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Ratio of dietary rumen degradable protein to rumen undegradable protein affects nitrogen partitioning but does not affect the bovine milk proteome produced by mid-lactation Holstein dairy cows

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Abstract

Little is known about the bovine milk proteome or whether it can be affected by diet. The objective of this study was to determine if the dietary rumen degradable protein (RDP):rumen undegradable protein (RUP) ra-tio could alter the bovine milk proteome. Six Holstein cows (parity: 2.5 ± 0.8) in mid lactation were blocked by days in milk (80 ± 43 d in milk) and milk yield (57.5 ± 6.0 kg) and randomly assigned to treatment groups. The experiment was conducted as a double-crossover design consisting of three 21-d periods. Within each period, treatment groups received diets with either (1) a high RDP:RUP ratio (RDP treatment: 62.4:37.6% of crude protein) or (2) a low RDP:RUP ratio (RUP treatment: 51.3:48.7% of crude protein). Both diets were isonitrogenous and isoenergetic (crude protein: 18.5%, net energy for lactation: 1.8 Mcal/kg of dry matter). To confirm N and energy status of cows, dry matter intake was determined daily, rumen fluid samples were collected for volatile fatty acid analysis, blood samples were collected for plasma glucose, β-hydroxybutyrate, urea nitrogen, and fatty acid analysis, and total 24-h urine and fecal samples were collected for N analysis. Milk samples were collected to determine the general milk composition and the protein profile. Milk samples collected for high-abundance protein analysis were subjected to HPLC analysis to determine the content of α-casein, β-casein, and κ-casein, as well as α-lactalbumin and β-lactoglobulin. Samples collected for low-abundance protein analysis were fractionated, enriched using ProteoMiner treatment, and separated using sodium dodecyl sulfate-PAGE. After excision and digestion, the peptides were analyzed using liquid chromatography (LC) tandem mass spectrometry (MS/MS). The LC-MS/MS data were analyzed using PROC GLIMMIX of SAS (version 9.4, SAS Institute Inc., Cary, NC) and adjusted using the MULTTEST procedure. All other parameters were analyzed using PROC MIXED of SAS. No treatment differences were observed in dry matter intake, milk yield, general milk composition, plasma parameters, or rumen volatile fatty acid concentrations, indicating no shift in total energy or protein available. Milk urea N and plasma urea N concentrations were higher in the RDP group, indicating some shift in N partitioning due to diet. A total of 595 milk proteins were identified,

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with 83% of these proteins known to be involved in cellular processes. Although none of the low-abundance proteins identified by LC-MS/MS were affected by diet, feeding a diet high in RUP decreased β-casein, κ-casein, and total milk casein concentration. Further investigations of the interactions between diet and the milk protein profile are needed to manipulate the milk proteome using diet.

Keywords

milk protein; proteomics; bioactive; low-abundance protein

INTRODUCTION

It is well established that milk plays an important role in neonatal nutrition; however, research investigating the concept that milk proteins are a source of bioactive compounds that have physiological importance beyond AA provision is relatively sparse. Many of the identified bioactive peptides are released after cleavage of the high-abundance milk protein fraction, which include all casein isoforms, as well as whey proteins α-LA and β-LG. Identified low-abundance milk proteins, which include all other whey proteins, are known to have functionality as either cleaved peptide fragments or as entire intact proteins that can withstand gastric cleavage. These bioactive proteins and peptides, derived from both the low- and high-abundance protein fraction, have been identified to have a large breadth of activity and play a role in human health, modulating physiological functions by various binding interactions with target cells and organs inducing beneficial physiological responses. Various functional properties associated with bioactive proteins and peptides include antimicrobial, antihypertensive, opioid, immunomodulatory, mineral binding, and antioxidative activities (Korhonen and Pihlanto, 2006; Sharma et al., 2011; Park and Nam, 2015). Investigation of human breast milk has identified several bioactive proteins and peptides that can influence infant health, particularly gut physiology and motility (Chatterton et al., 2013). Milk proteins present in bovine milk have also been identified to have bioactivity and cross-reactivity with human cells (Buccigrossi et al., 2007; Lönnerdal et al., 2011; Raikos and Dassios, 2014). Understanding secretion profiles of bovine milk proteins as well as mechanisms to manipulate this protein profile are important steps in further enhancing the healthfulness of bovine milk products.

The profile of proteins in bovine milk is influenced by animal factors such as breed, mastitis, and stage of lactation (Boehmer et al., 2010b; Zhang et al., 2015a; Tacoma et al., 2016). The mechanisms that affect the milk proteome at the cellular level within the mammary could be a result of AA supply or energy status on the transcriptional or translational efficiencies or rates within the cell (Osorio et al., 2016), or a result of posttranslational regulation such as changes in protein folding or intracellular protein transport (Ghazalpour et al., 2011). However, extracellular protein shifts may play just as important a role in determining the makeup of the milk proteome. Gene ontology (**GO**) analysis of the low-abundance bovine milk proteome fractions has consistently demonstrated that the majority of the identified proteins are extracellular in origin (Tacoma et al., 2016; Wang et al., 2017), suggesting that not only cell-specific regulation within the mammary gland as outlined above could affect

the milk proteome, but that nonsecretory cell specific metabolic changes could also lead to shifts in the milk proteome via junction leakage, or para- or trans-cellular passage of proteins.

Altering the milk protein profile and bioactive properties of the milk by manipulating the diet of the dairy cow offers a promising approach to naturally enhance the healthfulness of milk products. Research examining the relationship between nutrition and the bovine milk protein profile is limited and nutrition is a significant management factor that has the potential to alter milk protein composition (Kennelly et al., 2005; Tripathi, 2014). Christian et al. (1999) altered the proportions of high abundance bovine milk proteins by feeding a lupin-wheat-based diet, a high RUP source, to lactating dairy cows compared with cows fed spring-pasture, a high RDP source. Cows offered the lupin-wheat-based diet had higher concentrations of α_{S1}-CN, α_{S2}-CN, and γ-CN in the milk compared with cows on the high pasture diet, whereas concentrations of β-CN and κ-CN were present at higher concentrations in milk from cows fed spring pasture compared with cows on the lupin-based diet. More recently, a study was published outlining changes in high abundance milk protein expression patterns in response to inclusion of different corn and soybean feedstuffs in the ration. Although the type of corn included in the diet did not influence the milk protein profile, inclusion of heat-treated soybean meal resulted in a decrease in β-CN and zinc-α−2-glycoprotein fragments indicating the availability of RDP to influence secretion of specific milk proteins. These authors also reported differential expression of α-LA and zinc-α−2-glycoprotein due to diet, suggesting that ruminal microbial protein synthesis could affect the milk protein profile (Li et al., 2015). Mechanistically, shifts in the MCP versus diet-derived digestible RUP fractions reaching the small intestine are known to alter postabsorptive N metabolism, particularly affecting intestinal, hepatic, renal, and muscular metabolism (Hristov et al., 2004; Reynal and Broderick, 2005; Brito and Broderick, 2007), and would alter the blood proteome and N available for uptake and use for mammary protein synthesis but also the profile of nonmammary derived extracellular proteins within the milk via the mechanisms described above. Upon closer investigation of diets used in the research outlined by Christian et al. (1999) and Li et al. (2015), it is clear that they include diets with different RDP:RUP ratios; however, other nutrient differences between diets have made the interpretation of the effect of dietary protein content on the milk proteome difficult.

We hypothesize that it is the difference in diet RDP:RUP protein fraction that ultimately leads to a change in the bovine milk proteome. The goal of our study was to create 2 isonitrogenous and isoenergetic dairy rations with at least a 10% difference in the RDP:RUP ratio and examine the bovine milk proteome in milk samples collected from cows consuming these different diets.

MATERIALS AND METHODS

Experimental Design

Six mid-lactation Holstein dairy cows (parity: 2.5 ± 0.8) were blocked by DIM (80 ± 43 DIM) and milk yield (57.5 ± 6.0 kg) and then were randomly divided into 2 experimental groups in a double-crossover de-sign. Each of the 3 periods lasted 21 d and consisted of 16 d for diet adaptation and the last 5 d for sample collection. Cows were maintained in the same

tie-stall facility with sawdust bedding at the Paul R. Miller Re-search and Educational Center (University of Vermont, Burlington). All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

Diet and Feeding

All animals had free access to water throughout the trial and were fed to target 10% refusals. Cows were fed the same partial mixed ration once daily (0600 h) and a pelleted top-dress that was mixed thoroughly into the ration thrice daily (0330, 1100, and 1800 h) that was formulated to contain either (1) a higher RDP:RUP ratio (RDP diet), or (2) a high RUP:RDP ratio (RUP diet). The treatment groups switched between the RDP or RUP topdress after each period. Diets were formulated to be isonitrogenous and isoenergetic, and included urea, soybean meal, canola meal, and commercially available bypass AA sources to create the different RDP:RUP ratio (see Table 1 for nutrient profile and complete formulation). Feed samples were collected thrice weekly and stored at −20°C. Feed samples were later composited within feedstuff over each period throughout the experiment and analyzed by wet chemistry (DairyOne, Ithaca, NY). Daily feed refusals from each animal were collected and weighed each morning before feeding for the duration of the trial. A subsample of these refusals were stored at −20°C until analysis and subsequently dried at 65°C for 48 h to calculate individual daily DMI.

Milk Production and Milk Sample Collection

Cows were milked twice daily (0700 and 1600 h). Milk yield was recorded daily and milk samples were collected on d 0 as baseline samples and on d 16 to 19 at the end of each experimental period from the morning and afternoon milking. One set of milk subsamples were collected and preserved with bronopol and nata-mycin (Broad Spectrum Microtabs, D & F Control Systems Inc., Dublin, CA) and stored at 4°C. Samples were analyzed commercially (DHIA, Lancaster, PA) within 3 d after collection for general milk composition. A second set of milk subsamples collected for analysis of high abundance proteins were immediately put on ice and skimmed within 2 h of collection at 4,000 × *g* for 10 min at 4°C. The fat layer was removed and the skim milk samples were stored at −20°C until further analysis. A third set of milk subsamples collected for low abundance protein analysis were immediately frozen in a dry-ice ethanol bath after collection and stored at −80°C. Milk samples collected for high abundance protein analysis were analyzed individually, whereas milk samples collected for low abundance protein analysis were composited during the last week of each period by individual animal based on milk weights recorded at each milking.

