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The Effects of Diet on the Bovine Milk Proteome

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THE EFFECTS OF DIET ON THE BOVINE MILK PROTEOME

A Thesis Presented

by

Richard Anthony Scuderi

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The Faculty of the Graduate College

of

The University of Vermont

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ABSTRACT

Protein is an important fraction within bovine milk. This milk protein is not only vital for calf growth and development, but also includes bioactive proteins and peptides that have been shown to enhance the health of animals and humans. Research efforts are focusing on factors, such as nutrition, that can influence the quantity and profile of proteins within the bovine milk proteome. The research outlined herein investigated the impact of diet on the bovine milk proteome. The first experiment examined whether dietary inclusion of grape marc (GM), a condensed tannin (CT) containing by-product from the viticulture industry, could alter the bovine milk proteome through altered nitrogen (N) metabolism. In this experiment, 10 lactating Holstein cows were fed either 2.0 kg dry matter (DM)/ cow/ day of beet pulp: soy hulls in a 50% mixture (control), or 1.5 kg DM/ cow/ day of GM as part of a balanced dairy cow ration for a 28-d trial. Milk samples were obtained for analysis of the high- and low-abundance protein fractions. Skimmed milk samples collected for high-abundance protein analysis were measured using high performance liquid chromatography (HPLC), and liquid-chromatography tandem mass spectrometry (LC-MS/MS) was used to identify proteins in the low-abundance protein enriched fraction. Skimmed milk samples collected for low-abundance milk protein analysis were fractionated and enriched to remove higher abundance proteins. Enriched milk samples were then digested and labeled with isobaric tandem mass tags (TMT) prior to protein identification using LC-MS/MS analysis. There were no changes in the high-abundance protein fraction in response to diet; however, 16 of 127 low-abundance proteins were identified at different relative-abundances due to diet ($P \leq 0.05$). While there were no alterations in the metabolic or N status of animals due to GM supplementation, the 12% change in the low-abundance milk protein fraction highlighted the potential for dietary alteration of the bovine milk proteome.

A second experiment evaluated the inclusion of alternative forage crops (AFC) as a means to alter the bovine milk proteome. In this experiment, both the skimmed milk and milk fat globule membrane (MFGM) protein fractions were included in analysis. Milk samples were collected from 16 lactating Jersey cattle included in a 21-d grazing experiment, where cows were offered one of two diets. The control group (CON, n=8) grazed a grass-legume pasture mixture containing orchardgrass (*Dactylis glomerata*), timothy (*Phleum pratense*), Kentucky bluegrass (*Poa pratensis*), and white clover (*Trifolium repens*). The treatment group (AFC, n=8) grazed a similar base pasture that was strip-tilled with oat (*Avena sativa*), buckwheat (*Fagopyrum esculentum*), and chickling vetch (*Lathyrus sativus*) so that the AFC species comprised 10% of the AFC group's pasture DM intake (DMI). Milk samples were collected for HPLC analysis of the high abundance milk proteins, and LC-MS/MS analysis of the low abundance protein enriched skim milk fraction and MFGM-associated protein fraction. Cows that grazed pastures containing AFC had higher α 1-CAS content ($P = 0.005$), and higher relative-abundances of 7 low-abundance proteins within the skim milk and MFGM fractions ($P \leq 0.05$). While it is plausible that the inclusion of AFC in pasture increased nutrient availability to the mammary gland, the specific mechanisms that could have caused the shifts observed remain unclear. Further investigation is necessary to fully understand the role of diet and the milk protein profile.

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LIST OF ABBREVIATIONS

α -s1 CAS	Alpha-s1 casein
α -s2 CAS	Alpha-s2 casein
α -LA	Alpha-lactalbumin
β -CAS	Beta casein
β -LG	Beta lactoglobulin
β -LGA	Beta lactoglobulin variant A
β -LGB	Beta lactoglobulin variant B
κ -CAS	Kappa casein
γ CAS	Gamma casein
2DE	2-Dimensional gel electrophoresis
ABCG2	ATP-binding cassette sub-family G member 2
ACE	Angiotensin converting enzyme
AFC	Alternative forage crops
ALB	Serum albumin
APOE	Apolipoprotein E
B2M	Beta 2-microglobulin
BCA	Bicinchoninic acid assay
BLAST	Basic local alignment search tool
BTN	Butyrophilin
C9	Complement component C9
CAS	Casein
CD36	Platelet glycoprotein 4

CH ₃ CN	Acetonitrile
CH ₄	Methane
CLU	Clusterin
CON	Control
CP	Crude protein
CSG	Cool-season grass
CST3	Cystatin-C
CT	Condensed tannin
D0	Covariate, day 0
DHIA	Dairy herd improvement association
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H ₂ SO ₄	Sulfuric acid
HSP90AA1	Heat shock protein HSP 90-alpha
GIT	Gastrointestinal tract
GO	Gene ontology
GM	Grape marc
HPLC	High-performance liquid chromatography

IACUC	Institutional Animal Care and Use Committee
iTRAQ	Isobaric tags for relative and absolute quantification
KNG1	Kininogen-1
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MCP	Methyl Cellulose Precipitable assay
MEC	Mammary epithelial cell
MFGM	Milk fat globule membrane
MHC	Major histocompatibility complex
MS/MS	Tandem mass spectrometry
MUN	Milk urea nitrogen
N	Nitrogen
NEFA	Non-esterified fatty acids
PIgR	Polymeric immunoglobulin receptor
PUFA	Polyunsaturated fatty acids
PUN	Plasma urea nitrogen
QSOX1	Sulfhydryl oxidase
RDP	Rumen degradable protein
RUP	Rumen undegradable protein
SAA3	Mammary-associated Serum Amyloid A3 protein
SCC	Somatic Cell Count
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SILAC	Stable isotope labeling with amino acids in cell culture
TMR	Total mixed ration

TMT	Tandem mass tags
WSC	water soluble carbohydrates
ZAG	Zinc-alpha-2-glycoprotein

CHAPTER 1: LITERATURE REVIEW

There is growing interest to further elucidate factors that influence the bovine milk proteome due to the presence of bioactive proteins and peptides. Nutrition is one of many known factors that can alter milk composition, and there is growing evidence suggesting a relationship between diet and the composition of the milk protein profile, (also known as the milk proteome). This relationship between nutrition and the milk proteome is of particular interest as it could allow producers the opportunity to naturally influence the presence of bioactive proteins and peptides in milk; however, the mechanisms and drivers of this relationship are unclear. This review will discuss the composition of the bovine milk proteome, summarize findings relating to dietary alteration of the bovine milk proteome, and discuss some potential feeding strategies that may impact the proteomic composition of milk.

1.1 Bovine milk

1.1.1 Composition

Bovine milk is recognized as a nutritionally-significant food source for the neonatal calf. Total solids account for approximately 12% of bovine milk with roughly 4% fat content, 3.2% protein content, 5% lactose, and the remaining content comprising of vitamins and minerals (Mills et al., 2011); however, content is variable with a reported average of 3.2% protein, 3.6% fat, and 4.7% lactose (Pereira, 2014). In addition to the nutritional significance of milk for calf nutrition, it also serves an important medium for transferring other components involved in calf growth and development. Colostrum, for example, is the only source for transfer of the adaptive

immune response in newborn ruminants since their placental structure does not allow for the transfer of immunoglobulins from maternal blood flow (Larson et al., 1980). Considering the composition of bovine milk, it has also been considered to be a plentiful source of nutrition for human nutrition. For example, dairy products constitute one of the largest sources of dietary saturated fat in human diets (Shingfield et al., 2008). Bovine milk is also an important source for protein, which has been augmented in terms of proportion in human diets through the use of different nutritional supplements and products (Korhonen, 2009; Mills et al., 2011). While the protein content is only around 3.2% in bovine milk, it is the composition within the protein profile itself that is of significant interest due to the content of bioactive proteins and peptides. Hence, further attention has been paid towards the bovine milk proteome.

1.1.2 Bioactive proteins and peptides in milk

Bioactive proteins can be defined as proteins that possess roles beyond nutrition (Lonnerdal, 2013). In bovine milk, many different bioactive proteins and peptides have been identified. Bioactive peptides in milk are released from their native protein structure post-consumption in the gastrointestinal tract (GIT) through hydrolysis via proteolytic enzymes (Mills et al., 2011). Examples of bioactive peptides include those derived from casein (CAS) proteins, such as α 1-Casokinin-5, which has exhibited angiotensin converting enzyme (ACE) activity (Clare and Swaisgood, 2000). Irascidin is another casein peptide derived from α 1-CAS that has been shown to exhibit antimicrobial properties (Lopez-Exposito and Recio, 2008). Some bioactive proteins present in milk are entirely resistant to proteolysis, further suggesting biological significance (Korhonen, 2009; Mills et al., 2011; Lonnerdal, 2013). Lactoferrin and lactadherin are two examples

of proteins that are largely resistant to mediated enzymatic degradation in the GIT, and are known to exhibit biological responses post-consumption (Mills et al., 2011; Vanderghem et al., 2011; O’Riordan et al., 2014). Furthermore, it has been established that post-translational modification of proteins and peptides can further enhance their functionality. In bovine milk for example, the glycosylation of bioactive proteins lactoferrin and lactadherin, has been suggested to provide some resistance to hydrolysis in the GIT (O’Riordan et al., 2014). Bioactive proteins and peptides in bovine milk have been reported in the literature to play significant roles in the body. Characterized functions include, but are not limited to, growth, cellular signaling, antimicrobial, immunomodulatory, and anti-carcinogenic properties (FitzGerald and Meisel, 2003; Kilara and Panyam, 2003; Yamamoto et al., 2003; FitzGerald et al., 2004; Gauthier et al., 2006; Lopez-Fandino et al., 2006; Pihlanto, 2006; Lopez-Exposito and Recio, 2008). More comprehensive characterization of the bioactive proteins and peptides within bovine milk is ongoing with effort to further understand the functions and classifications of specific bioactive proteins and peptides.

1.1.3 Bovine milk protein profile (proteome)

1.1.3.1 High-abundance proteins

CAS proteins comprise 80% of bovine milk protein content with the remaining 20% being classified as whey proteins (D’Amato et al., 2009). CAS are phosphoproteins that are synthesized within mammary epithelial cells (MECs), and are present in unique micelle formations due to their hydrophobic properties (Livney, 2009). There is a number of different CAS isoforms including α CAS (α -s1 CAS, and α -s2 CAS), β , and κ CAS, as well as γ -CAS which is released in milk via hydrolysis with the enzyme plasmin

from the β -CAS structure (DePeters and Cant, 1992). Within the whey fraction, α -lactalbumin (α -LA), and the A and B variants of β -lactoglobulin (β -LGA, β -LGB) are present in the highest concentrations comprising of 25% and 50% of the total whey fraction, respectively (Roncada et al., 2012). Due to their higher concentrations, CAS proteins, α -LA, and both variants of β -LG are classified as high-abundance proteins in bovine milk.

1.1.3.2 Low-abundance proteins

The remaining proteins found within the whey fraction of bovine milk constitute the low-abundance protein fraction. Low-abundance proteins include a diverse protein fraction with origins ranging from within and outside of the MEC as well as possessing an array of functions and properties, some of which are known bioactives even at minor concentrations as was compiled in a review by Mills et al. (2011). Examples of low-abundance proteins include mammary-associated serum amyloid A (SAA3), lactoferrin, zinc-alpha-2-glycoprotein (ZAG), and lactoperoxidase, all of which have been consistently identified in the bovine milk proteome. It has been demonstrated in the literature that MECs will utilize peptides circulating in blood as a source of amino acids for protein synthesis, of which requires specific transport mechanisms that could provide a route of transfer for low-abundance proteins to enter into milk if not utilized or synthesized by the MEC (Bequette et al., 1998). Additionally, it has been established that paracellular and transcellular pathways are utilized by the MEC in order to transport nutrients, predominately the latter form using secretory vesicles, which could be a feasible mode of entry for non-mammary derived proteins to enter into milk (Shennan and Peaker, 2000).

1.1.3.3 Milk fat globule membrane (MFGM) associated proteins

When milk fat is synthesized on or within the outer surface of the smooth endoplasmic reticulum within the MEC, it is released into the cytosol as a lipid droplet with an outer layer of proteins before further aggregating into larger droplets (Cavaletto et al., 2008). The larger lipid droplets are then secreted from the apical surface of the MEC into the alveolar lumen (Figure 1.1). While being released from the MEC into the alveolar lumen, the phospholipid bilayer membrane of the MEC along with integral proteins envelopes the milk fat droplet in an exocytotic manner (Cavaletto et al., 2008). The milk fat globule membrane (MFGM) surrounds the triacylglycerol core and contains a diverse number of low-abundance proteins within the MFGM itself (Spitsberg, 2005; Cavaletto et al., 2008; Vanderghem et al., 2011). Cytosolic contents can also be occasionally captured and incorporated into the MFGM during apical secretion from the MEC, this cytosolic inclusion is commonly known as a cytoplasmic crescent (Cavaletto et al., 2008). High-abundance proteins have been identified within the MFGM; while Reinhardt et al. (2006) suggested that the identification of major milk proteins in the cytosolic crescent were contaminants, it could be possible for high-abundance proteins to be entrapped within the MFGM as a result from cytoplasmic crescent formation. The MFGM proteome is largely populated with butyrophilin, mucins, xanthine dehydrogenase/ oxidase, and CD36, all of which are known to exhibit bioactive properties (Spitsberg, 2005; Cavaletto et al., 2008; Dewettinck et al., 2008). While only constituting 1-4% of the total bovine milk proteome (Cavaletto et al., 2008; Yang et al., 2015), the bioactivity of several MFGM proteins have been

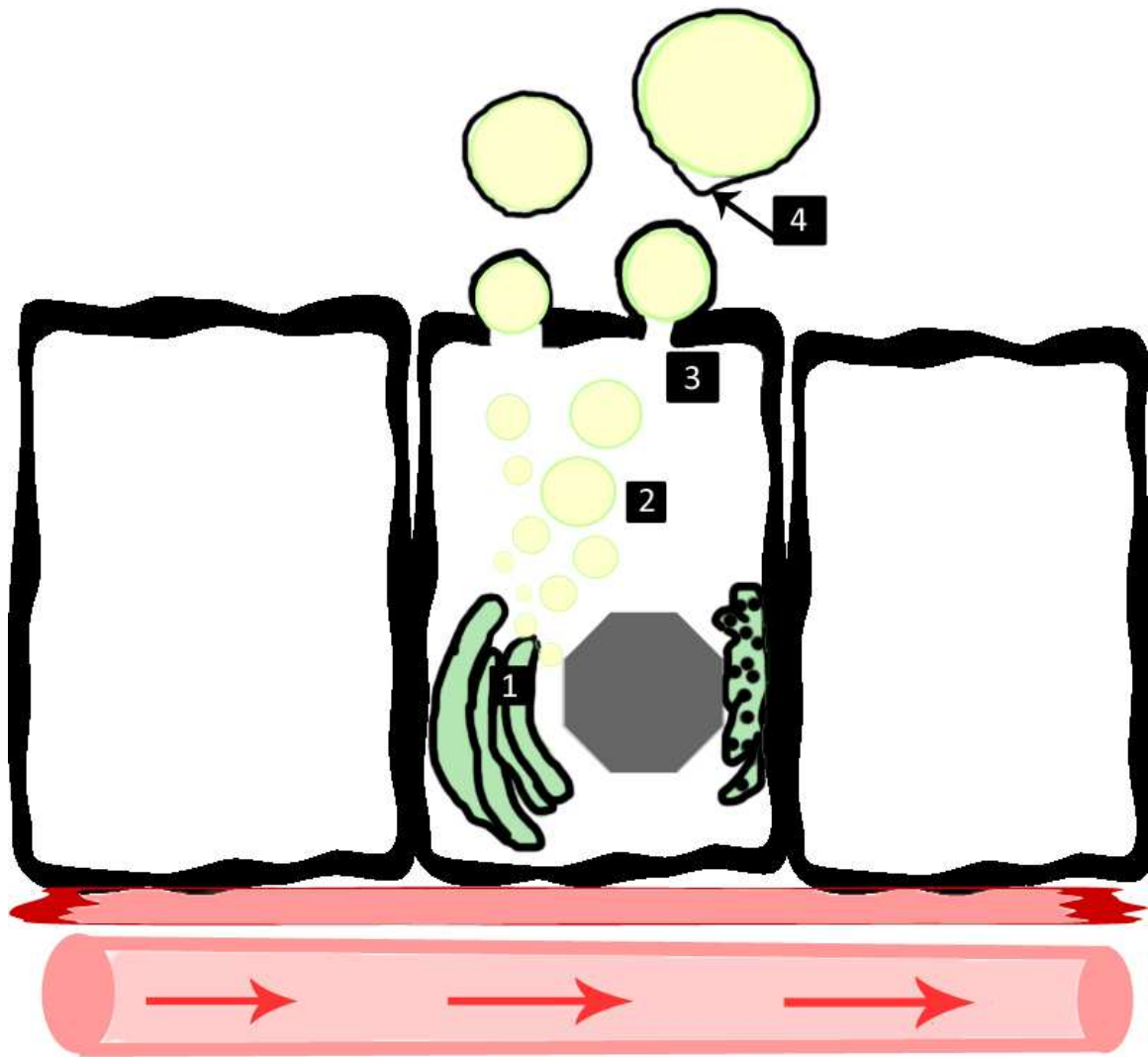


Figure 1.1. Milk fat globule membrane (MFGM) synthesis in a mammary epithelial cell (MEC). Milk fat synthesis begins in the mammary epithelial cell (MEC) on the surface of the smooth endoplasmic reticulum (SER) where 1) lipid droplets are released into the cytosol surrounded by a phospholipid monolayer with polar proteins. The micro droplets will 2) adjoin in the cytosol while migrating to the apical surface of the MEC. After which, the milk fat globule is 3) surrounded by the MEC's phospholipid bilayer membrane originating from the apical surface. The resulting secretion is the milk fat globule membrane (MFGM) which comprises of a lipid droplet surrounded by a tri-layer membrane (green layer, SER; black layer, MEC cell membrane) that contains integral and surface proteins. Incorporation of cytosolic contents during secretion into the alveolar lumen is known as the 4) cytoplasmic crescent (adapted from Cavaletto et al. 2008, and Cebo 2012).

extensively outlined in the literature (Spitsberg, 2005, O'Riordan et al., 2014).

Additionally, researchers have demonstrated the diversity and complexity of the MFGM proteome by identifying hundreds of different proteins. Reinhardt et al. (2006) identified 120 proteins in MFGM, whereas Yang et al. (2015) identified 520 proteins in the MFGM across multiple mammalian species including dairy cattle.

The unique tri-layer membrane structure of the MFGM has been suggested to offer partial protection for the proteins and peptides from hydrolysis in the GIT post-consumption, and could consequently provide an effective route to the small intestine for absorption (Dewettinck et al., 2008; Vanderghem et al., 2011). The structure and organization of some proteins within the MFGM can partially prevent exposure to hydrolytic enzymes within the GIT; including lactadherin, xanthine dehydrogenase and oxidase, which were only partially hydrolyzed when MFGM was digested *in-vitro* (Vanderghem et al., 2011; Ye et al., 2011).

1.1.4 Milk proteomic approaches

Due to its' complexity, several different analytical techniques have been developed to characterize the bovine milk proteome. Proteomics has been emerged as an entire research discipline in protein biology, and has been effectively used as a technique to examine the bovine milk proteome (Roncada et al., 2012). Additionally, the use of techniques such as bottom-up proteomics, where proteins are broken down into peptides before identification, has allowed researchers to expand the number of proteins identified in experiments (Twyman, 2014). To date, researchers have been successful in identifying thousands of proteins in bovine milk using proteomic approaches; however, the

methodology has been variable across studies. Important factors to consider when using proteomics are sample preparation to isolate the proteins within a sample, as well as further separation of the proteins and subsequent identification (Twyman, 2014). Steps to fractionate bovine milk are needed to enhance the identification of low-abundance proteins (Roncada et al., 2012). Most analytical methods now begin with a light centrifugation step that separates milk into three distinct layers: the cream layer, the fluid phase, and a pellet; where the fat layer comprises of low-abundance proteins within the MFGM, high abundant and low abundant proteins are contained in the fluid phase, and the pellet mainly comprises of cells (Nissen et al., 2013). Following the sample preparation, the fractionation of the selected layer into more specific fractions prior to physical separation and identification of proteins occurs. Isolation of the low-abundance proteins in the fluid phase can be accomplished either through acid precipitation, ultracentrifugation, microfiltration, or a combination thereof (Nissen et al., 2013). Nissen et al. (2013) characterized the proteins present in two of these distinct layers (the fluid phase, the cell pellet) in addition to using a non-fractionated sample as a control. Further characterization of the whey fraction was also conducted to compare three different fractionation techniques: acidification, microfiltration, and centrifugation. Using 2D-LC-MS/MS analysis, 376 distinct proteins were identified in total, and ultracentrifugation at 100,000xg was the most effective method to expand the identification of low-abundance proteins in the fluid phase (Nissen et al., 2013).

