cancer (*2*). Although various carcinogens are present in foods, their effects may be minor when compared with dietary components that inhibit the cancer process (*2*). As a consequence, many dietary treatments have been tested specifically for their ability to inhibit colon cancer.

Previous studies have demonstrated that purified sphingolipids, such as sphingomyelin, are protective against colon cancer in animal models (*3*, *4*). Sphingolipids are composed of a ceramide core, which, in turn, is composed of a sphingosine backbone with a fatty acid covalently bonded via an amide linkage. Several different head groups may be covalently attached to the ceramide, each resulting in a different class of sphingolipid. Examples include sphingomyelin, with a phosphocholine headgroup, glycosphingolipids with one or more monosaccharides in the headgroup, and gangliosides, which have at least three sugars in the headgroup including at least one sialic acid. The anticancer activity of sphingolipids is primarily associated with their metabolites, ceramide and sphingosine (*5*). Ceramide is an important second messenger implicated in various physiological functions, like apoptosis, and has recently been associated with targeting mitochondrial activity in colon cancer cells (*6*, *7*). Sphingolipid digestion is slow and occurs along the entire length of both the small intestine and the colon, which results in high levels of ceramide and sphingosine in the lumen producing the potentially beneficial effects (*8*). Dietary sphingomyelin and glycosphingolipids isolated from milk have been shown to inhibit chemically induced colon cancer in a mouse model (*9*, *10*), and administration either before or after tumor initiation reduced tumor formation (*11*).

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Published on Web 01/25/2010

Table 1. Major Components of MFGM (Adapted from Ref *12*)

lipids	%	polar lipids	%	proteins
triacylglycerols	62	sphingomyelin	22	mucin 1
diacylglycerols	9	phosphatidylcholine	36	xanthine oxidoreductase
monoacyl-glycerols	trace	phosphatidyl-ethanolamine	27	PAS III
sterols	0.2−2	phosphatidylinositol	11	CD 36
sterol esters	0.1−0.3	phosphatidylserine	4	butyrophilin
free fatty acids	0.6−6	lysophosphatidylcholine	2	PAS 6/7
hydrocarbons	1.2			adipophilin
phospholipids	26−31			fatty acid binding protein

One significant source of dietary sphingolipids is the milk fat globule membrane (MFGM), a protein−lipid complex that is derived from the apical surface of mammary epithelial cells and surrounds the fat globules in milk. During the synthesis of milk, fat droplets originate in the endoplasmic reticulum and transit directly to the apical surface of the cell. As they transit out of the epithelial cells and into the alveolar lumen, they pass through the apical membrane and are encapsulated in the plasma membrane complete with the exterior glycocalyx (*12*). A result of this process is that milk fat is present as discrete globules, which range from 0.1 to about 15 μm in diameter (*13*). The globules are composed of a nonpolar lipid core (mainly triglycerides) surrounded by the MFGM, which is composed of phospholipids and membrane glycoproteins (see **Table 1** for major components of MFGM). Triglycerides are the dominant lipid class, and account for approximately 98% of milk fat, while the balance is composed of phospholipids (0.8%), diglycerides (0.3%), monoglycerides (0.03%), cholesterol (0.3%), and free fatty acids (0.1%) (*14*).

While there is some MFGM in all dairy fats, it is especially enriched in churn buttermilk, a coproduct of butter manufacture. When cream is churned to make butter, the MFGM is released from the surface of milk fat globules, and it is recovered in the aqueous phase. Because of its unique lipid profile, relative sphingolipid enrichment, and widespread availability, MFGM is a good candidate for a colon chemopreventive, bioactive ingredient. MFGM has an interesting profile of carbohydrates, lipids and proteins, as has recently been demonstrated in several proteomic and lipidomic characterizations (*15*−*17*), and as such it is the most diverse fraction of milk. The unique compositional feature has led to the suggestion that MFGM may have interesting nutraceutical properties (*18*), and several research groups have conducted studies to facilitate its recovery from buttermilk (*19*−*30*).

