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The effects of dietary peuNDF-240 and rumen fermentable starch on the milk proteome of dairy cows

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Abstract

The milk proteome is affected by many factors, including diet, and characterizing the impact of diet on the milk proteome can aid in the identification of potential biomarkers that can be used as indicators of cow health and production in dairy systems. The objectives of this study were to 1) identify proteins that were affected by changes in dietary physically effective undegraded neutral detergent fiber (peuNDF-240) and rumen fermentable starch (RFS) levels and 2) determine if milking time affected any proteins due to proximity to feeding. Sixteen Holsteins cows were included in a 4x4 Latin square design experiment, including 4 28-d periods. Cows were milked thrice daily (4:30, 12:30, and 20:30 h). Samples collected from cows receiving two of the diets were analyzed in the current trial: diets were a high peuNDF-240 high RFS diet (HFHS; 8% peuNDF-240, 19.0 ±0.7% RFS) and a low peu-NDF-240 low RFS diet (LFLS; 6.35% peuNDF-240, 16.7±1.0% RFS). Milk samples were collected from each cow during 6 consecutive milkings on d 26-28 of each period. Samples were snap frozen and stored until analysis, and subsequently fractionated for protein isolation. Isolated proteins were quantified and labeled using TMT labels before being analyzed for low abundance proteins using LC-MS/MS. The results were analyzed using PROC MIXED in SAS (v 9.4) to identify the effect of treatment, time, and the interaction of treatment x time. There were 13 proteins identified that were either being affected by time, treatment, and the interaction of treatment x time. Proteins affected by diet, time, or the interaction of diet x time included serpin A3-1, a protease inhibitor, xanthine dehydrogenase/oxidase, which is involved in lipid droplet formation and secretion, and zinc-alpha-2-glycoprotein, which is involved in defense/immunity. Milk proteomics can help to further our understanding of how diet and other factors affect the cow in ways that might not be observed from looking at the cow.

Keywords: peuNDF, rumen fermentable starch, milk proteomics

Introduction

The proportions of the different carbohydrate fractions in the diet can affect dairy productivity. One important carbohydrate fraction in dairy diets is fiber, which encompasses the cell wall components of plant-based feedstuffs and can be measured using neutral detergent fiber (NDF) analysis (NRC, 2001). While NDF is an accurate measure of fiber in a feed based on cell wall components, it does not account for differences in digestibility of feeds (Mertens, 2015). A newer, more comprehensive term, peuNDF-240, combines particle size and the physically effective component (pe) fraction of the diet with the measured undigested NDF at 240 hours of fermentation (uNDF-240; Grant et al., 2018) to assess how particle size and uNDF-240 interact and affect fiber digestibility. Research has shown that through these shifts in digestibility, different levels of dietary peuNDF-240 can affect milk and milk protein yields. In one study examining the impact of dietary peuNDF-240 on dairy cow performance, the true milk protein produced by cows decreased as the peuNDF-240 of their diet increased (Smith et al., 2020). Similarly, another study that focused on uNDF-240 observed that cows fed higher uNDF-240 diets produced milk with lower protein percentages (Fustini et al., 2017). Another study by Smith (2021) reported that diets with lower levels of peuNDF-240 saw slightly higher daily milk yields.

Starch is another carbohydrate fraction that is highly fermentable in the rumen (NRC, 2001). Because of this, dairy systems often feed diets high in starch as the primary energy source (Giuberti et al., 2014) as a means to increase productivity, including milk production. The total starch content of the diet depends on the type of feeds being fed and can be influence by several factors, including grain type, location and climate, plant maturity, and farming practices

(Huntington, 1997). Once ingested, the extent and rate of starch fermentation can be affected by many different factors, including what part of the plant is being fed, the starch fraction, and the feed processing method (Giuberti et al., 2014; Huntington, 1997) Rumen fermentable starch (RFS) is the starch fraction that is fermented in the rumen by microbes. Apart from being an important energy source, it is also an important determinant of rumen health and environment. Research investigating dietary starch levels in the diet have demonstrated that dietary starch may affect milk and milk protein yields. One dairy study incorporated dietary straw that had been treated with sodium hydroxide to be more digestible observed that high total starch diets increased milk protein yields (Hanlon et al., 2020). Another study, however, observed that dietary total starch levels had no effect on milk yield or milk protein yield (Dann et al., 2014). A third study observed that cows in early lactation that were fed high total starch diets had lower true protein percentages (McCarthy et al., 2015). These disparities could be due to differences in lactation stage and in the starch levels and types in the diet.