To further fractionate samples, several different approaches have been used to separate proteins in complex biological samples, including two-dimensional gel electrophoresis (2DE) SDS-PAGE and ion-exchange both cationic and anionic in nature.

2DE methodology was first applied to fractionate bovine milk in 1982 (Roncada et al., 2012), and has been used by many investigators, including Reinhardt and Lippolis (2006) who identified 120 proteins in the MFGM (Reinhardt and Lippolis, 2006). Some disadvantages to 2DE include issues with resolution, sensitivity, and accurate representation (Twyman, 2014). Reinhardt and Lippolis (2006) described previous research where 2DE resulted in limited number of unique MFGM proteins identified (6-45 different MFGM proteins) which was likely related to the loss of membrane proteins due to either difficulties with getting proteins into solution prior to isoelectric focusing, or precipitation of membrane proteins at the isoelectric focus point; however, they addressed those problems by using SDS-PAGE to improve solubility issues with membrane proteins. Furthermore, they utilized high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) to improve resolution that is normally lost during 2DE workflows (Reinhardt and Lippolis, 2006). More recently, Li et al. (2015) identified 13 unique protein spots using 2DE that varied in abundance from different dietary treatment groups, and further validated their results with western blotting. Another proteomics separation approach includes multi-dimensional liquid chromatography (Le et al. 2011), which is often coupled to mass spectrometry instruments (Twyman, 2014). There are several different types of chromatography that utilize different biochemical properties to allow for separation of proteins including affinity chromatography based on ligand-binding properties, size exclusion chromatography, ion exchange chromatography in varying different degrees, and reversed phase chromatography (Twyman, 2014). For example, Le et al. (2011) analyzed the bovine milk and colostrum proteome while utilizing an ion-exchange approach

resulting in the identification of 293 proteins, 36 and 40 of which were uniquely identified in colostrum and milk samples, respectively.

More recently, an enrichment technique that utilizes affinity based ligand binding has been used to fractionate the higher-abundance proteins from the low-abundance proteins through the use of combinatorial peptide ligands. The ProteoMiner kit (Biorad; Hercules, CA) utilizes a library of combinatorial peptide ligands in bead form to concentrate low-abundance proteins in samples. Higher abundance proteins will bind with high affinity to specific ligands in the beads, and are eluted through a series of washes; hence, leaving behind a concentrated sample of low-abundance proteins (Righetti and Boschetti, 2008; Fonslow et al., 2011). D'Amato et al. (2009) identified 149 low-abundance proteins using ProteoMiner as a step in their proteomic approach. Tacoma et al. (2016) also incorporated ProteoMiner treatment prior to SDS-PAGE separation, and identified 935 low-abundance proteins in the skim milk fraction.

Protein identification in recent years has significantly improved through the use of mass:charge ratio (m/z) determination by mass spectrometry, which allows for accurate calculation and determination of molecular masses (Twyman, 2014). It is common to analyze samples using a label-free approach; however, the use of isobaric labeling has become increasingly used. Yang et al. (2015) identified 520 proteins in the MFGM of samples obtained from several mammalian species, which were labeled with isobaric tags for relative and absolute quantification (iTRAQ; Applied Biosystems, Foster City, CA) and were separated using strong cationic-exchange chromatography before further separation and identification using LC-MS/MS. Much like iTRAQ, Tandem Mass Tags (TMT; Thermo Scientific, Rockford, IL) have also been used in other proteomic-based

experiments with success. Recently, Tacoma et al. (2017b) utilized TMT labeling to identify 162 proteins in skimmed colostrum samples that were subjected to different heat treatment times. Isobaric tagging has provided researchers a better method by which to distinguish differences between multi-plexed samples by evaluating the relative-abundance of labeled proteins when compared to a specific label. This is a particularly useful tool in treatment comparisons, for example when comparing the relative abundance of proteins in milk samples collected from cows consuming different diets.

1.2 Dietary factors influencing milk composition

Nutrition is one factor that can influence milk composition. The mechanisms of nutritional impact on the composition of bovine milk are important to understand in order to create predicted and consistent responses. Producers are often times compensated based on specific milk components, including protein (Jones and Heinrichs, 2017). Therefore, having a better understanding of how feeding can affect milk composition can provide financial gains for producers, as well as provide producers with an avenue to naturally alter their milk composition. There are numerous studies and reviews on the effects of diet and specific milk components (Sutton 1989; DePeters and Cant, 1992). A review by Sutton (1989) summarized the effects of diet on milk composition, and identified milk fat as having the greatest potential for dietary alteration citing forage:concentrate as one of the possible mechanisms. For example, it had been demonstrated elsewhere that reductions of the forage:concentrate ratio resulted in decreased milk fat concentration with pronounced reductions when forage proportions fell below 50% on a dry matter (DM) basis (Journet and Chilliard, 1985; Thomas and Martin, 1988). The review further noted that milk protein was marginally

impacted by dietary protein, noting that severe deficits in dietary protein generally resulted in lower (0.1 - 0.2% decreases) milk protein content (Gordon, 1977). Similarly, Jenkins and McGuire (2006) outlined research that aligned with Sutton's hypotheses: milk fat has the greatest potential for change as a result from alterations in diet, followed by milk protein; however, lactose is very unlikely to change. Another extensive review by DePeters and Cant (1992) also highlighted the important role of dietary energy in milk protein concentration, identifying a positive correlation between energy intake and milk protein percent ($r = 0.42$) with 0.015% increases in milk protein percent for every megacalorie increase in net energy (Emery, 1978). These increases in milk protein percent were also in conjunction with increases in milk yield as well (Emery, 1978). It appears that total milk protein content and yield can be difficult to influence through diet; however, research has demonstrated changes in the composition of the milk protein fraction as a result from diet.

1.3 Dietary factors influencing the bovine milk proteome

In 1999, Christian et al. reported changes in the CAS and whey protein profiles when animals consumed diets that contained varying forms of available energy and protein (Christian et al., 1999). Friesian as well as Friesian-Jersey cross-bred cattle were used in three different 34-day experiments, where they were fed varying degrees of energy and protein. In the first experiment, cows were either fed a control diet of silage and pasture hay, and treatment cows were fed energy as oats, wheat, or barley and protein in the form of protected sunflower meal in the first experiment. During the second experiment, cows were fed different protein supplements including canola meal, cottonseed, and lupin with wheat as the energy source. Varying degrees of lupin and

wheat were fed to cows during the third experiment (Christian et al., 1999). In this series of experiments, these researchers observed changes in the CAS of milk with increases in total CAS concentration (g/kg) from cows consuming wheat in the first experiment, and lupin in the second experiment when compared to the control, as well as increases in the whey concentration (g/kg) when cows consumed diets that contained wheat in the first experiment, and lupin in the second experiment compared to the control diet (Christian et al., 1999). Additional differential changes occurred in the CA composition as a result from diet, including shifts in α -s1 CAS and α -s2 CAS. The changes observed in milk protein composition from these experiments suggest that they occurred as a result from dietary alteration of protein availability and energy supplementation.

More recently, Li et al. (2015) observed changes in protein composition through offering dairy cattle varying combinations of energy and protein fractions in the diet. Holstein cows were included in 21 day periods as part of a 4x4 latin square design, and were fed diets that were 1) synchronized for rapid fermentation using steam-flaked corn (SFC) and solvent-extracted soybean meal (SSBM) (diet B), 2) fine ground corn (FGC) and heat-treated soybean meal (HSBM) were synchronized for slow fermentation (diet C), 3) unsynchronized for rapid fermentation using SFC and HSBM (diet D), or 4) slow fermentation using FGC and SSBM (diet A; Li et al., 2015). The authors reported increases in proteins including zinc-alpha-2-glycoprotein (ZAG) and α -LA when diets were synchronized for rapid energy and N (diets B and D), suggesting a dietary role in the composition of the bovine milk proteome (Li et al., 2015).

Tacoma et al. (2017a) further examined the effects of varying proportions of dietary RDP and RUP on the bovine milk proteome. Six Holstein cows were used in a

double-crossover design with 3 periods lasting 21 days each, and were fed diets that were formulated with either a higher dietary inclusion of RDP through the use of urea, soybean meal, and canola meal or RUP through the use of bypass amino acid supplements. Total casein content was higher from cows fed a higher RDP diet, suggesting that altering RDP and RUP can affect downstream nutrient availability for MEC utilization and synthesis of mammary milk proteins (Tacoma et al., 2017a). Despite the alterations in the diet and changes in the high-abundance proteins, it appeared that diet had no effect on the low-abundance protein fraction in this study. When considering that the animals in both treatment groups were fed isonitrogenous and isoenergetic diets, it appears that protein type alone may not be sufficient to alter the bovine milk proteome (Tacoma et al., 2017a). The data from Tacoma et al. (2017a) aligns with the suggestion by Sutton (1989) where it was suggested that no direct connection between dietary RDP and RUP fractions on milk protein (Sutton, 1989), which is contrary to the work by Christian et al. (1999) and Li et al. (2015) when examining solely the dietary protein alterations.

However, based on the conclusions from Christian et al. (1999) and Li et al. (2015), it appears that the potential for altering the proteomic content of milk through dietary means cannot be changed through dietary protein type alone, but could potentially occur by also altering the amounts of energy as well. Although it is not well understood, Li et al. (2015) cited N-synchronization, or the establishment of a synchrony pattern between energy and nitrogen in the diet (Cabrita et al., 2006), as a possible dietary mechanism for altering bovine milk protein composition. It is plausible, and hence important to consider, that the relationship between dietary protein and available energy

could be responsible for altering the proteomic composition of bovine milk, not simply the individual components.

1.4 Nitrogen metabolism in ruminants

1.4.1 Overview

Aside from the unique process of microbe-mediated carbohydrate fermentation, the rumen microbes also impact protein digestion absorptive profiles and subsequent metabolism. The RDP portion of dietary crude protein is susceptible to degradation by microbial proteolytic enzymes, resulting in polypeptides and free amino acids. The free amino acids also face two fates in the rumen: incorporation into microbial protein, or deamination resulting in ammonia and carbon-backbones in the form of carboxylic acids (Van Soest, 1994). The released ammonia can be absorbed from the rumen and into the bloodstream where it is either recycled or excreted, or the microbes can utilize this ammonia along with the carboxylic acids and ATP as substrates for microbial protein synthesis. While RDP undergoes drastic changes in amino acid profile due to microbial degradation and yields microbial protein, RUP remains intact and untouched as it passes through the rumen. Both microbial protein and digestible RUP are then digested in the abomasum and upper small intestine before amino acids, peptides, and proteins in some instances are absorbed across the lower small intestine.

1.4.2 Microbial protein

The resulting flow of amino acids from digestion, also known as duodenal amino acid flow, derives its' amino acid composition from RUP, endogenous protein, and microbial protein (Sok et al., 2017). Microbial protein accounts for a significant

contribution of amino acid supply for ruminant animals (Firkins, 1996), where some authors have claimed microbial protein accounting for at least 65% of the amino acid supply being absorbed in the small intestine (Clark et al., 1992). In an attempt to understand the composition of microbial protein, researchers are beginning to consider the importance of different fractions contributing to microbial protein in the rumen including protozoa, which may be contributing 10-30% of the total amino acid supply in the duodenal flow (Sok et al., 2017). Considering the importance of microbial protein's contribution to the total amino acid duodenal flow available for absorption, focus has been turned towards utilizing diet to influencing the output of microbial protein for consequent improvements in production. Including feeding practices that allow for maximum microbial protein synthesis as a result from substrate availability in the rumen with dietary N and energy.

1.4.3 Nitrogen synchronization theory

The establishment of a synchronization pattern between energy and nitrogen (N) availability in the diet is defined as the N Synchronization Theory (Cabrita et al., 2006), and it can be achieved by changing dietary ingredients, altering feeding patterns and frequencies, or a combination of these options. It has been described that establishing a synchronized diet can potentially increase microbial protein output by increasing microbial capture of N. Furthermore, if such theory holds true, N synchronization may increase fermentation efficiency and ultimately improve feed utilization (Cabrita et al., 2006). Although there is evidence to support this theory, there are also a number of extraneous variables and contradictory results surrounding this theory. Dewhurst et al. (2000) cited an experiment conducted by Rooke et al. (1987) that supported N

synchronization theory with increases in microbial protein synthesis through the supplementation of glucose and casein; however, they summarized a number of other studies that yielded mixed results, including one from Kim et al. (1999) where there were no changes in microbial N as a result from synchrony. Furthermore, another review by Cabrita et al. (2006) cited a number of studies that evaluated the alteration of forage and concentrate proportions in the diet on rumen N synchronization, and it was concluded that factors including forage intake and subsequent fermentation rate, the chemical composition of the concentrate that was used, and the interaction between the forage and concentrate added confounding variables, thereby making it difficult to assess whether the effects observed were truly a result of synchrony (Hagemeister et al., 1981; Rode et al., 1985; Sniffen and Robinson, 1987; McAllan et al., 1988; Yang et al., 2001). While the research on N synchronization is limited and inconclusive, there is some speculation that ruminants may have developed a mechanism to avert the inefficiencies in N capture as a result from asynchronization; however, further research is needed (Cabrita et al., 2006).

1.5 Altered protein digestion by dietary inclusion of condensed tannins

Collectively, tannins are polyphenolic compounds in plants; however, they are well studied for their ability of precipitating proteins in solution (Cabrita et al., 2006). Tannins have two distinct forms: hydrolysable and condensed tannin(s) (CT) (Acamovic and Brooker, 2005; Waghorn, 2008; Nudda et al., 2015). While hydrolysable tannins induce toxicity after degradation in the GIT, CT in moderate concentrations can alter N absorption patterns in ruminant animals without adverse effects (Acamovic and Brooker, 2005). CT have been found in pasture grasses and legumes, as well as by-products such

as grape marc (Waghorn et al., 1987; Makkar, 2003). When consumed, unbound CT can form a stable CT-protein complex through binding in a neutral pH environment (ranging from 3.5-7.0) in the GIT ultimately making them unavailable for further degradation and absorption until the pH deviates from a neutral range (< 3.5, > 8.5), in which case dissociation has been shown to occur (Waghorn, 2008; Woodward et al., 2009; Greenwood et al., 2012; Nudda et al., 2015). This CT-protein binding has been observed in ruminant species, and it is hypothesized that some CT may bind to proteins in the reticulorumen making proteins unavailable for microbial degradation due to the lack of enzymes necessary to inhibit or degrade the binding process and associated complex respectively (Waghorn et al., 1987; Nudda et al., 2015). It has been further proposed that pH changes further along the GIT can cause the dissociation of CT-protein complexes, and previous research has further supported this theory (Waghorn et al., 1987; Makkar, 2003; Nudda et al., 2015). By binding to proteins in the rumen, CT-inclusive diets alter N metabolism by decreasing RDP available to the rumen microbes, and effectively increasing the degradable RUP fraction available for proteolysis by the animal in the abomasum and small intestine (Waghorn et al., 1987; Makkar, 2003).

1.6 Impact of Grape marc on nitrogen metabolism

Grape marc (GM) is defined as the byproduct of viticulture consisting of seeds, skins, and occasionally stems resulting from grape pressing (Moate et al., 2014). In viticulture, 1 ton of grapes are required to produce 150 gallons of wine pressing (Gerling, 2011). Generally, 10-20% of the harvested grape weight remains after pressing (Ben-Gera and Kramer, 1969). To further place this into perspective, the US state of Vermont harvested an average of 376 tons (752,000 lbs) or roughly 341,818 kg

of grapes in the 2015 and 2016 seasons (Keough, 2017), leaving behind an estimated range of 34,182 – 68,364 kg of GM. On a national level in 2015, the United States produced around 425,533 - 851,066 tons of GM from 4,255,330 tons of grapes for wine production (USDA, 2017). The remaining GM faces two fates: repurpose, or disposal. When discarded or composted due to lack of use, GM can be detrimental to the environment (Tsiplakou and Zervas, 2008). Hence alternative uses have been implemented, such as the incorporation into livestock feeding practices in the dairy industry. Research has revealed that GM is an effective supplement for dairy producers due to its' availability, low-cost, and lack of known negative detriments to total milk production (Nudda et al., 2015). GM generally has a low dry matter (DM) content, but contains notable amounts of fiber, lignin, and varying degrees of ether extract (seeds) as well as water soluble carbohydrates (WSC) especially in instances when grape pressing is not thorough, resulting with remaining intact grapes (Nudda et al., 2015; Manso et al., 2016). In addition to being a source of fiber, and WSC in some cases, other compounds in GM have been identified with potentially beneficial nutritive effects.

GM is also rich in various different polyphenols, including CT, that have an array of bioactivity including antiatherosclerotic, antibacterial, anticarcinogenic, and anti-inflammatory properties (Moate et al., 2014; Santos et al., 2014). Furthermore, GM is rich in various forms of vitamin E and linoleic acid, which have known antioxidant characteristics (Nudda et al., 2015). Other potentially beneficial compounds are present in GM at smaller concentrations, including anthocyanins, which have been attributed to having anti-inflammatory, anti-carcinogenic, and even anti-mutagenic activities among

others (Yi et al., 2009). The varying abundances of these different bioactive components in feedstuffs such as GM can provide other beneficial implications beyond improving the health and production of animals, including potential environmental benefits. Moate et al. (2014), for example, reported 20% and 23% lower methane (CH₄) production in cows that were fed dried GM and ensiled GM, respectively. While CT have been proposed to cause the changes in N-metabolism, Moate et al. (2014) were unable to definitively conclude which component caused the decreases in CH₄; however, they hypothesized that either the fat content, lignin, DL-tartaric acid, or the CTs could be responsible for their observations (Moate et al., 2014). Furthermore, Greenwood et al. (2012) reported a lower output of urinary N due to altered N partitioning in cows that consumed GM on pasture (Greenwood et al., 2012). The observations made in that experiment were likely a result from the CTs in the GM, which provides another example for the potential of CT-containing feeds to alter animal metabolism including N partitioning in ruminants.

1.7 Inclusion of alternative forage crops to alter nitrogen partitioning in grazing livestock

While by-products like GM are effective in altering N metabolism, there are other feedstuffs that can reach levels of efficacy, including different forage species. Alternative forage crops (AFC) are warm-season grasses and legumes that are incorporated into pastures, some of which contain secondary plant compounds that can alter N metabolism in ruminants (Ramirez-Restrepo and Barry, 2005). The practice of including AFC species into pasture has been increasingly utilized by pastoral dairy systems, especially organic dairy farms in the northeastern United States, where

producers are challenged with a decreased period of growth in cool-season grass species during the late spring and early summer (known as the summer slump). Considering the grazing requirements established by the USDA for organic producers where 30% of an animal's total dry matter intake (DMI) needs to originate from pasture during the 120 day grazing season (USDA 2010), maintaining pasture mass for optimal DMI can prove to be challenging during the summer months. Along with increased pasture biomass available for consumption, the incorporation of AFC species in a traditional grass-legume pasture has a number of potential benefits for pastoral dairy farmers including an increased drought resistance due to their genetic adaptations in warmer climates (Tilman and Downing, 1994).

Since warm season AFC species are able to adapt and thrive in warmer climates, the opportunity for improved chemical composition during the summer months can allow for the opportunity to improve animal production. Totty et al. (2013) utilized alternative pasture species as means for improving N utilization in grazing animals through the use of high-sugar ryegrass, chicory, plantain, and birdsfoot trefoil. Three different treatment groups were allocated in this experiment: a perennial ryegrass and white clover group (RG), a high-sugar perennial ryegrass and white clover group (HS), and a diverse pasture group using alternative pasture species as described (HSD). Cows grazing HSD pasture produced more milk at 16.9 L/d (compared to 15.2 L/d RG and 14.7 L/d HS), had a higher milk protein content at 655g/d (628 g/d RG and 616 g/d HS), and had a reduced urine N percent at 0.34% (0.57% RD and 0.58% HS) (Totty et al., 2013). The work presented by Totty et al. (2013) outlines the potential for improving milk production through the use of AFC. In addition to providing improved nutrient supply during the

summer months, some AFC-species are known to contain other phytochemicals that can influence production, including CT (Ramirez-Restrepo and Barry, 2005), perhaps allowing for the opportunity to naturally influence milk composition through feeding. For example, Kalber et al. (2013) reported improved milk quality when cows consumed buckwheat silage, a CT-containing AFC species, including a higher content of polyunsaturated fatty acids (PUFA) and improved cheese making properties (Kalber et al., 2013). These experiments provide further evidence for the opportunity to influence milk production in animals through the inclusion of CT-containing feeds in the diet. To date, the proteomic evaluation of milk protein composition as a result from incorporating AFC species in pasture has yet to be investigated.