Blood Collection

Blood samples were collected from the tailhead of each cow into heparinized and EDTA-coated tubes (Becton Dickinson and Company, Franklin Lakes, NJ) after milking (0800 and 1900 h) on d 0, and again on d 17, 19, and 21 of each period. Samples were placed on ice immediately after collection and plasma was isolated within 2 h of blood collection by centrifugation at 3,000 × *g* for 15 min at 4°C. Plasma was transferred into polypropylene tubes and frozen at −20°C until analysis. Plasma samples were later thawed and aliquoted into 0.5-mL centrifuge tubes and plasma concentrations of BHB (Sigma, St. Louis, MO),

plasma urea nitrogen (**PUN**; Teco Diagnostics, Anaheim, CA), glucose (Sigma), and fatty acids (ZenBio Inc., Research Triangle Park, NC) were determined using commercially available kits. Samples were analyzed according to the manufacturer's instructions and all coefficients of variation were <5%.

Rumen Fluid Collection

Rumen fluid samples were collected by esophageal intubation, which was performed at 1300 h on d 0, and again on d 19 and 21 of each period to determine rumen VFA profiles and verify that no major shifts in rumen energetics occurred as a result of treatment. Rumen fluid samples were centrifuged at 14,000 × *g* for 20 min at 8°C and the supernatant was filtered through a 25-mm hardened ashless filter (Whatman 540). The extracted supernatant was mixed with equal parts of an internal standard (50 μmol/mL of trimethyl acetic acid in 0.06 *M* oxalic acid). The samples were analyzed as per methods similar to those previously described by Dann et al. (2008). Nitrogen was used as the carrier gas at a flow rate of 15 mL/min, where the other gases were purified air at 300mL/min and hydrogen gas at 30 mL/min to the flame ionization detector. The oven temperature was held at 175°C for 25min and the injector and detector temperature were held at 200°C. Star Chromatography software (v. 6, Agilent Technologies, Santa Clara, CA) was used to analyze peaks based on the flame ionization detector response. Peaks were identified using individual VFA standards (Supelco, Sigma-Aldrich, St. Louis, MO) and molar proportions were calculated using molecular weights and sample volume.

Urine and Fecal Collection

Urine and fecal samples were collected for 24 h on d 0 and again on d 19 of each period to assess changes in N partitioning as a result of diet treatment. Urine and feces was collected using buckets and weights of each event were recorded before the sample was thoroughly mixed and a subsample collected. Four drops of sulfuric acid was added immediately to each urine subsample to acidify the sample to a pH <4. Fecal and acidified urine subsamples were placed on ice after collection and stored at −20°C until further analysis. All urine and fecal samples were thawed overnight at 4°C, and composited within animal based on the volume of each event in proportion to their total daily urine and fecal weights. The composite fecal samples were dried for 48 h at 65°C and ground. Composite urine and dried fecal samples were then submitted for N analysis to a commercial laboratory (University of Vermont Agricultural and Environmental Testing Laboratory, University of Vermont, Burlington). The estimated N balance of each cow was determined through the following calculation: N retained = N content of feed (g/d; g of CP intake/d/6.25) – [urine N output (g/d) + fecal N out-put (g/d) + milk N output (g/d)]. The milk N content was assumed to be milk protein/ 6.38.

Analysis of the High Abundance Milk Proteins

The skim milk samples stored for high abundance milk protein determination were thawed at 4°C over- night and mixed thoroughly by vortexing and then sonication at 33 W for 15 min at less than 25°C, cooled in an ice bath (Bransonic Model 220, Branson Ultrason-ics, Danbury, CT). Following sonication, a 0.5-mL aliquot of milk was pipetted into a

borosilicate test tube. An equal amount of reducing buffer (6.0 *M* guanidine hydrochloride, 5.0 m*M* trisodium citrate dehydrate, 20.0 m*M* dithiothreitol) was then added to the sample before incubation at room temperature for 1 h. The milk/buffer mixture was allowed to react for at least 1 h at room temperature, a further weighed volume of the buffer without the dithiothreitol reducing agent was added, and the sample transferred to a syringe and passed through a 0.45-μm regenerated cellulose mem-brane syringe filter (Sartorius, Goettingen, Germany) into an HPLC autosampler vial.

Samples were analyzed using a Shimadzu (Kyoto, Japan) HPLC with the following solvent gradient protocol, outlined by Bordin et al. (2001) with minor modifications: linear gradient from 26.5 to 28% eluent B in 2.5 min (0.60% B/min), an isocratic elution at 28.0% B for 4 min then from 28.6 to 30.6% B in 3.4 min (0.70% B/min), and from 30.6 to 33.5% B in 2.9 min (1.0% B/min), followed by an isocratic elution at 33.5% B for 3 min, an increase from 33.5 to 36.1% B in 2.6 min (1.0% B/min), an isocratic elution at 36.1% B for 5 min, an increase from 36.1 to 37% B in 1.5 min (0.6% B/min), an isocratic elution at 37% B for 2 min, and a final increase to 41% B in 6.5 min (0.60% B/min), for a total run time of 42 min at a flow rate of 0.50 mL/min.

For each analysis, 4 μL of sample was injected into the HPLC. Chromatograms were obtained at 214 nm and individual protein peaks were identified by comparison to injections of standard protein solutions prepared in our laboratory from purchased isolated proteins (Sigma), and integrated using Shimadzu LC-solution software (version 1.22, 2006) to determine the area under the peak. For quantification of total α-CN, as well as β-CN, κ-CN, α-LA, and β-LG, standard curves were directly determined by injecting known concentrations of the standard protein solutions. The constituent α_{S1}-CN and α_{S2}-CN proteins are not readily available as isolates; therefore, quantification of α_{S1}-CN and α_{S2}-CN was performed by interpolating the results from the total α-CN standard curve for semiquantitative comparisons between experimental groups.

Low Abundance Protein Isolation, Digestion, and Identification

Milk samples collected for low abundance protein analysis were thawed overnight at 4°C. To obtain a representative sample, milk samples from the morning and afternoon milking were composited within cow from d 16 to 19 within each period according to milk weights at each milking. The resulting 50-mL composite samples were subjected to fractionation and proteomic techniques as previously described (Tacoma et al., 2016). Briefly, protease inhibitor cocktail (Sigma) was added (0.24 mL/g of protein) to each composite sample. Samples were then centrifuged at 4,000 × *g* for 15 min at 4°C, and the skim milk samples were then combined with 60 m*M* $CaCl_2$ and adjusted to a pH of 4.3 using 30% acetic acid solution (Fisher Scientific, Fair Lawn, NJ). The whey-containing supernatant was collected after centrifugation of samples at 189,000 × *g* at 4°C for 70 min, and subsequently stored at −80°C. Samples were lyophilized, reconstituted in PBS, and the protein content of each sample was determined using the bicinchoninic acid assay kit (Pierce, Rockford, IL) using BSA as the standard. The low-abundance protein fraction was enriched using a ProteoMiner kit (BioRad, Hercules, CA) and proteins were eluted in 20 μL of 4× Laemmli sample buffer (8% SDS, 40% glycerol, 250 m*M* Tris pH 6.8, 400 m*M* dithiothreitol with trace amounts of

bromophenol blue) after heating the samples to 95°C for 10 min. The eluted samples were then separated by SDS-PAGE in precast 8 to 16% polyacrylamide gels (BioRad) using Tris-glycine (pH 8.3) containing 0.1% SDS as the running buffer. Gels were stained by incubating in Coomassie Brilliant Blue (BioRad) overnight, and destaining was performed using a solution of 10% acetic acid, 40% methanol, and 50% water. The Gel Doc XR + system (BioRad) was used to scan gels before gel excision. Each sample was cut into 15 segments, cut into 1 mm cubes, de-stained with 50 m*M* ammonium bicarbonate in 50% acetonitrile, and reduced by 10 m*M* dithiothreitol at 55°C for 1 h. Samples were then alkylated with 55 m*M* iodoacetamide in the dark at room temperature for 45 min before being washed and dehydrated twice with 100 m*M* ammonium bicarbonate and 100% acetonitrile. The gel segments were dried in a SpeedVac (Scientific Support, Hayward, CA) and digested by reaction with 7 μg/μL of trypsin for 18 h at 37°C. The digestion was ceased using 50 uL of 5% formic acid, and the resulting peptide solution was dried in a SpeedVac (Scientific Support). Samples were resuspended in 10 μL of a solu-tion containing 2.5% acetonitrile and 2.5% formic acid for liquid chromatography (**LC**)-MS/MS analysis.

Samples were analyzed by LC-MS/MS on a LTQ MS (Thermo Fisher Scientific, Waltham, MA), and 5 μL of the digest was loaded onto a 100 μm × 120 mm capillary column packed with MAGIC C18 (5 μm particle size, 20 nm pore size, Michrom Bioresources, Auburn, CA) at a flow rate of 500 nL/min. Peptides were separated by a gradient of 5 to 35% acetonitrile/0.1% formic acid over 98 min, 40 to 100% acetonitrile/0.1% formic acid in 1 min, and 100% acetonitrile for 10 min, followed by an immediate return to 2.5% CH_3CN/0.1% formic acid, and a hold at CH_3CN/0.1% formic acid. 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 spectrom-etry data were acquired in a data-dependent “Top 10” acquisition mode, in which a survey scan from *m*/*z* 400 to 1,600 is followed by 10 collision-induced dissociation MS/MS scans of the most abundant ions. Tandem mass spectrometry 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. Dynamic exclusion was enabled (repeat count: 2; repeat duration: 30 s; exclusion list size: 180; exclusion duration: 60 s). The minimum threshold was 500. Product ion spectra were searched using the SEQUEST search engine on Proteome Discoverer 1.4 (Thermo Fisher Scientific) against a curated Bovine Uniprot [*Bos taurus* database (24,206 entries) down-loaded July 9, 2014]. The 15 raw files from each sample (6 samples per period: 24 samples total) were searched as one contiguous input file and a single result file was generated for each sample. Search parameters were as follows: full trypsin enzymatic activity, 2 missed cleavages, and peptides between the molecular weight of 350 to 5,000; mass tolerance at 2 Da 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: carbamido-methylation). The result files were then further analyzed by Scaffold 4.3 (Proteome Software, Portland, OR) to compare the unique peptide counts and to identify GO functions of the identified proteins. Cross-correlation significance and minimum peptide cutoff filters were applied to limit the false positive rates to less than 1% in the data sets.