Milk proteomics, or the study of the complete milk protein profile, could provide further insight into the impacts of peuNDF-240 and RFS in the diet if assessed for biomarkers of animal health and production (Greenwood and Honan, 2019; Nissen et. al., 2013). Previous research has demonstrated the rich matrix of proteins in bovine milk, including lactose synthesis-related proteins, and immune associated proteins (e.g. lactoperoxidase, serum amyloid A, immunoglobulins; Delosière et al., 2019; Fahey et al., 2019).

The milk proteome can be affected by a few factors, including breed, lactation stage, animal health status, milking time and diet (Bondan et. al. 2019; Greenwood and Honan, 2019). Specifically, shifts in dietary protein, starch and NDF have been found to impact the milk proteome (Li et al., 2015; Scuderi et al, 2020). These dietary shifts can occur due to processing methods of feeds, forages in a pasture, or different feed compositions (Li et al, 2015; Scuderi et al., 2020). The changes in the proteome due to diet can show how changes in the diet affects the animals, both in health and production.

In Smith (2021), cows fed high starch diets saw increased lactose levels ($P=0.04$), low fiber diets saw increased milk yields ($P=0.01$), and there were no observed health challenges. And increased milk yields should mean higher calcium levels in the milk due to increased calcium metabolism. Proteomic analysis is needed, however, to determine how these changes could impact individual milk proteins. Considering this previous research, the overarching hypothesis for this study was that the milk proteome produced by the cows would be differentially affected when fed different amounts of peuNDF-240 and RFS. It was hypothesized that the cows consuming the high fiber high starch (HFHS) diet would secrete a higher relative abundance of lactose synthesis-related proteins in their milk, cows fed the low fiber low starch (LFLS) diet would have a higher relative abundance of calcium binding proteins in their milk, and that the abundance of immune-associated milk proteins would be unaffected by diet. Given the different rates of rumen fermentation and nutrient releases of the RFS and the peuNDF-240, it was hypothesized that there would be a difference in milk protein profile between the milking times due to their different proximities relative to feeding schedule.

Materials and Methods

Sample Collection:

The animal study was completed at the William H. Miner Institute (Chazy, NY) according to approved protocols, as outlined by Smith (2021). A total of 16 multiparous Holstein cows were enrolled in this study, with 8 being ruminally cannulated. The study was done as a 4x4 Latin square design, with cows being blocked into one of four groups based on parity (2.69±0.58), days in milk (DIM; 84.8±15.2 days), and milk production. Cows were housed in a tie stall barn and were fed a total mixed ration (TMR) once a day at 14:00 h, at 1.1 times the expected daily intake. Four different diets were formulated based on an expected production of 61 kg milk/day: 1) a high peu-NDF-240 high RFS diet (HFHS; 8% peuNDF-240, 19.0±0.7% RFS), 2) a high peu-NDF-240 low RFS diet, 3) a low peu-NDF-240 high RFS diet, and 4) a low peu-NDF-240 low RFS diet (LFLS; 6.35% peuNDF-240, 16.7±1.0% RFS). Cows were milked thrice daily (4:30, 12:30 and 20:30 h). Milk samples were collected at six consecutive milkings on d 26-28 of each of the four study periods, with the first sample being collected at 20:30 h on d 26. Milk samples were snap frozen in a dry ice – ethanol bath, transported to the University of Vermont (Burlington, VT) on dry ice after collection, and stored at -80^{o} C until analysis.

Milk Proteomics:

