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Characterization of the bovine milk proteome in early-lactation Holstein and Jersey breeds of dairy cows

Rinske Tacoma^a, Julia Fields^b, David B. Ebenstein^a, Ying-Wai Lam^b, and Sabrina L. Greenwood^{a,*}

Sabrina L. Greenwood: Sabrina.Greenwood@uvm.edu

^aThe University of Vermont, Department of Animal & Veterinary Sciences, 570 Main Street, Burlington, VT 05405

^bThe University of Vermont, Vermont Genetics Network Proteomics Facility, 109 Carrigan Drive, Burlington, VT 05405

1. Introduction

Milk is a valuable, natural product that provides a matrix of essential nutrients, growth factors and immune protection to offspring. Within the last five years, there has been a dramatic increase in the amount of published research focused on characterizing the milk proteome within different milk fractions, particularly in human milk [1-6]. Traditionally, milk proteins are categorized into three major groups: caseins, whey proteins and milk fat globule membrane (MFGM) proteins [6, 7]. By using a wide range of fractionation techniques, whey proteins can be separated from caseins and further processed to allow extraction and identification of the low-abundance protein fraction in milk. Bioactive proteins and peptides contained in, or derived from, the whey fraction are involved in a wide range of physiological activities, including antioxidant activity, immuno-stimulating functions, anti-inflammatory effects, and protection against pathogen-induced intestinal inflammation [7-10]. Many of the milk bioactive proteins and peptides are also known to exhibit multifunctional physiological properties [11]. Thus, milk proteins are currently considered the most important source of bioactive peptides.

Cow's milk is an important nutrient for much of the human population, and studies have begun to characterize the bovine milk proteome, its bioactive profile, and the extent of cross-reactivity of bovine bioactive milk peptides on human biological function [12-14]. In bovine milk, the caseins (α_{S1} , α_{S2} , β and κ -casein) comprise approximately 80% of the total milk protein content, while whey proteins (primarily α -lactalbumin (α -LA), β -lactoglobulin (β -LG) and serum albumin) represent the remainder. However, these highly abundant whey and casein proteins are far outnumbered by low-abundance proteins within the whey fraction. Reindhardt et al. [15] identified over 700 low-abundant whey proteins in skim bovine milk using quantitative proteomic techniques, including many with known immunological functions [15]. Lactoferrin is an important low-abundant protein involved in immune system development and is present in both human and bovine milk [16]. Positive health benefits

*Corresponding Author: Tel: +1802 656 0145; Fax: +1802 656 8196.

from lactoferrin in human breast milk are well documented [7, 8, 17-19] and recent studies show similar responses when infants are fed formula supplemented with bovine lactoferrin. [9, 12, 13, 20, 21]. Osteopontin, another bioactive protein present in both human and bovine milk, is recognized for its involvement in intestinal and immunological development in infants [8, 22]. Despite there being only 63% amino acid similarity between bovine and human osteopontin, osteopontin in bovine milk exerts effects on human intestinal cell proliferation *in vitro* similar to osteopontin in human breast milk [8, 22, 23]. The cross-reactive nature of milk bioactive proteins provides opportunity to use bovine milk derived bioactive proteins as potential ingredients for health promoting foodstuffs and biopharmaceuticals.

As with human breast milk, many external and genetic factors influence the composition of bovine milk. A recent study characterized and compared the MFGM proteins within several different species including two bovine breeds, the Jersey and Holstein breeds of dairy cattle [24]. Using quantitative proteomic techniques, protein profiles were examined and principal component analysis scored the two breeds sharing similar proteomic patterns but also showing that each breed had distinctive MFGM proteins that were present at different concentrations. The Jersey MFGM contained a higher abundance of proteins with antimicrobial and angiogenic activities, whereas the Holstein MFGM contained proteins involved in immune system modulatory processes including antioxidant, anti-apoptotic, anticancer, and host cell protection activities [24]. Breed differences in alveolar dynamics [25], feed conversion efficiency [26, 27], susceptibility to heat stress [28, 29], and genetic variants existing for protein types [30] could have contributed to the observed differences in MFGM protein profile. However, additional factors known to affect milk composition in cattle, including diet, cow health, parity, environment, management practices and stage of lactation [31-33], could have also contributed to the observed breed differences. While genetics are estimated to contribute 55% of the variation observed in milk composition between breeds, the remaining 45% is explained by differing management factors [34]. Feeding different breeds of cows the same diet while being maintained in the same environment under the same management practices allows for a more direct comparison of true breed differences in the milk proteome. We hypothesize that when Holstein and Jersey breeds of dairy cattle are fed the same balanced diet and maintained under the same management and environmental conditions, there will be significant differences in the low abundance milk protein profile contained within the skim fraction of milk from the two breeds of dairy cows. The objective of this study is to characterize and differentiate the low abundance protein profile within the skim milk fraction produced by Holstein and Jersey dairy cows that are maintained under the same management practices and environmental conditions.

2. Materials and methods

2.1. Animals and diet

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont (Burlington, VT, USA). Six Jersey cows (80 ± 49 days in milk (DIM)) and six Holstein cows (75 ± 21 DIM) were housed in the same tie-stall barn at the

Paul R. Miller Research and Educational Center (University of Vermont, Burlington, VT). All cows had free access to water and offered the same diet *ad libitum* (Table 1) for 7 days. All cows were fed individually for the duration of the trial. The diet consisted of a base forage ration that was fed twice daily (0600 and 1500 h) and a grain-based top-dress, which was thoroughly mixed into the offered forage three times a day at 0330, 1100, and 1800 h. Feed refusals from each animal were removed and weighed each morning and samples were stored at -20°C until analysis. Refusal samples were subsequently dried at 65°C for 48 h to calculate daily dry matter intake for each animal. Additional fresh feed and grain samples were collected and composited across the 7-d collection period for wet chemistry analysis (DairyOne, Ithaca, NY, USA).