Statistical Analysis

Statistical analysis of DMI, plasma parameters, milk composition, VFA, N balance, and high abundance protein data were performed using a linear mixed model for repeated measures ANOVA. The analyses were carried out with SAS software (version 9.4, SAS Institute Inc., Cary, NC). Preliminary data screening using PROC UNIVARIATE revealed that all dependent variables were approximately normally distributed. Data were analyzed by MIXED procedure of SAS with baseline values used as covariates and day as the repeated measure. All data are presented as least squares means ± standard error of the mean and were considered to be significantly different at$P < 0.05$. Trends were recognized at$P < 0.10$.

Analysis of spectral abundance from the low abundance protein data was performed using a generalized linear mixed model. Data were analyzed by GLIM-MIX procedure of SAS with baseline values used as covariates and day included as the repeated measure. A Poisson distribution was assumed because all dependent variables were count data. Low-abundance protein results were adjusted by the false discovery rate using the MULTTEST procedure of SAS. All data were presented as least squares means ± standard error of the mean and were considered to be significantly different at $P < 0.05$. Trends were recognized at $P < 0.10$.

RESULTS

Diet and DMI

Total CP (% of DM) was similar in both diets and a 13% difference (% of CP) in the RDP:RUP ratio between diets was achieved while maintaining similar NDF, NFC, and NE_L content (% of DM; Table 1). Dry matter intake was not different between the 2 treatment groups (Table 2).

Milk Yield and General Composition

Total milk yield as well as concentrations and yields of the individual milk constituents were not different between the 2 treatment groups (Table 2). Similarly, SCC was not significant between groups. The MUN was higher ($P = 0.04$) in milk samples analyzed from the RDP group (15.7 mg/dL) compared with those from the RUP group (14.6 mg/dL).

Plasma Metabolites

Plasma glucose, BHB, and fatty acid concentrations did not differ between treatment groups (Table 3). The PUN concentrations were higher ($P = 0.01$) from cows fed the RDP diet (1.02 mmol/L) compared with samples from cows fed the RUP diet (0.98 mmol/L). A significant period effect was observed on the concentrations of plasma BHB ($P \leq 0.01$) and PUN ($P \leq 0.01$).

Rumen VFA

Rumen propionate concentrations tended to be higher in cows that were fed the RUP diet ($P = 0.06$; Table 4); however, this response was primarily due to an outlier measured in the second period. For the remaining discussion, this trend was not included in the data interpretation.

N Partitioning

Nitrogen balance data are presented in Table 5 as total N excreted as well as proportion of N intake. Nitrogen intake was similar between the RDP and RUP diets (718 and 717 g/d, respectively; $P = 0.94$). Total fecal, urine, and milk N output (g/d or g/g of N intake) was not different between treatment groups. No effect of diet on calculated N retention was observed. Urine N excretion was affected by period.

High Abundance Milk Proteins

Concentrations of both κ-CN ($P = 0.04$) and total casein ($P < 0.01$) was lower in milk samples from the RUP group (5.39 and 36.3 mg/mL, respectively) compared with the RDP group (5.61 and 37.8 mg/ mL, respectively). Total α-CN ($P = 0.06$) concentration tended to be higher in milk samples collected from the RUP group (16.3 mg/mL) compared with the RDP group (15.7 mg/mL). A period effect was present on β-CN, κ-CN, and total casein concentrations. No difference was found between treatment groups in the skim milk whey fraction (Table 6).

Low Abundance Milk Proteins

Analysis of the skim milk samples from both dietary groups resulted in identification of 595 low abundance proteins (complete list in Supplemental File S1; https://doi.org/10.3168/jds.2017-12647). Of these, 292 were present at high enough peptide counts to be compared statistically across treatments. Using a correction for multiple tests, no treatment, day, or treatment by day effects were observed to affect the protein count of any of the proteins (Table 7).

DISCUSSION

Effect of Diet RDP:RUP Ratio on N Partitioning

Isonitrogenous and isoenergetic diets were formulated and used in this experiment with a 13% (% of CP) difference in the RDP:RUP ratio between the 2 diets. The lack of difference between DMI, rumen VFA concentration, and milk yield observed between the 2 treatment groups supports the suggestion that diets supplied similar nutrient profiles to the cows. The aim of providing a different RDP:RUP ratio to the cows was to create a divergence in how the protein was de-graded and consequently how the N was absorbed and used by the animal. We hypothesized that by altering the proportion of RDP and RUP in the diet for a lactating dairy cow would alter N utilization patterns, ultimately leading to changes in milk protein secretion profiles. Though the diet CP inclusion in this experiment (approximately 18.5% of DM) was relatively high for a lactating dairy cow by the NRC standards (NRC, 2001), lower MUN and PUN concentrations in milk and plasma samples collected from cows fed a diet higher in RUP compared with those that were fed the RDP diet highlights that the diet composition successfully altered the N utilization patterns between treatment groups and is in agreement with previous research (Brito and Broderick, 2007; Totty et al., 2013).

Milk Proteins Affected by Diet RDP:RUP Ratio

The significant increase in total casein concentrations measured from cows fed the RDP diet could be related to a more efficient N and energy capture by microbes with higher MCP synthesis and hindgut MCP utilization and uptake as a result. Increased energy and N availability to the cow would likely increase mammary protein synthesis capacity, which would also result in a higher mammary casein synthesis rates. This would support our observation of increased milk total casein content from cows fed the RDP diet compared with those on the RUP diet.

Cows on the RUP diet also had lower individual β-CN and κ-CN concentrations in the skim milk fraction com-pared with those on the RDP diet. These results suggest that, at least in part, the results observed by Christian et al. (1999) and Li et al. (2015) are due to changes in ruminal protein availability and consequent animal N partitioning. The mechanisms of action could be due to specific AA availability to the mammary gland (**MG**), which is known to affect total protein secretion in the milk (DePeters and Cant, 1992; Rius et al., 2010). How-ever, the diets used in the current study were predicted to satisfy all AA requirements, and without the observation of increased total milk protein output, it would indicate an additional requirement of specific AA above the current estimated AA requirements for synthesis of specific casein isoforms. Though this prospect is feasible, further investigation of mammary AA supply during differential RDP:RUP feeding with focus on its relationship to mammary function and casein isoform secretion is needed to address this mechanistic hypothesis. Hormone signaling, particularly that of insulin on the mTOR complexes, is another mechanism by which diet could affect mammary protein synthesis (Bionaz and Loor, 2011). Though the diets were designed to be isoenergetic and no significant changes in rumen VFA profile or plasma glucose concentration were observed, no plasma hormones were directly analyzed and there-fore cannot be discounted to have played a role in the observed shift in casein due to diet.

Unfortunately, the current known functions of β-CN and κ-CN provide little aid in development of a secondary hypothesis as to why this differential regulation might occur. The calcium-insensitive κ-CN is known to play an important role in micelle stability (Shekar et al., 2006), whereas the function of β-CN is unclear. Through a lactation, β-CN knockout mice secrete less milk protein, despite maintaining a normal lactation. The lower milk protein due to β-CN knockout is partially compensated through increased secretion of other casein isoforms (Kumar et al., 1994), indicating no crucial role in protein function or secretion.

Over 590 low abundance proteins were identified using a combination of fractionation and enrichment techniques. Although no effect of dietary treatment on the low abundance protein profile was observed, the identified proteins are known to have a large breadth of functions and included known bioactive proteins such as lactadherin, lactoperoxidase, lactoferrin, and osteopontin. Gene ontological analysis revealed that 83% of the low abundance proteins with identified GO functions were involved in cellular processes such as protein folding and stabilization, signal transduction, cell adhesion, complement activation pathways, and glycolytic and catabolic processes. Additionally, 73% of low abundance

proteins identified with known GO functions involved in binding processes were predominately proteins involved in metal-ion binding such as calcium, copper, magnesium, manganese, and zinc as well as ATP and GTP binding. Twenty-five percent of the low abundance proteins identified with known GO functions were involved in immune system regulation and these proteins were involved in activation of the complement proteins, the innate immune response, and antibacterial activities. Many of the low abundance proteins have multifunctional properties contributing to the complex regulation of cellular metabolism.

In agreement with previous research, many of the low abundance proteins identified in the current study are characterized generally as common nonmammary specific cellular proteins and more research is needed in this field to understand the pathways that lead to presence of these proteins in milk, as well as the physiological and metabolic factors affecting milk protein expression within the MG.

Effect of DIM on Milk Protein Profile

Using a double-crossover design, otherwise known as a switchback design, also allowed for investigation into the effect of DIM on the milk protein profile. The concentrations of β-CN, κ-CN, and total milk casein in-creased with increasing DIM. These results are consistent with previous studies (see review by Barber et al., 2005) and may be due to increased synthesis of casein in response to the positive energy status, reduced mam-mary protease activity, hormonal control, or regulation by an advancing pregnancy rather than directly due to stage of lactation (Barber et al., 2005).

None of the identified low abundance proteins were influenced by DIM. This was not expected considering that the trial encompassed 63 d and many whole-animal metabolic and physiologic changes occur through the progression of lactation, including changes at the tissue level as the MG undergoes involution, and inflammation in the MG subsides (Zhang et al., 2015a,b). Two factors may play into this lack of day effect. First, the animals were in mid lactation, which encompasses a period of less drastic mammary- and whole-animal-level shifts: energy balance has positively stabilized, milk production has peaked, and many of the postpartum diseases have subsided. This stabilization of the milk proteome in the mid-lactation stage is supported by recent work outlined by Zhang et al. (2017), who reported stable percentages of low-abundance proteins involved in enzymatic activity, immunity, and transport functions throughout the mid-lactation period of dairy cattle. A second factor that could come into play is animal variation. Though the research described herein used a robust switchback design to address the stated hypothesis, individual animal variation in parameters such as lactation persistency, rates of alveolar senescence, and nutrient utilization efficiency could prevent a true portrait of the effect of time on the milk proteome, particularly that of the lower abundance milk proteins that are present at low concentrations.