For this study, a subset of the collected milk samples was used for analysis. Milk proteomic analysis was performed according to Fahey et. al. (2020), using milk samples from the HFHS and LFLS diets from periods 2 and 3 of the study. For analysis, samples were thawed overnight at 4^{o}C in the refrigerator. Milk samples collected at the same milking time (i.e. 4:30, 12:30 or 20:30 h) were equally combined to create a composite milk sample for each milking time, to create 3 composite samples per animal per period. If a sample was missing or the tube was destroyed in transport, then a composite was not created, and milk from the single milking was used to represent the milking time. In total, 4 animal replicates per treatment, 8 between the two treatments, were assessed, with 3 milk samples per animal (total of 24 individual milk samples for proteomic analysis). For fractionation of the milk samples, a protease inhibitor cocktail (Protease Inhibitor Cocktail for use with mammalian cell and tissue extracts, Sigma-Aldrich, St. Louis, MO) was added, and the samples were centrifuged at 4000 x g for 15 min at 4 ^{o}C. The fat layer was then removed, and the samples were centrifuged again to remove any residual fat. Approximately 0.11 g of calcium dichloride ($CaCl_2$) was added to each 12 mL composite sample to deplete caseins, followed by acetic acid (Thermo Fisher Scientific, Fair Lawn, NJ) to acidify the sample to a pH of less than 4.4. Ultracentrifugation of the samples was performed at 189,000 x g for 70 min at 4^{o}C, and the supernatant was collected and frozen at -80^{o}C overnight. The protein concentration of each sample was determined using a bicinchoninic assay (BCA) kit as per the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). An 80 μg sample was digested with trypsin and labeled via amine-reactive compounds using Tandem Mass Tag 10plex Isobaric Labeling Kits as per the manufacturer's instructions (Pierce Biotechnology). Samples were then submitted to the Vermont Genetics Network Core Proteomics Facility (Burlington, VT) for LC-MS/MS analysis. Peptide identification analyses were performed according to the Vermont Genetics Network Proteomics Facility protocol as outlined by Fahey et al. (2020). Product ion spectra were searched against the *Bos taurus* data base. Raw files were searched as one continuous file.

Statistical Analysis:

Proteomic results were analyzed using the PROC MIXED procedure of SAS (v 9.4) to determine the impact of diet (HFHS vs LFLS), milking time (4:30, 12:30, 20:30 h), and the interaction of diet x time. Diet, milking time, and the interaction of diet x time were used as fixed effect, and cow ID was used as a random effect in the model. Significance was declared if $P< 0.05$, and a trend was declared if $0.05<P<0.1$.

Results

Characterized Proteome:

In this study, 40 proteins (Table 1) were identified through LC-MS/MS analysis, including 13 that were affected by diet, time, or their interaction.

Proteins Affected by Diet:

Six proteins were affected by diet (Table 2). Beta-1,4-galactosyltransferase 1 (P08037) tended to have a higher relative abundance due to the LFLS diet *(P*=0.08). Serpin family G member 1 (E1BMJ0) tended to have a higher relative abundance in samples from cows fed the HFHS diet *(P*=0.08). G3MXB5, an uncharacterized protein, had higher relative abundances at 4:30 and 12:30 h for HFHS fed cows and a higher relative abundance within the LFLS diet at 20:30 h *(P*=0.04). Serpin A3-1 (Q9TTE1) had higher relative abundances in samples from cows fed the LFLS diet (P=0.04). Folate receptor alpha (P02702) tended to have a higher relative abundance in samples from cows fed the LFLS diet at 4:30 and 20:30 h and a higher relative abundance due to the HFHS diet at 12:30 h *(P*=0.09). Kappa-casein (A0A140T8A9) had higher relative abundances in samples from cows fed the HFHS diet at 4:30 and 12:30 h and higher relative abundance due to the LFLS diet at 20:30 h *(P*=0.09).

Proteins Affected by Time:

Eight proteins were affected by time (Table 3). Xanthine dehydrogenase/oxidase (F1MUT3) had a higher relative abundance at the 12:30 h milking time *(P*=0.01) for both diets. Apolipoprotein A-I (P15497) tended to have a higher relative abundance at the 12:30 h milking *(P*=0.07). Osteopontin (P31096) also tended to have a higher relative abundance at the 12:30 h *(P*=0.07). Zinc-alpha-2-glycoprotein (Q3ZCH5) tended to have a lower relative abundance for samples collected at 12:30 h *(P*=0.07). Serotransferrin (G3X6N3) had a higher relative abundance in the 12:30 h milking time *(P*=0.04). G3MXB5, an uncharacterized protein, had higher relative abundance due to the HFHS diet at both 4:30 and 12:30 h, with a much higher relative abundance in samples from cows fed the HFHS diet at 12:30 h *(P*=0.001). Kappa-casein (A0A140T8A9) tended to have a much higher relative abundance in samples from cows fed the LFLS diet at 20:30 h *(P*=0.09). Serpin A3-1 (Q9TTE1) tended to have a much higher relative abundance in samples from the LFLS fed cows at 20:30 h *(P*=0.09).