1.8 Conclusions

Total milk protein content is difficult to change using diet; however, it is apparent that the milk proteomic composition itself has the potential to change as a function of diet. Moreover, there are more studies suggesting that milk proteomic composition can be altered by changing dietary availability of N and energy; however, there has yet to be any research examining the effects of the bovine milk proteome in response to supplementing feeds that contain CT, which present the opportunity to alter N partitioning in ruminants.

1.8.1 Hypothesis

Dietary intervention through the inclusion of either GM (Chapter 2) or AFC (Chapter 3) will alter the bovine milk proteome fractions. It can be further hypothesized that these changes will occur due to changes in N partitioning (Chapter 2).

1.8.2 Objectives

Our objectives include measuring the high and low-abundance protein profiles in milk samples (Chapters 2 and 3), as well as measuring changes in metabolic and N-status from lactating dairy cows (Chapter 2).

1.9 References

- Acamovic, T., and J. D. Brooker. 2005. Biochemistry of plant secondary metabolites and their effects in animals. *Proc. Nutr. Soc.* 64:403-412.
<https://dx.doi.org/10.1079/PNS2005449>
- Ben-Gera, I., and A. Kramer. 1969. The utilization of food industries wastes. *Adv. Food Res.* 17:77-152. [https://dx.doi.org/10.1016/S0065-2628\(08\)60309-2](https://dx.doi.org/10.1016/S0065-2628(08)60309-2)
- Bequette, B. J., F. R. C. Backwell, and L. A. Crompton. 1998. Current concepts of amino acid and protein metabolism in the mammary gland of the lactating ruminant. *J. Dairy Sci.* 81:2540-2559. [https://doi.org/10.3168/jds.S0022-0302\(98\)70147-X](https://doi.org/10.3168/jds.S0022-0302(98)70147-X)
- Cabrita, A. R. J., J. Dewhurst, J. M. F. Abreu, and A. J. M. Fonseca. 2006. Evaluation of the effects of synchronising the availability of N and energy on rumen function and production responses of dairy cows – a review. *Anim. Res.* 55:1-24.
<https://doi.org/10.1051/animres:2005045>
- Cavaletto, M., M. G. Giuffrida, and A. Conti. 2009. Milk fat globule membrane components – A proteomic approach. *Adv. Exp. Med. Biol.* 606: 129-141.
https://doi.org/10.1007/978-0-387-74087-4_4
- Cebo, C. 2012. Milk fat globule membrane proteomics: A 'snapshot' of mammary epithelial cell biology. *Food Technol. Biotechnol.* 50:306-314.
- Christian, M. P., C. Grainger, B. J. Sutherland, J. J. Mayes, M. C. Hannah, and B. Kefford. 1999. Managing diet quality for cheddar cheese manufacturing milk. 1. The influence of protein energy supplements. *J. Dairy Res.* 66:341-355.
- Clare, D. A., and H. E. Swaisgood. 2000. Bioactive milk peptides: a prospectus. *J. Dairy Sci.* 83:1187-1195. [https://doi.org/10.3168/jds.S0022-0302\(00\)74983-6](https://doi.org/10.3168/jds.S0022-0302(00)74983-6)
- Clark, J. H., T. H. Klusmeyer, and M. R. Cameron. 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. *J. Dairy Sci.* 75:2304-2323. [https://doi.org/10.3168/jds.S0022-0302\(92\)77992-2](https://doi.org/10.3168/jds.S0022-0302(92)77992-2)
- D'Amato, A., A. Bachi, E. Fasoli, E. Boschetti, G. Peltre, H. Senechal, and P. G. Righetti. 2009. In-depth exploration of cow's whey proteome via combinatorial peptide ligand libraries. *J. Proteome Res.* 8:3925-3936.
<https://doi.org/10.1021/pr900221x>
- DePeters, E. J., and J. P. Cant. 1992. Nutritional factors influencing the nitrogen composition of bovine-milk - a review. *J. Dairy Sci.* 75:2043-2070.
[https://dx.doi.org/10.3168/jds.S0022-0302\(92\)77964-8](https://dx.doi.org/10.3168/jds.S0022-0302(92)77964-8)

- Dewettinck, K., R. Rombaut, N. Thienpont, T. T. Le, K. Messens, and J. Van Camp. 2008. Nutritional and technological aspects of milk fat globule membrane material. *Int. Dairy J.* 18:436-457. <https://doi.org/10.1016/j.idairyj.2007.10.014>
- Dewhurst, R., D. Davies, and R. Merry. 2000. Microbial protein supply from the rumen. *Anim. Feed Sci. Technol.* 85:1-21. [https://doi.org/10.1016/S0377-8401\(00\)00139-5](https://doi.org/10.1016/S0377-8401(00)00139-5)
- Emery, R. 1978. Feeding For Increased Milk Protein. *J. Dairy Sci.* 61:825-828. [https://doi.org/10.3168/jds.S0022-0302\(78\)83656-X](https://doi.org/10.3168/jds.S0022-0302(78)83656-X)
- Firkins, J. L. 1996. Maximizing microbial protein synthesis in the rumen. *J. Nutr.* 126:1347S-1354S.
- FitzGerald, R., and H. Meisel. 2003. Milk protein hydrolysates and bioactive peptides. Pages 675-698 in Volume 1: Proteins. *Advanced Dairy Chemistry*. 3rd ed. Part B. P. F. Fox and P. L. H. McSweeney, ed. Kluwer Academic/ Plenum Publishers, New York, NY.
- FitzGerald, R. J., B. A. Murray, and D. J. Walsh. 2004. Hypotensive peptides from milk proteins. *J. Nutr.* 134:980S-988S.
- Fonslow, B. R., P. C. Carvalho, K. Academia, S. Freeby, T. Xu, A. Nakorchevsky, A. Paulus, and J. R. Yates III. 2011. Improvements in proteomic metrics of low abundance proteins through proteome equalization using ProteoMiner prior to MudPIT. *J. Proteome Res.* 10:3690-3700. <https://doi.org/10.1021/pr200304u>
- Gauthier, S. F., Y. Pouliot, and D. Saint-Sauveur. 2006. Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. *Int. Dairy J.* 16:1315-1323. <https://doi.org/10.1016/j.idairyj.2006.06.014>
- Gerling, C. 2011. Conversion Factors: From Vineyard to Bottle. Accessed Mar. 30th, 2017. <https://grapesandwine.cals.cornell.edu/newsletters/appellation-cornell/2011-newsletters/issue-8/conversion-factors-vineyard-bottle>
- Gordon, F. 1977. The effect of protein content on the response of lactating cows to level of concentrate feeding. *Animal Science.* 25:181-191. <https://doi.org/10.1017/S0003356100039404>
- Greenwood, S. L., G. R. Edwards, and R. Harrison. 2012. Short communication: supplementing grape marc to cows fed a pasture-based diet as a method to alter nitrogen partitioning and excretion. *J. Dairy Sci.* 95:755-758. <https://dx.doi.org/10.3168/jds.2011-4648>

- Hagemeister, H., W. Luppig, and W. Kaufmann. 1981. Microbial protein synthesis and digestion in the high-yielding dairy cow. Page 31 in Recent Developments in Ruminant Nutrition. W. Haresign, and D. J. A. Cole. Butterworths, London, Engl.
- Jenkins, T. C. and M. A. McGuire. 2006. Major advances in nutrition: Impact on milk composition. *J. Dairy Sci.* 89:1302-1310.
[https://doi.org/10.3168/jds.S0022-0302\(06\)72198-1](https://doi.org/10.3168/jds.S0022-0302(06)72198-1)
- Jones, C. M., and A. J. Heinrichs. 2017. Milk components: understanding milk fat and protein variation in your dairy herd. Accessed Sept. 5, 2017.
<https://extension.psu.edu/milk-components-understanding-milk-fat-and-protein-variation-in-your-dairy-herd>
- Journet, M. and Y. Chilliard. 1985. Influence de l'alimentation sur la composition du lait. 1. Taux butyreux: facteurs généraux. *Bull. Tech. CRZV Theix, INRA* 60:13-23.
- Kalber, T., M. Kreuzer, and F. Leiber. 2013. Effect of feeding buckwheat and chicory silages on fatty acid profile and cheese-making properties of milk from dairy cows. *J. Dairy Res.* 80:81-88. <https://doi.org/10.1017/S0022029912000647>
- Keough, G. 2017. New England Berries, Tree Fruit, and Grapes, 2016 Crop. Pages 1-4. USDA, Concord, NH.
- Kilara, A., and D. Panyam. 2003. Peptides from milk proteins and their properties. *Crit. Rev. Food Sci. Nutr.* 43:607-633.
<http://dx.doi.org/10.1080/10408690390251138>
- Kim, K. H., Y. G. Oh, J. J. Choung, and D. G. Chamberlain. 1999. Effects of varying degrees of synchrony of energy and nitrogen release in the rumen on the synthesis of microbial protein in cattle consuming grass silage. *J. Sci. Food Agric.* 79:833-838.
- Korhonen, H. 2009. Milk-derived bioactive peptides: from science to applications. *J. Funct. Food* 1:177-187. <https://dx.doi.org/10.1016/j.jff.2009.01.007>
- Larson, B. L., H. L. Heary, Jr., and J. E. Devery. 1980. Immunoglobulin production and transport by the mammary gland. *J Dairy Sci* 63:665-671.
[https://doi.org/10.3168/jds.S0022-0302\(80\)82988-2](https://doi.org/10.3168/jds.S0022-0302(80)82988-2)
- Le, A., L. D. Barton, J. T. Sanders, and Q. Zhang. 2011. Exploration of bovine milk proteome in colostral and mature whey using an Ion-Exchange approach. *J. Proteome Res.* 10:692-704. <https://dx.doi.org/10.1021/pr100884z>

- Li, S. S., J. S. Shen, D. X. Ren, and J. X. Liu. 2015. Effects of the processing methods of corn grain and soybean meal on milk protein expression profiles in dairy cows. *Animal*. 9:267-274. <https://dx.doi.org/10.1017/S1751731114002225>
- Lonnerdal, B. 2013. Bioactive proteins in breast milk. *J. Paediatr. Child Health*. 49(S1):1-7. <https://dx.doi.org/10.1111/jpc.12104>
- Lopez-Exposito, I., and I. Recio. 2008. Protective effect of milk peptides: antibacterial and antitumor properties. *Adv. Exp. Med. Biol*. 606:271-293.
- López-Fandiño, R., J. Otte, and J. Van Camp. 2006. Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. *Int. Dairy J*. 16:1277-1293. <https://doi.org/10.1016/j.idairyj.2006.06.004>
- Makkar, H. P. S. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small Ruminant Research* 49:241-256. [https://dx.doi.org/10.1016/S0921-4488\(03\)00142-1](https://dx.doi.org/10.1016/S0921-4488(03)00142-1)
- Manso, T., B. Gallardo, A. Salva, C. Guerra-Rivas, A. R. Mantecon, P. Lavin, and M. A. de la Fuente. 2016. Influence of dietary grape pomace combined with linseed oil on fatty acid profile and milk composition. *J. Dairy Sci*. 99:1111-1120. <https://dx.doi.org/10.3168/jds.2015-9981>
- McAllan, A., J. Cockburn, A. Williams, and R. Smith. 1988. The degradation of different protein supplements in the rumen of steers and the effects of these supplements on carbohydrate digestion. *Br. J. Nutr*. 60:669-682. <https://doi.org/10.1079/BJN19880136>
- Mills, S., R. P. Ross, C. Hill, G. F. Fitzgerald, and C. Stanton. 2011. Milk intelligence: mining milk for bioactive substances associated with human health. *Int. Dairy J*. 21:377-401. <https://dx.doi.org/10.1016/j.idairyj.2010.12.011>
- Moate, P. J., S. R. Williams, V. A. Torok, M. C. Hannah, B. E. Ribaux, M. H. Tavendale, R. J. Eckard, J. L. Jacobs, M. J. Auldist, and W. J. Wales. 2014. Grape marc reduces methane emissions when fed to dairy cows. *J. Dairy Sci*. 97:5073-5087. <https://doi.org/10.3168/jds.2013-7588>
- Nissen, A., E. Bendixen, K. L. Ingvarsten, and C. M. Rontvedt. 2013. Expanding the bovine milk proteome through extensive fractionation. *J. Dairy Sci*. 96:7854-7866. <https://dx.doi.org/10.3168/jds.2013-7106>
- Nudda, A., F. Correddu, A. Marzano, G. Battacone, P. Nicolussi, P. Bonelli, and G. Pulina. 2015. Effects of diets containing grape seed, linseed, or both on milk

- production traits, liver and kidney activities, and immunity of lactating dairy ewes. *J. Dairy Sci.* 98:1157-1166. <https://dx.doi.org/10.3168/jds.2014-8659>
- O'Riordan, N., M. Kane, L. Joshi, and R. M. Hickey. 2014. Structural and functional characteristics of bovine milk protein glycosylation. *Glycobiology* 24:220-236. <https://doi.org/10.1093/glycob/cwt162>
- Pereira, P. C. 2014. Milk nutritional composition and its role in human health. *J. Nut.* 30:619-627. <https://doi.org/10.1016/j.nut.2013.10.011>
- Pihlanto, A. 2006. Antioxidative peptides derived from milk proteins. *Int. Dairy J.* 16:1306-1314. <https://doi.org/10.1016/j.idairyj.2006.06.005>
- Ramirez-Restrepo, C. A., and T. N. Barry. 2005. Alternative temperate forages containing secondary compounds for improving sustainable productivity in grazing ruminants. *Anim. Feed Sci. Technol.* 120:179-201. <https://doi.org/10.1016/j.anifeedsci.2005.01.015>
- Reinhardt, T. A., and J. D. Lippolis. 2006. Bovine milk fat globule membrane proteome. *J Dairy Res.* 73:406-416. <https://doi.org/10.1017/S0022029906001889>
- Righetti, P. G., and E. Boschetti. 2008. The ProteoMiner and the FortyNiners: searching for gold nuggets in the proteomic arena. *Mass Spectrom. Rev.* 27:596-608. <https://doi.org/10.1002/mas.20178>
- Rode, L., D. Weakley, and L. Satter. 1985. Effect of forage amount and particle size in diets of lactating dairy cows on site of digestion and microbial protein synthesis. *Can. J. of Anim. Sci.* 65:101-111. <https://doi.org/10.4141/cjas85-011>
- Roncada, P., C. Piras, A. Soggiu, R. Turk, A. Urbani, and L. Bonizzi. 2012. Farm animal milk proteomics. *J Proteomics.* 75:4259-4274. <https://doi.org/10.1016/j.jprot.2012.05.028>
- Rooke, J. A., N. H. Lee, and D. G. Armstrong. 1987. The effects of intraruminal infusions of urea, casein, glucose syrup and a mixture of casein and glucose syrup on nitrogen digestion in the rumen of cattle receiving grass-silage diets. *Br. J. Nutr.* 57:89-98. <https://doi.org/10.1079/BJN19870012>
- Santos, N. W., G. T. D. Santos, D. C. Silva-Kazama, P. A. Grande, P. M. Pintro, F. E. de Marchi, C. C. Jobim, and H. V. Petit. 2014. Production, composition and antioxidants in milk of dairy cows fed diets containing soybean oil and grape residue silage. *Livestock Science* 159:37-45. <https://dx.doi.org/10.1016/j.livsci.2013.11.015>

- Shennan, D. B., and M. Peaker. 2000. Transport of milk constituents by the mammary gland. *Physiol. Rev.* 80:925-951.
- Shingfield, K. J., Y. Chilliard, V. Toivonen, P. Kairenius, and D. I. Givens. 2008. Trans fatty acids and bioactive lipids in ruminant milk. *Adv. Exp. Med. Biol.* 606:3-65.
- Sniffen, C. and P. Robinson. 1987. Microbial growth and flow as influenced by dietary manipulations. *J. Dairy Sci.* 70:425-441.
[https://doi.org/10.3168/jds.S0022-0302\(87\)80027-9](https://doi.org/10.3168/jds.S0022-0302(87)80027-9)
- Van Soest, P. J. 1994. *Nutritional Ecology of the Ruminant*. Second Edition. Pages 258-259. 2nd ed. Cornell University Press.
- Sok, M., D. R. Ouellet, J. L. Firkins, D. Pellerin, and H. Lapierre. 2017. Amino acid composition of rumen bacteria and protozoa in cattle. *J. Dairy Sci.* 100:5241-5249. <https://doi.org/10.3168/jds.2016-12447>
- Spitsberg, V. L. 2005. Invited review: Bovine milk fat globule membrane as a potential nutraceutical. *J. Dairy Sci.* 88:2289-2294.
[https://dx.doi.org/10.3168/jds.S0022-0302\(05\)72906-4](https://dx.doi.org/10.3168/jds.S0022-0302(05)72906-4)
- Sutton, J. D. 1989. Altering milk composition by feeding. *J. Dairy Sci.* 72:2801-2814.
[https://dx.doi.org/10.3168/jds.S0022-0302\(89\)79426-1](https://dx.doi.org/10.3168/jds.S0022-0302(89)79426-1)
- Tacoma, R., J. Fields, D. B. Ebenstein, Y.-W. Lam, and S. L. Greenwood. 2016. Characterization of the bovine milk proteome in early-lactation Holstein and Jersey breeds of dairy cows. *J. Proteomics* 130:200-210.
<https://dx.doi.org/10.1016/j.jprot.2015.09.024>
- Tacoma, R., J. Fields, D. B. Ebenstein, Y. W. Lam, and S. L. Greenwood. 2017a. 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. *J. Dairy Sci.* 100:7246-7261.
<https://doi.org/10.3168/jds.2017-12647>
- Tacoma, R., S. L. Gelsinger, Y. W. Lam, R. A. Scuderi, D. B. Ebenstein, A. J. Heinrichs, and S. L. Greenwood. 2017b. Exploration of the bovine colostrum proteome and effects of heat treatment time on colostrum protein profile. *J Dairy Sci.* *In-press*.
<https://doi.org/10.3168/jds.2017-13211>
- Thomas, P., and P. Martin. 1988. The influence of nutrient balance on milk yield and composition. Page 97. *Nutrition and Lactation in the dairy cow*. P. C. Garnsworthy. Butterworths, London, Engl.

