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## Comparative analysis of the skim milk and milk fat globule membrane proteomes produced by Jersey cows grazing pastures with different plant species diversity

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### Abstract

The objective of this experiment was to identify and characterize the bovine milk proteome within the skim milk fraction and milk fat globule membrane (MFGM)-associated fraction from 16 organically certified lactating Jersey cows after a short term of grazing pastures with or without annual forage crops (AFC). Cows were offered a partial mixed ration (~60% of dry matter intake) and approximately 40% of their total dry matter intake as herbage. Eight cows were offered a cool-season grass–legume herbage (GLH), which included orchardgrass (*Dactylis glomerata*), timothy (*Phleum pratense*), Kentucky bluegrass (*Poa pratensis*), and white clover (*Trifolium repens*). The other 8 cows were offered the same GLH strip-tilled with the AFC, including oat (*Avena sativa*), millet (*Pennisetum glaucum*), teff (*Eragrostis tef*), buckwheat (*Fagopyrum esculentum*), and chickling vetch (*Lathyrus sativus*). Milk samples were collected from each cow during a.m. and p.m. milkings on d 19 to 21 of grazing, and composite milk samples per cow were analyzed for (1) the high-abundance milk protein profile, (2) the skim milk low-abundance protein-enriched proteome, and (3) the MFGM proteome. Of the 443 proteins identified in the skim and MFGM proteomes, 433 were included in statistical analysis, including 68 proteins identified in the skim milk fraction and 365 in the MFGM-associated fraction. Analysis of the skim and MFGM proteomes encompassed unique gene ontology profiles and proportions of functional classifications. In response to diet,  $\alpha_{S1}$ -casein as well as 8 low-abundance proteins were present in higher concentration or abundance in milk from cows grazing the GLH strip-tilled with the AFC compared with milk from cows grazing GLH, suggesting that even short-term grazing of pastures including some AFC may affect the milk proteome.

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## Keywords

alternative forage crop; milk proteomics; liquid chromatography–tandem mass spectrometry; gene ontology

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## INTRODUCTION

The bovine milk proteome encompasses a broad array of proteins and is understood to be important in calf and human nutrition due to its bioactive profile (Vargas-Bello-Pérez et al., 2019). Bovine milk comprises high-abundance proteins, including caseins,  $\alpha$ -LA, and  $\beta$ -LG, as well as thousands of low-abundance proteins (D'Amato et al., 2009). As outlined by Shennan and Peaker (2000), milk proteins can be transported and secreted into the alveolar or ductal lumen via several routes, including transport by the Golgi apparatus, secretion as a component of the milk fat globule membrane (**MFGM**) or within a cytosolic crescent captured within a milk fat droplet, transcytotic transport involving organelle participation, or direct paracellular transport of proteins from the interstitial fluid to milk. The proportional contribution of these routes toward creation of the resulting milk proteome secreted by the cow appears to be affected by numerous factors, including stage of lactation (Wall et al., 2015), hormonal status (Stelwagen and Singh, 2014), and mammary inflammation, including mastitis (Wellnitz et al., 2016).

The skim fraction of the secreted milk contains an array of biologically active proteins (Greenwood and Honan, 2019). The MFGM is another protein-containing fraction that contains a rich bioactive profile (Lee et al., 2018). Butyrophilin (**BTN**), for example, is a common MFGM-associated protein that is a member of the Ig superfamily and is involved in immune T cell responses (Demmelmair et al., 2017). The MFGM is integral and correlated with milk fat secretion; thus, it is feasible that increases in milk fat output could increase the abundance of bioactive proteins and peptides in milk.

Inclusion of herbage and alternative forages such as annual crops or non-alfalfa legumes in the diet, particularly in pasture-based diets, are known to affect milk yield and milk composition, particularly milk fat content, even after only a short term of grazing the alternative pasture (Christian et al., 1999; Totty et al., 2013; Bryant et al., 2018). The inclusion of annual forage crops (**AFC**) in grazing pastures is increasing in popularity in the United States as a method to effectively increase pasture biomass when cool-season grass growth is limited in the summer months (Juntwait et al., 2016). Many of these AFC have unique nutrient profiles, often including secondary compounds that affect animal metabolism and milk production (Totty et al., 2013; Bryant et al., 2018). Given that the inclusion of AFC in pasture can affect milk production and general milk composition produced by grazing dairy cattle and that inclusion of plant-based secondary compounds, such as condensed tannins, in the diet of lactating dairy cattle can affect the skim milk proteome (Scuderi et al., 2019), it is feasible that inclusion of AFC in grazing diets could alter the skim milk- or MFGM-associated proteomes.

To date, very few studies have investigated the effect of pasture profile on the milk proteome produced by grazing cattle. It is hypothesized that the inclusion of AFC in pastures will alter

the skim- and MFGM-associated proteomes produced by cattle grazing these pastures as part of their daily ration over a short-term grazing period in the summer. The objectives of this study were to use proteomic approaches to characterize the skim milk- and MFGM-associated milk proteomes produced by cows grazing cool season grass–legume herbage (GLH) or GLH strip-tilled with AFC (GLAH) during the summer.

## MATERIALS AND METHODS

The University of New Hampshire Institutional Animal Care and Use Committee approved all experimental procedures (IACUC no. 150302). The experiment was carried out at the University of New Hampshire Burley-Demeritt Organic Dairy Research Farm (Lee, NH; 43°10′N, 70°99′W) from July 14 to August 2, 2015.