Proteins Affected by Diet x Time:

There were six proteins that were affected by the interaction of diet x time (Table 4). Kappa-casein (A0A140T8A9) had higher relative abundance in samples from cows fed the HFHS diet at 4:30 h and 12:30 h samples, and a higher relative abundance in the LFLS diet at 20:30 h *(P*=0.02). Serpin A3-1 (Q9TTE1) had a higher relative abundance in samples from cows fed the LFLS diet when compared to the HFHS diet *(P*=0.04) and is much higher at the 20:30 h for the LFLS diet *(P*=0.05). Angiogenin-1 (P10152) tended to higher relative abundance in the HFHS diet at 4:30 and 20:30 h, and a higher relative abundance in the LFLS diet at 12:30 h *(P*=0.08). Folate receptor alpha (P02702) tended to have a higher relative abundance in samples from the HFHS fed cows at 12:30 h and a higher relative abundance in samples from cows fed the LFLS diet at 4:30 and 20:30 h *(P*=0.06). G5E5T5 had a higher relative abundance in cows fed the LFLS diet for the 4:30 and 20:30 h milkings and a higher relative abundance in samples collected from cows fed the HFHS diet at 12:30 h *(P*=0.05). G3MXB5 had a higher relative abundance for the HFHS treatment at 4:30 and 20:30 h, and a higher relative abundance for LFLS fed cows at 12:30 *(P*=0.001)

Discussion

Proteins Affected by Diet:

Two proteins were affected only by diet, and they were beta-1,4-galactosyltransferase 1 and serpin family G member 1. Smith (2021) observed that HF diets had higher lactose in milk

(*P*=0.04), and beta-1,4-galactosyltransferase 1 is an enzyme involved in lactose synthesis (D'Ambrosio et al., 2008; Qasaba et al., 2008) but we observed a higher abundance of this protein in milk from LFLS fed cows, hence our result was not expected. One reason for this apparent disparity may be because beta-1,4-galactosyltransferase 1 is one part of the lactose synthase, and because of this, lactose synthesis is reliant on the levels of all parts of the synthase (Keenan et al., 1972). The other protein involved in the lactose synthase is alpha-lactalbumin (Keenan et al, 1972), and in this study alpha-lactalbumin was not identified as we removed higher abundance proteins through fractionation. Therefore, the alpha-lactalbumin levels could have remained unchanged in the LF diets, which would mean that less lactose would be produced, even with an increase in beta-1,4-galactosyltransferase 1. Beta-1,4-galatsyltransferase could be a candidate to pursue as a biomarker of animal intake and rumen health since it increases due to LF diets.

In this study, Serpin family G member 1 also tended to have a higher relative abundance in milk from the cows fed the HFHS diet. Serpin family G member 1 (E1BMJ0) functions include activation of immune response (Felice et al., 2021). While little research is available to explain this correlation, in Smith (2021), cows fed HS diets had reduced meal size and more meals over the course of a day. There was no effect on rumen pH, but this change in meal size and frequency could impact the relative abundance of serpin family G member 1.

Proteins Affected by Time:

Five proteins were affected only by time in this study: xanthine dehydrogenase/oxidase, apolipoprotein A-I, osteopontin, zinc-alpha-2-glycoprotein, and serotransferrin. Xanthine dehydrogenase/oxidase (F1MUT3) was identified by Lu et al. (2014) as a protein that aided in lipid droplet formation and secretion, and this is related milk fat in the milk. This protein had a higher relative abundance at the 12:30 h milking time for both diets. This is not in agreement with Bondan et al. (2019), which observed that the morning milking had higher milk fat levels. It would be expected that the xanthine dehydrogenase/oxidase levels would be highest when milk fat levels were the highest. But, in this study, milk fat levels could have been higher at a different milking than in Bondan et al. (2019) due to differences in diet and the fact that the cows in Bondan et al. (2019) were fed in the morning and the cows in this study were fed at 14:00 h.