2.2. Measurements and sampling

Cows were milked twice daily at 0400 and 1600 h. Milk yield was recorded at each milking and milk samples were collected during the morning and afternoon milking throughout the 7-day experiment (Tru-Test WB Ezi-Test Meters, DHIA, Lancaster, PA, USA). Milk samples for general milk component analysis were collected and preserved with bronopol and natamycin (D & F Control Systems, Inc., Broad Spectrum Microtabs, Norwood, MA, USA) and stored at 4°C until commercial analysis (DHIA, Lancaster, PA, USA), which was performed within two days after collection. Milk subsamples collected for low abundance protein analysis were immediately frozen in a dry-ice ethanol bath after collection and stored at -80°C until further analysis. Additional subsamples were chilled on ice immediately after collection and centrifuged at $4,000 \times g$ for 10 min at 4°C within 2 h of collection. The fat layer was removed and the skimmed milk samples were stored at -20°C for high-abundance protein analysis.

Blood samples were collected by coccygeal venipuncture after milking (0800 and 1700 h) on day 0 and 7. Samples were collected into heparinized Vacutainers® (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and stored on ice until centrifugation at $3,000 \times g$ for 15 min at 4°C for plasma collection. Plasma was transferred into polypropylene tubes and frozen at -20°C until analysis. Commercially available kits were used to analyze the plasma concentrations of β -hydroxybutyrate (BHBA; Sigma, Saint Louis, MO, USA), urea nitrogen (PUN; Teco Diagnostics, Anaheim, CA, USA), glucose (Sigma, Saint Louis, MO, USA), and non-esterified fatty acids (NEFA; ZenBio, Inc., Research Triangle Park, NC, USA). Samples were analyzed according to manufacturer's instructions and all coefficients of variance were < 5%.

2.3. Analysis of the high-abundance milk proteins

The skimmed milk samples were thawed overnight at 4°C for analysis of high abundance proteins by HPLC using a method adapted from Bordin et al. [35]. Thawed samples were vortexed to mix thoroughly and 500 μ l of milk was transferred to a flint glass culture tube. An equal volume of reducing buffer (6.0 M guanidine hydrochloride, 5.0 mM trisodium citrate dehydrate, 20.0 mM DTT) was added to the milk in each tube. The reducing buffer was prepared daily. The milk/buffer mixture was allowed to react at room temperature for 1 h and then transferred to a syringe and filtered through a 0.45 μ m regenerated cellulose membrane syringe filter (Sartorius Stedim Biotech GmbH, 37070, Goettingen, Germany)

into an HPLC autosampler vial. For each analysis, 4 μ l of sample was injected into the HPLC (Shimadzu Corporation, Kyoto, Japan) for separation. The separations were performed on a C₄ reversed-phase microbore analytical column (150 $\times$ 2.1mm, 300 Å pore diameter and 5 μ m particle size, Yydac 214MS, Grace Davison, MD, USA). A two-component solvent system was used as the mobile phase for separations. Eluent A was composed of 10% (v/v) acetonitrile and 0.1% trifluoroacetic acid in ultrapure water and eluent B was composed of 90% acetonitrile and 0.1% trifluoroacetic acid in ultrapure water. The following solvent gradient program was used during the separations: linear gradient from 26.5 to 28.6% eluent B in 7 min (0.30% B min⁻¹), then from 28.6 to 30.6% B in 10 min (0.20% B min⁻¹), and from 30.6 to 33.5% B in 5.8 min (0.50% min⁻¹), followed by an isocratic elution at 33.5% B for 10 min, an increase from 33.5 to 36.1% B in 5.2 min (0.50% min⁻¹), an isocratic elution at 36.1% B for 10 min, an increase from 36.1 to 37% B in 5 min (0.18% min⁻¹), an isocratic elution at 37% B for 10 min, and a final increase to 41% B in 13 min (0.30% min⁻¹), at a flow rate of 0.25 ml min⁻¹. The column was maintained at 40°C and the autosampler was held at 15°C. A detection wavelength of 214 nm was used and chromatographs were integrated using LCSolution software from Shimadzu (Kyoto, Japan).