### Animals, Design, and Diets

A subset of 16 (12 multiparous and 4 primiparous) organically certified Jersey cows that were part of a larger study (Juntwait et al., 2016) were included in the current study. Animal number was determined to be appropriate by calculation of a power statistic at 80% power accounting for a 50% coefficient of variation and 80% treatment difference, which was based on changes in abundance of milk BTN subfamily 1 member A1 due to low-level grape marc supplementation to dairy cattle by Scuderi et al. (2019). At the beginning of the study, cows ( $143 \pm 58$  DIM) were producing (mean  $\pm$  standard deviation)  $18.1 \pm 3.9$  kg of milk/d. Cows were milked twice daily (0530 and 1630 h) throughout the trial. Cows were blocked by milk yield, DIM, and parity and then assigned to treatments according to a randomized complete block design with 14 d for diet adaptation followed by 7 d for data and sample collection. Animals were fed a partial TMR at 60% of their daily DMI comprising (as % of DM) 19.9% ADF, 32.8% NDF, 15.2% CP, 23.8% starch, 6.4% crude fat, 3.1% lignin, and 8.0% ash as outlined by Juntwait et al. (2016). For the remaining 40% of daily DMI, cows grazed GLH. At the onset of the trial, 8 cows continued to graze a GLH pasture that consisted of orchardgrass (*Dactylis glomerata*), timothy (*Phleum pratense*), Kentucky bluegrass (*Poa pratensis*), and white clover (*Trifolium repens*). The other 8 cows grazed GLAH, including oat (*Avena sativa*), millet (*Pennisetum glaucum*), teff (*Eragrostis tef*), buckwheat (*Fagopyrum esculentum*), and chickling vetch (*Lathyrus sativus*). All cows had access to their assigned pastures from 1800 h (after evening milking) to 0500 h the following morning in a strip grazing management system. Pastures were sampled weekly throughout the trial, and samples were taken at 10 random sites within each paddock on each sampling day. Pre-grazed pasture samples were clipped within a measured quadrat at approximately 7 cm from ground height for botanical composition, nutrient profile, and biomass measurements. Biomass measurements were completed on both pre- and post-grazed pasture areas. The herbage nutrient compositions, herbage biomass yield, and botanical composition of the GLH and GLAH pastures were reported previously by Juntwait et al. (2016). Briefly, the botanical composition (DM basis) for the GLH pastures averaged 69% grasses, 11% legumes, and 20% other (broadleaf, weeds, and dead), with a nutrient composition (as % of DM) of 35.0% ADF, 53.1% NDF, 12.9% CP, 1.0% starch, 5.2% crude fat, 4.3% lignin, and 7.8% ash. The botanical composition (DM basis) of the GLAH pastures averaged 61% grasses, 13% legumes, 1% AFC grasses, 2% AFC legumes, 12% AFC broadleaf, and 11%

other (non-AFC broadleaf, weeds, and dead). The nutrient composition of the GLAH pasture was (as % of DM) 38.8% ADF, 50.1% NDF, 14.8% CP, 2.2% starch, 5.0% crude fat, 9.1% lignin, and 6.2% ash.

## Sampling

Concurrent with forage sampling days, individual milk samples were collected on d 19 to 21 of the experiment from each milking for 4 consecutive milkings (d 19 p.m. milking, d 20 a.m. and p.m. milkings, and d 21 p.m. milking) and analyzed for protein percentage, fat percentage, lactose percentage, and urea-N as outlined by Juntwait et al. (2016). Milk production and composition results for each of the 16 cows included in this study were analyzed using statistical methods outlined below and are listed in Table 1. In addition, 3 milk subsamples were collected at each of these 4 consecutive milkings from each individual cow, flash frozen immediately after collection in a dry ice ethanol bath, and transported on dry ice to the University of Vermont (Burlington, VT) for analysis. At each sample collection, 3 subsamples were collected for each cow: 1 subsample (~15 mL) was collected for HPLC quantification of high-abundance proteins and was stored at  $-20^{\circ}\text{C}$ , whereas the 2 other subsamples (~100 mL total each) were collected for MS (liquid chromatography–tandem MS; LC-MS/MS) based proteomic profiling of the low-abundance proteins in the skim milk and MFGM fractions and were stored at  $-80^{\circ}\text{C}$  until further analysis.

## HPLC Quantification of High-Abundance Milk Proteins

Milk samples collected for HPLC analysis were thawed overnight at  $4^{\circ}\text{C}$ , pooled within cow according to milk yield to create 1 representative sample per cow ( $n = 16$  samples total), and centrifuged at  $4,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The skim milk was then prepared and analyzed as previously described (Tacoma et al., 2016), and the fat content of these samples was assumed to be in trace amounts.

## Preparation of the Low-Abundance Protein-Enriched Skim Milk Fraction

Subsamples collected for LC-MS/MS skim protein analysis were pooled within cow according to milk yield, creating 1 representative sample per cow ( $n = 16$  samples total). Additionally, a universal control was generated by pooling milk from each GLH cow according to milk yield, which was subsequently used to compare relative-fold abundance changes in proteins across all samples. Composite samples from each cow, as well as the universal control, were fractionated, enriched, digested, labeled with isobaric tandem mass tags (TMT; Thermo Fisher Scientific, Waltham, MA), and analyzed according to established methods as previously reported (Tacoma et al., 2017b). Two 9-plexes were generated in this experiment, each including the universal control and a random subset of 8 samples. The two 9-plexes were then submitted for LC-MS/MS analysis as previously described (Tacoma et al., 2017b).