CONCLUSIONS

Nutritional manipulation of the dairy cow’s diet to alter milk composition offers a promising approach to naturally enhance the milk profile and could provide an opportunity for future development into functional foods directed toward increased healthfulness of milk. Although

altering the RDP:RUP ratio of the diet did not induce any differences in the low abundance milk protein profile, the effect of this diet alteration on the casein profile produced by the cattle demonstrates the potential to influence specific mammary-derived milk proteins. Further investigation into the mechanisms of this interaction are needed to verify the outcomes observed in this smaller study and more accurately predict the effect of diet changes on the milk protein profile. Future studies could begin by gaining a deeper understanding on how the MG responds to changes in plasma composition along with examining the regulatory mechanisms behind AA transport across the mammary epithelia and how a change in AA availability to the MG influences protein synthetic pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.

Ingredient and nutrient composition of the diets[1]

	Treatment	
Item	**RDP**	**RUP**
Ingredient (% of DM)		
Corn silage	48.5	48.5
Haylage	6.4	6.4
Soybean meal	13.1	5.6
Molasses cane	1.4	1.1
Corn grain	14.2	14.0
Citrus pulp dry	1.1	1.2
Canola meal	3.6	—
Wheat middlings	0.7	—
Wheat red dog	1.4	—
Berga fat	0.9	—
Corn distillers	—	3.5
Vitamins/minerals	2.9	2.7
Amino max	4.8	13.9
Urea	0.4	0.14
Amino enhancer	—	1.4
Nutrient composition (% of DM)		
DM (%)	57.1	57.0
CP	18.5	18.5
RDP	11.7	9.0
RUP	6.8	9.5
ADF	20.3	21.7
NDF	30.8	32.7
NFC	40.1	39.1
NE_L (Mcal/kg)	1.8	1.8

[1]Supplier of feed: Poulin Grain (Newport, VT).

Table 2.

Daily DMI, milk yield, and milk components of Holstein dairy cattle fed diets with either a high RDP:RUP ratio (RDP) or a low RDP:RUP ratio (RUP)

Item	Treatment			*P*-value	
	RDP	RUP	SED[1]	Treatment	Period
DMI (kg/d)	24.2	24.2	0.7	0.94	0.99
Milk yield (kg/d)	58.3	58.7	3.5	0.87	0.05
Milk component yield (kg/d)					
Fat	1.56	1.48	0.04	0.80	0.99
Protein	1.71	1.72	0.07	0.83	0.25
Milk component (%)					
Fat	3.48	3.43	0.22	0.80	0.99
Protein	2.96	2.93	0.13	0.83	0.25
SCC (× 1,000)	55.5	78.2	23.7	0.33	0.17
MUN (mg/dL)	15.7	14.6	0.86	0.04	<0.01

[1]SED = standard error of the difference.

Table 3.

Plasma metabolites from Holstein dairy cattle fed diets with either a high RDP:RUP ratio (RDP) or low RDP:RUP ratio (RUP)

	Treatment			*P*-value	
Item (mmol/L)	**RDP**	**RUP**	**SED**[1]	**Treatment**	**Period**
Glucose	3.51	3.52	0.04	0.77	0.68
BHB	0.26	0.27	0.01	0.67	<0.01
Plasma urea N	1.02	0.98	0.01	0.01	<0.01
Fatty acids	0.10	0.09	0.01	0.66	0.96

[1]SE = standard error of the difference.

Table 4.

Rumen VFA from Holstein dairy cattle fed diets with either a high RDP:RUP ratio (RDP) or low RDP:RUP ratio (RUP)

Item	Treatment			*P*-value	
	RDP	RUP	SED[1]	Treatment	Period
Acetate (mmol/L)	19.9	21.9	0.86	0.60	0.13
Acetate (% of total)	59.3	65.4	5.10	0.89	0.55
Butyrate (mmol/L)	3.34	3.91	0.24	0.64	0.27
Butyrate (% of total)	10.9	10.8	0.85	0.91	0.27
Propionate (mmol/L)	6.96	8.05	0.45	0.06	0.39
Propionate (% of total)	19.7	18.1	3.50	0.63	0.64
Acetate:propionate ratio	2.85	2.76	0.06	0.22	0.17
Total VFA (mmol/L)	35.1	33.9	2.18	0.76	0.60

[1]SE = standard error of the difference.

Table 5.

Nitrogen partitioning in plasma, urine, feces, and milk from Holstein dairy cattle fed diets with either a high RDP:RUP ratio (RDP) or low RDP:RUP ratio (RUP)

Item	Treatment		SED[1]	*P*-value	
	RDP	RUP		Treatment	Period
N intake (g/d)					
Forage	193	193	5.99	0.94	0.99
Concentrate	524	523	16.2	0.94	0.99
Total	718	717	22.2	0.94	0.99
N output					
Feces (g/d)	196	210	10.4	0.26	0.77
Proportion of N intake	0.27	0.28	0.009	0.38	0.56
Urine (g/d)	253	237	9.31	0.23	<0.01
Proportion of N intake	0.35	0.32	0.01	0.14	<0.01
Milk (g/d)	249	257	9.31	0.47	0.93
Proportion of N intake	0.35	0.34	0.03	0.74	0.13
Retention (g/d)	38.8	43.2	29.7	0.88	0.02

[1]SE = standard error of the difference.

Table 6.

High abundance milk proteins from Holstein dairy cattle fed diets with either a high RDP:RUP ratio (RDP) or low RDP:RUP ratio (RUP)

Item	Diet		SED[1]	*P*-value	
	RDP	RUP		Treatment	Period
Casein (mg/mL of skim milk)					
β-CN	16.3	15.8	0.29	0.06	0.03
κ-CN	5.61	5.39	0.08	0.04	0.01
Total α-CN	15.7	16.3	0.33	0.06	0.22
α_{S1}-CN	13.7	13.3	0.45	0.10	0.22
α_{S2}-CN	1.93	1.93	0.07	0.95	0.20
Total casein	37.8	36.3	0.48	<0.01	0.04
Whey (mg/mL of skim milk)					
α-LA	1.48	1.50	0.09	0.66	0.26
β-LGA[2]	2.52	2.50	0.19	0.91	0.10
β-LGB[3]	1.60	1.64	0.11	0.72	0.52
Total α-LA, β-LGA, β-LGB	5.50	5.75	0.37	0.36	0.52

[1]SE = standard error of the difference.

[2]β-LGA = β-lactoglobulin variant A.

[3]β-LGB = β-lactoglobulin variant B.

Table 7.

Average peptide counts of low abundance milk proteins identified in samples from Holstein dairy cattle consuming either a high RDP:RUP ratio (RDP) or low RDP:RUP ratio (RUP) diet

		Treatment[1]		*P*-value		
Protein accession	**Description**	**RDP**	**RUP**	**Treatment**	**Day**	**Treatment × day**
A1AG_BOVIN	Alpha-1-acid glycoprotein	2.63 (0.61)	2.51 (0.61)	0.99	1.00	0.9972
A1AT_BOVIN	Alpha-1-antiproteinase	7.18 (0.92)	8.74 (1.01)	0.99	1.00	0.9972
A1BG_BOVIN	Alpha-1B-glycoprotein	6.60 (0.88)	5.06 (0.77)	0.99	1.00	0.9972
A1L528_BOVIN	RAB1A, member RAS oncogene family	0.70 (0.38)	0.64 (0.36)	0.99	1.00	0.9972
A2AP_BOVIN	Alpha-2-antiplasmin	3.21 (0.61)	2.07 (0.52)	0.99	1.00	0.9972
A2MG_BOVIN	Alpha-2-macroglobulin	23.81 (2.38)	15.94 (1.79)	0.99	1.00	0.9972
A4IFN4_BOVIN	LOC615589 protein	1.55 (0.42)	1.04 (0.35)	0.99	1.00	0.9972
A4IFP2_BOVIN	KRT4 protein	4.95 (0.75)	6.90 (0.88)	0.99	1.00	0.9972
A4IFP7_BOVIN	ARF5 protein	1.37 (0.40)	1.85 (0.47)	0.99	1.00	0.9972
A5D7M6_BOVIN	KRT5 protein	11.67 (1.14)	12.95 (1.22)	0.99	1.00	0.9972
A5PJE3_BOVIN	Fibrinogen α chain	3.47 (0.83)	4.53 (0.99)	0.99	1.00	0.9972
A5PKC2_BOVIN	SHBG protein	1.31 (0.63)	0.94 (0.48)	0.99	1.00	0.9972
A6QNL0_BOVIN	Monocyte differentiation antigen CD14	8.61 (1.00)	8.74 (1.01)	0.99	1.00	0.9972
A6QNZ7_BOVIN	Keratin 10	8.28 (0.96)	9.31 (1.02)	0.99	1.00	0.9972
A6QP32_BOVIN	KRT85 protein	0.74 (0.36)	0.95 (0.45)	0.99	1.00	0.9972
A7E350_BOVIN	PLG protein	2.44 (0.78)	1.91 (0.70)	0.99	1.00	0.9972
ACTC_BOVIN	Actin, α cardiac muscle 1	1.77 (0.47)	2.52 (0.57)	0.99	1.00	0.9972
ACTG_BOVIN	Actin, cytoplasmic 2	3.38 (0.64)	3.95 (0.71)	0.99	1.00	0.9972
ACTS_BOVIN	Actin, α skeletal muscle	1.77 (0.47)	2.52 (0.57)	0.99	1.00	0.9972
ALBU_BOVIN	Serum albumin	41.02 (2.15)	43.51 (2.20)	0.99	1.00	0.9972
AMBP_BOVIN	Protein AMBP	5.32 (0.78)	4.93 (0.74)	0.99	1.00	0.9972
ANG1_BOVIN	Angiogenin-1	1.08 (0.36)	1.88 (0.46)	0.99	1.00	0.9972
ANT3_BOVIN	Antithrombin-III	13.69 (1.24)	11.75 (1.15)	0.99	1.00	0.9972
ANXA1_BOVIN	Annexin A1	1.02 (0.46)	0.37 (0.24)	0.99	1.00	0.9972
ANXA2_BOVIN	Annexin A2	2.22 (0.58)	2.66 (0.65)	0.99	1.00	0.9972
AOC3_BOVIN	Membrane primary amine oxidase	1.99 (0.48)	2.28 (0.51)	0.99	1.00	0.9972