- Tilman, D. and J. A. Downing. 1994. Biodiversity and Stability in Grasslands. *Nature* 367:363-365.
- Totty, V. K., S. L. Greenwood, R. H. Bryant, and G. R. Edwards. 2013. Nitrogen partitioning and milk production of dairy cows grazing simple and diverse pastures. *J. Dairy Sci.* 96:141-149. <https://dx.doi.org/10.3168/jds.2012-5504>
- Tsiplakou, E., and G. Zervas. 2008. The effect of dietary inclusion of olive tree leaves and grape marc on the content of conjugated linoleic acid and vaccenic acid in the milk of dairy sheep and goats. *J. Dairy Res.* 75:270-278. <https://dx.doi.org/10.1017/S0022029908003270>
- Twyman, R. M. 2014. *Principles of Proteomics*. Pages 15-20, 23-54. Garland Science, New York, New York.
- USDA. 2010. National Organic Program: Access to Pasture (Livestock). Pages 7153-7195. Vol. 75. Office of the Federal Register, National Archives and Records Administration, Washington DC.
- USDA. 2017. Noncitrus Fruits and Nuts 2016 Summary. Pages ISSN: 1948-2698.
- Vanderghem, C., F. Francis, S. Danthine, C. Deroanne, M. Paquot, E. De Pauw, and C. Blecker. 2011. Study on the susceptibility of the bovine milk fat globule membrane proteins to enzymatic hydrolysis and organization of some of the proteins. *Int. Dairy J.* 21:312-318. <https://doi.org/10.1016/j.idairyj.2010.12.006>
- Van Soest, P. J. 1994. *Nutritional Ecology of the Ruminant*. Second Edition. Pages 258-259. 2nd ed. Cornell University Press.
- Waghorn, G. 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production-progress and challenges. *Anim. Feed Sci. Technol.* 147:116-139. <https://dx.doi.org/10.1016/j.anifeedsci.2007.09.013>
- Waghorn, G. C., M. J. Ulyatt, A. John, and M. T. Fisher. 1987. The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *Lotus corniculatus* L. *Br. J. Nutr.* 57:115-126. <https://dx.doi.org/10.1079/BJN19870015>
- Woodward, S. L., G. G. Waghorn, K. A. Watkins, and M. A. Bryant. 2009. Feeding birdsfoot trefoil (*Lotus corniculatus*) reduces the environmental impacts of dairy farming. *Proc. N.Z. Soc. Anim. Prod.* 69:179-183.
- Yamamoto, N., M. Ejiri, and S. Mizuno. 2003. Biogenic peptides and their potential use. *Curr. Pharm. Des.* 9:1345-1355. <https://doi.org/10.2174/1381612033454801>

- Yang, W., K. Beauchemin, and L. Rode. 2001. Effects of grain processing, forage to concentrate ratio, and forage particle size on rumen pH and digestion by dairy cows. *J. Dairy Sci.* 84:2203-2216.
[https://doi.org/10.3168/jds.S0022-0302\(01\)74667-X](https://doi.org/10.3168/jds.S0022-0302(01)74667-X)
- Yang, Y., N. Zheng, X. Zhao, Y. Zhang, R. Han, L. Ma, S. Zhao, S. Li, T. Guo, and J. Wang. 2015. Proteomic characterization and comparison of mammalian milk fat globule proteomes by iTRAQ analysis. *J. Proteomics* 116:34-43.
<https://dx.doi.org/10.1016/j.jprot.2014.12.017>
- Ye, A., J. Cui, and H. Singh. 2011. Proteolysis of milk fat globule membrane proteins during in vitro gastric digestion of milk. *J. Dairy Sci.* 94:2762-2770.
<https://doi.org/10.3168/jds.2010-4099>
- Yi, C., J. Shi, J. Kramer, S. Xue, Y. M. Jiang, M. W. Zhang, Y. Ma, and J. Pohorly. 2009. Fatty acid composition and phenolic antioxidants of winemaking pomace powder. *Food Chem.* 114:570-576. <https://dx.doi.org/10.1016/j.foodchem.2008.09.103>

CHAPTER 2: DIETARY GRAPE MARC ALTERS THE BOVINE MILK PROTEOME

2.1 Abstract

Grape marc (GM) is a viticulture by-product comprising of the remaining skins, stems, and seeds after pressing that has been used as a supplement in livestock feeding practices due to its' low-cost. The objective of this experiment was to determine whether feeding GM, a condensed tannin (CT)-containing feed, to lactating dairy cows would alter the milk proteome through changes in nitrogen (N) partitioning. Ten lactating Holstein cows blocked by days in milk (141 ± 37 d) in a complete randomized block design, and fed a total mixed ration (TMR) diet top-dressed with either 1.5 kg dry matter (DM)/cow/day GM (GM group; n=5) or 2.0 kg DM/cow/day of a 50: 50 beet pulp: soy hulls mix (control group; n=5). Dry matter intake (DMI) was recorded daily, milk yield was recorded thrice weekly at AM and PM milkings along with sample collections for general components analysis through the Dairy Herd Improvement Association (DHIA; Lancaster, PA). Blood samples were collected once weekly after AM and PM milkings for analysis of metabolite concentrations including glucose, urea nitrogen, and non-esterified fatty acids (NEFA). 24-h urine and fecal collections were obtained on d 28 for additional analysis of N parameters. Milk samples collected for HPLC analysis of high-abundance proteins were collected on the covariate (D0) as well as the sample collection period (d 21-28). Additional samples from the collection period were used for subsequent liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of the low-abundance enriched protein fraction. Milk samples were pooled within cow according to

yield and skimmed; one skimmed sample was subjected to HPLC analysis whereas the other was used for LC-MS/MS analysis followed by fractionation and enrichment for the low-abundance protein fraction using ProteoMiner prior to trypsin digestion and isobaric tandem mass tag labeling. SEQUEST and Mascot databases were used to search the resulting product ion spectra on Proteome Discoverer 1.4. Results were analyzed using PROC MIXED in SAS 9.3. Despite the lack of changes observed in DMI, metabolic, and N status, 16 of the 127 proteins were different across treatments. Roughly 12% of the bovine milk proteome changed in this study, suggesting that feeding GM to lactating dairy cattle can alter the milk protein profile, though the mechanisms are still unclear. Additionally, our study aligns with other literature emphasizing the use of GM as an effective supplement without having any detriments on milk production.

2.2 Introduction

In bovine milk, there are a number of bioactive proteins and peptides that can elicit responses when consumed, including growth, cellular signaling, immunomodulation, antimicrobial, and even anti-cancer functions (Korhonen, 2009; Mills et al., 2011). Bioactive peptides are released from their native protein after being hydrolyzed by proteolytic enzymes in the digestive tract during digestion of the dietary milk, while bioactive proteins remain functionally intact as they have been shown to be resistant to proteolysis (Korhonen, 2009; Mills et al., 2011). There has been growing interest for enhancing proteins and peptides present in milk for human consumption due to their bioactivity; hence, much attention has been turned towards understanding the factors influencing this proteome.

Stage of lactation, genetics, and nutrition are known to influence the composition of the bovine milk proteome (Christian et al., 1992; Le et al., 2011; Yang et al., 2015; Tacoma et al., 2016). Nutritional factors are of particular interest since animal nutrition is a variable and important component in farm management. Ruminants possess a unique digestive tract that allows for pre-gastric fermentation and degradation of feedstuffs that are chemically available for the microbial population within the rumen, while any digestible feed components that are resistant to microbial breakdown in the rumen are not digested until the gastric compartment of the stomach, known as the abomasum. Hence, the proportions of nutrients that are available or unavailable for rumen-microbial utilization within the diet can substantially alter the absorbed nutrient profile and post-absorptive metabolism. One such example of a differentially utilized dietary nutrient is protein, in which the rumen degradable protein (RDP) fraction is available for microbial degradation unlike the rumen undegradable protein (RUP) fraction, which remains intact until gastric digestion in the abomasum.

Recently, Li et al. (2015) observed that offering dairy cattle varying degrees of ruminally-available diets by way of energy and RDP resulted in an increase in specific milk proteins, including zinc-alpha-2-glycoprotein (ZAG) and alpha-lactalbumin (α -LA), implying that diet impacts the bovine milk proteome. Earlier, Christian et al. (1999) observed changes in milk casein fractions when cows were fed diets with varying degrees of lupin and wheat, effectively altering the proportions of RDP and RUP; and a similar trend was observed by Li et al. (2015). The work done by Christian et al. (1999) and Li et al. (2015) highlights the potential for altering the proteomic composition of bovine milk

as a result of dietary RDP and RUP, likely by altering post-absorptive nitrogen (N) metabolism.

Grape marc (GM), or pomace, is a byproduct of the viticulture industry consisting of the remaining skins, stems, and seeds of the grapes after pressing for wine. The United States harvested 4,561,040 tons of grapes for wine-making in 2014 (USDA, 2015); of this, 10-20% of the original grape weight is typically leftover after processing (Ben-Gera and Kramer, 1969). With a calculated 827,536,853 kg of GM to dispose of annually, the wine-making industry has sought out ways to utilize the byproduct; however, much of the grape pomace is composted and it is considered a pollutant (Tsiplakou and Zervas, 2008; Santos et al., 2014). As a result, GM has been incorporated livestock feeding practices, including the dairy industry, due to its low-cost and regional availability (Nudda et al., 2015; Manso et al., 2016). Supplementing GM in dairy diets also appears to have no impact on milk production. Nudda et al. (2015) reported no change in milk production when feeding 300 g GM, and Santos et al. (2014) also reported no differences in milk composition when diets included ensiled GM at amounts of 50, 75, and 100 g/kg DM (Santos et al., 2014; Nudda et al., 2015).

GM contains polyphenolic compounds, including condensed tannin(s) (CT) that provide an array of bioactivity (Waghorn, 2008; Nudda et al., 2015). CT are secondary plant metabolites that bind to proteins in the digestive tract, making them unavailable for further degradation and absorption (Waghorn et al., 1987; Nudda et al., 2015). This CT-protein binding capability is known to occur in the rumen, and the binding has been reported as irreversible by microbial activity since the enzymes necessary to degrade CT

are not produced by the rumen microbes (Makkar, 2003). It has been hypothesized, however, that some CT may release the bound protein as a result of pH changes further downstream in the gastrointestinal tract, such as in the abomasum (Waghorn et al., 1987; Nudda et al., 2015). Various polymers of CT will bind to proteins at a pH range of 3.5-7.0 to form a stable complex, and have been shown to dissociate when the pH deviates from the ideal range (< 3.5 or > 8.5) (Waghorn et al., 1987). By binding to proteins in the rumen, CT-inclusive diets alter the N metabolism in ruminants by decreasing the RDP fraction, and thereby increasing the RUP fraction available for proteolysis by the animal in the abomasum and small intestine (Waghorn, 2008). Shifting sites of N uptake, as well as the amount and profile of protein available in the small intestine for absorption also impacts animal-derived products, such as milk, as has been observed when feeding other CT-rich feeds, including birdsfoot trefoil (Woodward et al., 2009), red clover (Lee et al., 2009), and chicory (Totty et al., 2013) for example (Acamovic and Brooker, 2005). Moreover, we propose that feeding a CT-containing feedstuff, like GM, may alter outputs of specific proteins into milk such as described by Li et al. (2015) by decreasing the proportion of RDP as a result of CT-binding.

The hypothesis of the research described herein was that dietary supplementation of GM to lactating dairy cows would alter the bovine milk proteome, including ZAG and α -LA, via changes in N partitioning. The objectives of this study were to i) determine the high- and low-abundance protein enriched profiles in milk using proteomic approaches and summarize ontological functions of the identified proteome, and ii) assess concurrent changes in N partitioning by measuring indicators of N status in milk, plasma, urine, and feces.

2.3 Materials and Methods

2.3.1 Animals, Diet, and Experimental design

This trial and all of the procedures were done in accordance with the Institutional Animal Care and Use Act (IACUC) at The University of Vermont (Burlington, VT; IACUC approval number 16-064). Ten mid-lactation Holstein cows were blocked based upon parity (1.2 ± 0.4), DIM (147 ± 37 d), and daily milk yield (41.3 ± 5.4 kg) and included in a randomized complete block design for a 28 d trial. The experiment consisted of a 21 d adaptation period followed by a 7 d sample collection period. All cows were housed in tie-stalls at the Paul R. Miller Research and Education Center (The University of Vermont, Burlington, VT). All cows were milked at 4:00 h and 16:00 h daily.

Cows had free access to water and were fed a total mixed ration (TMR) diet. Each cow was given their entire weighed daily base ration, which included grass silage, corn silage, and a mash, once daily at 5:00 h. A weighed amount of concentrate pellet was mixed into the base ration four times daily (3:30 h, 10:00 h, 15:30 h, and 22:00 h). Total daily feedstuff allowances per cow are listed in Table 1. Treatments were dietary supplements, where cows received once a day either 1) a beet pulp:soy hulls (50:50, BP) mixture at 2.0 kg DM/cow/day, or 2) GM at 1.5 kg DM/cow/day (Table 1) at 5:00 h for the duration of the 28 d trial. GM was sourced from a local vineyard (Shelburne Vineyard, Shelburne, VT), and stored on-farm under a covered landing in 1 tonne harvest bags.

Diet refusals from each cow were collected daily before feeding (5:00 h) and weighed, then a subsample was stored at -20°C for further analysis. Samples were later dried at 65°C for 48-72 h for determination of individual DMI. Feed samples were also collected weekly across the 28 d period and composited within feedstuff for wet-chemistry analysis (Dairy One Lab, Ithaca, NY; Table 2.1).

2.3.2 Condensed Tannin content of GM

GM samples, collected once weekly, were stored at -20°C until later analysis of CT content as per methods previously described (Sarneckis et al., 2006; Mercurio et al., 2007) with minor modifications. Briefly, a composite of the weekly samples was created and then blended using a Bella Rocket Blender (Sensio, Montreal, QC, Canada), and homogenized using a Qiagen TissueLyserII at 30 Hz for 3 min (Qiagen, Hilden, Germany). Ten mL of 50% ethanol solution were then added to 1 g of the homogenate, vortexed, and placed on a shaker for 1 h. Centrifugation immediately followed at 4,695xg for 10 min at room temperature. The supernatant was removed and analyzed using a Methyl Cellulose Precipitable Tannin Assay (MCP) as previously outlined (Sarneckis et al., 2006; Mercurio et al., 2007).

2.3.3 Plasma

Blood samples were collected from the coccygeal vessel in heparinized and ethylenediaminetetraacetic acid-coated (EDTA) vacutainers (BD, Franklin Lakes, NJ) after AM and PM milking during the covariate period (D0), and d 21 and 28 of the sample collection period. Samples were immediately placed on ice and centrifuged at 3000xg for 15 min at 4°C. Plasma was harvested and stored at -20°C until further

analysis. Samples were analyzed using commercially available kits for plasma urea nitrogen (PUN; Teco Diagnostics, Anaheim, CA), glucose (Sigma Aldrich, St. Louis, MO), and non-esterified fatty acid (NEFA; Zenbio, Research Triangle Park, NC) concentrations.

2.3.4 Urine and Feces

Total urine and fecal collections from each cow were completed on d 28. Urine was collected using modified urine cup collectors as previously described (Lascano et al., 2010). Briefly, cows were fitted with urine collection devices attached to 40 L carboys containing 100% sulfuric acid (H_2SO_4 ; Fisher Scientific, Pittsburgh, PA) to acidify the urine to a $pH < 4$ as it was collected from each animal. The H_2SO_4 was incrementally added to the carboys during the sampling period, totaling 350 mL. At the end of the 24 h, the urine collected from each animal was mixed thoroughly, the total weight was recorded, and a subsample was collected. Feces was collected via free-catch onto tarps behind the cows, and transferred to holding bins for each animal during the 24 h collection period. The fecal matter was mixed thoroughly, the total weight was recorded, and a subsample for each cow was collected. All urine and fecal subsamples were stored at $-20^\circ C$ until being submitted for wet-chemistry analysis (Dairy One, Ithaca, NY). Endpoint measures included urine urea, urine ammonia, urine CP, fecal N, and fecal ammonia N.

2.3.5 Milk Sampling

Milk yield was recorded at each milking, and samples were collected from each cow using continuous in-line samplers at AM and PM milkings. Three milk samples were

collected from each cow during D0 and during the endpoint sample collection period (d 21-28) for further analyses. One set of milk samples were transferred into tubes containing the preservative bronopol at the time of milking, stored at 4°C, and submitted for commercial analysis of milk fat, protein, somatic cell count (SCC) and milk urea nitrogen (MUN) content to the DHIA (Lancaster, PA). The two additional samples collected, one for HPLC analysis (5 mL) and one liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis (30 mL), were immediately placed into a dry ice/ethanol bath on-farm before being stored at -20°C (HPLC analysis) and -80°C (LC-MS/MS analysis).

2.3.6 Analysis of high-abundance milk proteins using HPLC methodology

Milk samples collected for HPLC analysis of the high-abundance milk proteins, including α -s1, α -s2, β and κ -caseins (CAS), α -LA, and the A and B variants of β -lactoglobulin (β -LGA, and β -LGB, respectively) were thawed overnight at 4°C. Samples collected during D0 were pooled within day according to milk yield, while the samples collected on d 21-28 of the experiment were pooled within cow as a proportion of milk yield. Samples were then centrifuged at 4,000xg for 10 min at 4°C to allow for separation of the cream layer. The skim milk fraction was processed and analyzed using HPLC as previously described (Bordin et al., 2001; Tacoma et al., 2016). Briefly, a reducing buffer containing dithiothreitol (DTT), 6 M guanidine-HCl, and 5 mM trisodium citrate in water was added to each sample. Samples were then vortexed and left in the fridge to incubate overnight. After incubation, a volume of buffer without the reducing agent DTT was added to each sample and the solution was filtered through a 0.45 μ m syringe filter (Sartorius. Göttingen, Germany) into a borosilicate test tube. The filtrate was transferred

to autosampler vials for subsequent HPLC analysis (Shimadzu Corporation, Kyoto Japan). Separations were completed according to methods previously described by Bordin et al. (2001) on a C₄ reversed-phase microbore analytical column (150 × 2.1 mm, 300 Å pore diameter and 5 µm particle size, Yydac 214 MS, Grace Davison, MD, USA).

2.3.7 Preparation of low-abundance protein enriched milk fraction

Milk samples collected during the sampling period (d 21-28) for the identification of low-abundance enriched proteins through LC-MS/MS analysis were thawed overnight at 4°C and were composited by milk yield. Protein fractionation and enrichment were performed as previously described (Tacoma et al., 2016). Briefly, a protease inhibitor (Protease Inhibitor Cocktail, Sigma, Milwaukee, WI) was added at 0.24 mL per g of protein, followed by centrifugation at 4,000xg for 10 min at 4°C to allow for separation of the cream layer. Skim milk samples were depleted of casein by calcium dichloride precipitation followed by ultracentrifugation at 189,000xg for 70 min at 4°C. The supernatant was stored at -80°C prior to lyophilization and reconstitution in PBS. The protein concentration of the reconstituted samples was determined using the bicinchoninic acid assay (BCA; Pierce, Rockford, IL) using bovine serum albumin as the standard. Samples were enriched using a ProteoMiner kit (BioRad, Hercules, CA) as per manufacturer's instructions. Eluted samples were analyzed for protein concentration using BCA. 1 µg of baker's yeast GAPDH (*S. cerevisiae*; Glyceraldehyde-3-phosphate Dehydrogenase; Sigma-Aldrich, St. Louis, MO) was added to 99 µg of each sample, and each of the samples (100 µg total) were digested with trypsin followed by labeling using isobaric Tandem Mass Tags (TMT) as per manufacturer's instructions (product #90113;

Thermo Scientific, Rockford, IL). Samples were then combined in equal parts, and the 10plex was kept at -80°C until subsequent LC-MS/MS.

2.3.8 Liquid Chromatography – Mass Spectrometry

Four μL of the TMT reaction mixture were dried under vacuum and labeled peptides were resuspended in 10 μL of 2.5% acetonitrile (CH_3CN) and 2.5% formic acid (FA) in water for subsequent liquid chromatography-mass spectrometry (LC-MS) analysis. LC-MS-based peptide identification and quantification was performed on the Q-Exactive mass spectrometer coupled to an EASY-nLC (Thermo Fisher Scientific, Waltham, MA). Five μL of the sample was loaded onto a 100 μm x 120 mm capillary column packed with Halo C18 (2.7 μm particle size, 90 nm pore size, Michrom Bioresources, CA, USA) at a flow rate of 300 nL/min. Peptides were separated using a gradient of 2.5-35% $\text{CH}_3\text{CN}/0.1\%$ FA over 150 min, 35-100% $\text{CH}_3\text{CN}/0.1\%$ FA in 1 min and then 100% $\text{CH}_3\text{CN}/0.1\%$ FA for 8 min, followed by an immediate return to 2.5% $\text{CH}_3\text{CN}/0.1\%$ FA and a hold at 2.5% $\text{CH}_3\text{CN}/0.1\%$ FA. A nanospray ionization source introduced the peptides into the mass spectrometer through the use of a laser pulled ~ 3 μm orifice with a spray voltage of 2.0 kV. Mass spectrometry data was acquired in a data-dependent manner using “Top 10” acquisition mode with lock mass function activated (m/z 371.1012; use lock masses: best; lock mass injection: full MS), in which a survey scan from m/z 350-1600 at 70,000 resolution (AGC target $1e^6$; max IT 100 ms; profile mode). Following data acquisition, 10 higher-energy collisional dissociation MS/MS scans were performed on the most abundant ions at 35,000 resolution (AGC target $1e^5$; max IT 100 ms; profile mode). An isolation width of 1.2 m/z and a normalized collisional energy of 35% was used to obtain MS/MS scans, and dynamic exclusion was

enabled (peptide match: preferred; exclude isotopes: on; underfill ratio: 1%; exclusion duration: 30 sec). SEQUEST and Mascot search engines were used for the subsequent product ion spectra on Proteome Discoverer 1.4 (Thermo Fisher Scientific, Waltham, MA, USA) against a curated Bovine Uniprot (*Bos taurus* database; UP000009136; 24,346 entries; downloaded Dec. 9, 2015) with sequences in forward and reverse orientations. To verify effective tryptic digestion and subsequent labeling of peptides, the product ion spectra were re-searched against a *saccharomyces cerevisiae* database. Search parameters were as follows: full trypsin enzymatic activity, maximum missed cleavages = 2, and peptides MW between 350 to 5000; mass tolerance at 20 ppm for precursor ions and 0.02 Da for fragment ions, dynamic modifications on methionines (+15.9949 Da: oxidation), Dynamic TMT6plex modification (The TMT6plex and TMT10plex have the same isobaric mass) on N-termini and lysines (229.163 Da), as well as static modification on cysteines (+57.021 Da). Percolator node was used to limit the false positive (FP) rates to less than 1% in the data set. Reporter Ion Quantification Node in Proteome Discoverer 1.4 was used for quantification purposes. All of the acquired protein identification and quantification information (< 1% FP; with protein grouping enabled) was exported to Excel spreadsheets. Relative fold-change values of the proteins identified within each animal were compared against values from each of the control cows for data generated using both the *bos taurus* and *saccharomyces cerevisiae* databases. *Saccharomyces cerevisiae* GAPDH (Accession numbers: P00359 and P00360) and *bos taurus* GAPDH (Accession number: P10096) search results shared one common amino acid sequence (LTGMAFR); therefore, protein P10096 was excluded from bioinformatics.