## Isolation of the MFGM-Associated Protein Fraction

The third set of subsamples collected for protein profiling in the MFGM fraction was thawed overnight at  $4^{\circ}\text{C}$  and pooled within cow according to milk yield to create 1 representative sample per cow ( $n = 16$  samples total). Of the milk samples retained for MFGM proteome

analysis, 1 milk sample was excluded from processing due to improper storage. To the remaining 15 samples (8 GLH, 7 GLAH), a protease inhibitor cocktail (Sigma, Milwaukee, WI) was added at 0.24 mL/g of protein. The MFGM was isolated according to previously published methods (Yang et al., 2015) with some modifications. Briefly, samples were centrifuged at  $3,000 \times g$  at  $4^{\circ}\text{C}$  for 15 min. The skim layer was discarded, and the cream layer was incubated with 5 volumes of phosphate-buffered saline at  $37^{\circ}\text{C}$  for 20 min, followed by centrifugation at  $3,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ . This wash and centrifugation step was repeated 3 times to remove residual CN proteins. The recovered cream was incubated with 10 volumes of lysis buffer [50 mM Tris-HCl at pH 7.4, 4% SDS (wt/vol) solution] for 1 h at room temperature with periodic vortexing every 10 to 15 min for 10 to 15 s. After incubation, samples were then placed in a water bath at  $95^{\circ}\text{C}$  for 5 min followed by centrifugation at  $12,000 \times g$  at  $4^{\circ}\text{C}$  for 15 min. The residual fat was removed and lysates were centrifuged again. The lysates were then precipitated in acetone at a 1:6 ratio (lysate:acetone) at  $-20^{\circ}\text{C}$  for 20 h. A universal control was generated before this step by pooling an equal volume of supernatant from each GLH sample. Following the precipitation, samples were centrifuged at  $14,000 \times g$  at  $4^{\circ}\text{C}$  for 40 min, and the pellet was resuspended in radioimmunoprecipitation assay buffer (Sigma-Aldrich, St. Louis, MO) to maximize protein suspension and concentration (Subedi et al., 2019) and protease inhibitor cocktail (Sigma) to inhibit degradation of suspended proteins followed by storage at  $-80^{\circ}\text{C}$  until protein quantification. The bicinchoninic acid assay (Pierce, Rockford, IL) was used to quantify protein concentrations with bovine serum albumin as the standard.

### SDS PAGE Separation of the MFGM-Associated Protein Fraction

Quantified samples were separated by SDS-PAGE using a total of 2 gels. A random subset of 8 samples and a universal control were loaded on one gel, and a random subset of 7 samples and a universal control were loaded on the other. Reducing sample buffer (5 $\times$ ; Thermo Scientific, Rockford, IL) was added to each sample in a 1:5 ratio to 49  $\mu\text{g}$  of sample plus 1  $\mu\text{g}$  of *Saccharomyces cerevisiae* GAPDH (Sigma-Aldrich), and the mixture was heated at  $90^{\circ}\text{C}$  for 3 min. The *S. cerevisiae* GAPDH was added to verify the efficiencies of subsequent digestion and TMT-labeling steps across samples. After cooling to room temperature, the samples were loaded onto a precast 12% polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins were separated through electrophoresis, which was run for 15 min at 200 V. Gels were stained in Coomassie blue (Bio-Rad) overnight, and gel images were obtained from scanning before excision.

### In-Gel Digestion of the MFGM-Associated Protein Fraction

The gel lanes were excised into 3 segments according to their molecular weights (I = heavier in weight, upper portion of the gel; II = medium weight, middle portion of the gel; III = lighter in weight, lower portion of the gel; see Figure 1 for example of gel image and segmentation) and subjected to trypsin digestion protocols as described previously (Tacoma et al., 2016), except for using triethylammonium bicarbonate as buffer instead of ammonium bicarbonate. This gel-based separation strategy allowed the high-abundant proteins to be confined to gel slice II-mid, whereas the relatively low-abundant proteins were localized in gel slices I-upper and III-lower. Gel slices I, II, and III were each analyzed separately in 3 MS runs to increase the proteome coverage.

### Isobaric TMT Labeling of the MFGM-Associated Protein Fraction

Isobaric TMT labeling of the protein samples was performed according to the manufacturers' protocols with minor modifications. Briefly, dried peptides in gel slices I and III and gel slice II from each sample were resuspended in 25 and 50  $\mu\text{L}$  of triethylammonium bicarbonate, respectively. After resuspension, 10 and 20  $\mu\text{L}$  of TMT reagents (0.8 mg dissolved in 41  $\mu\text{L}$  of acetonitrile) were added to gel slices I and III and gel slice II, respectively, followed by brief vortexing and incubation for 1.5 h at room temperature. Then, 5% hydroxylamine was added to quench the reactions. One-third of the total reactions was combined, resulting in three 9-plex reactions (I, II, III) from gel 1 (TMT-1) and three 8-plex reactions (I, II, III) from gel 2 (TMT-2). The mixtures were dried and stored at  $-80^{\circ}\text{C}$  until LC-MS/MS analysis.

### LC-MS/MS

The LC-MS/MS analysis was conducted as previously described (Tacoma et al., 2017b). Briefly, a Q-Exactive mass spectrometer coupled to an EASY-nLC (Thermo Fisher Scientific) was used for peptide identification and quantification.

The resulting product ion spectra were searched using the SEQUEST and Mascot search engines on Proteome Discoverer 2.2 (Thermo Fisher Scientific) against a curated Uniprot *Bos taurus* protein database (UP000009136; downloaded on Aug. 31, 2017). Common processing and consensus workflows for reporter-based quantification were used with minor modifications. In the processing workflow, the parameters were set as follows: (1) full trypsin enzymatic activity; (2) maximum missed cleavages = 2; (3) minimum peptide length = 6, (4) mass tolerance at 10 ppm for precursor ions and 0.02 Da for fragment ions; (5) dynamic modifications on methionines (+15.9949 Da: oxidation), dynamic TMT6plex modification (the TMT6plex and TMT10plex have the same isobaric mass) on N-termini and lysines (229.163 Da); and (6) static carbamidomethylation modification on cysteines (+57.021 Da). Percolator node was included in the workflow to limit the false-positive rates to less than 1% in the data set.