Although very few studies have been conducted to determine any nutritional benefits of MFGM, some indicate positive effects. A recent study concluded that digestion products of MFGM may have antimicrobial activity (*31*). Because of its unique biochemical nature, sphingolipid enrichment, and resulting putative chemoprotective properties, we investigated whether diets containing MFGM are protective against colon cancer in Fischer-344 rats using the aberrant crypt foci (ACF) model.

MATERIALS AND METHODS

Isolation of Milk Fat Globule Membrane. Sweet cream was obtained from Cal Poly Dairy Farm milk by centrifugation after pasteurization. It was churned after a waiting period of 16 h at 4 °C using a continuous pilot scale butter churn (Egli AG, Bütschwil, Switzerland). Buttermilk was recovered, and butter fines were removed by filtration through cheese cloth.

A pilot plant scale system (R-12 model, GEA-Niro Filtration, Hudson, WI) using two spiral polymeric membranes fitted in parallel on the module (10 kDa molecular weight cutoff, 11.33 m2 total surface area) was used for buttermilk concentration. The process was carried out at 25 °C, the transmembrane pressure was 6 bar, and the linear velocity was approximately 1 m/s. The ultrafiltration was conducted until a 10-fold volumetric concentration factor was reached. Diafiltration was completed by adding tap water continuously at 25 °C to the feed tank to replace the removed permeate until reaching a 5-fold diafiltration factor. In each step of the filtration, samples of retentates were collected for composition analysis and the permeate flux was measured. The final retentates were spray-dried (Niro Filterlab Spray-drier, Hudson, WI) to obtain whey buttermilk powders.

Table 2. Composition of Dietary Treatments

	control[a]	AMF[a]	MFGM[a]
	Protein (g/kg diet)		
casein	183	183	183
whey	17	17	17
DL-methionine	3	3	3
	Carbohydrate[b](g/kg diet)		
sucrose	495	495	495
lactose	5	5	5
cornstarch	150	150	150
cellulose	50	50	50
	Fat (g/kg diet)		
corn oil (control)	50		
AMF		50	25
MFGM isolate[c]			25
	Vitamins and Minerals[d](mg/kg diet)		
vitamin mix	10000	10000	10000
Zn	30	30	30 (12.6)[e]
Mg	507	507	507 (24.6)[e]
Ca	5000	5000	5000 (88)[e]
P	1561	1561	1561 (14.5)[e]
Cu	6	6	6 (2.5)[e]
choline bitartrate	2000	2000	2000
	Sphingolipid Content (% by Weight)		
total phospholipids	0.9%	0.09%	0.53%
sphingomyelin	0.03%	0.03%	0.11%

[a] Diets were prepared by Dyets.com. [b] Four grams of lactose was added to the control and AMF diets to balance lactose in MFGM isolate. [c] MFGM isolate is 68% protein, 20% fat, 4% ash, and 4% lactose. The casein to whey ratio is 80:20. The triglyceride to polar lipid ratio is 3:1. [d] Mineral composition of MFGM isolate was determined by ICP-AAS. Minerals were adjusted in MFGM diet accordingly. [e] Amount derived from MFGM portion of the diet in parentheses (mg/kg diet).

Diet Formulation. Diets were formulated to differ only in fat composition. This was achieved by analyzing the composition of the MFGM isolate, selecting an appropriate amount to add to the diets to achieve a 0.1% (w/w) sphingomyelin concentration, and then adjusting the other nutrients accordingly. The measurement of protein, total fat, ash, and lactose were conducted as previously described (*21*). Ash was further analyzed for specific minerals by inductively coupled plasma spectroscopy (ICP-AES) at a core facility on the Utah State University campus. The composition of the three experimental diets is shown in **Table 2**.

Animals and Diets. Sixty-three male, weanling Fischer-344 rats (Charles River Laboratories) were randomly assigned to one of three dietary treatments that differed only in the fat source as previously described. The diets were based on the AIN-76A rodent diet, and the fat sources were (a) corn oil, (b) anhydrous milk fat, and (c) a combination of AMF and MFGM. After a 7-day acclimation period on standard chow diets, the rats were individually housed in a room controlled for temperature, humidity, and light cycle and were given free access to experimental diets and deionized water. Food intake and weight were measured weekly, and the animals were monitored for signs of disease. All experimental protocols involving animals were approved by the Utah State University Institutional Animal Care and Use Committee.