Apolipoprotein A-I (P15497), a protein linked to milk fat synthesis (Larsen et al., 2007), also tended to have a higher relative abundance at 12:30 h when compared to 4:30 and 20:30 h. In the research reported by Larsen et al. (2007) the abundance of apolipoprotein A-I increased as milk yield increased, and in Bondan et al. (2019) milk fat was greatest in the morning. In the current study, Apolipoprotein A-I was at its highest abundance at 12:30 h, which is different from what was reported by Bondan et al. (2019). Again, this could be due to differences in diets and feeding times.

Osteopontin (P31096) is a bioactive protein that is involved in immune and developmental processes (Christensen et al, 2021). Osteopontin tended to have a higher relative abundance at 12:30 h when compared to 4:30 and 20:30 h. There has been little research that explains this correlation, but it could be related to release of nutrients. Further research is needed to understand this correlation. Osteopontin could be a candidate for a biomarker for rumen health since an unhealthy rumen environment can lead to an inflammatory response.

Zinc-alpha-2-glycoprotein (Q3ZCH5), also known as ZAG, has functions that include antigen processing and presentation and defense/immunity (D’Ambrosio et al, 2008; Felice et al, 2021). In this study, ZAG tended to have a lower relative abundance in samples collected at 12:30 h and was not affected by either the HFHS or LFLS diets. Previous research investigating

ZAG has identified that it is impacted by dietary protein profile (Li et al, 2015), milking number after calving, and the interaction of milking number and parity (Fahey et al., 2020). In relation to this trial, it could be that ZAG was impacted due the proximity to feeding time, with higher relative abundances in the milkings that were closer to the feeding time.

Serotransferrin (G3X6N3) had a higher relative abundance in samples collected at 12:30 h when compared to 4:30 and 20:30 h. Serotransferrin, which is encompassed within the larger family of transferrin glycoproteins, has been previously identified in milk (Schanbacher et al., 1993) and has iron-binding properties. This is distinct from lactoferrin; research in zebu suggests that its presence in milk is related to be related to immune regulation and host defense (Chopra et al., 2020).

Proteins Affected by Diet x Time:

There were six proteins that were affected by the interaction of diet x time. These included kappa-casein, serpin A3-1, angiogenin-1, folate receptor alpha, as well as two uncharacterized proteins: G5E5T5, and G3MXB5. Kappa-casein (A0A140T8A9) was identified; however, this is a residue remaining after precipitating the caseins and is unlikely a reliable indicator correlated to the treatments in the current trial.

Serpin A3-1 (Q9TTE1), a protease inhibitor (Larsen et al., 2007), had a higher relative abundance in milk from cows fed the LFLS diet when compared to samples from the cows fed the HFHS diet and higher relative abundance at 20:30 h in samples from cows fed the LFLS diet. This is consistent with previous research. Smith (2021) observed that LF diets had increased milk yields, and in Larsen et al. (2007) Q9TTE1 increased as milk yield increased. Due to this relationship of increased protein abundance with increased milk yields, Serpin A3-1 could be used as a biomarker for production.

Angiogenin-1 tended to a higher relative abundance in the HFHS diet at 4:30 and 20:30 h, and a higher relative abundance in the LFLS diet at 12:30 h. Angiogenin-1 (P10152) functions include modulating immune response (Komolova and Fedorova, 2002) and has been previously identified in bovine milk (Fahey et al, 2020). In this study, cows were fed at 14:00 h, so the relative abundances for angiogenin-1 could be related to the release of nutrients of the different diets.

Folate receptor alpha (P02702) function is associated with fat metabolism and transport (D’Ambrosio et al, 2008). Folate receptor alpha tended to have a higher relative abundance for the HFHS diet at 12:30 h, and a higher relative abundance in samples from cows fed the LFLS diet at 4:30 and 20:30 h. Folate receptor alpha has been consistently identified in milk and colostrum in multiple species, including cows and water buffalo (D’Ambrosio et al., 2008; Nissen et al., 2017).

G5E5T5 is an uncharacterized protein but has been suggested to be Immunoglobulin heavy constant mu, which functions as an antigen processing protein that supports presentation and activation of immune response (Felice et al, 2008; Scuderi et al., 2020). G5E5T5 had a higher relative abundance in samples from LFLS fed cows at both 4:30 and 20:30 h, and a higher relative abundance in the HFHS diet at 12:30 h. G3MXB5 is another uncharacterized protein, and Scuderi et al. (2019) identified G3MXB5 and reported this protein as IgA heavy chain constant region (partial). G3MXB5 had a higher relative abundance in samples from cows fed the HFHS treatment at 4:30 and 20:30 h, and a higher relative abundance for the LFLS treatment at 12:30 h. Both proteins are associated with immune response and should be further investigated in terms of their function and presence before being considered as a biomarker.