In the consensus workflow, parameters were set as follows: (1) both unique and razor peptides were used for quantification; (2) reject quan results with missing channels: false; (3) apply quan value corrections: false; (4) co-isolation threshold: 50; (5) average reporter S/N threshold = 10; (6) no normalization was used; and (7) scaling mode was set "on all average." The raw files of the 3 separate runs of MFGM fraction (I, II, and III) were searched together, resulting in 2 results files (TMT-1 for gel 1 and TMT-2 for gel 2). Protein identification and quantification information (<1% false positive) were exported from the msf result files to Excel (Microsoft, Redmond, WA) spreadsheets. Only the proteins that were identified in both TMT-1 and TMT-2 runs were kept. The ratios (relative to the respective universal control in each TMT run) were used for further statistical analyses.

### Statistical and Bioinformatic Analysis

For statistical analysis, PROC MIXED of SAS 9.4 (SAS Institute Inc., Cary, NC) was performed, including treatment (GLH vs. GLAH) as the fixed effect and milk parameter as the dependent variable. Treatment least squares means were compared using the probability



of differences (PDIFF) option and included a Tukey adjustment test. Of the 76 proteins identified in the skim milk fraction and 367 proteins identified in the MFGM-associated fraction through Proteome Discoverer 2.2 (Thermo Fisher Scientific), 68 skim milk-associated proteins and 365 MFGM-associated proteins were present in enough samples to be statistically analyzed, and proteins that were included in statistical analysis were included in bioinformatic characterization. For bioinformatic analysis, proteins that were uncharacterized in Proteome Discoverer 2.2 (Thermo Fisher Scientific) were identified by obtaining their FASTA sequence through UniProt (Chen et al., 2017) and searching this sequence in BLAST (Camacho et al., 2009). Identified proteins were then annotated according to their biological processes, molecular functions, and cellular components through gene ontology (GO) using the PANTHER classification system (Mi et al., 2017). All GO data presented are the percentage of gene hits against the total number of genes identified. Statistical differences were declared significant if  $P < 0.05$ .

## RESULTS AND DISCUSSION

In this study, the skim milk- and MFGM-associated milk proteomes produced by cows grazing GLH were compared with the proteomes produced by cows grazing GLAH pastures. From a production perspective, the altered pasture profile and nutrient density of the GLAH pasture did not result in higher milk or lactose yields but did support higher milk fat and protein secretion (both % and kg/d) from cows grazing this GLAH pasture, as outlined in Table 1. This resulted in a positive increase in milk solids (total sum of lactose, protein, and fat) and ECM yield (kg/d), and would generally be perceived as beneficial from a commercial standpoint and would be a result of increased mammary metabolism. Focusing on the high-abundance milk protein profile, the concentration of  $\alpha_{S1}$ -CN was 18% higher ( $P = 0.005$ ) in milk from cows grazing GLAH (mean = 14.4 mg/mL) compared with those grazing GLH (mean = 12.2 mg/mL; Table 1), whereas there were no differences in  $\alpha_{S2}$ -CN,  $\alpha$ -LA,  $\beta$ -CN,  $\kappa$ -CN,  $\beta$ -LG variant A, and  $\beta$ -LG variant B concentrations across treatments (Table 1). The reason for this increase is not currently understood. A tentative hypothesis could be that the shift in  $\alpha_{S1}$ -CN may be a result of higher nutrient density in the GLAH, which contained greater concentrations of CP (14.8% in GLAH vs. 12.9% in GLH) and starch (2.2% in GLAH vs. 1.0% in GLH). Tacoma et al. (2017a) reported higher concentrations of  $\kappa$ -CN and a trend toward higher concentrations of CN isoforms  $\alpha_{S1}$  and  $\beta$  produced by the cows fed a diet containing 18.5% CP (as % of DM) that had a higher RDP:RUP ratio compared with Holstein cows fed an isonitrogenous diet that had a lower RDP:RUP ratio. The proportion of RDP:RUP was not measured in the current trial, and post-grazing botanical composition was not analyzed to determine whether cows were being selective of specific herbage species; however, the higher milk and plasma urea-N concentrations reported by Juntwait et al. (2016) from the cows grazing GLH compared with GLAH suggest that the total CP available was the more influential factor in this current trial.

To examine the proteomes associated with the skim milk- and MFGM-associated fractions, we used different techniques to isolate these groups of proteins from the milk samples collected. Using LC-MS/MS techniques to characterize the skim milk- and MFGM-associated protein fractions, 8 of the 433 proteins characterized (1.8%) were identified at greater relative abundance from cows that were grazing GLAH compared with those grazing