		Treatment[1]		*P*-value		
Protein accession	**Description**	**RDP**	**RUP**	**Treatment**	**Day**	**Treatment × day**
AOCX_BOVIN	Primary amine oxidase, liver isozyme	4.55 (0.76)	5.50 (0.83)	0.99	1.00	0.9972
AOCY_BOVIN	Primary amine oxidase, lung isozyme	2.20 (0.50)	2.27 (0.50)	0.99	1.00	0.9972
APOA1_BOVIN	Apolipoprotein A-I	6.34 (0.85)	6.10 (0.85)	0.99	1.00	0.9972
APOE_BOVIN	Apolipoprotein E	4.22 (0.70)	5.45 (0.79)	0.99	1.00	0.9972
ARF1_BOVIN	ADP-ribosylation factor 1	2.67 (0.55)	3.29 (0.62)	0.99	1.00	0.9972
ARF2_BOVIN	ADP-ribosylation factor 2	1.67 (0.44)	2.23 (0.50)	0.99	1.00	0.9972
ARF3_BOVIN	ADP-ribosylation factor 3	2.67 (0.55)	3.29 (0.62)	0.99	1.00	0.9972
B4GT1_BOVIN	Beta-1,4-galactosyltransferase 1	4.10 (0.87)	4.11 (0.89)	0.99	1.00	0.9972
B8Y9S9_BOVIN	Embryo-specific fibronectin 1 transcript variant	16.78 (1.86)	15.81 (1.81)	0.99	1.00	0.9972
BMP3_BOVIN	Bone morphogenetic protein 3	0.67 (0.34)	0.71 (0.37)	0.99	1.00	0.9972
BT1A1_BOVIN	Butyrophilin subfamily 1 member A1	1.30 (0.42)	1.63 (0.48)	0.99	1.00	0.9972
C1GLC_BOVIN	C1GALT1-specific chaperone 1	0.93 (0.36)	0.51 (0.26)	0.99	1.00	0.9972
C4BPA_BOVIN	C4b-binding protein α chain	3.22 (0.66)	2.01 (0.74)	0.99	1.00	0.9972
CAB45_BOVIN	45 kDa calcium-binding protein	6.78 (0.90)	8.42 (1.01)	0.99	1.00	0.9972
CASA1_BOVIN	Alpha-S1-casein	4.13 (0.69)	3.62 (0.64)	0.99	1.00	0.9972
CASA2_BOVIN	Alpha-S2-casein	2.34 (0.53)	1.42 (0.42	0.99	1.00	0.9972
CASB_BOVIN	Beta-casein	1.73 (0.49)	1.89 (0.51)	0.99	1.00	0.9972
CASK_BOVIN	Kappa-casein	2.49 (0.62)	3.12 (0.72)	0.99	1.00	0.9985
CATB_BOVIN	Cathepsin B	2.69 (0.61)	2.88 (0.64)	0.99	1.00	0.9972
CATS_BOVIN	Cathepsin S	1.40 (0.40)	1.90 (0.46)	0.99	1.00	0.9972
CBPB2_BOVIN	Carboxypeptidase B2	2.15 (0.50)	2.22 (0.50)	0.99	1.00	0.9972
CD14_BOVIN	Monocyte differentiation antigen CD14	8.61 (1.0)	8.74 (1.01)	0.99	1.00	0.9972
CFAB_BOVIN	Complement factor B	17.18 (1.39)	15.33 (1.31)	0.99	1.00	0.9972
CFAH_BOVIN	Complement factor H	9.49 (1.54)	7.04 (1.22)	0.99	1.00	0.9972
CH3L1_BOVIN	Chitinase-3-like protein 1	7.50 (0.98)	5.97 (0.85)	0.99	1.00	0.9972
CHP1_BOVIN	Calcineurin B homologous protein 1	0.34 (0.20)	0.63 (0.30)	0.99	1.00	0.9972
CLUS_BOVIN	Clusterin	6.77 (0.88)	6.42 (0.85)	0.99	1.00	0.9972
CO2_BOVIN	Complement C2	1.75 (0.47)	1.35 (0.39)	0.99	1.00	0.9972
CO3_BOVIN	Complement C3	49.97 (4.99)	50.21 (5.01)	0.99	1.00	0.9811
CO6_BOVIN	Complement component C6	4.75 (0.91)	5.48 (1.00)	0.99	1.00	0.9972

		Treatment[1]		*P*-value		
Protein accession	**Description**	**RDP**	**RUP**	**Treatment**	**Day**	**Treatment × day**
CO7_BOVIN	Complement component C7	9.06 (1.85)	6.82 (1.46)	0.99	1.00	0.9972
CO9_BOVIN	Complement component C9	2.58 (0.95)	1.60 (0.71)	0.99	1.00	0.9972
COL12_BOVIN	Collectin-12	3.89 (0.69)	2.75 (0.59)	0.99	1.00	0.9972
CTNB1_BOVIN	Catenin β−1	0.57 (0.28)	0.62 (0.28)	0.99	1.00	0.9972
CYTC_BOVIN	Cystatin-C	1.46 (0.41)	1.15b (0.36)	0.99	1.00	0.9972
DSG1_BOVIN	Desmoglein-1	1.26 (0.38)	1.30 (0.38)	0.99	1.00	0.9972
E1B748_BOVIN	Hypoxia upregulated protein 1 isoform X1	0.96 (0.62)	2.25 (1.24)	0.99	1.00	0.9972
E1B991_BOVIN	keratin, type II cytoskeletal 2 epidermal	3.76 (0.65)	4.22 (0.69)	0.99	1.00	0.9972
E1B9H5_BOVIN	Transforming growth factor β receptor type 3 isoform X2	2.87 (0.59)	1.86 (0.50)	0.99	1.00	0.9972
E1B9K1_BOVIN	Polyubiquitin-C	1.00 (0.34)	1.21 (0.37)	0.99	1.00	0.9972
E1BA17_BOVIN	Collagen α−1 (XIV) chain isoform X2	13.09 (1.42)	15.45 (1.58)	0.99	1.00	0.9972
E1BC58_BOVIN	Ras-related protein Rab-2B	0.69 (0.30)	0.86 (0.31)	0.99	1.00	0.9972
E1BD43_BOVIN	Amine oxidase	1.99 (0.48)	2.28 (0.51)	0.99	1.00	0.9972
E1BF27_BOVIN	Putative sodium-coupled neutral amino acid transporter 10 isoform X1	2.09 (0.50)	2.18 (0.51)	0.99	1.00	0.9972
E1BGW1_BOVIN	Mucin-15	0.41 (0.26)	0.36 (0.24)	0.99	1.00	0.9972
E1BGX8_BOVIN	HHIP-like 2	0.97 (0.34)	0.70 (0.31)	0.99	1.00	0.9972
E1BH06_BOVIN	Complement C4-A-like isoform X1	13.04 (2.04)	15.48 (2.36)	0.99	1.00	0.9972
E1BHI7_BOVIN	Butyrophilin subfamily 1 member A1	0.90 (0.33)	1.35 (0.44)	0.99	1.00	0.9972
E1BI82_BOVIN	Serotransferrin-like	1.18 (0.39)	2.20 (0.50)	0.99	1.00	0.9972
E1BI98_BOVIN	Collagen α−1(VI) chain precursor	1.78 (0.47)	1.25 (0.40)	0.99	1.00	0.9972
E1BJN3_BOVIN	Amine oxidase	1.21 (0.37)	1.06 (0.35)	0.99	1.00	0.9972
E1BKT9_BOVIN	Desmoplakin	5.96 (1.17)	7.22 (1.35)	0.99	1.00	0.9972
E1BLR9_BOVIN	Low quality protein: carboxypeptidase D	2.82 (0.57)	2.74 (0.57)	0.99	1.00	0.9972
E1BMJ0_BOVIN	Factor XIIa inhibitor isoform X1	8.53 (1.00)	8.11 (0.97)	0.99	1.00	0.9972
E1BNY3_BOVIN	Semaphorin-6D precursor	0.76 (0.33)	0.87 (0.36)	0.99	1.00	0.9972
ECHD1_BOVIN	Ethylmalonyl-CoA decarboxylase	2.07 (0.50)	3.11 (0.59)	0.99	1.00	0.9972
ENOA_BOVIN	Alpha-enolase	0.71 (0.43)	0.72 (0.44)	0.99	1.00	0.9972
ENPP3_BOVIN	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	2.51 (0.77)	1.64 (0.58)	0.99	1.00	0.9972
F12AI_BOVIN	Factor XIIa inhibitor	8.34 (1.01)	7.97 (0.98)	0.99	1.00	0.9972
F1MAV0_BOVIN	Fibrinogen β chain	3.56 (1.20)	3.60 (1.33)	0.99	1.00	0.9972