Table 3. Fatty Acid Composition of Diets (%)

	control	AMF	MFGM
C4:0	0	1.8	2.7
C6:0	0	2.4	2.5
C8:0	0	1.7	1.6
C10:0	0	3.5	3.3
C12:0	0	3.8	3.3
C14:0	0.5	11.6	10.3
C15:0	0.1	1	1
C16:0	11.4	27.3	27.4
C16:1 n7	0.2	1.9	1.6
C18:0	2.2	10.5	11.5
C18:1 n7t	0.1	2.6	2.7
C18:1 n9	27.4	24.3	23.6
C18:2 n6	56.4	4	4.8
C18:2 9c, t11	0	0.6	0.5
C18:3 n3	1.0	0.6	0.51

Animals were fed experimental diets for three weeks and then injected (intraperitoneal) with 1,2-dimethylhydrazine (25 mg/kg of body weight, Sigma) in phosphate-buffered saline with 1 mmol/L EDTA (Sigma) once per week for two consecutive weeks. Seventeen or sixteen rats per treatment were injected with the carcinogen, and four rats per treatment were injected with a saline vehicle control. The colonic mucosa, red blood cells, and plasma from these animals were used for gene expression studies and fatty acid analysis. Following injections, animals were fed experimental diets for nine additional weeks. After MRI analysis of body composition (EchoMRI-900), rats were sacrificed by cardiac puncture following ketamine/xylazine anesthesia. Tissues and organs (except lower bowel) were removed, flash frozen in liquid nitrogen, and stored at −80 °C for future analysis. The lower bowel was removed, washed with saline, split open, laid flat, and stored in 70% ethanol at 4 °C. To avoid bias, colons were randomized and ACF in the entire colon were counted by light microscopy following staining with methylene blue.

RNA Isolation and Gene Expression. Mucosal cells from animals injected with the vehicle control were obtained by scraping saline-washed, split colons with a glass slide, and then flash frozen in liquid nitrogen. Mucosal scrapings were homogenized in Trizol with a tissue homogenizer, and total RNA was extracted using the RNAqueous kit (Ambion) according to the manufacturer's protocol. Total RNA was frozen and sent to Genome Quebec for analysis using the Illumina platform (Illumina, Inc.). Results were analyzed using a software analysis program, FlexArray, developed by Genome Quebec to normalize data. Data was then subjected to pathway analysis using Panther (SRI International). Pathway analysis did not reveal differentially regulated cancer pathways that might explain fewer aberrant crypts in MFGM fed animals compared to the control and AMF treatments.

Lipid Profiling. Lipids were quantified in diets, plasma, red blood cells, and mucosa using a combination of thin layer chromatography separation and gas chromatographic analysis of fatty acid methyl ester derivatives (FAMEs). Lipids were extracted using the method of Folch (*32*), and separated on silica TLC plates according to the method of Watkins (*33*). Bands were visualized using the fluorescent dye primulin (*34*), and scraped from the TLC plates into 4 mL vials fitted with Teflon caps. FAMEs were prepared according to the method of Curtis (*35*) and analyzed using a Shimadzu GC-2010 equipped with a BPX-70 capillary column (10 m × 0.1 mm i.d. × 0.2 μm film thickness, SGE Inc., Austin, TX). One microliter of sample was injected out of a volume of 100 μL. The injector was maintained at 250 °C and all samples were injected in split mode (triglyceride samples at a 250:1 ratio and all other samples 25:1). Hydrogen was used as the carrier gas at a linear velocity of 56.4 cm/s, the total flow was 16 mL/min with a 3 mL/min purge flow. The oven program was as follows: initial temperature of 50 °C for 0.29 min, 82 °C/min ramp to 180 °C, hold for 1.45 min, ramp at 13.8 °C/min to 220 °C, ramp to 250 at 35.54 °C/min, hold for 2 min. The detector was maintained at 250 °C; the detector gases were as follows: air at 400 mL/min; hydrogen at 39 mL/min. Total run time was 9.04 min. Fatty acids were identified according to retention time to authentic standards, and quantified using surrogate spikes and experimentally determined response factors.