2.4. Enrichment of the low abundance proteins

Milk samples collected for low abundance protein analysis were thawed overnight at 4°C. To obtain a representative sample for each animal, aliquots were composited within animal across the week according to milk yield at each milking. A mammalian protease inhibitor cocktail (Protease Inhibitor Cocktail, Sigma, Milwaukee, WI, USA) was added at 0.24 ml inhibitor per gram of protein in the milk to a 50 ml aliquot sample which was then centrifuged at 4,000 $\times g$ for 15 min at 4°C. The cream layer was then removed and skimmed samples were depleted of casein using a previously described method [36]. Briefly, addition of CaCl₂ (60 mM) was mixed into the skimmed sample and the pH was adjusted to 4.3 using 30% acetic acid (Fisher Scientific, Fair Lawn, NJ, USA). Samples were then centrifuged at 189,000 $\times g$ at 4°C for 70 min and the supernatant was collected and stored at -80°C [1, 36]. Samples were lyophilized and reconstituted to 500 mg whey powder in 1 ml PBS. The samples were analyzed for their protein content using the bicinchoninic acid assay kit (Pierce, Rockford, IL, USA), using bovine serum albumin as the standard. The low-abundance minor proteins were enriched by the ProteoMiner Kit (BioRad Laboratories, Hercules, CA, USA) and 32 mg of whey protein was added to 100 μ l of ProteoMiner beads. The whey samples were gently shaken with individual ProteoMiner columns for 2 h at room temperature and columns were washed thoroughly using HPLC grade water to remove excess proteins [37, 38]. The low abundance proteins were eluted off the beads by addition of 20 μ l 4x Laemmli sample buffer (8% SDS, 40% glycerol, 250 mM Tris, pH 6.8, 400 mM DTT with trace amount of bromophenol blue). The mixture of the protein solution with the beads was heated at 95°C for 10 min and the supernatant was subjected to SDS-PAGE on a precast 8-16% polyacrylamide gel to separate proteins (BioRad, Hercules, CA, USA). Tris-glycine (pH 8.3) was used as the running buffer containing 0.1% SDS. Electrophoresis was performed for approximately 35 min at 200 V until the protein bands reached the bottom of the gel. Staining was performed using Coomassie Brilliant Blue (BioRad, Hercules, CA, USA) overnight. To visualize the protein bands, destaining with a solution of 10% acetic acid, 40% methanol, and 50% water was performed until the background was clear. The

SDS-PAGE stained gels were scanned with a Gel Doc XR+ system (BioRad, Hercules, CA, USA).

2.5. In-gel Digestion

Gel lanes were cut into 15 slices along the migration path and cut into small 1 mm cubes. The gel pieces were destained with 50 mM ammonium bicarbonate in 50% acetonitrile. Protein samples were reduced by 10 mM DTT at 55°C for 1 h and alkylated with 55 mM iodoacetamide in the dark at room temperature for 45 min. The gel pieces were then washed/rehydrated and dehydrated twice alternately with 100 mM ammonium bicarbonate and 100% acetonitrile. The gel pieces were dried in a SpeedVac (Scientific Support, Hayward, CA, USA) and trypsin digestion was carried out for 18 h at 37°C with 7 ng μl^{-1} of trypsin. The tryptic peptides were acidified with 50 μl of 5% formic acid to stop the reaction, extracted, and dried in a SpeedVac (Scientific Support, Hayward, CA, USA). The dried peptide samples were re-suspended in 10 μl of a solution of 2.5% acetonitrile and 2.5% formic acid in water for analysis by LC-MS.

2.6 Protein Identification by nano-scale LC/MS

LC-MS based protein identification was performed on a linear ion trap (LTQ)-Orbitrap Discovery mass spectrometer coupled to a Surveyor MS Pump Plus (Thermo Fisher Scientific, Waltham, MA, USA). Samples (5 μl) were loaded onto a 100 $\mu\text{m} \times 120$ mm capillary column packed with MAGIC C18 (5 μm particle size, 20 nm pore size, Michrom Bioresources, CA, USA) at a flow rate of 500 nl min^{-1} . Peptides were separated using a gradient of 5-35% acetonitrile/ 0.1% formic acid over 98 min followed by 35-100% acetonitrile/ 0.1% formic acid in 1 min and then 100% acetonitrile for 10 min, followed by an immediate return to 2.5% $\text{CH}_3\text{CN}/0.1\%$ FA and a hold at $\text{CH}_3\text{CN}/0.1\%$ FA. Peptides were introduced into the linear ion trap via a nanospray ionization source and a laser pulled ~ 3 μm orifice with a spray voltage of 1.8 kV. Mass spectrometry data was acquired in a data-dependent “Top 10” acquisition mode with lock mass function activated (m/z 371.1012216635), in which a Orbitrap survey scan from m/z 360-1600 at 30,000 (FWHM) resolution was paralleled by 10 collision-induced dissociation (CID) MS/MS scans of the most abundant ions in the LTQ. MS/MS scans were acquired with the following parameters: isolation width: 2 m/z , normalized collision energy: 35%, Activation Q: 0.250 and activation time = 30 ms. Review mode for FTMS master scans was enabled. Dynamic exclusion was enabled (repeat count: 2; repeat duration: 30 sec; exclusion list size: 180; exclusion duration: 60 sec). The minimum threshold was 500. Singly charged ions were excluded for MS/MS. Product ion spectra were searched using the SEQUEST search engine on Proteome Discoverer 1.4 (Thermo Fisher Scientific, Waltham, MA, USA) against a curated Bovine Uniprot (-*Bos taurus* database (24,206 entries) downloaded July 9, 2014-) with sequences in forward and reverse orientations. The 15 raw files from each Holstein (6 samples) and the 15 raw files from each Jersey (6 samples) cows were searched as one contiguous input file and a single result file was generated for each (12 results files in total). Search Parameters were as follows: full trypsin enzymatic activity, two missed cleavages, and peptides between the MW of 350-5000; mass tolerance at 20 ppm for precursor ions and 0.8 Da for fragment ions, dynamic modifications on methionine (+15.9949 Da: oxidation) (4 maximum dynamic modifications allowed per peptide); and static modification on cysteine (+57.0215 Da:

carbamidomethylation). The result files were then further analyzed by Scaffold 4.3 (Proteome Software, Portland, OR, USA) to compare the unique peptide counts and to identify gene ontology functions of the identified proteins. Cross-correlation (Xcorr) significance filters were applied to limit the false positive rates to less than 1% in both data sets. The Xcorr values were as follows: (+1): 1.5, (+2): 2.2, (+3): 2.8, (+4): 3.5. Other filters applied were a minimum peptide cutoff of 2 as well as DeltaCN >0.1. Ultimately, the confidence parameters resulted in less than 1% false discovery.