		Treatment[1]		*P*-value		
Protein accession	**Description**	**RDP**	**RUP**	**Treatment**	**Day**	**Treatment × day**
F1MC11_BOVIN	Keratin, type I cytoskeletal 14	15.67 (1.32)	16.86 (1.37)	0.99	1.00	0.9972
F1MCF8_BOVIN	IGL@ protein	4.10 (0.68)	4.11 (0.68)	0.99	1.00	0.9972
F1MD77_BOVIN	Laminin B2-like	15.13 (1.31)	13.45 (1.23)	0.99	1.00	0.9972
F1MEA1_BOVIN	Transmembrane protein 132A	0.93 (0.54)	0.56 (0.36)	0.99	1.00	0.9972
F1MH40_BOVIN	IGK protein	5.52 (0.79)	4.98 (0.75)	0.99	1.00	0.9972
F1MI18_BOVIN	Alpha-2-macroglobulin	3.81 (1.76)	0.80 (0.47)	0.99	1.00	0.9972
F1MJK3_BOVIN	Alpha-2-macroglobulin	3.81 (1.76)	0.80 (0.47)	0.99	1.00	0.9972
F1MJQ1_BOVIN	Ras-related protein Rab-7a	2.81 (0.57)	3.41 (0.62)	0.99	1.00	0.9972
F1MK08_BOVIN	Tripeptidyl-peptidase 1	0.89 (0.34)	1.40 (0.44)	0.99	1.00	0.9972
F1MKC4_BOVIN	Actin, gamma-enteric smooth muscle	1.35 (0.39)	2.01 (0.51)	0.99	1.00	0.9972
F1MLW7_BOVIN	Uncharacterized protein	4.31 (0.69)	4.22 (0.68)	0.99	1.00	0.9972
F1MLW8_BOVIN	IGL@ protein	3.87 (0.66)	4.00 (0.67)	0.99	1.00	0.9972
F1MM32_BOVIN	Sulfhydryl oxidase	12.95 (1.70)	9.21 (1.34)	0.99	1.00	0.9972
F1MM86_BOVIN	Complement component C6	4.75 (0.91)	5.48 (1.00)	0.99	1.00	0.9972
F1MMD7_BOVIN	Inter-α-trypsin inhibitor heavy chain H4	7.01 (1.34)	7.26 (1.38)	0.99	1.00	0.9811
F1MMK9_BOVIN	Protein AMBP	5.32 (0.78)	4.93 (0.74)	0.99	1.00	0.9972
F1MMP5_BOVIN	Inter-α-trypsin inhibitor heavy chain H1	7.05(1.36)	6.62 (1.29)	0.99	1.00	0.9811
F1MNN7_BOVIN	Lipopolysaccharide-binding protein	3.57 (0.64)	2.70 (0.61)	0.99	1.00	0.9972
F1MNT5_BOVIN	Mucin-20	1.58 (0.45)	1.58 (0.41)	0.99	1.00	0.9972
F1MNV5_BOVIN	Kininogen-1	9.34 (1.04)	8.70 (1.00)	0.99	1.00	0.9972
F1MNW4_BOVIN	Inter-α-trypsin inhibitor heavy chain H2	9.72 (1.42)	8.29 (1.26)	0.99	1.00	0.9972
F1MPE1_BOVIN	CD109 antigen isoform X1	16.41 (1.36)	14.96 (1.30)	0.99	1.00	0.9972
F1MR22_BOVIN	Polymeric immunoglobulin receptor precursor	19.64 (1.50)	19.72 (1.49)	0.99	1.00	0.9972
F1MRA6_BOVIN	Uncharacterized protein	1.30 (0.38)	0.76 (0.28)	0.99	1.00	0.9972
F1MRD0_BOVIN	Actin, cytoplasmic 1	3.14 (0.61)	3.21 (0.63)	0.99	1.00	0.9972
F1MSZ6_BOVIN	Antithrombin-III	14.04 (1.26)	12.34 (1.18)	0.99	1.00	0.9972
F1MTI7_BOVIN	Protein CutA	0.38 (0.22)	0.61 (0.27)	0.99	1.00	0.9972
F1MU12_BOVIN	Keratin, type II cytoskeletal 8	1.54 (0.42)	1.59 (0.43)	0.99	1.00	0.9972
F1MU18_BOVIN	Oncostatin M receptor	2.15 (0.50)	2.30 (0.52)	0.99	1.00	0.9972
F1MUT3_BOVIN	Xanthine dehydrogenase/oxidase	25.81 (2.62)	26.82 (2.69)	0.99	1.00	0.9972

		Treatment[1]		*P*-value		
Protein accession	**Description**	**RDP**	**RUP**	**Treatment**	**Day**	**Treatment × day**
F1MUY2_BOVIN	Keratin, type II cytoskeletal 59 kDa, component IV	8.21 (0.96)	7.94 (0.95)	0.99	1.00	0.9972
F1MWN3_BOVIN	Nidogen-1 precursor	0.40 (0.25)	0.62 (0.31)	0.99	1.00	0.9972
F1MX50_BOVIN	Cellular repressor of E1A-stimulated genes	2.33 (0.54)	1.62 (0.48)	0.99	1.00	0.9972
F1MXP8_BOVIN	Prosaposin	7.17 (0.90)	7.39 (0.93)	0.99	1.00	0.9972
F1MYX9_BOVIN	Laminin subunit α−4 precursor	6.13 (1.05)	4.79 (0.90)	0.99	1.00	0.9972
F1MZ96_BOVIN	IGK protein	5.51 (0.79)	5.09 (0.76)	0.99	1.00	0.9972
F1N045_BOVIN	Complement component C7	9.43 (1.96)	6.99 (1.52)	0.99	1.00	0.9972
F1N076_BOVIN	Ceruloplasmin isoform X1	23.41 (2.34)	24.12 (2.40)	0.99	1.00	0.9811
F1N1I6_BOVIN	Gelsolin	13.60 (1.24)	13.91 (1.25)	0.99	1.00	0.9972
F1N2D5_BOVIN	Iduronate 2-sulfatase precursor	0.99 (0.35)	0.70 (0.31)	0.99	1.00	0.9972
F1N362_BOVIN	Keratin, type II cuticular Hb4	2.62 (0.54)	2.18 (0.50)	0.99	1.00	0.9972
F1N3A1_BOVIN	Thrombospondin-1	18.75 (1.47)	17.97 (1.42)	0.99	1.00	0.9972
F1N4M7_BOVIN	TPA: complement factor I	6.70 (0.87)	6.55 (0.83)	0.99	1.00	0.9972
F1N4X7_BOVIN	Suppressor of tumorigenicity 14 protein homolog	1.94 (0.47)	1.92 (0.47)	0.99	1.00	0.9972
F1N5M2_BOVIN	Vitamin D-binding protein	14.07 (1.27)	14.47 (1.29)	0.99	1.00	0.9972
F1N614_BOVIN	78 kDa glucose-regulated protein precursor	4.54 (1.14)	4.09 (1.07)	0.99	1.00	0.9972
F1N650_BOVIN	Annexin	1.02 (0.46)	0.37 (0.24)	0.99	1.00	0.9972
F1N6W9_BOVIN	Collagen α−1 (XVIII) chain isoform X1	9.07 (1.01)	8.50 (0.98)	0.99	1.00	0.9972
F1N726_BOVIN	Pancreatic secretory granule membrane major glycoprotein GP2 precursor	8.70 (1.01)	7.87 (0.95)	0.99	1.00	0.9972
F6PQI6_BOVIN	Polymeric immunoglobulin receptor precursor	16.93 (1.42)	17.82 (1.45)	0.99	1.00	0.9972
F6R3I5_BOVIN	Cysteine-rich secretory protein 3 precursor	1.32 (0.38)	1.22 (0.37)	0.99	1.00	0.9972
FABPH_BOVIN	Fatty acid-binding protein, heart	4.32 (0.71)	4.85 (0.75)	0.99	1.00	0.9972
FETUA_BOVIN	Alpha-2-HS-glycoprotein	4.50 (0.71)	4.09 (0.68)	0.99	1.00	0.9972
FETUB_BOVIN	Fetuin-B	1.56 (0.54)	1.53 (0.49)	0.99	1.00	0.9972
FGFP1_BOVIN	Fibroblast growth factor-binding protein 1	3.33 (0.62)	3.44 (0.63)	0.99	1.00	0.9972
FIBA_BOVIN	Fibrinogen α chain	3.47 (0.83)	4.53 (0.99)	0.99	1.00	0.9972
FIBB_BOVIN	Fibrinogen β chain	3.57 (1.19)	3.53 (1.29)	0.99	1.00	0.9972
FIBG_BOVIN	Fibrinogen gamma-B chain	3.72 (0.67)	3.31 (0.66)	0.99	1.00	0.9972
FINC_BOVIN	Fibronectin	16.48 (1.79)	15.54 (1.75)	0.99	1.00	0.9972