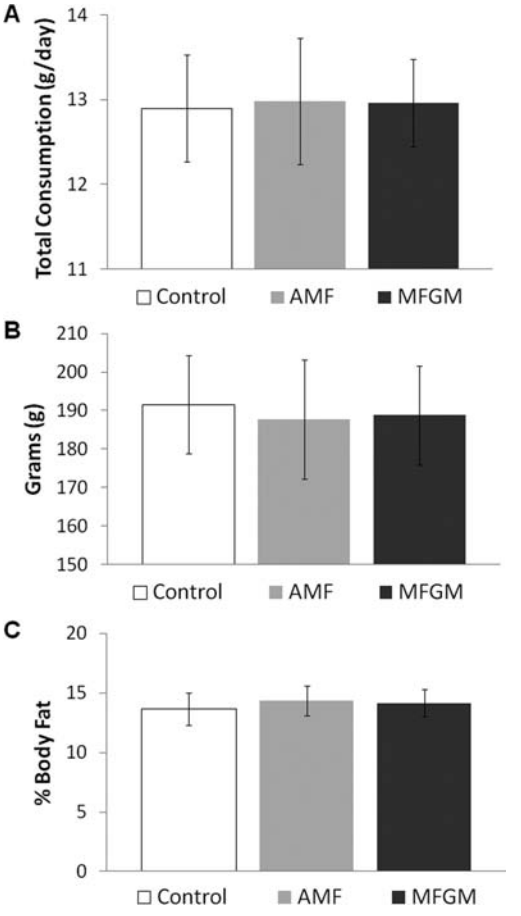

Figure 1. Effect of experimental diets on consumption (**A**), total weight gain (**B**) and body fat composition (**C**). Values are means (n = 17 control diet, n = 16 anhydrous milk fat diet, n = 16 milk fat globular membrane diet) ± standard deviation. Significant differences were determined by ANOVA. Experimental diets did not significantly affect consumption, weight gain or body fat percentage.

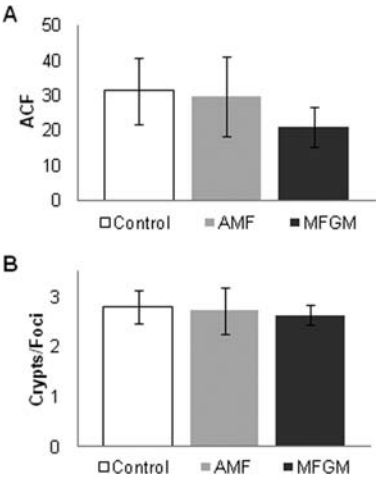

Figure 2. Effect of experimental diets on (**A**) 1,2-dimethylhydrazine induced aberrant crypt foci (ACF) and (**B**) ACF size. Values are means (n = 17 control diet, n = 16 anhydrous milk fat diet, n = 16 milk fat globular membrane diet) ± standard deviation. Differently lettered columns are significantly different (P < 0.05). Significant differences were determined by ANOVA and Fisher's LSD test.

Statistical Analysis. Differences in feed intake, body weight, body composition, ACF, lipid profiles of total plasma triglycerides, total plasma phospholipids, mucosa sphingomyelin and RBCs were determined by

ANOVA and Fisher's LSD test. Significant differences in gene expression were determined by FlexArray 1.3 analysis software. Expression was normalized using the lumi method (a pipeline for processing Illumina microarray) and differences in relative gene expression were determined using the EB (Wright and Simon) method (*36*).

RESULTS AND DISCUSSION

MFGM Composition and Diet Formulation. The composition of isolated MFGM was 68% protein, 20% fat, 4.5% ash and 4% lactose. It was determined that adding the MFGM isolate at 12.5% of the diet (w/w) would supply approximately 1/2 of the fat in the AIN-76A diet (2.5% out of 5%) and would provide approximately 10% of the fat as membrane lipids with about 0.1% (w/w) as sphingomyelin. As the MFGM extract also contains a significant portion of milk protein (68% of powder with a casein:whey ratio of 8:2), addition of 12.5% MFGM also contributed 1.7% whey protein. To prevent potential confounding effects from different protein sources, we adjusted the control diet and AMF diet to also contain 1.7% whey protein. Additionally, some of the proteins of the MFGM powder are membrane proteins associated with the material itself. Compositional analyses of highly purified MFGM indicate the material is approximately 50% protein and 50% lipid (*37*). Therefore, the contribution of MFGM membrane proteins to the MFGM diet may be as high as 12.5% of protein, and were unique to this diet. We also adjusted the carbohydrate content of the control and AMF diets to accommodate a small amount of lactose in the MFGM powder. Finally, we balanced diets to account for the mineral profile of MFGM powder, see **Table 2** for diet compositions.