2.6. Statistical analysis

Statistical analyses on daily dry matter intake (DMI), plasma parameters, and milk composition data were performed using a linear mixed model of repeated measures ANOVA. The analyses were carried out with SAS software (9.4) (SAS Institute, Cary, NC, USA) and preliminary data screening revealed that all dependent variables were normally distributed. Data were analyzed by MIXED procedure of SAS with breed and day as fixed effects. All data were presented as least square means (LSM) $\pm$ standard error of means (SEM) and were considered to be significantly different at $P < 0.05$. The peptide count data was analyzed in SAS (9.4) using an independent sample t-test. The heat maps were generated by cluster analysis using Ward's method in JMP (Pro 10) (SAS Institute, Cary, NC, USA).

3. Results

3.1 Diet and dry matter intake

The chemical composition of the diet is listed in Table 1. Daily DMI was significantly different between the two dairy breeds (Table 2; $p = 0.011$) where the Holstein cows consumed an average of 21.8 kg DM cow⁻¹ day⁻¹ and the Jersey cows consumed on average 17.3 kg DM cow⁻¹ day⁻¹.

3.2 Plasma

Blood plasma samples collected on day 0 and day 7 were used to monitor the energy and nitrogen status from each animal. Such biological tests are commonly used in the dairy industry to investigate the general health of animals and provide a diagnostic tool to identify problems in nutritional management, diet formulation or disease incidence [39, 40]. Results from the blood data confirm that all twelve animals were in positive energy balance as highlighted by the normal ranges observed in the plasma glucose, NEFA and BHBA concentrations measurements of each cow (Table 3). Plasma NEFA, typically elevated in circumstances of under nutrition and adipose tissue mobilization, was not significantly different between the Holstein (0.17 mM) and Jersey (0.23 mM) breeds and was lower than the recommended maximum concentration threshold value of 0.33 mM in early lactation cows [41], indicating adequate dietary energy intake amongst both breeds of cows. Plasma BHBA was also within the normal physiological range for both Holsteins (0.31 mM) and Jersey (0.34 mM) dairy cows [41]. The plasma glucose concentrations in both the Holstein (3.60 mM) and Jersey (3.59 mM) breeds indicated a high level of gluconeogenesis in both breeds, in agreement with the predicted rumen volatile fatty acid concentrations from our diet [42]. PUN was measured to assess nitrogen status within each animal. Mean PUN

concentrations in Holstein cows (1.19 ± 0.03 mM) and Jersey cows (1.25 ± 0.03 mM) were above the normal PUN range of 0.66-0.88 mM [41]; however, the mean PUN concentrations and variation measured in the present study were similar to those reported previously in cows on high protein diets [43, 44] and was expected.

3.3 Milk composition and yield

Milk yield, as well as concentrations and yield of the individual major milk constituents, were significantly different between the two dairy breeds (Table 2). Holstein cows produced 49.9 kg milk per day containing 3.24% milk fat and 2.54% milk protein. The Jersey breed produced significantly less milk (34.6 kg milk per day) but had significantly higher percentages of milk fat (4.28%) and milk protein (3.24%). Despite milk from Jersey cows having higher concentrations of milk fat and protein in milk, Holstein cows produced significantly more total milk per day and more kg of milk fat and protein per day (Table 2). Optimum milk urea nitrogen (MUN) concentrations are typically between 10-14 mg dl⁻¹. The MUN concentrations from Holstein (17.13 mg dl⁻¹) and Jersey (15.45 mg dl⁻¹) cattle in this trial were slightly above the optimum MUN concentrations [45]. These higher MUN concentrations are tightly correlated to PUN concentrations, and both support the observed high dietary protein and nitrogen intake.

3.3.1 High-Abundance Milk Proteins—Identification and quantification of high-abundance milk proteins was achieved using HPLC. The results were generally expected, with significant differences existing between the concentrations of casein and whey proteins produced by the two breeds as previously observed (Table 4) [46]. Total casein and whey concentrations were significantly higher in Jersey milk (58.0 mg ml⁻¹ and 5.3 mg ml⁻¹, respectively) compared to Holstein milk (46.4 mg ml⁻¹ and 4.0 mg ml⁻¹, respectively). The most abundant casein protein, total α -casein, was found at significantly higher concentrations in Jersey milk (17.6 mg ml⁻¹) as compared to Holstein milk (14.0 mg ml⁻¹). Similarly, the most abundant whey protein, β -LG variant A, was present at much higher concentrations in Jersey milk (3.3 mg ml⁻¹) compared to milk from Holstein cows (1.8 mg ml⁻¹). β -LG variant B was present at similar concentrations in milk from the Holstein (1.3 mg ml⁻¹) and Jersey (0.96 mg ml⁻¹) breeds. This is most likely explained by high variation existing between individuals.