2.3.9 Bioinformatics

Milk proteins identified by LC-MS/MS analysis that were classified as uncharacterized through Proteome Discoverer 1.4 were identified using basic local alignment search tool (BLAST) (Camacho et al., 2009). Proteins that were identified as affected by dietary treatment through statistical analysis were matched to their associated annotated functions using gene ontology (GO) through The PANTHER Classification System (Mi et al., 2017). Proteins were annotated to their biological process, molecular function, cellular component, and protein class and graphed in Prism 7 (GraphPad Software Inc, La Jolla, CA) according to their percent of gene hits against total number of function hits as calculated from PANTHER. Additional protein-protein interaction analysis was conducted through STRING (Szklarczyk et al., 2015) within Cytoscape (Cytoscape 3.5.1; La Jolla, CA) (Shannon et al., 2003) using a STRING app (Szklarczyk et al., 2017) to generate a network of interactions.

2.3.10 Calculation of nitrogen intake, excretion, and retention

Daily N intake (g) of each cow was calculated by multiplying DMI (g) by the CP content (% of DM) of the feed, and dividing by 6.25 to determine g N/d intake. Daily fecal N output (g) of each cow was calculated by multiplying the weight of feces collected after 24 h by the fecal N %. Daily urine N output (g) of each cow was obtained by multiplying the weight of urine collected after 24 h by the urine CP %, and dividing by 6.25 to obtain the estimated g N/d excretion in urine. Daily milk N output (g) of each cow was calculated by dividing the milk protein yield (g) of the cow by 6.38 to obtain g N/d secretion in milk. The N retention of each cow was then calculated by subtracting the g N/d excreted in urine, feces and milk from the g N/d intake.

2.3.11 Statistical Analysis

The replicate number for this study was calculated at 80% power, whereby 5 cows per treatment were required to detect a 45% difference in urine N content at 20% CV as observed in previous research examining intake of polyphenolic containing feeds on N output of lactating dairy cattle (Lee et al., 2009). The PROC MIXED procedure was used in SAS version 9.4 (SAS Institute, Cary, NC) to perform repeated measures ANOVA on DMI, milk components, and plasma results. Treatment, day, and a day x treatment interaction were used as fixed effects. D0 was included as a covariate in each of these models. PROC MIXED model was also used in SAS version 9.4 for analysis of the endpoint values for high-abundance proteins and urine and fecal parameters with treatment included as a fixed effect. Fold change of each milk protein identified by LC-MS/MS was calculated relative to each control sample, and milk proteins were then statistically analyzed as repeated measures with treatment included as a fixed effect. Conditional formatting was performed in Excel (v.14.2.2.) to generate a three-way color scale heat map hybridized with the table listing relative fold change values. Significant differences were declared if $P \leq 0.05$.

2.4 Results

2.4.1 Nutrient composition, DMI, and CT content of GM

There were no differences in DMI between treatment groups (Table 2.4). The CT concentration was 4.29 g/kg of GM, equaling an estimated total intake of 6.38 g CT daily (Table 2.1).

2.4.2 Urine and Fecal Samples

Urine urea, ammonia, and CP were not different between treatment groups (Table 2.2). Additionally, there were no differences in fecal N and fecal ammonia N concentrations (Table 2.2).

2.4.3 Plasma

There were no differences in PUN concentrations (Table 2.3). Plasma glucose levels, as well as NEFA concentrations were not different between treatment groups (Table 2.3).

2.4.4 Milk yield and components

Total milk yield (kg/d), as well as the content (%) and yield (kg/d) of milk fat and protein were not affected by treatment (Table 2.4). There were no additional differences observed in other milk parameters including MUN and somatic cell count.

2.4.5 Nitrogen partitioning

There was no difference in N intake (g N/d), g N/d in urine, feces, or milk, or g N/d retained across treatments (Table 2.2).

2.4.6 High-abundance milk protein concentrations determined by HPLC analysis

There were no differences in the milk concentrations of α -s1, α -s2, β or κ - CAS, α -LA, β -LGA, or β -LGB across treatment groups (Table 2.5).

2.4.7 Low-abundance protein enriched milk fraction

A total of 127 proteins were identified (Supplementary Table 2.1); and of those, 16 were affected by treatment (Table 2.6).

2.4.8 Bioinformatic analysis

Gene ontology analysis of the 16 affected proteins revealed cellular process (GO term: 0009987; 25.6%) as the most prominent term for biological process, followed by metabolic process (GO term: 0008152; 12.8%), response to stimulus (GO term: 0050896; 12.8%), and localization (GO term: 0051179; 12.8%; Figure 2.1, Table 2.7). Accounting for 50% of the 16 proteins, catalytic activity (GO term: 0003824) was the most annotated molecular function term. Cellular component analysis categorized 28.6% of the proteins as extracellular (GO term: 0005576) and an additional 21.4% were identified to be of membrane origin (GO term: 0016020). Additionally, the most prominent protein classes included: transporter (GO term: PC00227; 23.1%), oxidoreductase (GO term: PC00176; 15.4%), transfer/ carrier protein (GO term: PC00219; 15.4%), and enzyme modulator (GO term: PC00095; 15.4%) classifications.

STRING analysis identified a network interaction between 9 of the proteins affected by dietary treatment (Figure 2.2): complement component C9 (C9), clusterin (CLU), sulfhydryl oxidase (QSOX1), serum albumin (ALB), kininogen-1 (KNG1), cystatin-C (CST3), platelet glycoprotein 4 (CD36), apolipoprotein E (APOE), and ATP-binding cassette sub-family G member 2 (ABCG2) nodes.

2.5 Discussion

In this study, the objectives were to identify shifts in the bovine milk proteome of cows fed GM compared to control cows using proteomic approaches and to assess differences in N partitioning between the two groups of cows. There have been several studies evaluating the effects of diet on total bovine milk protein content (Depeters and Cant, 1992), and more recent research has confirmed that changes in milk protein composition itself can be achieved through dietary intervention (Li et al., 2015). It is evident from other studies that altering primary nutrient fractions, including the dietary protein fraction, can shift the milk proteome (Sutton, 1989; Christian et al., 1999; Li et al., 2015). To further validate those findings, the research described herein focused on the utilization of the byproduct GM as a means to alter the milk proteome, since it is a byproduct that has shown promising results in altering N metabolism (Greenwood et al., 2012).

No changes in N partitioning were observed in the current study, and this is likely due to a low-dietary inclusion of GM as well as a low CT content in the GM in the diet. Feeding 1.5 kg GM/d was likely not enough to elicit changes in N metabolism as seen in previous studies, for example where GM was fed to grazing cows (Greenwood et al., 2012). With a concentration of 4.29 g CT/kg GM, the CT concentration of the GM used in our study was very low compared to other studies (Waghorn et al., 1987; Greenwood et al., 2012). Previous studies have also outlined the potential variability of CT binding (Acamovic and Brooker, 2005), and this variability could also have been a factor in the current results. While there is evidence suggesting that protein-CT complexes dissociate in the abomasum of ruminants, it has also been hypothesized that

some CT can re-associate with proteins downstream in the digestive tract such as the small intestine, and thus inhibiting intestinal proteolysis and absorption (Acamovic and Brooker, 2005). Alternatively, the CT may be binding the proteins with such astringency that prevents dissociation entirely. While it has also been suggested that CT can inhibit carbohydrate digestion in the rumen (Susmel and Stefanon, 1993), protein binding is the predominate effect, which further demonstrates that the binding properties of CT *in vivo* are not entirely predictable because of differential substrate preferences (Acamovic and Brooker, 2005). Furthermore, there could also be other interactions with free tannins in the small intestine limiting nutrient absorption by affecting digestive tract permeability and enzymatic activity (Acamovic and Brooker, 2005).

Although there were no changes in metabolic or N status, the current study did identify 16 proteins that were affected by dietary treatment, including the higher relative abundance of two bioactives defined in literature, serum amyloid A protein (SAA3) (Mills et al., 2011) and butyrophilin subfamily 1 member A1 (BTN) (Spitsberg, 2005).

SAA3 in blood has been classically known as a marker of inflammation; however, there is also evidence suggesting direct antimicrobial activity (Molenaar et al., 2009; Mills et al., 2011). It is now accepted that SAA3 also originates from the mammary gland, and it has been shown that mammary-derived SAA3 exhibits an extra-mammary protective response against microbial infection (Molenaar et al., 2009). It has been further proposed that the antimicrobial role of SAA3 is included in a more generalized host response that affects the binding properties of pathogens (Mills et al., 2011). Similarly to SAA3, the relative abundance of BTN was also higher in milk samples from GM-fed cows compared to control cows. Though BTN is the major protein associated with the

milk-fat globule membrane (MFGM) (Spitsberg, 2005), its presence in non-fat milk fractions has previously been reported (Nissen et al., 2013) and is in agreement with the current study. Characterization of BTN functionality apart from its function in milk fat globule stability has led to the identification of numerous roles of BTN family members, including an important role in immune cell activation (Arnett and Viney, 2014). The higher relative abundance of these two immunomodulatory proteins (2.13 fold increase in BTN, 1.76 fold increase in SAA3) occurs alongside with shifts in known blood proteins, including von Willebrand factor A domain-containing protein, platelet glycoprotein 4, kininogen-1 and serum albumin. While all of these proteins have previously been identified in ProteoMiner-treated milk fractions, (Molinari et al., 2012; Tacoma et al., 2016) the current finding that GM treatment affected their relative abundance in milk suggests that perhaps paracellular or transcytotic passage of these extracellular proteins across the mammary epithelium was changed, which are known mechanisms of blood-milk protein connectivity (Shennan and Peaker, 2000; Monks and Neville, 2004; Kobayashi et al., 2013). Of the 16 milk proteins altered by dietary treatment, 28.6% were of extracellular origin, with another 21% of membrane origin (Figure 2.1), 15% of the treatment affected proteins being annotated as transfer/carrier proteins, which include proteins that carry substances and do not involve transmembrane transport, and 23% of the proteins annotated as protein class transporter, which do include proteins with transmembrane activity (Figure 2.1; Mi et al., 2017).. The immune- and transport- related protein classification suggested by The PANTHER Classification System is further supported by STRING analysis, which revealed that 9 of the treatment-affected proteins had one or multiple interactions with other proteins (nodes) altered by the diet (Figure 2),

including immune-related proteins such as complement component C9 (C9), clusterin (CLU), and apolipoprotein E (APOE).

The mechanism(s) driving the shifts in the milk proteome observed in the current study are less clear. Scrutiny of the diet and treatment impacts yields three potential mechanistic hypotheses. One dietary fraction that could have contributed to the differences observed in the milk proteome is the fiber content, particularly the lignin content. Lignin is an indigestible phenolic compound often associated with the cellulose fraction of the feed, (Susmel and Stefanon, 1993) and GM-fed cows consumed approximately 400 g/d more lignin from their supplement as compared to the control cows (Table 2.8). It is plausible that lignin could have had a small impact on DMI and nutrient uptake, ultimately impacting mammary nutrient availability. However, plasma metabolites, including glucose and NEFA, were not affected by treatment and these parameters would reflect any deficit.

A second hypothesis addressing the possible mechanisms of diet-induced milk proteome shifts in the current study is that other phytochemicals apart from CT, such as anthocyanins, which are rich in grape pomace (Yi et al., 2009) but were not measured in this study. Anthocyanins have been documented to have several human health impacts, including immunomodulation (He and Giusti, 2010), which could in part explain the observed differences in the milk proteome of GM-fed cows compared to control cows. Based on research by Yi et al. (2009), the anthocyanin content of the GM would be approximately 0.1%, equating to an intake of roughly 1.8 g anthocyanins per treatment cow per day in the current study. According to Hosoda et al. (2012), no difference in DMI or milk production was observed when lactating cows consumed an additional 14.9

g/d of anthocyanin in an enriched corn silage. Though there were some effects of treatment on digestibility in the study by Hosoda et al. (2012), the anythocyanin intake was an estimated 88% higher in their study compared to the current study. Therefore, the anthocyanin content was likely too low to alone induce the observed changes in the milk proteome observed in the research described herein.

A third possible mechanism is highlighted after more intense scrutiny of the results, and aligns with our original hypothesis. Despite conducting a statistical analysis to determine the appropriate number of animals to use for this study, statistical tendencies ($0.05 < P < 0.10$) were observed in the present study, including lower milk yield ($P = 0.07$; Table 4) from cows fed GM, and higher g/d fecal N output ($P = 0.09$; Table 2) from GM-fed cows. Higher fecal N output by GM-fed cows is in line with previous literature, (Greenwood et al., 2012) and agrees with the idea that highly astringent CT from GM may irreversibly bind with ingested proteins or quickly re-associate with proteins post-ruminally, ultimately making the bound proteins, peptides and amino acids unavailable for absorption in the small intestine (Acamovic and Brooker, 2005). A reduction in intestinal absorption of these N-containing compounds would create the higher fecal N loss observed, and would also explain the tendency for milk yield to decrease in cows that were fed GM. The lack of difference observed in plasma and milk N may be because these two fractions have larger N pools that are supplemented by hepatic, muscle, and renal metabolism. If a higher CT-concentration was used in the diet, it is plausible that a more clear relationship between N partitioning and changes in the milk proteome could be observed.

2.6 Conclusions

In the present study, the effect of dietary GM on the bovine milk proteome was evaluated. This study did not observe any changes in N partitioning as a result of GM intake. Despite the low CT content of the GM, 16 proteins in the low-abundance enriched milk protein fraction were affected by diet. Bioinformatic analysis of these 16 proteins suggested that many of these proteins are not of intracellular origin, and several plasma-associated proteins were identified in the milk. Our observation that dietary GM intake affected the abundance of these proteins could indicate a change in passage of these proteins across the blood – milk barrier. The hypothesized mechanisms relating dietary GM to the proteomic composition of milk could not be confirmed, and further investigation to clarify this relationship is needed.

Table 2.1 Ingredients and chemical composition of experimental diets

Ingredient (kg DM/day)	Control	GM
Corn silage	9.0	9.0
Concentrate	8.8	8.8
Grass silage	4.2	4.2
Mash	1.9	1.9
Grape marc (GM)	0.0	1.5
Beet pulp/ soy hulls (BP)	2.0	0.0
DM (%)	50.54	47.48
aNDFom (% DM)	34.33	33.85
Crude protein (% DM)	15.73	16.01
Non-fiber carbohydrate (% DM)	39.57	39.13
Ether extract (% DM)	4.13	4.77
GM CT content (g CT/kg GM)	0.00	4.29

Concentrate comprised of wheat middlings, pellet steam flaked corn, soybean meal, distillers grains, fine ground corn meal. Mash comprised of ground fine corn grain (28%), canola meal solvent (12.5%), soybean meal solvent (8.9%), and Rumensin[®] (270 mg/day). aNDFom, Ash-corrected Neutral Detergent Fiber (NDF). CT, condensed tannins.

Table 2.2 Urine and fecal N parameters of lactating Holstein dairy cows fed a diet supplemented with either grape marc (GM) or a beet pulp: soy hulls mixture (control)

	Control	GM	SE	<i>P</i> -value
urine urea (%)	0.06	0.02	0.027	0.34
urine ammonia (%)	0.01	0.01	0.002	0.20
urine crude protein (%)	5.36	6.06	0.680	0.49
fecal nitrogen (%)	0.37	0.39	0.021	0.41
fecal ammonia N (%)	0.03	0.03	0.002	0.29
dietary nitrogen intake (g N/d)	619.4	606.3	32.75	0.78
milk nitrogen output (g N/d)	193.4	194.3	16.28	0.97
fecal nitrogen output (g N/d)	136.6	175.0	14.24	0.09
urine nitrogen output (g N/d)	155.5	151.1	23.02	0.90
retained nitrogen (g N/d)	133.9	86.0	46.23	0.48

Least square means reported for Control and GM groups. Expressed as percent of DM.

SE, standard error.

Table 2.3. Plasma metabolite concentrations of lactating Holstein dairy cows fed a diet supplemented with either grape marc (GM) or a beet pulp: soy hulls mixture (control)

	Control	GM	SE	<i>P</i> -Value
PUN (mg/mL)	10.21	9.93	0.49	0.71
Plasma glucose (mg/mL)	54.66	55.00	0.02	0.90
NEFA (μ M)	27.37	26.46	2.82	0.83

Least square means reported for Control and GM groups. SE, standard error; PUN,

plasma urea nitrogen; NEFA, non-esterified fatty acids.

Table 2.4. Dry matter intake, milk yield, and milk components of lactating dairy Holstein dairy cows fed a diet supplemented with either grape marc (GM) or a beet pulp: soy hulls mixture (control)

	Control	GM	SE	<i>P</i> -value
DMI (kg/day)	25.83	26.61	0.93	0.57
Milk yield (kg/day)	39.87	36.22	1.22	0.07
Milk components (%)				
Milk fat	4.10	3.86	0.16	0.32
Milk protein	3.34	3.43	0.04	0.13
Milk components yield (kg/day)				
Milk fat	1.62	1.38	0.85	0.10
Milk protein	1.32	1.23	0.05	0.19
MUN (mg/dL)	12.80	13.46	0.43	0.32
SCC (Cells x1000)	83.15	76.12	24.99	0.85

Least square means reported for Control and GM groups. SE, standard error; DMI, dry matter intake; MUN, milk urea nitrogen; SCC, somatic cell count.

Table 2.5. High-abundance protein concentrations from lactating Holstein dairy cows fed a diet supplemented with either grape marc (GM) or a beet pulp: soy hulls mixture (control)

Milk protein (mg/mL skim milk)	Control	GM	SE	<i>P</i> -value
Casein				
α-S1 CAS	13.02	13.42	0.22	0.24
α-S2 CAS	1.78	1.78	0.05	1.00
β-CAS	17.94	18.48	0.28	0.23
κ-CAS	5.55	5.65	0.09	0.43
Whey				
α-LA	3.01	3.00	0.01	0.55
β-LGA	3.20	3.16	0.07	0.70
β-LGB	3.39	3.33	0.02	0.10

Least square means reported for Control and GM groups. SE, standard error. CAS, casein; β-LGA, β-Lactoglobulin variant A; β-LGB, β-Lactoglobulin variant B.