GLH (Table 2). Similar to protein identification rates in other studies using similar methods (e.g., Fahey et al., 2020), a total of 76 proteins were identified in the low-abundance protein-enriched skim milk fraction, and 68 were included in statistical analysis (Supplemental Table S1, <https://doi.org/10.3168/jds.2019-17726>). Whereas 66 of these proteins were not affected by dietary treatment, 2 of these proteins were higher in milk from cows grazing GLAH compared with the GLH cows (Table 2), including  $\beta$ -2-microglobulin ( $P=0.009$ ) and polymeric immunoglobulin receptor ( $P=0.04$ ). The protein 2-microglobulin is a component associated with the major histocompatibility complex I and has been detected in all body fluids as well as the surfaces of nucleated cells (Bourantas et al., 1999). Little information outlining the functionality of 2-microglobulin in milk has been reported, and the effect of diet on this protein is not understood; however, it is a component of the receptor responsible for IgG transfer across mammary epithelial cells, and polymorphisms of the gene coding for this protein result in suppression of IgG1 secretion in milk (Zhao et al., 2012). Polymeric immunoglobulin receptor is another immune-related protein that is associated with IgA production in the small intestine (Asano and Komiyama, 2011). A more extensive gel (SDS-PAGE)-based fractionation protocol was included to increase the separation and identification of proteins in the MFGM, resulting in identification of a total of 367 proteins in the MFGM-associated protein fraction, 365 of which were included in statistical analysis (Supplemental Table S2, <https://doi.org/10.3168/jds.2019-17726>). The number of proteins identified in the current trial is less than the published work by Yang et al. (2015), who used a similar MFGM isolation technique but used a non-gel-based iTRAQ downstream protocol. In the current research, only 6 proteins identified in the MFGM fraction were different ( $P=0.05$ ) in abundance across cows grazing GLH versus GLAH: acetyl-CoA carboxylase 1, aminopeptidase, mucin-16 isoform X4, volume regulated anion channel subunit LRRC8C, elongation factor 2, and oncostatin-M-specific receptor subunit  $\beta$  precursor. All of these proteins were higher in relative abundance within the MFGM-associated protein fraction from animals grazing GLAH compared with those grazing GLH (Table 2). Some of these proteins have known associations with mammary gland function and, in particular, milk fat synthesis. Although we did not measure indicators of milk fat synthesis shifts such as fatty acid profile or mammary gene expression as part of the experiment presented herein, milk fat (% and concentration) was higher in samples from cows grazing GLAH compared with samples from those grazing GLH, suggesting a shift in fatty acid metabolism within the mammary gland, and could include a combination of uptake, passage, and synthesis. Acetyl-CoA carboxylase is an important enzyme in mammary de novo fatty acid synthesis, and the coding region of the acetyl-CoA carboxylase gene (ACACA) has been proposed to be an important candidate gene to estimate milk fat content (Bionaz and Loo, 2008; Matsumoto et al., 2012). Acetyl-CoA carboxylase 1, mucin-16, and leucine-rich repeat-containing protein 8C (which was the predicted protein corresponding to accession A5PK13, which is now identified as volume regulated anion channel subunit LRRC8C) were all previously reported to be present at higher abundance in the proteome surrounding small milk fat globules compared with the proteome surrounding large globules (Lu et al., 2016). All cows used in this study were the Jersey breed; however, it is interesting to note that Jersey cattle have small milk fat globule sizes compared with some other breeds such as Holsteins (Fleming et al., 2017), and hence globule size-associated proteins may be more responsive in the Jersey breed of cows. Although the



literature outlining the importance of oncostatin-M receptor proteins in milk is limited, changes in milk fatty acid profile and milk fat synthesis in dairy cattle as a result of linseed or safflower oil supplementation did induce a shift in mammary mRNA expression of oncostatin-M receptor (Ibeagha-Awemu et al., 2016), suggesting at least some interaction between diet and this protein at the mammary level.

Apart from examination of the implications of pasture plant species on protein profile, this study examined the skim milk proteome alongside the MFGM-associated proteome and allowed for more extensive comparative analysis of the comprehensive proteome. In this experiment, a total of 68 proteins were characterized in the skim milk fraction and 365 proteins were characterized in the MFGM-associated protein fraction. Furthermore, of the proteins identified by LC-MS/MS analysis in the skim milk- and MFGM-associated protein fractions, 34 proteins were identified in both fractions (Table 3). Much of this overlap is likely a result of the inability of the methodology to purely isolate the 2 fractions from each other as well as the biological origins of the fractions themselves. For instance, cytosolic crescents trapped within a milk fat droplet would likely include many cellular proteins that would be more highly abundant in the skim milk fraction due to cell sloughing, whereas the milk skimming protocol would likely leave a small amount of fat residue in the sample as well as lysed MFGM fragments. Although it is important to be mindful of these limitations, overall trends of the overlapping proteins may still be worthwhile to consider. Two keratin proteins (A6ANZ7 and G3N0V2) that have AA sequences that are similar across both humans and cattle are within this list and could be a result of keratin shedding by the mammary gland (Bitman et al., 1991) or some human contamination, which is commonly observed across proteomic studies. Apart from keratin, the other 32 proteins highlight some potentially interesting biology. Within this list of proteins identified across the 2 proteomes, both of the skim milk proteins affected by diet were included, and patterns of these 2 proteins in the MFGM followed the same numeric trend across treatment, though not significant. However, none of the MFGM-associated proteins affected by diet were identified in the skim milk proteome, suggesting some deviation in functionality across the 2 proteomes despite an overall similar pattern in the functionality profiles as GO revealed through PANTHER (Figure 2).

Nutrient profile of the pasture could also be driving the higher relative abundance of these low-abundance proteins in the milk from cows grazing GLAH compared with those grazing GLH; however, the lack of uniform increase across the whole proteome suggests that perhaps biological function, rather than simply dietary nutrient density, may also be an important consideration. Although milk itself is not a connected tissue, and the parent mammary gland is a somewhat unique tissue in and of itself, use of GO analysis to characterize the identified milk proteomes can be a useful mechanism to gain a sense of the effect of treatment on the secreted milk protein profile in terms of the likely origin and function of the protein in the dam, or perhaps its bioactive potential post-consumption. Cellular process (GO term: 0009987) was the most annotated term for biological process across both the skim milk- and MFGM-associated protein fractions, being annotated to 22.7 and 40.9% of the proteins identified in the skim milk- and MFGM-associated protein fractions, respectively (Figure 2A). A recent milk proteomics review (Greenwood and Honan, 2019) included a small meta-analysis using several data sets to compare the skim