3.3.2 Low-Abundance Milk Whey Proteins—A total of 935 low-abundant proteins were identified in the skim milk fraction of the two breeds, including 43 proteins that were present at significantly different abundance (Fig. 1, Fig. 2, Table 5). Bioactive proteins that were found at significantly different abundance between the breeds in the skim milk samples include lactotransferrin ($P=0.003$), a number of complement proteins (complement C2, $P=0.0001$; complement C1, $P=0.007$; complement component 1, $P=0.015$) and chitinase domain-containing protein 1 ($P=0.045$; Tables 5 and 6). However, the majority of the low abundance proteins within the skim milk fraction were found at relatively similar abundances between the two breeds (Fig. 1). By using a range of extensive fractionation techniques including casein removal and ProteoMiner treatment, the highly abundant proteins were removed to allow for enrichment of the low abundance proteins within the skim milk fraction for subsequent proteomics analysis. Supplemental Tables 1 & 2 contain the

complete list of the low-abundance proteins identified in this study as well as total number of distinct sequenced peptides and gene ontology functions for each protein. To ensure statistical validity, six animals were included in each group. A homoscedastic t-test was performed on the peptide count data of each protein for each breed generating P-values to compare abundance of the proteins between the two breeds. The relative abundance of a select few proteins was demonstrated using the SIEVE software, which plot the ion elution profile on the chromatographic time scale (extracted ion chromatograms) of the target identified peptides (Supplemental Fig. 1.). Identification of gene ontology functions of the full protein profile highlights the similarities of the different breed proteomes (Supplemental Table 1), while identification of the ontological functions of the differentially expressed proteins (Table 5; Fig. 2) highlighted the lack of categorical specificity of these proteins.

4. Discussion

The aim of the present study was to investigate, characterize and compare the bovine milk proteome between Holstein and Jersey dairy cows. Over the past 30 years there has been growing interest to unravel the dynamic framework of the milk proteome, and there has also been development in the techniques used to fractionate and identify these proteins (Fig. 3.). Murakami et al. [47] used ultracentrifugation and immunoabsorption techniques on human colostrum and mature milk samples to remove the high abundance proteins. These researchers employed 2-DE protein separation and silver staining techniques, identifying 28 proteins in human colostrum and milk [47]. In 2002, Yamada et al. [48] identified 29 low abundance proteins in bovine milk samples using immunoabsorption techniques and 2-DE to fractionate and separate the low abundance proteins, which were subsequently identified using microsequencing and mass spectrometry for protein identification [48]. Improvements made in LC-MS based proteomic technologies and high abundance protein precipitation techniques has since enabled the expansion of milk proteome characterization, resulting in the isolation and identification of over 150 low abundance milk proteins in human colostrum [49]. Further method development ensued, where pasteurized bovine skim milk was subjected to ultracentrifugation and the first use of peptide library treatment for protein separation was reported [50]. In this research, ProteoMiner columns were used to deplete the pasteurized milk samples from high abundance proteins followed by SDS-PAGE for low abundance protein separation. Enrichment of the low abundance proteins resulted in the identification of 149 low abundance proteins, including 100 proteins that had not previously been reported in proteomic studies [50]. The ProteoMiner technique had also been used to examine the human milk protein profile over a twelve-month lactation period, resulting in identification of 115 low abundance whey proteins ; however, ultracentrifugation was not performed in this method which might explain the lower number of protein identifications [2]. A combination of ProteoMiner depletion, electrophoretic techniques, and isobaric tags for relative and absolute quantitation (iTRAQ) techniques were then used to identify over 400 low abundance whey proteins within skimmed human milk [51]. Proteomic methods continued to be developed by Gao et al. [52] where ion-exchange and SDS-PAGE based protein fractionation methods were used to extend the human milk proteome to 976 low abundance proteins [52]. Permutations of these methodologies were utilized to examine changes in the bovine milk MFGM, whey and exosome proteome induced by mammary

bacterial infection. Over 740 low abundance whey proteins were identified using casein precipitation, ultracentrifugation, immunoglobulin depletion, acetone precipitation and iTRAQ labeling for protein identification and quantification [15]. In the research described herein, we used a combination of fractionation techniques followed by SDS-PAGE and mass spectrometry sequencing to comprehensively elucidate and compare the differential milk proteome produced by two breeds. Peptide counting approaches was applied to obtain statistically significant semi-quantitative data, rather than using isotopic tagging techniques, which can be used in future studies involving within-breed accurate quantification. We successfully identified a total of 935 low abundance whey proteins in bovine skim milk from two dairy cattle breeds (Fig.1). This allowed us to explore the diversity of the bovine milk proteome from two prominent North American dairy breeds maintained under the same diet, environment and management conditions in order to better assess true breed differences.

In agreement with past literature, total milk protein including all casein and whey fractions were all found at significantly different concentrations between the two breeds (Table 4) [53]. A significant proportion of the bioactive peptides found in human milk have been identified in bovine skim milk in the current study. Some key bioactive proteins identified in our study that are known to exert beneficial effects on human health include lactotransferrin, osteopontin, lactoperoxidase, and cathelicidin [8, 12, 22, 54, 55]. However, contrary to our hypothesis, there were very few differences in the low abundance protein profile between breeds (Fig. 1). A total of 43 identified proteins were found at difference abundances between breeds, 81% of which were higher in samples from Jersey cattle compared to Holstein cattle (Fig.2). Complements C1 and C2, ectonucleotide pyrophosphatase, and chitinase domain-containing protein 1 are described as having functions involved in the immune response and were all identified to exist at higher abundance in samples from Jersey cattle compared to Holstein cattle; however, whether they can elicit bioactivity upon consumption is yet unknown [24, 56, 57]. Of the 8 proteins identified to be higher in Holstein cattle, the only one thus far known to possess bioactive properties was lactotransferrin, which is a well characterized multifunctional bioactive protein involved in many biological functions [58-60]. Osteopontin, lactoperoxidase, and growth factors including insulin-like growth factor (IGF) and transforming growth factor- β are all important bioactive proteins known to affect human health [8, 9], and these were all found to be at similar abundances in both breeds of cattle (Supplementary table 1). Overall, these results suggest that the bovine milk proteome, at least in skim milk fraction, is similar across breeds. This is valuable information, as it allows for more direct comparison between research being performed using different breeds of dairy cattle, and can allow for general extrapolation and application of results seen in one breed to another. However, the knowledge this research also highlights is the need for caution of cross-breed comparisons, particularly if focusing on the select few proteins that were found to be differentially expressed between the two breeds in the current study.