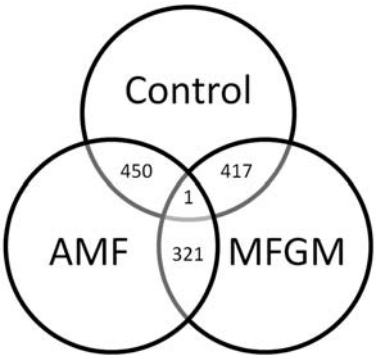

Figure 3. Venn diagram of differentially regulated genes in the mucosa between dietary treatments. Out of 21,792 genes, a total of 417, 450, and 321 genes were differentially regulated with significance in the mucosa between the MFGM vs control, AMF vs control, and MFGM vs AMF dietary treatments, respectively. See Supplementary Table 1 in the Supporting Information for a listing of the most differentially regulated genes between treatments.

Upon receipt, the diets were analyzed for total fatty acid composition (**Table 3**), phospholipid composition, and sphingomyelin content (**Table 2**). As was planned, the MFGM powder contributed approximately 10% phospholipids (0.53% w/w of diet) to the lipid fraction, of which sphingomyelin made up 20% (or 0.11% w/w of diet). One surprising finding in the diet analysis was that the sphingomyelin concentration of the control and AMF diets was not insignificant (0.03% w/w for both diets). As the values are the same and as the fatty acid profiles of the sphingomyelin are the same despite the different fat sources, we conclude that sphingomyelin is likely contributed by either the casein or the whey, or a combination of both. Nonetheless, previous studies have found significant effects with such low levels of sphingomyelin, and therefore the protective effects provided by MFGM in this study may be somewhat underestimated.

Effect of Diet on Consumption, Weight Gain and Body Composition. Dietary treatment did not significantly affect consumption or weight gain. MRI analysis of whole animals indicated no significant effect on body fat percentage (**Figure 1**). Given that feed intake can affect carcinogenesis, confounding factors are possible when feeding different diets even though the diets are isocaloric. Since the difference in fat source between the three diets used in this study did not affect consumption, weight gain, or body fat, feeding behaviors in each group cannot account for differences in ACF.