Conclusions

Milk proteomics can allow us to understand how factors, such as change in diet or time of milking, can affect the milk proteome. It can also be a tool to identify biomarkers that one day could be used to non-invasively assess the health of lactating cows. In this study, 13 proteins were affected by diet, time, and/or their interaction. These included proteins related to immune response and function, fat metabolism and transport, and lactose synthesis. No calcium binding proteins were identified to be affected by diet in this study, and immune-associated proteins were affected by diet, time, and their interaction. Proteins that should be considered as candidates for biomarkers and should have further research include Serpin A3-1, G5E5T5, G3MXB5, and beta-1,4-galactosyltransferase 1. The mechanism behind changes in the proteome related to both diet and time is not completely understood and needs to be further studied.

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Table 1. Identified milk proteins with their relative abundances in samples collected at 4:30, 12:30, and 20:30 hours from cows fed a diet of either high fiber high starch (HFHS) or low fiber low starch (LFLS).

	4:30 h				12:30 h				20:30 h						
	Abundance		SE		Abundance		SE		Abundance		SE		P-Values		
Accession	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	TXD	D	T
A0A140T897	100.5	97.2	14.6	15.3	110.1	118.6	20.4	12.6	103.4	96.9	13.5	20.3	0.89	0.97	0.55
A0A140T8A9	96.5	91.5	19.4	20.2	107.4	98.8	27.3	16.2	84.6	200.5	17.7	27.3	0.02	0.09	*0.09
A0JNP2	93.0	75.8	49.0	50.9	121.1	85.3	62.3	46.0	115.9	115.3	47.0	62.0	0.91	0.67	0.68
E1BMJ0	100.1	55.1	21.1	22.0	137.0	98.2	29.8	17.6	86.3	56.2	19.3	29.7	0.95	0.08	0.14
F1MCF8	117.5	90.1	27.7	28.4	143.7	118.2	39.2	21.6	114.8	180.3	24.9	39.2	0.28	0.87	0.38
F1MLW8	92.0	119.4	21.6	22.7	89.5	110.7	30.1	18.8	114.4	122.8	20.0	30.1	0.91	0.38	0.72
F1MUT3	82.2	81.3	17.2	18.1	155.4	110.1	23.7	15.4	78.8	69.1	16.1	23.6	0.42	0.28	**0.01
F1MZ96	123.6	114.7	25.7	26.8	132.2	129.9	36.2	21.6	121.5	72.6	23.6	36.2	0.69	0.43	0.50
F1N726	82.3	64.0	18.0	18.4	121.3	86.9	25.4	14.0	71.9	50.9	16.1	25.4	0.91	0.16	0.14
F6R3I5	87.2	98.5	11.5	11.9	103.7	91.4	16.3	9.4	91.9	101.2	10.5	16.3	0.61	0.80	0.92
G3MXB5	104.7	87.6	23.1	23.6	194.5	108.5	26.5	22.5	85.4	90.9	22.7	26.4	0.01	0.04	**0.001
G3MXZ0	104.8	93.1	20.9	22.0	91.6	108.1	29.1	18.3	104.5	116.7	19.4	29.1	0.76	0.78	0.83
G3N0V0	127.9	116.2	25.0	26.2	174.3	131.4	34.6	22.0	121.8	82.7	23.2	34.6	0.78	0.22	0.19
G3N2D7	118.8	123.5	19.5	19.9	112.3	114.3	27.5	15.2	120.9	152.4	17.5	27.5	0.77	0.48	0.59
G3X6N3	102.0	121.1	25.0	25.6	167.1	162.9	35.3	19.4	115.2	46.5	22.5	35.3	0.30	0.44	**0.04
G3X7A5	119.2	118.6	20.8	21.3	147.3	130.8	29.4	16.2	112.7	64.7	18.7	29.4	0.59	0.27	0.15
G5E513	123.0	135.3	27.1	27.9	143.4	111.6	32.6	26.0	124.3	87.5	26.3	32.5	0.28	0.36	0.36
G5E5H7	144.2	155.9	65.7	69.0	74.4	103.6	90.4	58.5	140.6	82.8	61.4	90.1	0.80	0.93	0.62
G5E5T5	106.3	116.1	21.0	22.0	168.2	90.2	29.4	17.9	100.7	150.3	19.3	29.4	0.05	0.76	0.68
O02853	140.9	114.6	40.7	42.4	186.8	94.2	52.7	37.9	131.5	112.4	38.9	52.4	0.53	0.22	0.86
P00711	117.8	118.5	19.4	19.8	113.0	115.5	27.4	15.1	106.1	143.9	17.4	27.4	0.65	0.45	0.89
P01888	77.8	81.4	37.4	38.3	53.0	122.1	52.9	29.1	61.7	28.9	33.6	52.9	0.51	0.70	0.60
P02662	97.7	95.4	41.0	42.0	82.6	134.0	58.0	31.9	92.9	95.0	36.9	58.0	0.81	0.65	0.95