5. Conclusion

The current research compares the skim milk proteome produced by the two dominant dairy cattle breeds in the USA and provides insight into what protein profiles these different breeds produce when maintained under the same environmental and nutritional conditions.

Elucidation of the protein profile was accomplished using a combination of enrichment and fractionation techniques, followed by mass spectrometry-based proteomic analysis. The Holstein and Jersey proteomes were assessed using a peptide counting approach, while bioinformatic tools such as gene ontology were used to understand biological functions in which these proteins are involved. Our findings expand the current knowledge on low abundance proteins present in bovine milk and offers insight for future research exploring the bovine milk proteome, providing an analytical platform for future use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Investigated the milk protein profile produced by Holstein and Jersey dairy cows
- High similarity in the low abundance protein profile in skim milk fraction produced by both breeds of dairy cattle
- Expanded the current knowledge on low abundance proteins present in bovine milk
- Research offers insight for future research exploring the bovine milk proteome

Fig. 1.

Heat map presentation of peptide counting data of the 935 proteins identified. Cluster analysis was performed in JMP (Pro 10) using the ward method where the differentially expressed low abundant proteins were assembled into their GO functional groups. Clustering was based on peptide counts in Holstein and Jersey cow skimmed milk. Bar color represents peptide count data where dark red represents higher expression and blue color represents lower expression.

Fig. 2.

Hierarchical clustering of the 43 significantly differentially expressed protein within bovine skim milk between Holstein and Jersey cattle. Analysis was performed in JMP (Pro 10) using multivariate clustering. Bar color represents peptide count data where dark red represents higher expression and blue color represents lower expression.

Fig. 3.

Summary of methods and results from proteomic studies analyzing the low abundant whey protein profile in milk.

Table 1
Ingredient and chemical composition of diets

	Diet
Ingredient	
Corn silage	36.6
Haylage	18.3
Soybean meal	7.4
Canola meal solvent	4.9
Citrus pulp dry	8.5
Amino max	7.3
Corn grain ground fine	10.8
Vitamin-mineral mix ^a	5.9
Nutrient composition	
DM ^b (%)	59.6
NDF ^c (% of DM)	35.7
CP ^d (% of DM)	18.7
NFC ^e (% of DM)	34.9

^aVitamin-mineral mix contained (DM basis): 5.5% PGI amino enhancer, 2.3% Sodium Sesquinate , 2.6% Calcium carbonate, 1.3% salt, 1.4% PGI vitamin premix, 0.7% Magnesium, 0.05% Zinpro 40, 0.02% Rumensin, 0.31% Diamune trace mineral.

^bDM: dry matter.

^cNDF: neutral detergent fiber.

^dCP: crude protein.

^eNFC: non-fiber carbohydrate.

Table 2
Daily dry matter intake (DMI), milk yield and milk components by Holstein and Jersey dairy cows

	Breed		SE	P value
	Holstein	Jersey		
DMI (kg day ⁻¹)	21.78	17.34	1.02	0.0119
Milk yield (kg day ⁻¹)	49.90	34.61	1.45	<0.0001
Milk component yields (kg day ⁻¹)				
Fat	1.56	1.48	0.04	ns
Protein	1.24	1.11	0.03	0.0068
Milk components (%)				
Fat	3.24	4.28	0.12	<.0001
Protein	2.54	3.24	0.12	0.0021
Somatic cell count (×1000)	54.31	139.05	57.02	ns
Milk urea nitrogen (mg dl ⁻¹)	15.45	17.13	0.86	ns

Table 3
Plasma metabolite concentrations of Holstein and Jersey dairy cows

	Breed		SE	P value
	Holstein	Jersey		
Glucose (mM)	3.60	3.59	0.134	ns
BHBA ^a (mM)	0.31	0.34	0.034	ns
PUN ^b (mM)	1.19	1.25	0.033	ns
NEFA ^c (mM)	0.17	0.23	0.039	ns

^aBHBA: β -hydroxybutyrate.

^bPUN: Plasma urea nitrogen.

^cNEFA: Non-esterified fatty acid.

Table 4
High abundance milk protein concentration from Holstein and Jersey dairy cows

	Breed		SE	P value
	Holstein	Jersey		
Casein (mg ml ⁻¹)				
β-Casein	13.40	15.91	0.36	<0.001
κ-Casein	4.93	6.82	0.19	<0.001
Total α-Casein	13.94	17.60	0.44	<0.001
α-s1	12.72	16.11	0.40	<0.001
α-s2	1.30	1.57	0.67	0.015
Total Caseins	46.39	58.03	1.39	<0.001
Whey (mg ml ⁻¹)				
α-Lactalbumin (α-LA)	0.93	1.05	0.02	0.0004
β-Lactoglobulin variant A (β- LGA)	1.81	3.31	0.29	0.002
β-Lactoglobulin variant B (β- LGB)	1.28	0.96	0.16	ns
Total α-LA, β-LGA, β-LGB	4.02	5.32	0.16	<0.001

Gene Ontology functions associated with the low abundant proteins present at significantly different peptide counts in bovine milk between Holstein and Jersey dairy cattle breeds