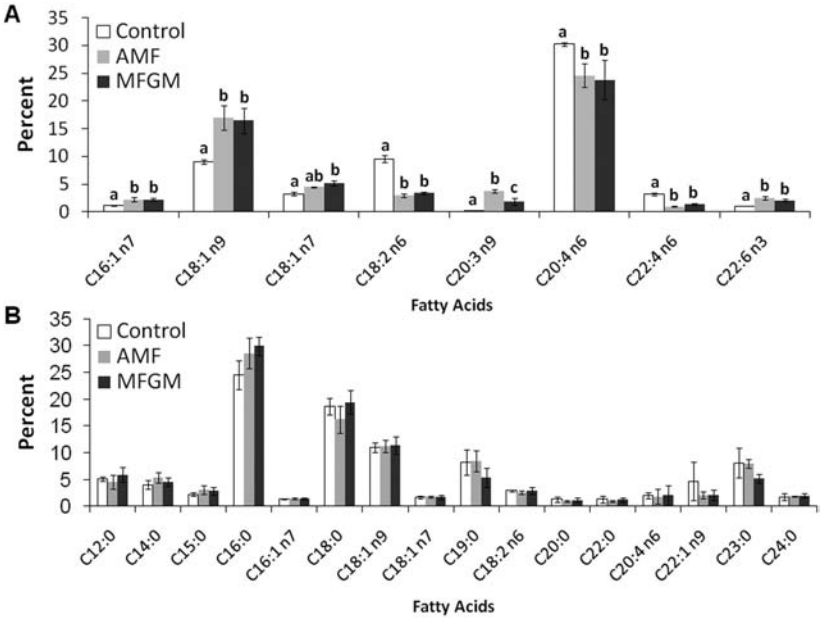

Figure 4. Effect of experimental diets on fatty acid profile of (**A**) red blood cells and (**B**) mucosal sphingomyelin. Samples were taken from saline injected control animals ($n = 4$ control diet, $n = 3$ anhydrous milk fat diet, $n = 4$ milk fat globular membrane diet). Values are the mean percent of total fatty acids ± standard deviation. Significant differences were determined by ANOVA and Fisher's LSD test. Differently lettered columns are significantly different ($P < 0.05$). Red blood cell fatty acids that comprise less 1% of total fatty acids are not shown.