Table 2.6. 16 Low-abundance enriched proteins identified in milk samples at significantly different relative-abundances collected from lactating Holstein dairy cows fed a diet supplemented with either grape marc (GM) or a beet pulp: soy hulls mixture (control)

Accession number	Protein	Control	GM	SE	<i>P</i> -Value
Q03247	Apolipoprotein E	1.01	1.43	0.083	0.002
Q3MHN2	Complement component C9	1.06	0.73	0.086	0.012
P17697	Clusterin	1.04	1.42	0.073	0.014
P18892	Butyrophilin subfamily 1 member A1	1.17	2.13	0.319	0.015
F1MM32	Sulfhydryl oxidase	1.07	0.77	0.088	0.019
Q4GZT4	ATP-binding cassette sub-family G member 2	1.11	1.62	0.210	0.021
F1MIT3	von Willebrand factor A domain-containing protein 8	1.41	13.97	3.519	0.022
M0QW03	TPA: prolactin-like protein	1.06	1.53	0.159	0.025
P01035	Cystatin-C	1.17	0.67	0.126	0.030
P02769	Serum albumin	1.30	0.66	0.180	0.032
F1MMW8	Serum amyloid A protein	1.09	1.76	0.235	0.033
F1MNV5	Kininogen-1	1.15	0.75	0.131	0.036
P26201	Platelet glycoprotein 4	1.44	2.79	0.518	0.040
F1N6D4	Sodium-dependent phosphate transport protein 2B	1.47	2.90	0.565	0.042
F1MUP9	synaptic vesicle membrane protein VAT-1 homolog	1.02	1.26	0.081	0.044
Q0V8M0	Protein KRI1 homolog	1.02	1.16	0.033	0.046

Least square means reported for Control and GM groups expressed as relative-abundance. SE, standard error.

Table 2.7. GO annotation of the 16 Low-abundance enriched proteins identified in milk samples at significantly different relative-abundances collected from lactating Holstein dairy cows fed a diet supplemented with either grape marc (GM) or a beet pulp: soy hulls mixture (control)

Accession number	Protein	Gene Ontology			
		Biological Process	Molecular Function	Cellular Process	Protein Class
Q03247	Apolipoprotein E	Anion transport, Biosynthetic process, Catabolic process, Cell differentiation, Cell growth, Cellular component biogenesis, Cellular component morphogenesis, Cholesterol metabolic process, Homeostatic process, Negative regulation of apoptotic process, Nervous system development, Protein metabolic process, Response to stress, Single-	Enzyme activator activity, Lipid binding, Lipid transporter activity, Oxio-reductase activity, Receptor binding, Transferase activity (transferring acyl groups)	Extracellular space, Macromolecular complex	

		multicellular organism process			
Q3MHN2 P17697	Complement component C9 Clusterin	Cell-cell adhesion, Immune system process, Signal transduction			
P18892	Butyrophilin subfamily 1 member A1	Cellular defense response, Cellular process, Exocytosis, Intracellular transport protein, Proteolysis, System development	Ubiquitin-protein ligase activity		Ubiquitin protein ligase
F1MM32	Sulfhydryl oxidase	Cellular process	Oxio-reductase activity, Protein disulfide isomerase activity	Golgi apparatus, Cytoplasm, Extracellular space, Integral to membrane	Oxidase

Q4GZT4	ATP-binding cassette sub-family G member 2 von Willebrand factor A domain-containing protein 8	Catabolic process, Cellular process, Nucleobase-containing compound metabolic process, Phosphate-containing compound metabolic process, Response to stimulus	ATPase activity (coupled to transmembrane movement of substances), Lipid transporter activity, Pyrophosphatase activity, Transmembrane transporter activity	Apical part of cell, Integral to membrane, Plasma membrane, Protein complex	ATP-binding cassette (ABC) transporter
F1MIT3	TPA: prolactin-like protein	Cellular process	Growth factor activity, Hormone activity		Growth factor, Peptide hormone
M0QW03	Cystatin-C	Proteolysis	Cysteine-type endopeptidase inhibitor activity, Protein binding		Cysteine protease inhibitor
P01035	Serum albumin	Transport			Transfer/ carrier protein
P02769	Serum amyloid A protein	Cellular component movement, Localization, Locomotion, Response to external stimulus	Extracellular space		Apolipoprotein, Defense/immunity protein, Transporter

F1MNV5	Kininogen-1	Blood coagulation, Cellular calcium ion homeostasis, Cellular process, Regulation of biological process, Response to stress, Single-multicellular organism process	Cysteine-type endopeptidase inhibitor activity	Extracellular space	
P26201	Platelet glycoprotein 4	Cell adhesion, Cellular process, Macrophage activation	Receptor activity		Receptor
F1N6D4	Sodium- dependent phosphate transport protein 2B	Anion transport, Cellular process, Homeostatic process, Phosphate ion transport	Cation transmembrane transporter activity	Apical part of cell, Cell projection, Cytoplasm, Organelle, Plasma membrane	Enzyme modulator, Transporter
F1MUP9	synaptic vesicle membrane protein VAT-1 homolog	Apoptotic process, Carbohydrate metabolism process	Oxio-reductase activity		Dehydrogenase, Reductase
Q0V8M0	Protein KRI1 homolog				

Table 2.8. Chemical composition of supplements

	Control	GM
dry matter (%)	90.1	30.2
crude protein (% DM)	9.9	12.8
ADF (% DM)	41.3	44.2
aNDFom (% DM)	50.5	53.2
lignin (% DM)	3.0	30.2
NFC (% DM)	30.8	20.1
starch (% DM)	6.8	19.9
crude fat (% DM)	1.4	9.4
ash (% DM)	7.45	4.52

aNDFom, ash-corrected neutral detergent fiber; ADF, acid-detergent fiber; NFC, non-fiber carbohydrate; GM, grape marc; BP, beet pulp: soyhulls mixture.

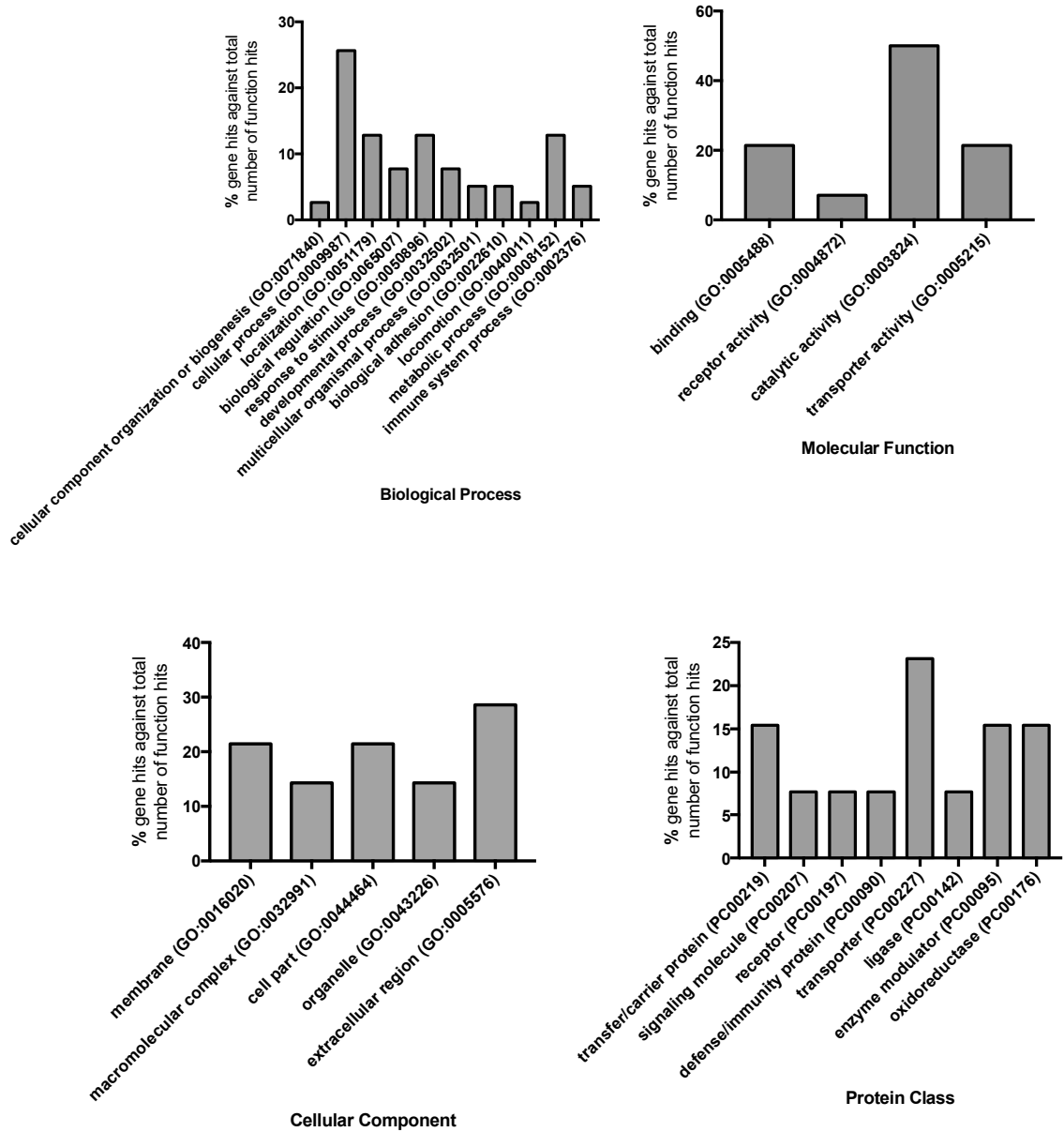


Figure 2.1. Gene ontology (GO) representing the biological processes, molecular functions, cellular components, as well as protein classes of proteins identified by LC-MS/MS that were different between treatment groups.

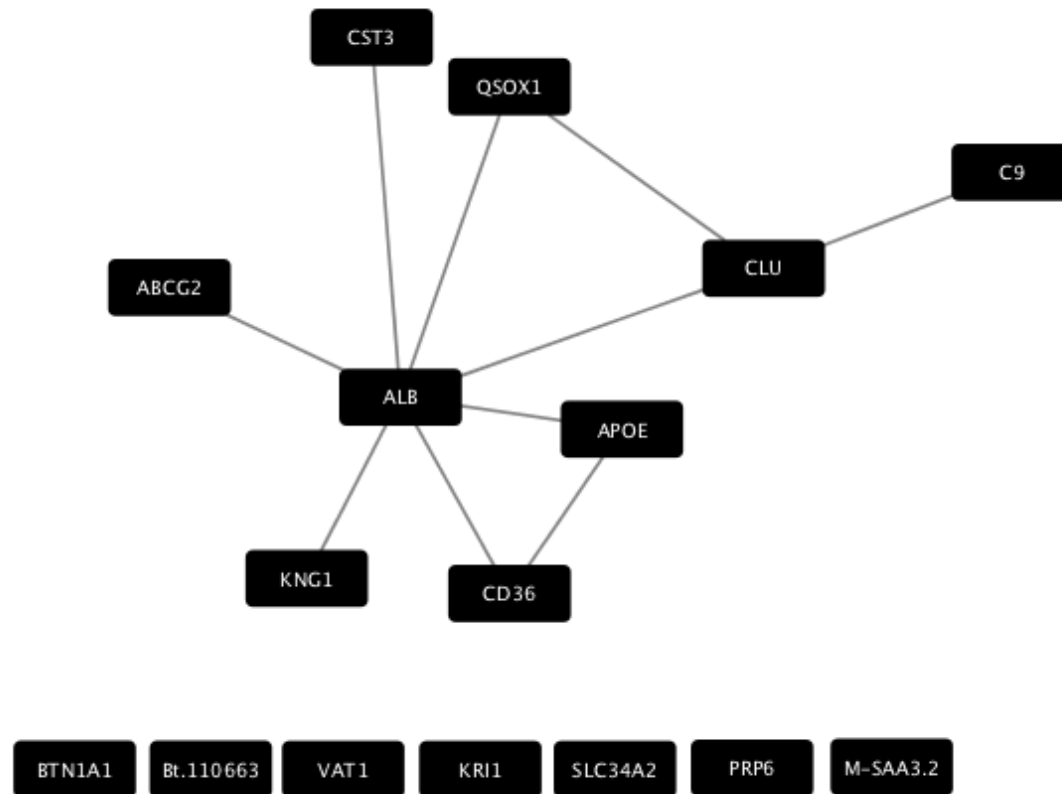


Figure 2.2. STRING map illustrating the relationship between proteins characterized by GO analysis, including complement component C9 (C9), clusterin (CLU), sulfhydryl oxidase (QSOX1), serum albumin (ALB), kininogen-1 (KNG1), cystatin-C (CST3), platelet glycoprotein 4 (CD36), apolipoprotein E (APOE), and ATP-binding cassette sub-family G member 2 (ABCG2).

2.7 References

- Acamovic, T., and J. D. Brooker. 2005. Biochemistry of plant secondary metabolites and their effects in animals. *Proc. Nutr. Soc.* 64:403-412.
<https://dx.doi.org/10.1079/PNS2005449>
- Arnett, H. A., and J. L. Viney. 2014. Immune modulation by butyrophilins. *Nat. Rev. Immunol.* 14:559-569. <http://dx.doi.org/10.1038/nri3715>
- Ben-Gera, I., and A. Kramer. 1969. The utilization of food industries wastes. *Adv. Food Res.* 17:77-152. [https://dx.doi.org/10.1016/S0065-2628\(08\)60309-2](https://dx.doi.org/10.1016/S0065-2628(08)60309-2)
- Bordin, G., F. C. Raposo, B. de la Calle, and A. R. Rodriguez. 2001. Identification and quantification of major bovine milk proteins by liquid chromatography. *J. Chromatogr. A.* 928:63-76. [https://dx.doi.org/10.1016/S0021-9673\(01\)01097-4](https://dx.doi.org/10.1016/S0021-9673(01)01097-4)
- Camacho, C., G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, and T. L. Madden. 2009. BLAST+: architecture and applications. *BMC Bioinf.* 10:421.
<https://dx.doi.org/10.1186/1471-2105-10-421>
- Christian, M. P., C. Grainger, B. J. Sutherland, J. J. Mayes, M. C. Hannah, and B. Kefford. 1999. Managing diet quality for Cheddar cheese manufacturing milk. 1. The influence of protein and energy supplements. *J. Dairy Res.* 66:341-355.
- Depeters, E. J., and J. P. Cant. 1992. Nutritional factors influencing the nitrogen composition of bovine-milk - a review. *J. Dairy Sci.* 75:2043-2070.
[https://dx.doi.org/10.3168/jds.S0022-0302\(92\)77964-8](https://dx.doi.org/10.3168/jds.S0022-0302(92)77964-8)
- Greenwood, S. L., G. R. Edwards, and R. Harrison. 2012. Short communication: supplementing grape marc to cows fed a pasture-based diet as a method to alter nitrogen partitioning and excretion. *J. Dairy Sci.* 95:755-758.
<https://dx.doi.org/10.3168/jds.2011-4648>
- He, J. A., and M. M. Giusti. 2010. Anthocyanins: natural colorants with health-promoting properties. *Annu. Rev. Food Sci. Technol.* 1:163-187.
<https://dx.doi.org/10.1146/annurev.food.080708.100754>
- Hosoda, K., B. Eruden, H. Matsuyama, and S. Shioya. 2012. Effect of anthocyanin-rich corn silage on digestibility, milk production and plasma enzyme activities in lactating dairy cows. *Anim. Sci. J.* 83:453-459.
<http://dx.doi.org/10.1111/j.1740-0929.2011.00981.x>

- Kobayashi, K., S. Oyama, A. Numata, M. M. Rahman, and H. Kumura. 2013. Lipopolysaccharide disrupts the milk-blood barrier by modulating claudins in mammary alveolar tight junctions. *PloS One* 8: e62187. <https://dx.doi.org/10.1371/journal.pone.0062187>
- Korhonen, H. 2009. Milk-derived bioactive peptides: from science to applications. *J. Funct. Food* 1:177-187. <https://dx.doi.org/10.1016/j.jff.2009.01.007>
- Lascano, G. J., G. I. Zanton, A. J. Heinrichs, and W. P. Weiss. 2010. Technical note: a noninvasive urine collection device for female cattle: modification of the urine cup collection method. *J. Dairy Sci.* 93:2691-2694. <https://dx.doi.org/10.3168/jds.2009-3027>
- Le, A., L. D. Barton, J. T. Sanders, and Q. Zhang. 2011. Exploration of bovine milk proteome in colostrum and mature whey using an Ion-Exchange approach. *J. Proteome Res.* 10:692-704. <https://dx.doi.org/10.1021/pr100884z>
- Lee, M. R., V. J. Theobald, J. K. Tweed, A. L. Winters, and N. D. Scollan. 2009. Effect of feeding fresh or conditioned red clover on milk fatty acids and nitrogen utilization in lactating dairy cows. *J. Dairy Sci.* 92:1136-1147. <https://dx.doi.org/10.3168/jds.2008-1692>
- Li, S. S., J. S. Shen, D. X. Ren, and J. X. Liu. 2015. Effects of the processing methods of corn grain and soybean meal on milk protein expression profiles in dairy cows. *Animal* 9:267-274. <https://dx.doi.org/10.1017/S1751731114002225>
- Makkar, H. P. S. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small Ruminant Research* 49:241-256. [https://dx.doi.org/10.1016/S0921-4488\(03\)00142-1](https://dx.doi.org/10.1016/S0921-4488(03)00142-1)
- Manso, T., B. Gallardo, A. Salva, C. Guerra-Rivas, A. R. Mantecon, P. Lavin, and M. A. de la Fuente. 2016. Influence of dietary grape pomace combined with linseed oil on fatty acid profile and milk composition. *J. Dairy Sci.* 99:1111-1120. <https://dx.doi.org/10.3168/jds.2015-9981>
- Mercurio, M. D., R. G. Damberg, M. J. Herderich, and P. A. Smith. 2007. High throughput analysis of red wine and grape phenolics-adaptation and validation of methyl cellulose precipitable tannin assay and modified somers color assay to a rapid 96 well plate format. *J. Agric. Food Chem.* 55:4651-4657. <https://dx.doi.org/10.1021/jf063674n>

- Mi, H., X. Huang, A. Muruganujan, H. Tang, C. Mills, D. Kang, and P. D. Thomas. 2017. PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. *Nucleic Acids Res.* 45:D183-D189. <https://dx.doi.org/10.1093/nar/gkw1138>
- Mills, S., R. P. Ross, C. Hill, G. F. Fitzgerald, and C. Stanton. 2011. Milk intelligence: mining milk for bioactive substances associated with human health. *Int. Dairy J.* 21:377-401. <https://dx.doi.org/10.1016/j.idairyj.2010.12.011>
- Molenaar, A. J., D. P. Harris, G. H. Rajan, M. L. Pearson, M. R. Callaghan, L. Sommer, V. C. Farr, K. E. Oden, M. C. Miles, R. S. Petrova, L. L. Good, K. Singh, R. D. McLaren, C. G. Prosser, K. S. Kim, R. J. Wieliczko, M. H. Dines, K. M. Johannessen, M. R. Grigor, S. R. Davis, and K. Stelwagen. 2009. The acute-phase protein serum amyloid A3 is expressed in the bovine mammary gland and plays a role in host defence. *Biomarkers* 14:26-37. <http://dx.doi.org/10.1080/13547500902730714>
- Molinari, C. E., Y. S. Casadio, B. T. Hartmann, A. Livk, S. Bringans, P. G. Arthur, and P. E. Hartmann. 2012. Proteome mapping of human skim milk proteins in term and preterm Milk. *J. Proteome Res.* 11:1696-1714. <https://dx.doi.org/10.1021/pr2008797>
- Monks, J., and M. C. Neville. 2004. Albumin transecytosis across the epithelium of the lactating mouse mammary gland. *J. Physiol. London* 560:267-280. <https://dx.doi.org/10.1113/jphysiol.2004.068403>
- Nissen, A., E. Bendixen, K. L. Ingvarsten, and C. M. Rontvedt. 2013. Expanding the bovine milk proteome through extensive fractionation. *J. Dairy Sci.* 96:7854-7866. <https://dx.doi.org/10.3168/jds.2013-7106>
- Nudda, A., F. Correddu, A. Marzano, G. Battacone, P. Nicolussi, P. Bonelli, and G. Pulina. 2015. Effects of diets containing grape seed, linseed, or both on milk production traits, liver and kidney activities, and immunity of lactating dairy ewes. *J. Dairy Sci.* 98:1157-1166. <https://dx.doi.org/10.3168/jds.2014-8659>
- Santos, N. W., G. T. D. Santos, D. C. Silva-Kazama, P. A. Grande, P. M. Pintro, F. E. de Marchi, C. C. Jobim, and H. V. Petit. 2014. Production, composition and antioxidants in milk of dairy cows fed diets containing soybean oil and grape residue silage. *Livestock Science* 159:37-45. <https://dx.doi.org/10.1016/j.livsci.2013.11.015>