milk and MFGM proteomes and identified the majority of proteins from both proteomes as being involved in cellular process. Whereas the review reported similar annotation proportion for the skim milk proteome, with a range of 21.7 to 29.0% of identified proteins being annotated to cellular process in the data sets used, the review reported that cellular process annotation comprised only 24.2 to 28.2% of the MFGM-associated proteome, which is lower than that observed in the current trial. This could again be a result of breed or globule size or could be due to differences in methodology, which are noted by Greenwood and Honan (2019) as a likely source of variation across proteomic research. In the current trial, response to stimulus (GO term: 0050896), localization (GO term: 0051179), biological regulation (GO term: 0065007), and metabolic process (GO term: 0008152) preceded cellular process for both the skim milk- and MFGM-associated proteins. Catalytic activity (GO term: 0003824) and binding (GO term: 0005488) were the most annotated molecular function terms for the skim milk and MFGM proteins, accounting for a combined total of 48.5 and 61.6% in the skim fraction and MFGM fraction, respectively (Figure 2B). The GO classification of catalytic activity encompasses enzymes, whereas the classification of binding encompasses receptors and other proteins that possess a binding site. Given the highly metabolic nature of the mammary gland and its reliance on nutrient uptake from the blood, it is not surprising to identify a high proportion of these protein classes within the milk proteome, which includes proteins secreted through numerous routes. There were a few examples of divergence in GO terms for both proteomes. Additional molecular function terms that were annotated for MFGM proteins and not the skim proteins included structural molecule activity (GO term: 0005198), translation regulator activity (GO term: 0005198), and transporter activity (GO term: 0005215). Although the higher number of classifications identified in the MFGM is likely due to the greater number of proteins identified within that proteome, comparison of the profiles of the identified proteins across the 2 proteomes (Figure 2) highlights that the skim milk proteome contained a greater proportion of proteins associated with response to stimulus, biological regulation, immune system process, and reproduction compared with the MFGM. Research exploring the bioactive properties of immune-associated proteins in milk has been at the forefront of scientific progress in the field of milk proteomics because of milk's important contribution to neonatal immunity, and the research herein affirms that the skim milk portion of milk may contain the higher number of immune-associated proteins for further study, though potency cannot be elucidated from this study. An interactomics-based study from Zhang et al. (2016) highlighted changes throughout lactation in human and bovine milk samples in both the skim and MFGM fractions, and GO analysis similarly revealed a larger proportion of proteins associated with immune-related activity in the skim milk protein fraction in both human and bovine samples compared with the MFGM fraction. In contrast, under the biological process annotations, many proteins with cell-associated process classifications were identified in higher proportions in the MFGM-associated proteome.

As expected, the skim milk and MFGM-associated proteomes were different in their cellular component profiles. According to cellular component annotation of the skim milk protein fraction, 28.8% were annotated with the extracellular region (GO term: 0005576) followed by cell (13.6%; GO term: 0005623), membrane (6.1%; GO term: 0016020), and protein-containing complex (6.1%; GO term: 0032991; Figure 2C). In contrast, more than 50% of

the MFGM proteins were annotated as cell or organelle associated, which is consistent with the literature outlining the formation of the MFGM itself (Cavaletto et al., 2008). The MFGM cellular component annotation included 38.7% of proteins as being from the cell (GO term: 0005623), followed by 15.7% being organelle associated (GO term: 0043226) and 15.7% being part of a protein-containing complex (GO term: 0032991). The higher inclusion of these protein classes in the MFGM-associated proteome is likely also a result of its inclusion of the apical membrane acquired during secretion and is in line with previous observations (Greenwood and Honan, 2019). Additional cellular component terms that were annotated for the MFGM-associated protein fraction, but not the skim milk fraction, included the cell junction (GO term: 0030054) and supramolecular complex (GO term: 0099080). This could be a result of simply having a higher protein number identified using a more intensive fractionation methodology, as biologically it would seem likely to identify proteins of these classes in both the skim milk and MFGM fractions. Despite the differences observed in cellular component annotation between the skim milk and MFGM fractions, membrane proteins (GO term: 0016020) were identified to be present at similar proportions in both skim milk and MFGM fractions (6.1 and 9.2%, respectively), again highlighting the complexity of interpreting these unique yet complimentary proteomes.