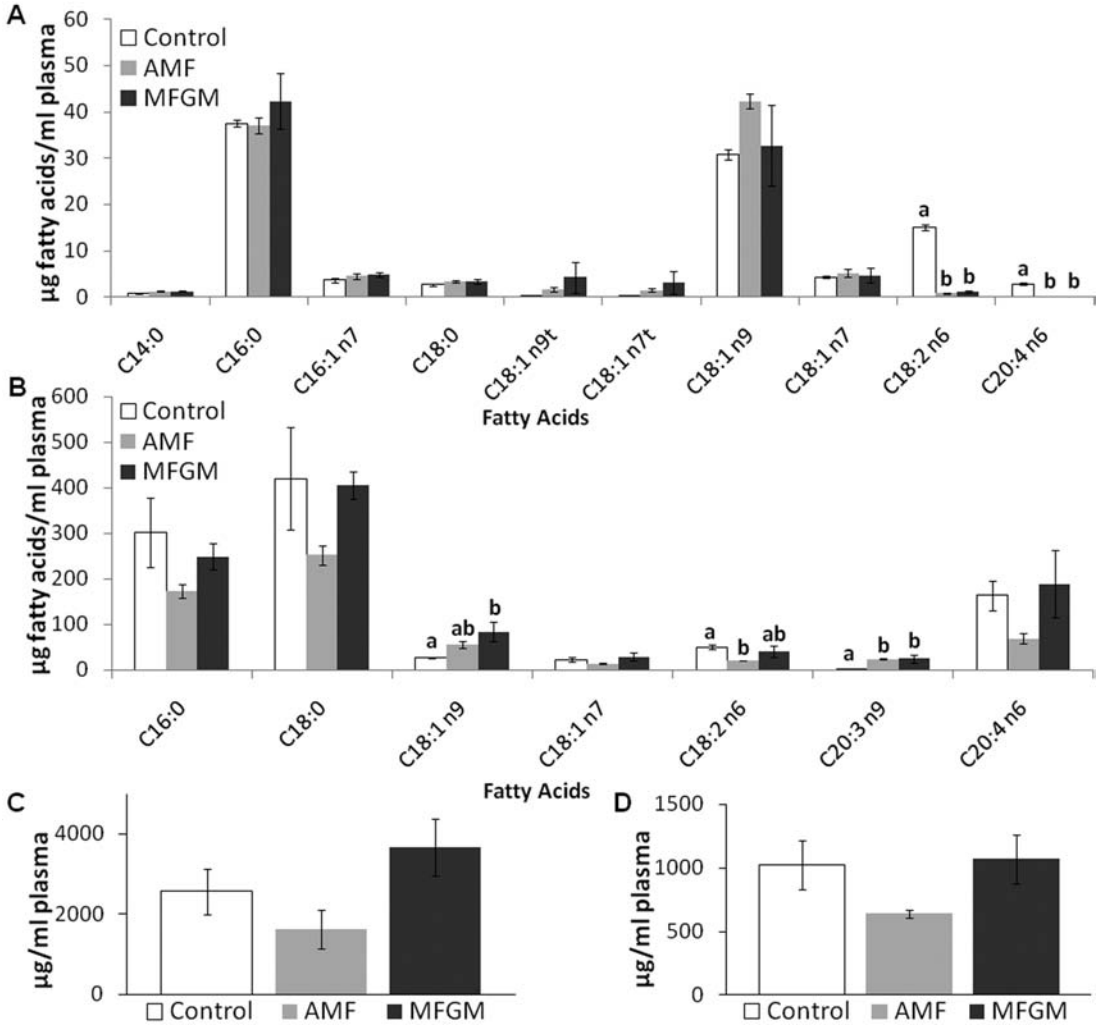

Figure 5. Effect of diet on fatty acid profile of (**A**) plasma triglycerides, (**B**) plasma phospholipids, (**C**) sum total of plasma triglycerides, and (**D**) sum total of phospholipid fatty acids. Samples were taken from saline injected control animals (n = 4 control diet, n = 3 anhydrous milk fat diet, n = 4 milk fat globular membrane diet). Values are means (μg fatty acids/mL of plasma) ± standard deviation. Significant differences were determined by ANOVA and Fisher's LSD test. Differently lettered columns are significantly different ($P < 0.05$). Triglyceride fatty acids with values less than 0.5 μg/mL and phospholipid fatty acids with values less than 10 μg/mL are not shown.

Effect of Dietary Treatment on Appearance of ACF. The ACF model is a well-established model of colon cancer and has been used extensively in nutritional studies (*9*−*11*,*38*−*41*). Rats fed the MFGM (n = 16) diet had a significantly reduced number of ACF ($P < 0.005$) compared to the control (n = 17) (20.9 ± 5.7 vs 31.3 ± 9.5) and AMF (n = 16) diets (29.8 ± 11.4), and ACF was not significantly different between control and AMF treatments (**Figure 2A**). Dietary treatment had no effect on ACF size (**Figure 2B**) nor was there a significant difference in the number of ACF with four or more crypts/focus (data not shown) possibly suggesting that MFGM treatment is more relevant to preventing ACF initiation as opposed to ACF growth progression.

Effect of Diet on Mucosal Gene Expression. Out of 21,792 genes, a total of 417, 450, and 321 genes were differentially regulated with significance in the mucosa between the MFGM and control, AMF and control, and MFGM and AMF dietary treatments, respectively (**Figure 3**). Despite the observation that MFGM treatment decreased ACF compared to both control and AMF; no common gene regulation or change in cancer pathways were observed between MFGM vs control and AMF.

Although dietary treatment did not influence the expression of common colon cancer genes, sphingomyelin and MFGM's ability to regulate post-transcriptional gene expression cannot be completely ruled out. A recent study demonstrated that sphingomyelin treatment did not significantly alter mRNA levels but had a significant effect on protein levels of genes critical to the early stages of colon cancer, such as beta-catenin, connexin-43 and Bcl-2 (*41*). Our results together with these recent findings suggest that the sphingolipids present in the MFGM may not be regulating transcription but may be regulating specific post-transcriptional events to reverse aberrant expression of individual proteins involved in colon carcinogenesis. Because many of the phospholipids found in MFGM are common second messengers, future studies utilizing proteomics to examine any relationships between MFGM and relevant metabolic pathways are needed.

Effect of Diet on Tissue and Plasma Lipids. The fatty acid composition of the AMF and MFGM diets were very similar at the fatty acid level and very different from that of the control diet (**Table 3**). To determine how the fatty acids in the three diets affected tissue levels, we measured the fatty acid compositions of the red blood cells (RBCs) as well as the mucosa (**Figure 4**). According to the data, the RBC fatty acid composition of animals reflected that of the diet, while the mucosa did not. Several notable differences are apparent in the RBCs. For example, the animals fed the control diets had a lower percentage of oleic acid (C18:1n9) in RBCs despite the fact that there was more of this

fatty acid in the diet on a percentage basis. In addition, there were significant differences in the metabolism of the n6 and n3 essential fatty acids. For example, the RBCs of the animals fed the control diets were enriched in linoleic acid (C18:2n6) and its elongation and desaturation products arachadonic acid (C20:4n6) and docosatetraenoic acid (C22:4n6). The animals fed the AMF and MFGM diets, on the other hand, had greater RBC fatty acid proportions of docosapentaenoic acid (C22:5n3) and docosahexaenoic acid (C22:6n3) despite the fact that the overall percentage of n3 fatty acid contribution to the diet was lower (1.0% for control diet vs 0.8% for both AMF and MFGM diets). It has been hypothesized that high tissue levels of arachadonic acid may affect susceptibility to cancer via inflammatory signaling (*43*). However, our results do not indicate that there is any effect as the animals fed the AMF and the control diets had similar levels of ACF.