P02663	79.4	99.1	39.3	40.2	57.2	109.1	55.6	30.6	87.3	59.2	35.3	55.6	0.69	0.69	0.93
P02666	88.3	43.0	28.5	29.1	62.5	79.3	40.3	22.2	99.4	83.1	25.6	40.3	0.62	0.57	0.71
P02702	83.4	103.7	29.4	30.8	112.9	106.6	39.5	26.8	69.9	205.1	27.8	39.3	0.06	0.09	0.26
P02754	112.0	125.7	12.0	12.3	115.1	105.0	16.9	9.3	104.1	96.0	10.8	16.9	0.60	0.89	0.39
P08037	91.9	128.1	21.0	21.4	110.3	123.4	29.6	16.3	96.9	156.5	18.8	29.6	0.65	0.08	0.78
P10152	154.8	84.3	25.0	25.9	83.1	120.2	35.3	20.3	88.4	138.6	22.7	35.3	0.08	0.81	0.80
P10790	84.0	116.0	17.0	17.7	103.5	106.2	24.0	14.1	82.0	111.6	15.6	24.0	0.70	0.20	0.91
P15497	84.2	104.5	24.0	25.0	145.1	114.8	33.9	19.8	70.4	50.7	21.9	33.9	0.58	0.67	**0.07
P18892	94.7	106.6	26.6	27.2	181.6	111.5	37.7	20.7	113.6	107.2	23.9	37.7	0.38	0.39	0.30
P24627	105.2	99.4	17.8	18.6	165.3	99.5	24.9	15.2	90.6	103.0	16.4	24.9	0.16	0.27	0.18
P31096	109.1	143.8	74.8	76.5	287.5	255.0	105.8	58.2	64.7	41.6	67.2	105.8	0.90	0.92	**0.07
P79345	108.5	140.3	14.8	15.1	105.5	98.2	20.9	11.5	100.3	101.9	13.3	20.9	0.45	0.53	0.29
P80195	113.1	125.2	22.6	23.1	127.0	99.2	31.9	17.6	123.3	172.3	20.3	31.9	0.38	0.60	0.39
P81265	102.0	111.4	14.8	15.2	120.7	111.4	21.0	11.5	106.6	109.7	13.3	21.0	0.85	0.94	0.83
Q0P569	138.2	121.9	33.7	34.4	97.2	167.8	47.6	26.2	97.4	110.7	30.3	47.6	0.51	0.47	0.72
Q3ZCH5	140.6	166.8	28.3	29.6	64.3	101.5	39.7	24.1	140.1	158.4	26.0	39.7	0.96	0.33	**0.07
Q95114	85.5	80.4	17.8	18.2	99.0	92.4	25.2	13.8	86.0	100.0	16.0	25.2	0.86	0.96	0.78
Q9TTE1	111.9	113.2	33.4	34.2	104.7	125.3	47.3	26.0	99.5	291.2	30.0	47.3	0.05	0.04	*0.09

*T=time, D=diet, * linear time effect, ** quadratic time effect, significance at P<0.5, trend at 0.05<P<0.1*

Table 2. Milk proteins affected by diet with cows either fed a high fiber high starch (HFHS) or low fiber low starch (LFLS) diet.