- Sarneckis, C. J., R. G. Damberg, P. Jones, M. Mercurio, M. J. Herderich, and P. A. Smith. 2006. Quantification of condensed tannins by precipitation with methyl cellulose: development and validation of an optimised tool for grape and wine analysis. *Aust. J. Grape Wine Res.* 12:39-49.
<https://dx.doi.org/10.1111/j.1755-0238.2006.tb00042.x>
- Shannon, P., A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski, and T. Ideker. 2003. Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13:2498-2504. <https://dx.doi.org/10.1101/gr.1239303>
- Shennan, D. B., and M. Peaker. 2000. Transport of milk constituents by the mammary gland. *Physiol. Rev.* 80:925-951.
- Spitsberg, V. L. 2005. Invited review: Bovine milk fat globule membrane as a potential nutraceutical. *J. Dairy Sci.* 88:2289-2294.
[https://dx.doi.org/10.3168/jds.S0022-0302\(05\)72906-4](https://dx.doi.org/10.3168/jds.S0022-0302(05)72906-4)
- Susmel, P., and B. Stefanon. 1993. Aspects of lignin degradation by rumen microorganisms. *J. Biotechnol.* 30:141-148.
[https://dx.doi.org/10.1016/0168-1656\(93\)90035-L](https://dx.doi.org/10.1016/0168-1656(93)90035-L)
- Sutton, J. D. 1989. Altering milk composition by feeding. *J. Dairy Sci.* 72:2801-2814.
[https://dx.doi.org/10.3168/jds.S0022-0302\(89\)79426-1](https://dx.doi.org/10.3168/jds.S0022-0302(89)79426-1)
- Szklarczyk, D., A. Franceschini, S. Wyder, K. Forslund, D. Heller, J. Huerta-Cepas, M. Simonovic, A. Roth, A. Santos, K. P. Tsafou, M. Kuhn, P. Bork, L. J. Jensen, and C. von Mering. 2015. STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* 43(Database issue):D447-D452. <https://dx.doi.org/10.1093/nar/gku1003>
- Szklarczyk, D., J. H. Morris, H. Cook, M. Kuhn, S. Wyder, M. Simonovic, A. Santos, N. T. Doncheva, A. Roth, P. Bork, L. J. Jensen, and C. von Mering. 2017. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res.* 45:D362-D368.
<https://dx.doi.org/10.1093/nar/gkw937>
- Tacoma, R., J. Fields, D. B. Ebenstein, Y.-W. Lam, and S. L. Greenwood. 2016. Characterization of the bovine milk proteome in early-lactation Holstein and Jersey breeds of dairy cows. *J. Proteomics* 130:200-210.
<https://dx.doi.org/10.1016/j.jprot.2015.09.024>

- Totty, V. K., S. L. Greenwood, R. H. Bryant, and G. R. Edwards. 2013. Nitrogen partitioning and milk production of dairy cows grazing simple and diverse pastures. *J. Dairy Sci.* 96:141-149. <https://dx.doi.org/10.3168/jds.2012-5504>
- Tsiplakou, E., and G. Zervas. 2008. The effect of dietary inclusion of olive tree leaves and grape marc on the content of conjugated linoleic acid and vaccenic acid in the milk of dairy sheep and goats. *J. Dairy Res.* 75:270-278. <https://dx.doi.org/10.1017/S0022029908003270>
- US Department of Agriculture. 2015. Noncitrus fruit and nuts 2014 preliminary summary. ISSN 1948-2698.
- Waghorn, G. 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production-progress and challenges. *Anim. Feed Sci. Technol.* 147:116-139. <https://dx.doi.org/10.1016/j.anifeedsci.2007.09.013>
- Waghorn, G. C., M. J. Ulyatt, A. John, and M. T. Fisher. 1987. The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *Lotus corniculatus* L. *Br. J. Nutr.* 57:115-126. <https://dx.doi.org/10.1079/BJN19870015>
- Woodward, S. L., G. G. Waghorn, K. A. Watkins, and M. A. Bryant. 2009. Feeding birdsfoot trefoil (*Lotus corniculatus*) reduces the environmental impacts of dairy farming. *Proc. N.Z. Soc. Anim. Prod.* 69:179-183.
- Yang, Y., N. Zheng, X. Zhao, Y. Zhang, R. Han, L. Ma, S. Zhao, S. Li, T. Guo, and J. Wang. 2015. Proteomic characterization and comparison of mammalian milk fat globule proteomes by iTRAQ analysis. *J. Proteomics* 116:34-43. <https://dx.doi.org/10.1016/j.jprot.2014.12.017>
- Yi, C., J. Shi, J. Kramer, S. Xue, Y. M. Jiang, M. W. Zhang, Y. Ma, and J. Pohorly. 2009. Fatty acid composition and phenolic antioxidants of winemaking pomace powder. *Food Chem.* 114:570-576. <https://dx.doi.org/10.1016/j.foodchem.2008.09.103>

CHAPTER 3: COMPARATIVE ANALYSIS OF THE SKIM MILK AND MILK FAT GLOBULE MEMBRANE PROTEIN FRACTIONS PRODUCED BY JERSEY COWS GRAZING DIFFERENT PASTURE FORAGE CROPS

3.1 Abstract

The objective of this experiment was to determine whether the inclusion of alternative forage crops in pasture grazed by lactating Jersey cattle would alter the bovine milk proteome they produce. Sixteen lactating Jersey cows were blocked by days in milk (143 ± 58 DIM) for a 21-day pasture-based experiment. Cows received a partial mixed ration (60% of total dry matter intake, DMI) and had access to one of two pasture treatments for grazing (40% of total DMI). The control animals (CON, n=8) were offered a grass-legume pasture mixture, which included orchardgrass (*Dactylis glomerata*), timothy (*Phleum pratense*), Kentucky bluegrass (*Poa pratensis*), and white clover (*Trifolium repens*). The treatment group (AFC, n=8) were offered the same base pasture strip-tilled with the AFC crops oat (*Avena sativa*), buckwheat (*Fagopyrum esculentum*), and chickling vetch (*Lathyrus sativus*) so as to represent 10% of their pasture DMI. Milk samples were collected during AM and PM milkings on d 19-21 for milk proteomic analysis. In total, three milk samples were composited within cow according to milk yield. One of these skimmed milk samples was used for high-abundance milk protein analysis using high-performance liquid chromatography. The second skimmed sample was subjected to low-abundance protein identification using liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. The fat layer was collected from the third sample via centrifugation at 3000xg for 15 min at 4° for analysis of the low-abundance enriched protein fraction within the milk fat globule membrane (MFGM)

using LC-MS/MS analysis. There was a higher concentration (mg/mL of skim milk) of the high-abundance protein α -s1 CAS in milk from AFC cows ($P = 0.005$). Using LC-MS/MS methodology, 53 proteins were identified in the skim milk fraction, and 245 in the MFGM-protein associated fraction. Two proteins were altered by diet in the skim fraction, and five in the MFGM fraction; these seven affected proteins were all higher in relative-abundance in milk from the AFC cows compared to the control cows. While the mechanism(s) responsible for the changes observed in the seven proteins remain unknown, the results of this study highlight the need for continued investigation into dietary influences of the bovine milk proteome.

3.2 Introduction

The northeastern United States faces challenges with maintaining optimal dry matter intake (DMI) of grazing dairy cattle due a decrease in the growth of cool season grasses (CSG) in the late spring and summer months. One method that has been implemented to alleviate this loss of DM availability is to increase pasture biomass through the inclusion of alternative forage crops (AFC) into pasture, which include non-traditional grasses, legumes, and grain crops. The incorporation of AFC species in pasture can effectively increase pasture biomass when CSG growth is limited in the summer months, but can also provide benefits such as drought resistance through increasing biodiversity (Tilman and Downing, 1994), and lower feed costs for pastoral producers (Pereira et al., 2013).

The inclusion of AFC in pasture can also affect the nutrients available to the animal. Milk production can be modified as a result from changes in the absorbed

nutrient profile and post-absorptive metabolism, which has been reported in other pasture based studies (Miller et al., 2001; Totty et al., 2013). While parameters, such as milk yield, can be influenced by the total energy and protein availability from the diet (Christian et al., 1999; Li et al., 2015), the milk profile may be more subject to changes induced by a specific dietary nutrient profile (Jenkins and McGuire 2006). Additionally, secondary phytochemicals, including condensed tannin(s) (CT) and other phenolic compounds within various AFC species, can modify nutrient absorption and metabolic patterns when consumed, which is considered to be a benefit depending on the base forage diet (Waghorn et al., 1987; Makkar, 2003; Acamovic and Brooker, 2005). Since some AFC species are known to contain a nutritive profile that is unique to traditional pasture grasses, the altered nutritive profile lends the potential for alterations in metabolism and production when consumed (Ramirez-Restrepo and Barry, 2005; Totty et al., 2013). For example, Christian et al. (1999) reported changes in the casein and whey content of bovine milk as a result of varying inclusions of wheat and lupin, two different AFC options, in the diet. Recent research has been devoted to investigating the effects of nutrient profile of the diet on the bovine milk proteome, which yielded variable results (Li et al., 2015; Tacoma et al., 2017a); however, none of these experiments were performed in grazing systems.

Growing interest in the bovine milk proteome stems from identification of the bioactivity of specific proteins within the milk protein fraction (Mills et al., 2011). Bovine milk is comprised of high-abundance proteins, including caseins (CAS), α -lactalbumin (α -LA), and β -lactoglobulin (β -LG), as well as low-abundance proteins

(D'Amato et al., 2009). Bioactive peptides are released after hydrolysis of native protein structures within both the high- and low- abundance fractions, while the low abundance protein fraction also includes bioactive proteins that are resistant to enteric proteolysis (Claire and Swaisgood, 2000; Lopez-Exposito and Recio 2008; Korhonen, 2009; Lonnerdal, 2013; O'Riordan et al., 2014). Immunomodulation, anti-microbial, growth, and anti-cancer properties are examples of the functions associated with bioactive proteins and peptides in milk (FitzGerald and Meisel, 2003; Kilara and Panyam, 2003; Yamamoto et al., 2003; FitzGerald et al., 2004; Gauthier et al., 2006; Lopez-Fandino et al., 2006; Pihlanto, 2006; Lopez-Exposito and Recio, 2008).

While the skim milk fraction contains an array of these bioactive compounds, the milk fat globule membrane (MFGM) is another specific protein-containing milk fraction of interest due to its known bioactive profile (Spitsberg, 2005). The unique structure of the MFGM provides some form of protection for the integral proteins against hydrolysis in the gastrointestinal tract (GIT) after consumption (Vanderghem et al., 2011; Ye et al., 2011). Although the MFGM proteome only constitutes roughly 1-4% of the overall bovine milk proteome (Yang et al., 2015), there are a number of potent bioactive proteins and peptides present (Spitsberg, 2005; Vanderghem et al., 2011). A review by Spitsberg (2005) described many of the MFGM associated bioactive proteins that have been shown to exhibit immunomodulatory, antimicrobial, anti-cancer, and other biological properties. Butyrophilin (BTN), for example, is a common MFGM-associated protein that is higher in abundance relative to other proteins in the MFGM fraction. Additionally, BTN has been characterized with properties including immunomodulation

where some research has suggested its role in decreasing the onset of multiple sclerosis in experimental models (Stefflerl et al., 2000; Mana et al., 2004). Since the MFGM is associated with milk fat production, it is feasible that increases in milk fat output could increase the abundance of bioactive proteins and peptides in milk. Furthermore, increases in milk fat production has been observed from animals grazing pasture (Stergiadis et al., 2015).

The hypothesis of this study is the inclusion of AFC species in pasture, which can alter the post-absorptive nutrient composition via secondary compounds such as CT, will alter the skim milk and the MFGM-associated protein fractions of the bovine milk proteome from lactating Jersey cattle as a result of the nutritive profile absorbed. The objective of this study was to utilize proteomic approaches to characterize changes in the composition of the bovine milk proteome when cows grazed pastures containing AFC.

3.3 Materials and Methods

3.3.1 Animals, Design, and Diets

From a larger experiment, a subset of sixteen lactating Jersey cattle were blocked by milk yield (18.1 ± 3.9 kg), days in milk (143 ± 58 ; DIM), parity (1.4 ± 53 lactations), and stratified within block across treatments in a 21-d randomized complete block design conducted in the summer months of 2015 as described previously (Juntwait et al., 2016). All procedures from this larger experiment were completed as approved by the University of New Hampshire's Institutional Animal Care and Use Committee. Cows were fed a partial mixed ration at 60% of their daily DMI, and for the remaining 40% of daily DMI, cows grazed pasture. Control cows (CON; n=8) grazed pasture that contained

orchardgrass (*Dactylis glomerata*), timothy (*Phleum pratense*), Kentucky bluegrass (*Poa pratensis*) along with the legume white clover (*Trifolium repens*). The treatment group (AFC; n=8) grazed CON pasture that was strip-tilled with buckwheat (*Fagopyrum esculentum*), chickling vetch (*Lathyrus sativus*), and oat (*Avena sativa*) to represent 10% of their daily DMI. PMR and pasture nutrient compositions, as well as botanical composition proportions are reported previously by Juntwait et al. (2016).

3.3.2 Sampling

Milk samples were obtained on d 19-21 of the experiment. Three subsamples were collected from each cow, flash frozen on farm in a dry ice/ethanol bath, and transported on dry ice to The University of Vermont for analysis. One subsample (~15 mL) collected for high-performance liquid chromatography (HPLC) quantification of high-abundance proteins was stored at -20°C, while two other subsamples (~100 mL total) were collected for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of the low-abundance proteins in the skim milk and MFGM fractions, and were stored at -80°C until further analysis.

3.3.3 HPLC quantification of high-abundance milk proteins

Milk samples collected for HPLC analysis were thawed overnight at 4°C, pooled within cow according to milk yield, and centrifuged at 4,000xg for 10 min at 4°C. The skimmed milk was then prepared and analyzed as previously described (Tacoma et al., 2016).

3.3.4 Preparation of the low-abundance protein enriched skim milk fraction

Subsamples collected for LC-MS/MS skim protein analysis were pooled within cow according to milk yield. Additionally, a universal control was also generated by pooling milk from each control cow according to milk yield; which was later used to compare relative-fold abundance changes in proteins across all samples. Composite samples from each cow, as well as the universal control, were fractionated, enriched, digested, labeled with isobaric tandem mass tags (TMT; Thermo Fisher Scientific, Waltham, MA), and analyzed according to established methods as previously outlined (Tacoma et al., 2017b). Two 9-plexes were generated in this experiment, each including the universal control, and a random subset of eight samples. The 9-plexes were then submitted to the Vermont Genetics Network Proteomics Facility (The University of Vermont, Burlington, VT) for LC-MS/MS analysis as previously described (Tacoma et al., 2017b).

3.3.5 Liquid chromatography – tandem mass spectrometry

LC-MS/MS analysis was conducted as previously described (Tacoma et al., 2017b). Briefly, a Q-Exactive mass spectrometer coupled to an EASY-nLC (Thermo Fisher Scientific, Waltham, MA) was used for peptide identification and quantification. Subsequent product ion spectra were searched against SEQUEST and Mascot search engines on Proteome Discoverer 1.4 (Thermo Fisher Scientific, Waltham, MA, USA) against a curated Bovine Uniprot (Bos taurus database; UP000009136; 24,346 entries; downloaded Dec. 9, 2015) with sequences in forward and reverse orientations. Samples were also searched against a yeast database (*S. cerevisiae*) for validation of the yeast

GAPDH (glyceraldehyde-3-phosphate dehydrogenase; *S. cerevisiae*; Sigma-Aldrich, St. Louis, MO) insertion. Quantification was performed using Reporter Ion Quantification Node in Proteome Discoverer 1.4. All of the protein identification and quantification information (<1% false positive; with protein grouping enabled) was exported to Excel spreadsheets. The universal control was used as a common denominator for expressing the relative-fold change values of the proteins identified within each animal.

3.3.6 Isolation of the MFGM-associated protein fraction

The third set of subsamples collected for protein profiling in the MFGM fraction was thawed overnight at 4°C and pooled within cow according to milk yield. Of the milk samples retained for MFGM proteome analysis, one AFC sample was not included due to improper thawing. To the remaining 15 samples (8 CON, 7 AFC), a Protease Inhibitor Cocktail (Sigma, Milwaukee, WI) was added at 0.24 mL per g of protein. The MFGM was isolated according to previously published methods (Yang et al., 2015) with some modifications. Samples were centrifuged at 3000xg at 4°C for 15 min. The skim layer was discarded, and the cream layer was incubated with 5 volumes of phosphate-buffered saline solution at 37°C for 20 min, followed by centrifugation at 3000xg for 30 min at 4°C. This wash and centrifugation step was repeated three times to remove residual CAS proteins. Following, the recovered cream was incubated with 10 volumes of lysis buffer (50mM Tris-HCl at pH 7.4, 4% SDS (w/v) solution) for 1 h at room temperature with periodic vortexing every 10-15 min for 10-15 s. After incubation, samples were then placed in a water bath at 95°C for 5 min followed by centrifugation at 12,000xg at 4°C for 15 min. The residual fat was removed and lysates were centrifuged again. The lysates

were then precipitated in acetone at a 1:6 ratio (lysate: acetone) at -20°C for 20 h. A universal control was generated prior this step by pooling an equal volume of supernatant from each control. Following the precipitation, samples were centrifuged at 14,000xg at 4°C for 40 min using a Sorvall BR-C4 centrifuge (Thermo Scientific, Waltham, MA), and the pellet was re-suspended in radioimmunoprecipitation assay buffer (Sigma Aldrich, Saint Louis, MO) as well as the re-addition Protease Inhibitor Cocktail and stored at -80°C until protein quantification. The bicinchoninic acid assay (Pierce, Rockford, IL) was used to quantify protein concentrations with bovine serum albumin as the standard.

3.3.7 SDS PAGE separation of MFGM-associated protein fraction

Quantified samples were separated by SDS-PAGE using a total of two gels. Reducing sample buffer (5 X; Thermo Scientific, Rockford, IL) was added to each sample in a 1:5 ratio to 49 µg of sample plus 1 µg of *S. cerevisiae* (baker's yeast) GAPDH (Sigma-Aldrich, St. Louis, MO) and the mixture was heated at 90°C for 3 min. After cooling to room temperature, the samples were loaded onto a precast 12% polyacrylamide gel (Biorad, Hercules, CA). Proteins were electrophoresed for 15 min at 200 V. Gels were stained in Coomassie Blue (Biorad, Hercules, CA) overnight and gel images were obtained from scanning prior to excision.

3.3.8 In-gel digestion of the MFGM-associated protein fraction

The gel lanes were excised into three segments according to their molecular weights (I, heavier in weight, upper portion of the gel; II, medium weight, middle portion of the gel; III, lighter in weight, lower portion of the gel) and subjected to trypsin

digestion protocols, as described previously (Tacoma et al., 2016) except for using triethylammonium bicarbonate as buffer instead of ammonium bicarbonate.

3.3.9 Isobaric TMT labeling of MFGM-associated protein fraction

Isobaric TMT labeling of the protein samples were performed according to the manufacturers' protocols with minor modifications. Briefly, dried peptides in gel slices I and III, and gel slice II from each sample were resuspended in 25 and 50 μL of triethylammonium bicarbonate, respectively. After resuspension, 10 and 20 μL of TMT reagents (0.8 mg dissolved in 41 μL of acetonitrile (CH_3CN)) were added to gel slices I and II, and gel slice II, respectively, followed by briefly vortexing and an incubation for 1.5 h at room temperature. Following, 5% hydroxylamine was added to quench the reactions. One-third of the total reactions were combined resulting in three 9-plex reactions (I, II, III) from gel 1 and three 8-plex (I, II, III) from gel 2. The mixtures were dried down and stored at -80°C until LC-MS/MS analysis at the Vermont Genetics Network Proteomics Facility (The University of Vermont, Burlington, VT) as described above.

3.3.10 Bioinformatic analysis of identified proteins

Uncharacterized protein sequences were identified using BLAST (Camacho et al., 2009). Identified proteins were then annotated according to their biological processes, molecular functions, and cellular components through gene ontology (GO) using the PANTHER Classification System (Mi et al., 2017). The percent of gene hits against total number of functional hits as calculated by PANTHER were used to generate grouped bar graphs in Prism 7 (GraphPad Software Inc, La Jolla, CA). A Venn diagram was

generated using the VennDiagram package in R (R Core Team, Vienna, Austria) using the accession numbers from the proteins identified in the skim and MFGM fractions (Team, 2015). Further STRING (Szklarczyk et al., 2015) analysis was also conducted to measure possible protein-protein interactions within a STRING plug-in app (Szklarczyk et al., 2017) for Cytoscape (Cytoscape 3.5.1; La Jolla, CA) (Shannon et al., 2003). Customized networks were generated within Cytoscape for the skim and MFGM fractions, as well as a network illustrating the proteins identified in both fractions.