We hypothesized that the higher content of spingomyelin in the MFGM would affect the mucosal spingomyelin fatty acid profile. As sphingomyelin is thought to be slowly digested along the length of the digestive tract (*8*), it stands to reason that it may be available in the gut lumen for absorption by the epithelia. Alternatively, as was noted with RBCs, if absorbed, sphingomyelin might also be provided to the mucosa via the systemic circulation. However, unlike RBCs, the sphingomyelin profile of the colonic mucosa was not significantly affected by the differences in dietary fatty acids (**Figure 4**). According to the diet analysis, the sphingomyelin fatty acids most abundant in the MFGM diet, compared to the control and AMF diets, were C16:0, C22:0, C23:0 and C24:0 (data not shown). Yet, the sphingomyelin of the MFGM diets does not seem to be enriched in the longer chain species, although there is a small effect with C16:0 (**Figure 4**).

Plasma triglycerides differed significantly only between animals fed the AMF (1,634 μg/mL plasma $\pm$ 477) and MFGM (3,666 μg/mL plasma $\pm$ 703; $P < 0.02$) dietary treatments (**Figure 5**). Plasma phospholipids were significantly different in animals fed the AMF (640 μg/mL plasma $\pm$ 35) diet from those fed the control (1,025 μg/mL plasma $\pm$ 194; $P < 0.04$) and MFGM (1,069 μg/mL plasma $\pm$ 194; $P < 0.04$) diets; however, no significant difference was seen between the animals fed the control and MFGM diets (**Figure 5**). Despite the similar fatty acid profiles of MFGM and AMF diets, they have very different effects on plasma triglycerides and phospholipids that are not related to differences seen in cancer protection.

Our results support the hypothesis that diets containing MFGM are protective against colon cancer in Fischer-344 rats, perhaps because of MFGM's high polar lipid content, namely, sphingomyelin. By incorporating MFGM into the diet, animals were provided (0.11% w/w) sphingomyelin. Previous studies using sphingomyelin concentrations ranging from 0.025% to 0.1% sphingomyelin (by weight) have clearly shown sphingomyelin's role in the prevention of colon carcinogenesis (*9*−*11*, *38*, *42*); however many of those studies used a very pure form of isolated sphingomyelin. In this study, the sphingomyelin is in a more practical form or more similar to how it would be incorporated into human diets, and the MFGM contains other important phospholipids such as phosphatidylcholine and phosphatidylethanolamine. One other major difference in the MFGM diet, compared to the control and AMF diets, was the contribution of MFGM proteins. Several of these membrane proteins have been hypothesized to provide biological effects, and we cannot rule out the fact that they may have contributed to the protective effect of the diet. In pilot studies conducted *in vitro*, we noted that these proteins are extensively proteolyzed by a combination of stomach and pancreatic proteases, but this does not rule out bioactivity mediated by the peptide fragments. Thus, the contribution of these proteins remains unknown. The results of this study demonstrate that MFGM, a readily available byproduct from dairy processing, can be incorporated into diets and is protective against colon cancer.

ABBREVIATIONS USED

MFGM, milk fat globule membrane; AMF, anhydrous milk fat; TLC, thin layer chromatography; ACF, aberrant crypt foci; FAMEs, fatty acid methyl esters; RBCs, red blood cells.

ACKNOWLEDGMENT

We thank Dr. Aaron Olsen and Kent Udy for assistance with animal studies.

Supporting Information Available: Tables containing gene expression from microarray including the five most up-regulated and down-regulated genes between MFGM diet vs control diet, MFGM diet vs AMF diet, and AMF diet vs control diet. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review October 14, 2009. Revised manuscript received January 4, 2010. Accepted January 11, 2010. Financial support for this project was provided by Utah State University Center for Integrated BioSystems Seed Grant and by the Utah Agricultural Experiment Station. This paper was approved by the Utah Agricultural Experiment Station as paper #8315.