## CONCLUSIONS

In this experiment, the inclusion of AFC in pasture affected 1.8% of the skim milk and MFGM proteomes, which may be related to the low proportion of AFC in the pasture or change in overall nutritional value of the feed. Of the proteins that were affected by diet, both of the proteins affected in the skim milk fraction are involved in immune function, whereas many of the 8 MFGM-associated proteins have roles in mammary cell function and milk fat synthesis or secretion. On a comparative level, bioinformatics examination of proteome associated with the skim milk fraction and MFGM fraction highlighted some potential differences in pre-secretory cellular localization of the proteins as well as known current roles of the proteins within the characterized proteomes. This trial and results reported herein provide support to mounting evidence that diet can, at least to some extent, alter the bovine milk proteome and that the different fractions of milk (namely, the skim- and MFGM-associated fractions) are unique in their proteome profiles.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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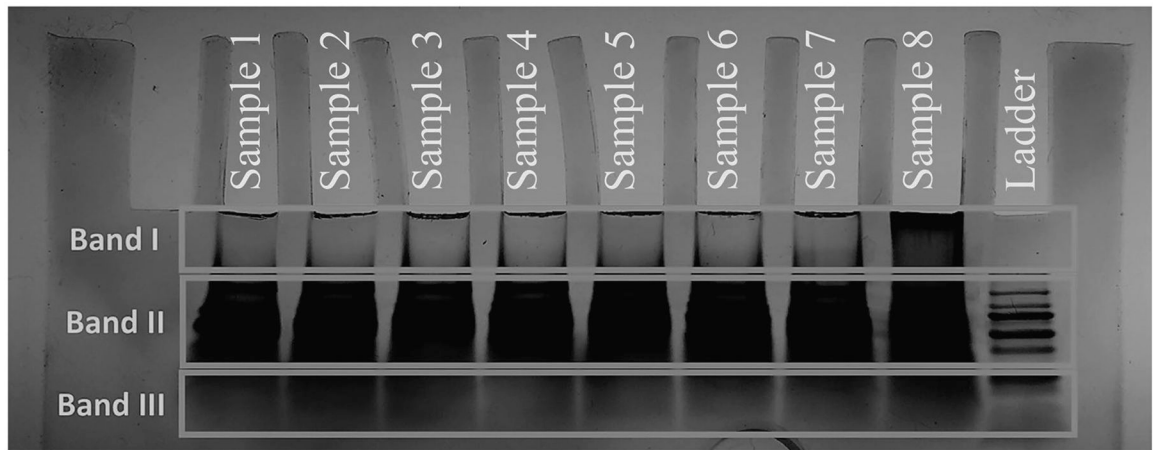
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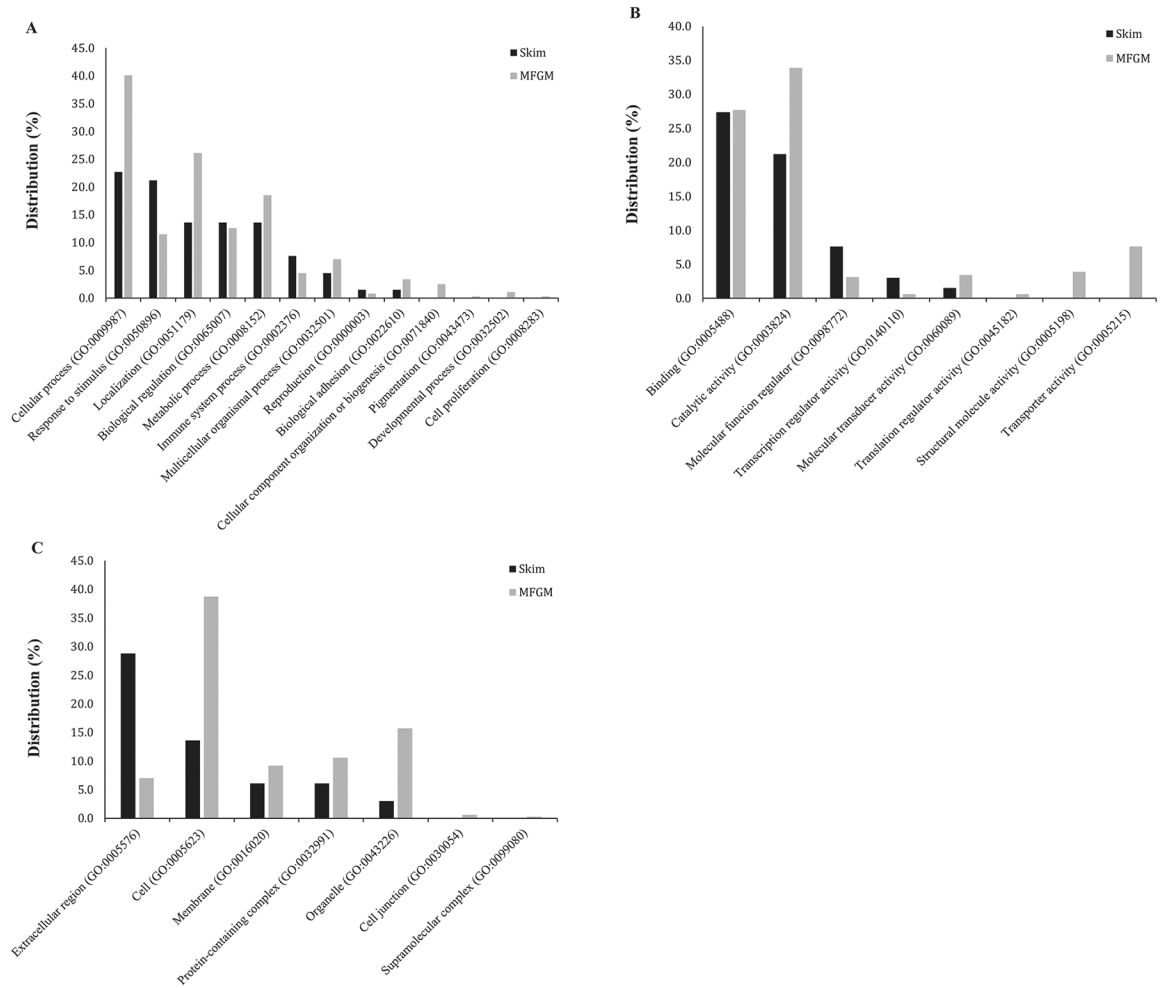
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**Figure 1.**

Gel image of samples (1 sample per lane) after protein separation by SDS-PAGE. After separation was complete, each lane was excised into 3 segments (depicted as bands I, II, and III), lanes were separated, and each segment from each lane was then analyzed separately by liquid chromatography–tandem MS.



**Figure 2.** The gene ontology (GO) annotations of the proteins identified by liquid chromatography–tandem MS analysis in both skim milk and milk fat globule membrane (MFGM) samples according to their (A) biological process, (B) molecular function, and (C) cell component.