Table 5

	Accession Number	Molecular Weight	biological regulation	cellular process	developmental process	immune system process	metabolic process	multi-organism process	response to stimulus
Complement C2	CO2_BOVIN	83 kDa	complement activation, classical pathway	complement activation, classical pathway	complement activation, classical pathway	antibacterial humoral response	antibacterial humoral response	antifungal humoral response	complement activation, classical pathway
Nidogen-1 precursor	F1MWN3_BOVIN	136 kDa							
Glucuronosyltransferase 1	Q3SZB0_BOVIN	37 kDa							
Embryo-specific Fibronectin 1 transcript variant	B8YS99_BOVIN	262 kDa	regulation of cell shape	cell adhesion	Angiogenesis			wound healing	
Fibronectin	F1NC_BOVIN	272 kDa	regulation of cell shape	cell adhesion	Angiogenesis			wound healing	
Leucine-rich alpha-2-glycoprotein 1	Q2KIF2_BOVIN	38 kDa							
Lactotransferrin	TRFL_BOVIN	78 kDa	positive regulation of NF-kappaB transcription factor activity	cellular iron ion homeostasis	bone morphogenesis	antibacterial humoral response		antibacterial humoral response	
Stromal cell-derived factor 2-like protein 1	SDF2L_BOVIN	24 kDa							
Fructose-1,6-bisphosphatase 1	F16P1_BOVIN	37 kDa	negative regulation of cell growth	protein homotrimerization			gluconeogenesis		
Vitamin D-binding protein O	VTDB_BOVIN	53 kDa							
Complement C1s subcomponent	C1S_BOVIN(+1)	77 kDa	complement activation, classical pathway	complement activation, classical pathway	complement activation, classical pathway	complement activation, classical pathway	complement activation, classical pathway	complement activation, classical pathway	
Protein kinase C-binding protein NELL2	NELL2_BOVIN	91 kDa							
Thrombospondin-4	TSBP4_BOVIN	106 kDa	positive regulation of cell division	myoblast migration	myoblast migration			behavioral response to pain	
Plectin-B2 precursor	G3X755_BOVIN	205 kDa							
Alanin precursor	G3MYZ3_BOVIN	70 kDa							
Collectin-43	CL43_BOVIN	34 kDa							
Cell division control protein 42 homolog	CDC42_BOVIN	21 kDa	regulation of filopodium formation	small GTPase mediated signal transduction	nervous system development				
Hypoxia up-regulated protein 1 isoform X1	E1B748_BOVIN	112 kDa							
Aspartate aminotransferase	AAATC_BOVIN	46 kDa	fatty acid homeostasis	oxaloacetate metabolic process			glutamate catabolic process to 2-oxoglutarate		cellular response to insulin stimulus
Vitamin D-binding protein	F1NSM2_BOVIN	53 kDa							
Complement component 1, r subcomponent	ASD9E9_BOVIN	80 kDa							
Suppressor of tumorigenicity 14 protein homolog	STI14_BOVIN	95 kDa		keratinocyte differentiation	keratinocyte differentiation				

	Accession Number	Molecular Weight	biological regulation	cellular process	developmental process	immune system process	metabolic process	multi-organism process	response to stimulus
Mamman-binding lectin serine protease 1	F1MV59_BOVIN	81 kDa							
Ectonucleotide pyrophosphatase	ENPP2_BOVIN	102 kDa	regulation of angiogenesis	phosphatidylethanolamine catabolic process		immune response	phosphatidylethanolamine catabolic process		chemotaxis
Alpha-mannosidase 2x	E1BGJ4_BOVIN	130 kDa							
SLC27A6 protein	A4QQD5_BOVIN	72 kDa							
Alpha-2-macroglobulin	F1MI18_BOVIN	166 kDa	negative regulation of complement activation, lectin pathway						
Transmembrane protein 132A	F1MEA1_BOVIN	111 kDa	signal transduction	signal transduction					response to cytokine stimulus
Integrin alpha 9 precursor	E1BFQ6_BOVIN	119 kDa	integrin-mediated signaling pathway	cell adhesion	blood vessel development			interspecies interaction between organisms	
IGL6 protein	F1MCF8_BOVIN	25 kDa							
Transaldolase	TALDO_BOVIN	38 kDa		pentose-phosphate shunt			pentose-phosphate shunt		
Aldehyde oxidase isoform X5	E1BEX5_BOVIN	149 kDa					oxidation reduction		
Sorpin B8	SPB8_BOVIN	42 kDa	regulation of proteolysis						
Malate dehydrogenase	MDHM_BOVIN	36 kDa		internal protein amino acid acetylation			tricarboxylic acid cycle		
Coagulation factor X	FA10_BOVIN	55 kDa	positive regulation of protein kinase B signaling cascade						blood coagulation
Cartilage oligomeric matrix protein	COMP_BOVIN	82 kDa		cell adhesion	apoptosis				
Chitinase domain-containing protein 1	CHID1_BOVIN	45 kDa		chitin catabolic process		immune immune response	chitin catabolic process		inflammatory response
SERPINA11 protein	A3PK77_BOVIN	47 kDa							
complement factor H-related 1-like isoform 2	F1MW79_BOVIN	31 kDa							
Hypoxanthine-guanine phosphoribosyltransferase	HPRT_BOVIN	24 kDa	positive regulation of dephamine metabolic process	protein homodimerization			GMP catabolic process		
hypoxanthine-guanine phosphoribosyltransferase isoform X2	GANTU_BOVIN	25 kDa							
Transaldolase	GAESC8_BOVIN	38 kDa		pentose-phosphate shunt			pentose-phosphate shunt		
Complement component 1, r subcomponent	Q3SYT3_BOVIN	45 kDa							