		4:30 h				12:30 h				20:30 h				
		Abundance		SE		Abundance		SE		Abundance		SE		P-Values
Accession	Description	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	D
Q9TTE1	Serpin A3-1	111.9	113.2	33.4	34.2	104.7	125.3	47.3	26.0	99.5	291.2	30.0	47.3	0.04
G3MXB5	Uncharacterized Protein	104.7	87.6	23.1	23.6	194.5	108.5	26.5	22.5	85.4	90.9	22.7	26.4	0.04
P08037	Beta-1,4-galactosyltransferase 1	91.9	128.1	21.0	21.4	110.3	123.4	29.6	16.3	96.9	156.5	18.8	29.6	0.08
E1BMJ0	Serpin family G member 1	100.1	55.1	21.1	22.0	137.0	98.2	29.8	17.6	86.3	56.2	19.3	29.7	0.08
A0A140T8A9	Kappa-casein	96.5	91.5	19.4	20.2	107.4	98.8	27.3	16.2	84.6	200.5	17.7	27.3	0.09
P02702	Folate receptor alpha	83.4	103.7	29.4	30.8	112.9	106.6	39.5	26.8	69.9	205.1	27.8	39.3	0.09

significance at $P<0.5$, trend at $0.05<P<0.1$

Table 3. Milk proteins affected by milking time.

		4:30 h				12:30 h				20:30 h				
		Abundance		SE		Abundance		SE		Abundance		SE		P-Values
Accession	Description	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	T
G3MXB5	Uncharacterized protein	104.7	87.6	23.1	23.6	194.5	108.5	26.5	22.5	85.4	90.9	22.7	26.4	**0.001
F1MUT3	Xanthine dehydrogenase/oxidase	82.2	81.3	17.2	18.1	155.4	110.1	23.7	15.4	78.8	69.1	16.1	23.6	**0.01
G3X6N3	Serotransferrin	102.0	121.1	25.0	25.6	167.1	162.9	35.3	19.4	115.2	46.5	22.5	35.3	**0.04
P15497	Apolipoprotein A-I	84.2	104.5	24.0	25.0	145.1	114.8	33.9	19.8	70.4	50.7	21.9	33.9	**0.07
P31096	Osteopontin	109.1	143.8	74.8	76.5	287.5	255.0	105.8	58.2	64.7	41.6	67.2	105.8	**0.07
Q3ZCH5	Zinc-alpha-2-glycoprotein	140.6	166.8	28.3	29.6	64.3	101.5	39.7	24.1	140.1	158.4	26.0	39.7	**0.07
A0A140T8A9	Kappa-casein	96.5	91.5	19.4	20.2	107.4	98.8	27.3	16.2	84.6	200.5	17.7	27.3	*0.09
Q9TTE1	Serpin A3-1	111.9	113.2	33.4	34.2	104.7	125.3	47.3	26.0	99.5	291.2	30.0	47.3	*0.09

*significance at $P<0.5$, trend at $0.05<P<0.1$, * linear time effect, ** quadratic time effect.*

Table 4. Milk proteins affected by the interaction of diet x time with cows fed either a high fiber high starch (HFHS) or low fiber low starch (LFLS) diet and milked at 4:30, 12:30 and 20:30 h daily.

		4:30 h				12:30 h				20:30 h				
		Abundance		SE		Abundance		SE		Abundance		SE		P-Values
Accession	Description	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	HFHS	LFLS	DXT
G3MXB5	Uncharacterized protein	104.7	87.6	23.1	23.6	194.5	108.5	26.5	22.5	85.4	90.9	22.7	26.4	0.01
A0A140T8A9	kappa-casein	96.5	91.5	19.4	20.2	107.4	98.8	27.3	16.2	84.6	200.5	17.7	27.3	0.02
G5E5T5	Uncharacterized protein	106.3	116.1	21.0	22.0	168.2	90.2	29.4	17.9	100.7	150.3	19.3	29.4	0.05
Q9TTE1	Serpin A3-1	111.9	113.2	33.4	34.2	104.7	125.3	47.3	26.0	99.5	291.2	30.0	47.3	0.05
P02702	Folate receptor alpha	83.4	103.7	29.4	30.8	112.9	106.6	39.5	26.8	69.9	205.1	27.8	39.3	0.06
P10152	Angiogenin-1	154.8	84.3	25.0	25.9	83.1	120.2	35.3	20.3	88.4	138.6	22.7	35.3	0.08

T=time, D=diet, significance at P<0.5, trend at 0.05<P<0.1