		Treatment[1]		*P*-value		
Protein accession	**Description**	**RDP**	**RUP**	**Treatment**	**Day**	**Treatment × day**
FOLR1_BOVIN	Folate receptor α	1.42 (0.41)	1.31 (0.41)	0.99	1.00	0.9972
FST_BOVIN	Follistatin	1.84 (0.48)	1.58 (0.43)	0.99	1.00	0.9972
G1K122_BOVIN	Retinol-binding protein 4	2.37 (0.52)	2.52 (0.54)	0.99	1.00	0.9972
G1K1L8_BOVIN	Leukocyte elastase inhibitor	1.38 (0.52)	1.31 (0.40)	0.99	1.00	0.9972
G3MX39_BOVIN	Uncharacterized protein	0.65 (0.35)	0.83 (0.37)	0.99	1.00	0.9972
G3MX98_BOVIN	Uncharacterized protein	1.03 (0.35)	1.33 (0.38)	0.99	1.00	0.9972
G3MXR2_BOVIN	Mucin-16	2.20 (0.51)	1.88 (0.46)	0.99	1.00	0.9972
G3MXS7_BOVIN	Uncharacterized protein	1.72 (0.47)	1.55 (0.43)	0.99	1.00	0.9972
G3MYU2_BOVIN	Keratin, type II cytoskeletal 1b	2.78 (0.56)	2.35 (0.52)	0.99	1.00	0.9972
G3MYW7_BOVIN	Lactadherin	13.21 (1.27)	13.89 (1.30)	0.99	1.00	0.9972
G3MZ71_BOVIN	Keratin, type II cytoskeletal 2 epidermal isoform X2	3.76 (0.65)	4.22 (0.69)	0.99	1.00	0.9972
G3N0V2_BOVIN	Keratin, type II cytoskeletal 1	8.97 (1.00)	9.91 (1.06)	0.99	1.00	0.9972
G3N156_BOVIN	Plakophilin-1	0.87 (0.36)	0.64 (0.30)	0.99	1.00	0.9972
G3N2D8_BOVIN	Gamma-glutamyltranspeptidase 1	1.10 (0.37)	1.26 (0.38)	0.99	1.00	0.9972
G3N2K4_BOVIN	Golgi glycoprotein 1 precursor	2.53 (0.58)	1.65 (0.43)	0.99	1.00	0.9972
G3N2P6_BOVIN	Keratin, type I cytoskeletal 42-like isoform X1	2.50 (0.16)	3.42 (.)	0.99	1.00	0.9972
G3X6N3_BOVIN	Serotransferrin	30.06 (1.84)	31.96 (1.90)	0.99	1.00	0.9972
G3X7A5_BOVIN	Complement C3	49.44 (4.98)	49.80 (5.02)	0.99	1.00	0.9811
G3X7D2_BOVIN	Chitinase-3-like protein 1	8.47 (1.04)	6.75 (0.91)	0.99	1.00	0.9972
G3X7N4_BOVIN	Phosphoglycerate kinase	0.53 (0.25)	0.72 (0.31)	0.99	1.00	0.9972
G3X8G9_BOVIN	Keratin, type II cytoskeletal 8	2.99 (0.58)	2.35 (0.54)	0.99	1.00	0.9972
G5E5H7_BOVIN	Latent-transforming growth factor β-binding protein 1 precursor	17.19 (1.40)	17.97 (1.43)	0.99	1.00	0.9972
G5E6M1_BOVIN	Polymeric immunoglobulin receptor	7.77 (0.96)	8.57 (1.00)	0.99	1.00	0.9972
G6PI_BOVIN	Glucose-6-phosphate isomerase	0.78 (0.33)	1.02 (0.42)	0.99	1.00	0.9972
G8JKW7_BOVIN	Serpin A3–5 isoform X2	8.81 (1.00)	11.38 (1.15)	0.99	1.00	0.9972
G8JKX4_BOVIN	Actin, aortic smooth muscle	1.65 (0.45)	2.51 (0.57)	0.99	1.00	0.9972
G8JKZ1_BOVIN	Ectonucleotide pyrophosphatase/phosphodiesterase family member 3	2.51 (0.77)	1.64 (0.58)	0.99	1.00	0.9972
GDIA_BOVIN	Rab GDP dissociation inhibitor α	1.86 (0.67)	1.51 (0.56)	0.99	1.00	0.9972
GDIB_BOVIN	Rab GDP dissociation inhibitor β	4.31 (1.59)	3.35 (1.30)	0.99	1.00	0.9972
GDIR1_BOVIN	Rho GDP-dissociation inhibitor 1	2.91 (0.57)	3.05 (0.59)	0.99	1.00	0.9972

		Treatment[1]		*P*-value		
Protein accession	**Description**	**RDP**	**RUP**	**Treatment**	**Day**	**Treatment × day**
GELS_BOVIN	Gelsolin	12.97 (1.21)	13.00 (1.21)	0.99	1.00	0.9972
GLCM1_BOVIN	Glycosylation-dependent cell adhesion molecule 1	3.74 (0.66)	2.60 (0.55)	0.99	1.00	0.9972
GNPTG_BOVIN	RecName: Full = *N*-acetylglucosamine-1-phosphotransferase subunit	1.07 (0.42)	0.95 (0.39)	0.99	1.00	0.9972
GPC1_BOVIN	Glypican-1	6.48 (0.86)	5.31 (0.79)	0.99	1.00	0.9972
GRP78_BOVIN	78 kDa glucose-regulated protein	6.31 (1.47)	5.56 (1.34)	0.99	1.00	0.9972
HEBP1_BOVIN	Heme-binding protein 1	0.96 (0.35)	0.71 (0.29)	0.99	1.00	0.9972
HP20_BOVIN	Protein HP-20 homolog	2.55 (0.54)	2.64 (0.55)	0.99	1.00	0.9972
HP251_BOVIN	Protein HP-25 homolog 1	1.84 (0.48)	1.82 (0.48)	0.99	1.00	0.9972
HP252_BOVIN	Protein HP-25 homolog 2	1.98 (0.47)	2.22 (0.51)	0.99	1.00	0.9972
HS71L_BOVIN	Heat shock 70 kDa protein 1-like	1.21 (0.37)	1.37 (0.42)	0.99	1.00	0.9972
HSP72_BOVIN	Heat shock-related 70 kDa protein 2	1.56 (0.42)	1.63 (0.48)	0.99	1.00	0.9972
HSP7C_BOVIN	Heat shock cognate 71 kDa protein	2.48 (0.73)	2.41 (0.81)	0.99	1.00	0.9972
HTRA1_BOVIN	Serine protease HTRA1	1.01 (0.42)	1.61 (0.57)	0.99	1.00	0.9972
IDHC_BOVIN	Isocitrate dehydrogenase (NADP) cytoplasmic	2.42 (1.27)	1.43 (0.78)	0.99	1.00	0.9972
ILEU_BOVIN	Leukocyte elastase inhibitor	2.85 (0.86)	3.37 (0.79)	0.99	1.00	0.9972
ITIH1_BOVIN	Inter-α-trypsin inhibitor heavy chain H1	6.93 (1.29)	6.58 (1.23)	0.99	1.00	0.9811
ITIH4_BOVIN	Inter-α-trypsin inhibitor heavy chain H4	7.01 (1.34)	7.26 (1.38)	0.99	1.00	0.9811
K1C17_BOVIN	Keratin, type I cytoskeletal 17	9.57 (1.05)	10.18 (1.08)	0.99	1.00	0.9972
K1C19_BOVIN	Keratin, type I cytoskeletal 19	3.88 (0.66)	3.93 (0.67)	0.99	1.00	0.9972
K1C25_BOVIN	Keratin, type I cytoskeletal 25	2.80 (0.57)	2.83 (0.58)	0.99	1.00	0.9972
K1C27_BOVIN	Keratin, type I cytoskeletal 27	2.80 (0.57)	2.83 (0.58)	0.99	1.00	0.9972
K1C28_BOVIN	Keratin, type I cytoskeletal 28	2.05 (0.49)	2.40 (0.53)	0.99	1.00	0.9972
K2C7_BOVIN	Keratin, type II cytoskeletal 7	4.44 (0.70)	3.68 (0.65)	0.99	1.00	0.9972
K2C71_BOVIN	Keratin, type II cytoskeletal 71	3.38 (0.62)	3.74 (0.65)	0.99	1.00	0.9972
K2C72_BOVIN	Keratin, type II cytoskeletal 72	2.71 (0.56)	4.23 (0.69)	0.99	1.00	0.9972
K2C73_BOVIN	Keratin, type II cytoskeletal 73	3.54 (0.63)	4.18 (0.69)	0.99	1.00	0.9972
K2C75_BOVIN	Keratin, type II cytoskeletal 75	7.87 (0.94)	7.64 (0.93)	0.99	1.00	0.9972
K2C78_BOVIN	Keratin, type II cytoskeletal 78	1.15 (0.38)	0.95 (0.33)	0.99	1.00	0.9972
K2C79_BOVIN	Keratin, type II cytoskeletal 79	6.00 (0.82)	5.67 (0.80)	0.99	1.00	0.9972
K2C8_BOVIN	Keratin, type II cytoskeletal 8	3.09 (0.59)	2.64 (0.56)	0.99	1.00	0.9972

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		Treatment[1]		*P*-value		
Protein accession	**Description**	**RDP**	**RUP**	**Treatment**	**Day**	**Treatment × day**
K2C80_BOVIN	Keratin, type II cytoskeletal 80	1.03 (0.35)	1.60 (0.42)	0.99	1.00	0.9972
KNG1_BOVIN	Kininogen I	9.68 (1.04)	9.27 (1.02)	0.99	1.00	0.9972
KNG2_BOVIN	Kininogen II	7.25 (0.91)	6.93 (0.89)	0.99	1.00	0.9972
KT222_BOVIN	Keratin-like protein KRT222	1.00 (0.34)	1.10 (0.36)	0.99	1.00	0.9972
LACB_BOVIN	Beta-LG	17.66 (1.40)	18.09 (1.42)	0.99	1.00	0.9972
LALBA_BOVIN	Alpha-LA	4.54 (0.72)	4.64 (0.72)	0.99	1.00	0.9972
LBP_BOVIN	Lipopolysaccharide-binding protein	3.57 (0.64)	2.70 (0.61)	0.99	1.00	0.9972
LUM_BOVIN	Lumican	1.02 (0.34)	0.89 (0.32)	0.99	1.00	0.9972
M0QVY0_BOVIN	Keratin 6A	10.72 (1.20)	11.25 (1.24)	0.99	1.00	0.9972
MA2B1_BOVIN	Lysosomal α-mannosidase	2.70 (0.57)	1.29 (0.41)	0.99	1.00	0.9972
MFGM_BOVIN	Lactadherin	17.55 (1.49)	18.55 (1.53)	0.99	1.00	0.9972
MUC1_BOVIN	Mucin-1	1.35 (0.41)	1.39 (0.40)	0.99	1.00	0.9972
MUC15_BOVIN	Mucin-15	0.41 (0.26)	0.36 (0.24)	0.99	1.00	0.9972
NELL2_BOVIN	Protein kinase C-binding protein NELL2	4.85 (0.98)	3.97 (0.88)	0.99	1.00	0.9972
NPC2_BOVIN	Epididymal secretory protein E1	2.45 (0.53)	2.92 (0.58)	0.99	1.00	0.9972
NUCB1_BOVIN	Nucleobindin-1	2.73 (1.02)	2.49 (1.01)	0.99	1.00	0.9972
PBIP1_BOVIN	Pre-B-cell leukemia transcription factor-interacting protein 1	0.66 (0.29)	0.56 (0.29)	0.99	1.00	0.9972
PEBP1_BOVIN	Phosphatidylethanolamine-binding protein 1	2.86 (0.58)	3.03 (0.60)	0.99	1.00	0.9972
PEDF_BOVIN	Pigment epithelium-derived factor	11.02 (1.12)	10.69 (1.10)	0.99	1.00	0.9972
PERL_BOVIN	Lactoperoxidase	18.59 (1.44)	17.22 (1.39)	0.99	1.00	0.9972
PGK1_BOVIN	Phosphoglycerate kinase 1	1.12 (0.46)	1.29 (0.52)	0.99	1.00	0.9972
PGRP1_BOVIN	Peptidoglycan recognition protein 1	0.50 (0.26)	0.59 (0.28)	0.99	1.00	0.9972
PGS1_BOVIN	Biglycan	0.83 (0.34)	0.40 (0.23)	0.99	1.00	0.9972
PIGR_BOVIN	Polymeric immunoglobulin receptor	21.79 (1.61)	22.61 (1.63)	0.99	1.00	0.9972
PKP1_BOVIN	Plakophilin-1	0.89 (0.41)	0.72 (0.34)	0.99	1.00	0.9986
PLAK_BOVIN	Junction plakoglobin	5.71 (1.11)	5.02 (1.03)	0.99	1.00	0.9972
PLMN_BOVIN	Plasminogen	3.29 (0.80)	2.71 (0.73)	0.99	1.00	0.9972
PNPH_BOVIN	Purine nucleoside phosphorylase	2.80 (0.85)	3.05 (0.92)	0.99	1.00	0.9972
PPIB_BOVIN	Peptidyl-prolyl *cis-trans* isomerase B	4.94 (0.76)	6.31 (0.85)	0.99	1.00	0.9972
PPIC_BOVIN	Peptidyl-prolyl *cis-trans* isomerase C	1.93 (0.47)	1.74 (0.44)	0.99	1.00	0.9972