Table 6
Peptide count data of the low abundant proteins present at significantly different abundance in bovine milk between Holstein and Jersey dairy cattle breeds

Accession Number	J1	J2	J3	J4	J5	J6	Av. J	H1	H2	H3	H4	H5	H6	Av. H	p-value
CO2_BOVIN	13	13	16	15	10	10	12.8	4	8	6	7	4	4	5.5	0.000
F1MWN3_BOVIN	15	10	9	11	14	8	11.2	5	9	2	6	4	3	4.8	0.002
Q3SZB0_BOVIN	4	3	3	2	2	1	2.5	1	1	0	0	1	0	0.5	0.002
B8Y9S9_BOVIN	63	58	59	51	48	42	53.5	40	38	41	39	30	42	38.3	0.002
FINC_BOVIN	62	57	58	50	47	41	52.5	39	38	41	39	30	41	38.0	0.003
Q2KIF2_BOVIN	3	4	2	4	3	4	3.3	5	5	5	5	4	6	5.0	0.003
TRFL_BOVIN	32	37	46	37	26	30	34.7	42	46	47	46	49	50	46.7	0.003
SDF2L_BOVIN	0	0	0		0		0.0	2	1	1				1.3	0.005
F16P1_BOVIN	5	4	3	5	4	5	4.3	2	4	0	1	0	3	1.7	0.005
VTDB_BOVIN	18	17	17	16	17	17	17.0	19	20	21	24	22	17	20.5	0.007
C1S_BOVIN (+1)	6	6	5	7	6	4	5.7	5	4	4	4	3	4	4.0	0.007
NELL2_BOVIN	23	12	18	18	16	17	17.3	16	11	12	12	9	9	11.5	0.009
TSP4_BOVIN	6	5	10	6	6	6	6.5	1	6	5	1	0	3	2.7	0.011
G3X755_BOVIN	9	5	7	10	6	4	6.8	2	5	2	5	1	4	3.2	0.011
G3MYZ3_BOVIN	1	3	1	0	2	2	1.5	2	4	2	4	5	5	3.7	0.012
CL43_BOVIN	5	2	2	3		3	3.0	1	2	2	0	0	1	1.0	0.012
CDC42_BOVIN	2	3	1	1	1	1	1.5	4	3	2	2	3	4	3.0	0.013
E1B748_BOVIN	11	10	17	12	8	9	11.2	8	8	8	7	2	4	6.2	0.013
AATC_BOVIN	2	2	1	1		1	1.4	0	1	0			0	0.3	0.014
F1N5M2_BOVIN	22	22	21	20	22	21	21.3	22	25	24	29	27	22	24.8	0.014
A5D9E9_BOVIN	6	3	6	6	3	1	4.2	1	3	1	1	0	2	1.3	0.015
ST14_BOVIN	7	5	5	8	5	4	5.7	8	9	7	10	6	9	8.2	0.016
F1MVS9_BOVIN	10	2	5	3	5	7	5.3	3	3	2	1	1	0	1.7	0.016
ENPP2_BOVIN	7	3	6	4	0	1	3.5	0	0		0	0	0	0.0	0.020
E1BGJ4_BOVIN	8	6	6	6	5	7	6.3	2	4	1	8	1	3	3.2	0.021
A6QQD5_BOVIN	1	1	1	1	2	0	1.0	0	0	1	0	0	0	0.2	0.022

Accession Number	J1	J2	J3	J4	J5	J6	Av. J	H1	H2	H3	H4	H5	H6	Av. H	p-value
F1MI18_BOVIN	30	13	19	13	17	12	17.3	5	10	7	11	12	11	9.3	0.023
F1MEA1_BOVIN	3	4	3	4	2	4	3.3	3	3	2	2	1	2	2.2	0.028
E1BFQ6_BOVIN	2	1	1	0	2	1	1.2		0	1	0	0	0	0.2	0.033
F1MCF8_BOVIN	5	6	4	6	7	5	5.5	4	5	5	4	4	4	4.3	0.035
TALDO_BOVIN	2	2		1	0	0	1.0	0	0	0	0	0	0	0.0	0.035
E1BBX5_BOVIN	1	1	1	0	0	0	0.5	1	1	1	2	1	1	1.2	0.038
SPB8_BOVIN	2	1	1	2	1	2	1.5	1	1	0	1	1	1	0.8	0.038
MDHM_BOVIN	3	4	3	2	0	0	2.0	0	1	0	0	1	0	0.3	0.042
FA10_BOVIN	1	0	0		1	2	0.8	0	0	0	0	0	0	0.0	0.042
COMP_BOVIN	1	1	1	1	2	2	1.3		1	0		0	1	0.5	0.044
CHID1_BOVIN	6	7	8	3	4	3	5.2	5	5	1	2	0	1	2.3	0.045
A5PK77_BOVIN	3	3	2	1	0	2	1.8	0	1	0	0	2	0	0.5	0.046
F1MW79_BOVIN	1	2	1	2	2		1.6	0	1	1			1	0.8	0.047
HPRT_BOVIN	1	1	2	0	1	2	1.2	0	1	0	0	1	0	0.3	0.049
G3N0T0_BOVIN	1	1	2	0	1	2	1.2	0	1	0	0	1	0	0.3	0.049
G5E5C8_BOVIN	2	2	0	2	0	0	1.0	0	0	0	0	0	0	0.0	0.049
Q3SYT3_BOVIN	3	1	2	2	1	1	1.7	1	1	1	1	0	1	0.8	0.049