Milk yield and components from cows that grazed either a grass–legume herbage (GLH) or pastures that included GLH plus annual forage crops (GLAH)

**Table 1.**

Item	Diet <sup>1</sup>		
	GLH	GLAH	P-value
Milk yield (kg/d)	17.31 ± 0.84	18.90 ± 0.84	0.20
ECM yield <sup>2</sup> (kg/d)	19.35 ± 1.00	22.87 ± 1.00	0.03
Milk solids yield <sup>3</sup> (kg/d)	2.16 ± 0.11	2.53 ± 0.11	0.03
Milk lactose (%)	4.79 ± 0.03	4.80 ± 0.03	0.77
Milk lactose yield (kg/d)	0.83 ± 0.04	0.91 ± 0.04	0.19
Milk fat (%)	4.29 ± 0.12	4.86 ± 0.12	0.01
Milk fat yield (kg/d)	0.74 ± 0.04	0.92 ± 0.04	0.02
Milk protein (%)	3.43 ± 0.08	3.72 ± 0.08	0.02
Milk protein yield (kg/d)	0.59 ± 0.03	0.70 ± 0.03	0.02
Milk protein (mg/mL of skim milk)			
κ-CN	7.37 ± 0.04	8.49 ± 0.04	0.06
α <sub>82</sub> -CN	1.50 ± 0.09	1.43 ± 0.09	0.56
α <sub>18</sub> -CN	12.17 ± 0.46	14.35 ± 0.46	0.01
β-CN	13.53 ± 0.32	14.43 ± 0.32	0.07
α-LA	1.00 ± 0.05	1.00 ± 0.05	1.00
β-LG variant B	1.97 ± 0.45	2.10 ± 0.45	0.85
β-LG variant A	2.90 ± 0.49	3.02 ± 0.49	0.86

<sup>1</sup>Least squares means expressed as value ± SE.

<sup>2</sup>Calculated as  $[12.82 \times \text{fat yield (kg/d)}] + [7.13 \times \text{protein yield (kg/d)}] + [0.323 \times \text{milk yield (kg/d)}]$ .

<sup>3</sup>Calculated as lactose yield (kg/d) + fat yield (kg/d) + protein yield (kg/d).

Proteins affected by diet as identified by liquid chromatography–tandem MS analysis in the skim milk fraction and milk fat globule membrane (MFGM)-associated protein fraction in cows that grazed either grass–legume herbage (GLH) or pastures that included GLH plus annual forage crops (GLAH)

**Table 2.**

Fraction	Accession	Protein name	Diet <sup>1</sup>		
			GLH	GLAH	P-value
Skim milk	P01888	β-2-Microglobulin	1.32 ± 0.14	1.91 ± 0.14	0.01
	P81265	Polymetric immunoglobulin receptor	1.35 ± 0.37	2.56 ± 0.37	0.04
MFGM	E1BGH6	Acetyl-CoA carboxylase 1	0.86 ± 0.09	1.21 ± 0.08	0.01
	E1BP91	Aminopeptidase	0.77 ± 0.09	1.13 ± 0.09	0.01
	G3MXR2	Mucin-16 isoform X4	0.84 ± 0.06	1.07 ± 0.06	0.03
	A5PK13	Volume-regulated anion channel subunit LRRC8C	0.95 ± 0.15	1.45 ± 0.14	0.03
	Q3SYU2	Elongation factor 2	0.90 ± 0.15	1.37 ± 0.14	0.04
	F1MGK8	Oncostatin-M-specific receptor subunit β precursor	0.99 ± 0.10	1.29 ± 0.09	0.05

<sup>1</sup>Least squares means expressed as abundance relative to the universal control ± SE.

**Table 3.**

The 34 common proteins identified by liquid chromatography–tandem MS analysis in both the skim milk- and milk fat globule membrane-associated protein fractions from cows that grazed either grass–legume herbage or pastures that included grass–legume herbage plus annual forage crops

Accession	Protein
A0A140T897	Serum albumin
A0A140T8A9	$\kappa$ -Casein
A0INP2	Secretoglobin family 1D member
A5PJE3	Fibrinogen $\alpha$ chain
A6QNL0	Monocyte differentiation antigen CD14
A6QNZ7	Keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris)
F1MUT3	Xanthine dehydrogenase/oxidase
F1MXX6	Lactadherin
F1NIN6	Perilipin
FIN726	Glycoprotein 2
G3MXXB5	Immunoglobulin IgA heavy chain constant region, partial
G3N0S9	Predicted: apolipoprotein R
G3N0V2	Keratin 1
G5E5I3	IgM heavy chain constant region, secretory form, partial
G5E5H7	Progesterone associated endometrial protein
G5E5T5	Immunoglobulin M heavy chain secretory form
P00711	$\alpha$ -Lactalbumin
P01888	$\beta$ -2-Microglobulin
P02662	$\alpha$ <sub>S1</sub> -Casein
P02663	$\alpha$ <sub>S2</sub> -Casein
P02666	$\beta$ -Casein
P08037	$\beta$ -1,4-Galactosyltransferase 1
P10790	Fatty acid-binding protein, heart
P11151	Lipoprotein lipase
P15497	Apolipoprotein A-I
P18892	Butyrophilin subfamily 1 member A1
P24627	Lactotransferrin

Accession	Protein
P26201	Platelet glycoprotein 4
P80025	Lactoperoxidase
P80195	Glycosylation-dependent cell adhesion molecule 1
P81265	Polymeric immunoglobulin receptor
Q28065	C4b-binding protein $\alpha$ chain
Q32PA1	CD59 molecule (CD59 blood group)
Q9MZX6	Fibroblast growth factor-binding protein 1

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