J Dairy Sci. Author manuscript; available in PMC 2019 January 29.

		Treatment[1]		*P*-value		
Protein accession	**Description**	**RDP**	**RUP**	**Treatment**	**Day**	**Treatment × day**
PRDX2_BOVIN	Peroxiredoxin-2	0.77 (0.32)	0.66 (0.29)	0.99	1.00	0.9972
Q08DN1_BOVIN	Tumor necrosis factor receptor superfamily, member 21	0.71 (0.46)	0.90 (0.55)	0.99	1.00	0.9972
Q0IIA2_BOVIN	Odorant-binding protein-like	1.35 (0.55)	1.41 (0.65)	0.99	1.00	0.9972
Q0IIH5_BOVIN	Nucleobindin 2	7.75 (0.95)	8.75 (1.04)	0.99	1.00	0.9972
Q0V7N2_BOVIN	Complement C2	1.75 (0.47)	1.35 (0.39)	0.99	1.00	0.9972
Q148J4_BOVIN	RAB2A, member RAS oncogene family	0.84 (0.35)	0.87 (0.32)	0.99	1.00	0.9972
Q17QC7_BOVIN	Poliovirus receptor-related 2 (herpesvirus entry mediator B)	6.42 (0.88)	5.30 (0.81)	0.99	1.00	0.9972
Q17QL7_BOVIN	KRT15 protein	4.99 (0.75)	5.15 (0.77)	0.99	1.00	0.9972
Q1RMM9_BOVIN	Alpha-N-acetylgalactosaminidase	2.39 (0.53)	1.91 (0.47)	0.99	1.00	0.9972
Q2KIF2_BOVIN	Leucine-rich α−2-glycoprotein 1	1.26 (0.48)	1.10 (0.43)	0.99	1.00	0.9972
Q2NKV1_BOVIN	Angiogenin, ribonuclease, RNase A family, 5	1.08 (0.36)	1.88 (0.46)	0.99	1.00	0.9972
Q2NKZ9_BOVIN	Serine carboxypeptidase 1	0.34 (0.26)	0.62 (0.41)	0.99	1.00	0.9972
Q2TBX9_BOVIN	Integrin α FG-GAP repeat containing 1	1.43 (0.54)	1.36 (0.53)	0.99	1.00	0.9972
Q32PA1_BOVIN	CD59 molecule, complement regulatory protein	0.91 (0.32)	0.75 (0.30)	0.99	1.00	0.9986
Q3SYR8_BOVIN	Immunoglobulin J chain	1.52 (0.42)	1.84 (0.48)	0.99	1.00	0.9972
Q3SZH5_BOVIN	Angiotensinogen	6.40 (0.85)	4.73 (0.74)	0.99	1.00	0.9972
Q3SZZ9_BOVIN	FGG protein	5.75 (1.06)	5.18 (1.02)	0.99	1.00	0.9811
Q3ZBD1_BOVIN	RAB1A, member RAS oncogene family	0.60 (0.29)	0.58 (0.29)	0.99	1.00	0.9972
Q3ZBY4_BOVIN	Fructose-bisphosphate aldolase	0.93 (0.41)	0.79 (0.40)	0.99	1.00	0.9972
Q58DP6_BOVIN	Ribonuclease 4	2.36 (0.52)	2.69 (0.57)	0.99	1.00	0.9972
Q58DT6_BOVIN	Rho GDP dissociation inhibitor (GDI) α	2.14 (0.49)	2.17 (0.50)	0.99	1.00	0.9972
Q5E9G7_BOVIN	Cadherin 16	1.11 (0.38)	0.68 (0.30)	0.99	1.00	0.9972
Q5GN72_BOVIN	Alpha-1-acid glycoprotein	2.63 (0.61)	2.51 (0.61)	0.99	1.00	0.9972
RAB18_BOVIN	Ras-related protein Rab-18	0.33 (0.27)	0.43 (0.33)	0.99	1.00	0.9972
RAB1B_BOVIN	Ras-related protein Rab-1B	0.58 (0.32)	0.62 (0.34)	0.99	1.00	0.9972
RAB7A_BOVIN	Ras-related protein Rab-7a	1.94 (0.47)	2.41 (0.52)	0.99	1.00	0.9972
RAP1A_BOVIN	Ras-related protein Rap-1A	1.81 (0.56)	1.42 (0.48)	0.99	1.00	0.9972
RAP1B_BOVIN	Ras-related protein Rap-1b	1.98 (0.47)	1.52 (0.43)	0.99	1.00	0.9972
RET4_BOVIN	Retinol-binding protein 4	2.37 (0.52)	2.52 (0.54)	0.99	1.00	0.9972
RL40_BOVIN	Ubiquitin-60S ribosomal protein L40	1.00 (0.36)	1.24 (0.44)	0.99	1.00	0.9972

J Dairy Sci. Author manuscript; available in PMC 2019 January 29.

		Treatment[1]		*P*-value		
Protein accession	**Description**	**RDP**	**RUP**	**Treatment**	**Day**	**Treatment × day**
RNAS4_BOVIN	Ribonuclease 4	1.10 (0.37)	1.50 (0.42)	0.99	1.00	0.9972
RS27A_BOVIN	Ubiquitin-40S ribosomal protein S27a	1.00 (0.34)	1.21 (0.37)	0.99	1.00	0.9972
SAP_BOVIN	Prosaposin	8.11 (0.96)	8.32 (0.99)	0.99	1.00	0.9972
SPA31_BOVIN	Serpin A3–1	10.50 (1.09)	11.18 (1.13)	0.99	1.00	0.9972
SPA33_BOVIN	Serpin A3–3	6.94 (0.89)	8.08 (0.96)	0.99	1.00	0.9972
SPA34_BOVIN	Serpin A3–4	6.93 (0.93)	8.10 (0.99)	0.99	1.00	0.9972
SPA35_BOVIN	Serpin A3–5	6.81 (0.88)	9.41 (1.03)	0.99	1.00	0.9972
SPA36_BOVIN	Serpin A3–6	4.16 (0.70)	4.43 (0.71)	0.99	1.00	0.9972
SPA37_BOVIN	Serpin A3–7	4.04 (0.69)	2.83 (0.59)	0.99	1.00	0.9972
SPA38_BOVIN	Serpin A3–8	2.15 (0.49)	2.06 (0.52)	0.99	1.00	0.9972
ST14_BOVIN	Suppressor of tumorigenicity 14 protein homolog	3.92 (0.69)	3.29 (0.63)	0.99	1.00	0.9972
TETN_BOVIN	Tetranectin	3.19 (0.62)	3.03 (0.59)	0.99	1.00	0.9972
THRB_BOVIN	Prothrombin	2.06 (0.99)	1.61 (0.79)	0.99	1.00	0.9972
TPP1_BOVIN	Tripeptidyl-peptidase 1	0.89 (0.34)	1.40 (0.44)	0.99	1.00	0.9972
TRFE_BOVIN	Serotransferrin	31.03 (1.87)	32.92 (1.93)	0.99	1.00	0.9972
TRFL_BOVIN	Lactotransferrin	39.18 (2.10)	36.41 (2.02)	0.99	1.00	0.9972
TSP1_BOVIN	Thrombospondin-1	16.69 (1.38)	15.84 (1.33)	0.99	1.00	0.9972
TSP4_BOVIN	Thrombospondin-4	0.94 (0.33)	1.51 (0.42)	0.99	1.00	0.9972
TTHY_BOVIN	Transthyretin	1.06 (0.40)	0.82 (0.34)	0.99	1.00	0.9972
UBB_BOVIN	Polyubiquitin-B	1.00 (0.36)	1.24 (0.44)	0.99	1.00	0.9972
UBC_BOVIN	Polyubiquitin-C	1.00 (0.34)	1.21 (0.37)	0.99	1.00	0.9972
VAS1_BOVIN	V-type proton ATPase subunit S1	1.77 (0.45)	2.49 (0.53)	0.99	1.00	0.9972
VNN1_BOVIN	Pantetheinase	2.20 (0.58)	1.11 (0.39)	0.99	1.00	0.9972
VTDB_BOVIN	Vitamin D-binding protein	12.67 (1.22)	12.91 (1.23)	0.99	1.00	0.9972
XDH_BOVIN	Xanthine dehydrogenase/oxidase	25.45 (2.42)	26.53 (2.50)	0.99	1.00	0.9972
YKT6_BOVIN	Synaptobrevin homolog YKT6	1.01 (0.33)	0.89 (0.37)	0.99	1.00	0.9972
ZA2G_BOVIN	Zinc-α−2-glycoprotein	12.43 (1.24)	13.68(1.31)	0.99	1.00	0.9972

[1]Standard error values are presented in parentheses adjacent to means.