3.3.11 Statistical Analysis

A PROC MIXED procedure was used in SAS 9.4 (SAS Institute, Cary, NC) to statistically analyze the protein data generated by HPLC and LC-MS/MS analysis with treatment used as the fixed effect.

3.4 Results

3.4.1 Milk yield and components

Milk yield (kg/d) was not altered by diet in this experiment (Table 3.1). Milk fat percentage was higher in milk from cows that grazed pastures containing AFC ($P = 0.01$) compared to CON cows. Milk protein percent and yield (kg/d) as well as fat yield (kg/d) were not affected by dietary treatment. Somatic cell count (SCC), was not different across treatment groups.

3.4.2 HPLC quantification of high-abundance proteins

The concentration of α -S1 CAS was 15% higher in milk from AFC cows at 14.4 mg/mL compared to 12.2 mg/mL in the CON cows ($P = 0.005$, Table 3.2). There were no

differences in α -s2, α -LA, β CAS, κ CAS, β -LGA, and β -LGB concentrations across treatments.

3.4.3 The low-abundance protein enriched fraction in skim milk

A total of 53 proteins were identified in the low-abundance protein enriched skim milk fraction (Supplementary Table 3.1). Two of these proteins were higher in cows grazing AFC pasture compared to the CON cows (Table 3.3), including beta-2-microglobulin ($P = 0.009$) and polymeric immunoglobulin receptor ($P = 0.04$).

3.4.4 MFGM-associated protein fraction

A total of 245 proteins were identified in the MFGM-associated protein fraction (Supplementary Table 3.2), 5 of which were present at higher abundance in cows grazing AFC pasture compared to the CON cows (Table 3.3; $P \leq 0.05$).

3.4.5 Bioinformatic analysis of the skim milk and MFGM-associated protein fractions

Cellular process (GO term: 0009987) was the most annotated biological process term of the proteins identified using GO. Cellular process was annotated to 15.1% and 26.9% of the proteins identified in the skim milk and MFGM-associated protein fractions, respectively (Figure 3.1). Biological regulation (GO term: 0065007), response to stimulus (GO term: 0050896), and metabolic process (GO term: 0008152), respectively, proceeded cellular process for the skim milk fraction proteins; whereas localization (GO term: 0051179), metabolic process, and response to stimulus followed cellular process for the MFGM-associated protein fraction. Catalytic activity (GO term: 0003824) and binding (GO term: 0005488) were the most annotated molecular function terms for the

skim milk and MFGM proteins, accounting for 45.7% and 34.3%, respectively, in the skim fraction and 38.1% and 30.5%, respectively, in the MFGM fraction. Additional molecular function terms that were annotated for MFGM proteins and not the skim proteins included structural molecule activity (GO term: 0005198), translation regulator activity (GO term: 0005198), and channel regulator activity (GO term: 0016247).

According to cellular component annotation of the skim milk protein fraction, 61.5% were annotated with the extracellular region (GO term: 0005576) followed by membrane (15.4%; GO term: 0016020), and macromolecular complex (11.5%; GO term: 0032991). The MFGM cellular component annotation noted 43% as having a cell part (GO term: 0044464) annotation, followed by organelle (24.7%; GO term: 0043226), and macromolecular complex (16.1%). Additional cellular component terms that were annotated for the MFGM-associated protein fraction, but not the skim milk fraction, included synapse (GO term: 0045202), and cell junction (GO term: 0030054).

Of the proteins identified by LC-MS/MS analysis in the skim milk and MFGM-associated protein fractions, 24 proteins were present in both fractions (Figure 3.2). Further STRING analysis revealed a network of proteins (nodes) with several interactions from curated databases in both the MFGM-associated and skim milk protein fractions, and also revealed the interactions between the shared proteins (nodes) identified in both fractions (Figure 3.3).

3.5 Discussion

This experiment examined the inclusion of buckwheat (*Fagopyrum esculentum*), chickling vetch (*Lathyrus sativus*), and oat (*Avena sativa*) as AFC species in grazed

pasture, which could be used as a means to alter the bovine milk proteome within both the skim milk and MFGM-associated protein fractions. These AFC species are known to contain varying degrees of polyphenolics (Ramirez-Restrepo and Barry, 2005; Kalber et al., 2013), which have been shown to alter animal metabolism and could thereby influence production, potentially including the milk protein profile (Greenwood et al., 2012; Li et al., 2015). The impact of pasture diversity has yielded mixed results in terms of its impact on milk production, general component content, as well as yield (Lee et al., 2009, Totty et al., 2013, Stergiadis et al., 2015). However, the present study aimed to investigate the impact of including AFC in pasture on the milk protein profile.

The milk components were largely unchanged when cows grazed AFC pasture compared to the CON cows, with exception to an increase in milk fat percent in cows grazing AFC pasture ($P = 0.01$; Table 3.1). This finding is in agreement with previous research that investigated the milk composition in response to dairy cows grazing different pasture allowances, where milk fat yield increased when animals consumed more pasture (Stergiadis et al., 2015). Since the remaining milk components, including milk protein percent and yield, as well as SCC were not different between treatment groups, only the proteomic compositional changes within the high and low-abundance protein fractions will be discussed.

The content of α -s1 CAS was higher in milk from cows that consumed AFC in pasture. Higher CAS content implies an increase in nutrient supply to the mammary gland for mammary protein synthesis; for example, a summary on diet and milk protein output highlighted how increased energy concentration in the diet can result in an

increased milk protein content (Emery, 1978). Furthermore, increases in milk casein content have also been observed in a recent report by Tacoma et al. (2017a) where varying degrees of rumen-degradable and rumen-undegradable protein were used. As reported by Juntwait et al. (2016), the nutrient composition of the summer AFC species on pasture showed some slight differences in protein content (14.5% DM versus 12.9% DM in CON pasture), as well as starch content (2.2% DM versus 1.0% DM in CON pasture). Although a higher starch content could have increased metabolizable energy availability and potentially microbial protein synthesis potential, the differences in starch at present were minimal; therefore, starch content is unlikely to be the sole factor explaining the differences observed. Furthermore, the possibility of a higher starch content increasing energy availability is conflicted when considering the higher lignin content in the AFC pasture (9.1% DM versus 4.3% DM in CON pasture). Lignin is known to inhibit fermentation in the rumen, which can ultimately limit downstream nutrient availability (Susmel and Stefanon, 1993). Therefore, it is difficult to determine which specific dietary-derived nutrients in this experiment could have contributed to the increased α -s1 CAS content

A total 53 proteins were identified in the skim milk fraction and 245 proteins were identified in the MFGM-associated protein fraction. Furthermore, a total of 24 proteins were present in both the skim milk and MFGM-associated protein fraction (Figure 3.2; Table 3.4). While there were a total of 7 proteins in both fractions that were higher in relative abundance from cows that grazed AFC pasture, the bovine milk majority of the proteome in this study was unaffected. It is plausible that a higher nutrient

availability could ultimately alter the quantity of low-abundance milk proteins; however, it is difficult to identify the specific nutrient(s) responsible for the changes observed in this experiment as aforementioned. For example, Li et al. (2015) reported increases in whey proteins when cows were fed diets that were formulated for fast energy and nitrogen release, citing nitrogen synchrony as a possible mechanism allowing for increases in microbial protein synthesis and therefore altering the amino acid profile absorbed in the small intestine. However, the exact underlying mechanisms relating diet to proteomic composition has yet to be characterized. Since the proteome was largely unchanged in this study, the proteomic composition of the skim milk fraction and MFGM-associated fraction was further investigated to better characterize and understand the identified proteins.

In the skim milk fraction, two proteins were identified in higher relative-fold abundance in the AFC group: beta 2-microglobulin (B2M; $P = 0.009$) and polymeric immunoglobulin receptor (PIgR; $P = 0.036$; Table 3.3). B2M is a component associated with the major histocompatibility complex I and has been detected in all body fluids as well as the surfaces of nucleated cells (Bourantas et al., 1999). PIgR is another immune-related protein that is associated with IgA production in the small intestine (Asano and Komiyama, 2011).

Sodium/ nucleoside cotransporter, endoplasmin, glycerol-3-phosphate acyltransferase 1, mitochondrial, lactoperoxidase, and puromycin-sensitive aminopeptidase were higher in relative-abundance within in the MFGM-associated protein fraction from animals grazing AFC-containing pasture (Table 3.3).

Lactoperoxidase is a known bioactive enzyme that exhibits antimicrobial properties, and has been identified in the skim fraction as well as the MFGM-associated protein fraction (Mills et al., 2011).

Between the two fractions, a total of 24 proteins were identified in the skim milk and MFGM-associated protein fractions. Additionally, both of these proteome fractions also appeared to have some similar functionality profiles as GO revealed through PANTHER (Figure 3.1). Cellular and metabolic process were two of the most annotated terms for biological process across both the skim milk and MFGM-associated protein fractions, and both catalytic activity and binding were prominent GO terms for molecular function in both skim and MFGM fractions. There were a few examples of divergence in GO terms for both proteomes where under biological process annotations such as immune system process (GO term: 0002376), which was higher in the skim proteome when compared to the MFGM proteome (7.5% vs 2.0%). This could imply that there are more proteins present in the skim milk fraction that are associated with immunity and immunomodulatory properties. An interactomics based study from Zhang et al. (2017) highlighted changes throughout lactation in human and bovine milk samples in both the skim and MFGM fractions, and GO analysis similarly revealed a higher biological process annotation for immune-related activity in the skim milk protein fraction in both human and bovine samples. Conversely under the biological process annotations, cellular component organization or biogenesis (GO term: 0071840) was annotated for 8.9% of the proteins in the MFGM fraction compared to 2.2% in the skim milk fraction; which aligns with the biochemistry of MFGM formation taking place

within the mammary epithelial cell starting on the apical surface of the smooth endoplasmic reticulum (Cavaletto et al., 2008). Due to the mechanism of secretion, it is probable for proteins that are associated with cell component organization or biogenesis to be incorporated into the MFGM during formation.

As expected, cellular component annotations were largely unique when comparing the skim milk and MFGM fractions. Over 60% of the skim milk proteins were annotated with the extracellular region. In contrast, 43% and 24% of the MFGM proteins were annotated with the cell part and organelle regions, respectively, which aligns with the literature outlining the formation of the MFGM itself (Cavaletto et al., 2008). Despite the differences observed in cellular component annotation between the skim milk and MFGM fractions, macromolecular complex (GO term: 0032991) was annotated with close similarity relative to the other terms in both skim milk and MFGM fractions (11.5% and 16.1% respectively). A macromolecular complex can refer to any macromolecule adjoining together in a stable assemblage, and it is possible for proteins to adjoin together in such a manner (Mi et al., 2017). This annotation supports the idea that protein-to-protein interactions are occurring in both the skim milk and MFGM fractions.

The customized networks generated by STRING within Cytoscape revealed a number of interactions between the proteins (nodes) in both skim milk and MFGM fractions (Figure 3.3). The skim milk proteins were largely shown to interact with serum albumin, annotated as ALB, where 19 out of the 38 nodes were displayed interaction(s) with ALB. Unlike the skim milk fraction, the interactions observed within the MFGM fraction were very diverse with little evidence for any clear patterns. Similarly to the

skim fraction, serum albumin (ALB) was a common denominator in many of the interactions displaying connections with 54 of the 194 nodes; however, there were other proteins that displayed several interactions including heat shock protein HSP 90-alpha (HSP90AA1) where 57 nodes displayed interaction(s). Protein HSP90AA1 works as a chaperone that assists other proteins with maturation, regulates target proteins, and provides structural maintenance according to STRING (Szklarczyk et al., 2015). Along with protein HSP90AA1, there are other proteins present within the MFGM STRING network that display a large number of interactions, which further iterates the complexity and diversity of the MFGM fraction.

In this experiment, the effects of including AFC in pasture on subsequent proteomic composition of the bovine skim milk and MFGM profiles were evaluated. With exception to milk fat percentage, changes were not observed in general milk components or yield; however, some changes in the milk protein profiles were observed across treatments with higher contents of high-abundance proteins and higher relative-abundances of low-abundance proteins in milk from AFC cows. This data does provide further evidence for dietary influence on the bovine milk protein profile. However, to date, a definitive mechanistic pathway(s) has yet to be identified and confirmed as the means for the changes observed. This experiment, along with the others aforementioned, aligns with the need for further scrutiny of the underlying physiology and possible mechanisms for further explanation of the changes observed in proteomic profiles.

Table 3.1. Milk yield and components from cows that grazed either CON or AFC pastures

	CON	AFC	SE	<i>P</i> -value	
				Block	Treatment
Milk Yield (kg/d)	17.6	18.6	0.89	0.631	0.507
Milk Components (%)					
Milk Fat	4.36	4.79	0.09	0.555	0.019
Milk Protein	3.52	3.63	0.04	0.141	0.152
Milk Components (kg/d)					
Milk Fat	0.78	0.88	0.04	0.540	0.215
Milk Protein	0.63	0.66	0.03	0.378	0.494
SCC (Cells x 1000)	123.8	200.8	61.41	0.079	0.409

Least square means reported for CON and AFC groups. SE, standard error; ECM, energy-corrected milk; SCC, somatic cell count.

Table 3.2. High-abundance protein concentrations from cows that grazed either CON or AFC- supplemented pastures

Casein (mg/mL)	CON	AFC	SE	P-value
κ -CAS	7.37	8.49	0.039	0.06
α -s2 CAS	1.50	1.43	0.085	0.56
α -s1 CAS	12.17	14.35	0.464	0.005
β -CAS	13.53	14.43	0.323	0.07
Whey (mg/mL)				
α -LA	1.00	1.00	0.045	1.00
β -LGB	1.97	2.10	0.453	0.85
β -LGA	2.90	3.02	0.486	0.86

Least square means reported for CON and AFC groups. SE, standard error; CAS, casein;

β -LGB, β -Lactoglobulin variant B; β -LGA, β -Lactoglobulin variant A.

Table 3.3. Proteins affected by diet as identified by LC-MS/MS analysis in the skim milk fraction and milk fat globule membrane (MFGM) associated protein fraction in cows that grazed either CON or AFC pastures

Fraction	Accession	Protein	Control	AFC	SE*	Control SE	AFC SE	P-value
Skim Milk	P01888	Beta-2-microglobulin	1.32	1.91	0.137			0.009
	P81265	Polymeric immunoglobulin receptor	1.35	2.56	0.368			0.036
MFGM	F1MGR1	Sodium/nucleoside cotransporter	1.08	1.33		0.066	0.058	0.027
	Q95M18	Endoplasmin	0.74	1.11		0.102	0.090	0.034
	F1MDT6	Glycerol-3-phosphate acyltransferase 1, mitochondrial	0.88	1.07		0.053	0.047	0.034
	P80025	Lactoperoxidase	0.82	1.28		0.127	0.112	0.034
	E1BP91	puromycin-sensitive aminopeptidase	0.87	1.27		0.122	0.108	0.049

Least square means reported for the CON and AFC groups expressed as relative-abundance. SE, standard error. SE column marked by asterisk (*) represents the SE from the skim milk fraction results.

Table 3.4. The 24 common proteins identified by LC-MS/MS analysis in both the skim milk and milk fat globule membrane (MFGM) associated protein fractions from cows that grazed either control (CON) pasture or pasture strip-tilled with alternative forage crops (AFC)

Accession	Protein
F1MMW8	Serum amyloid A protein
F1MUT3	Xanthine dehydrogenase/oxidase
F1N726	Pancreatic secretory granule membrane major glycoprotein GP2 precursor
G3MXB5	Immunoglobulin IgA heavy chain constant region, partial
G5E513	IgM heavy chain constant region, secretory form, partial
G5E5T5	immunoglobulin M heavy chain secretory form
P00711	Alpha-lactalbumin
P01888	Beta-2-microglobulin
P02662	Alpha-S1-casein
P02663	Alpha-S2-casein
P02666	Beta-casein
P02668	Kappa-casein
P02769	Serum albumin
P10790	Fatty acid-binding protein, heart
P11151	Lipoprotein lipase
P15497	Apolipoprotein A-I
P18892	Butyrophilin subfamily 1 member A1
P24627	Lactotransferrin
P80025	Lactoperoxidase
P80195	Glycosylation-dependent cell adhesion molecule 1
P81265	Polymeric immunoglobulin receptor
Q32PA1	CD59 molecule, complement regulatory protein
Q95114	Lactadherin
Q95122	Monocyte differentiation antigen CD14

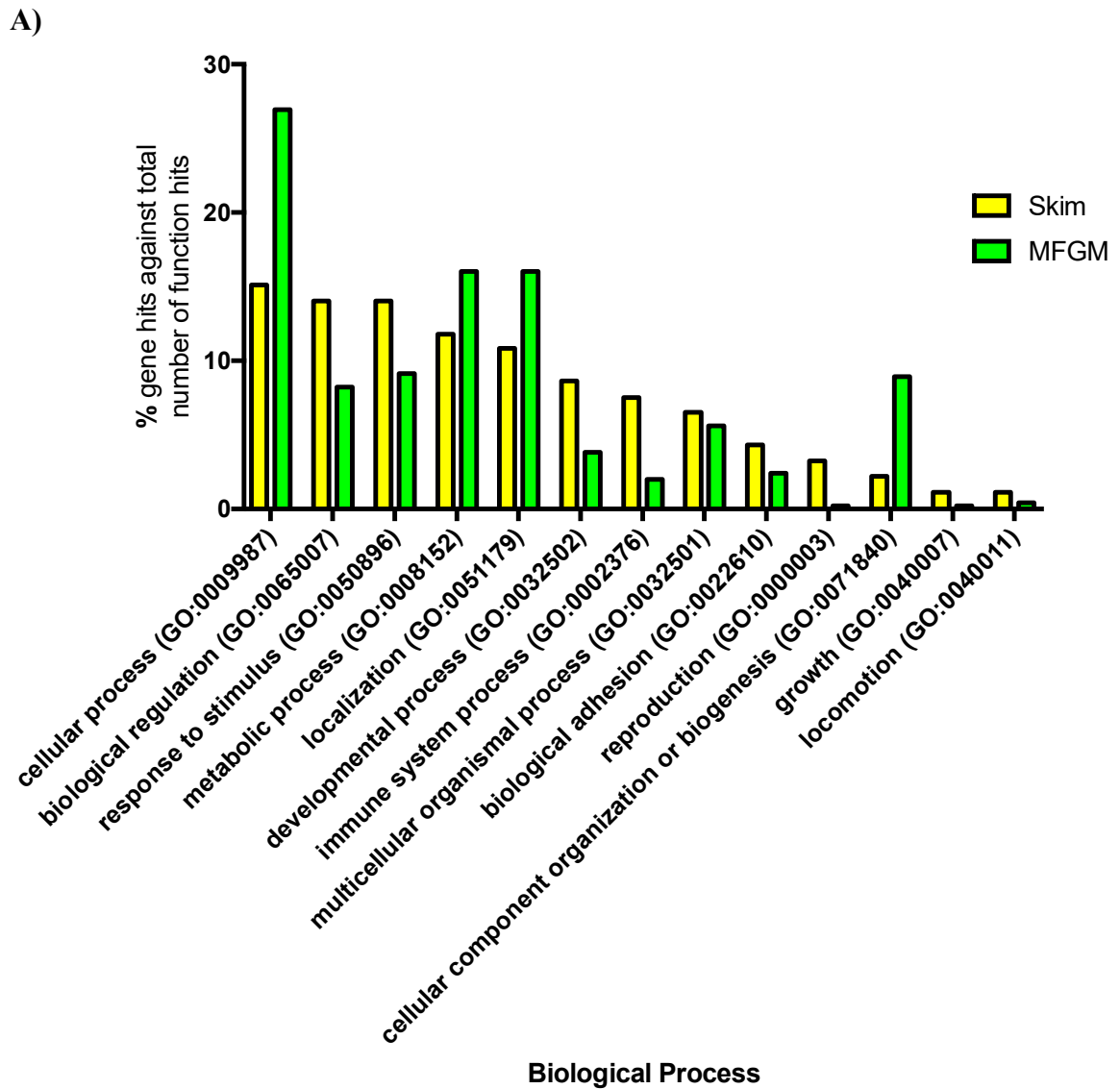


Figure 3.1 A. The gene ontology (GO) annotations of the proteins identified by LC-MS/MS analysis in both skim milk and milk fat globule membrane (MFGM) samples as according to their biological process.

B)

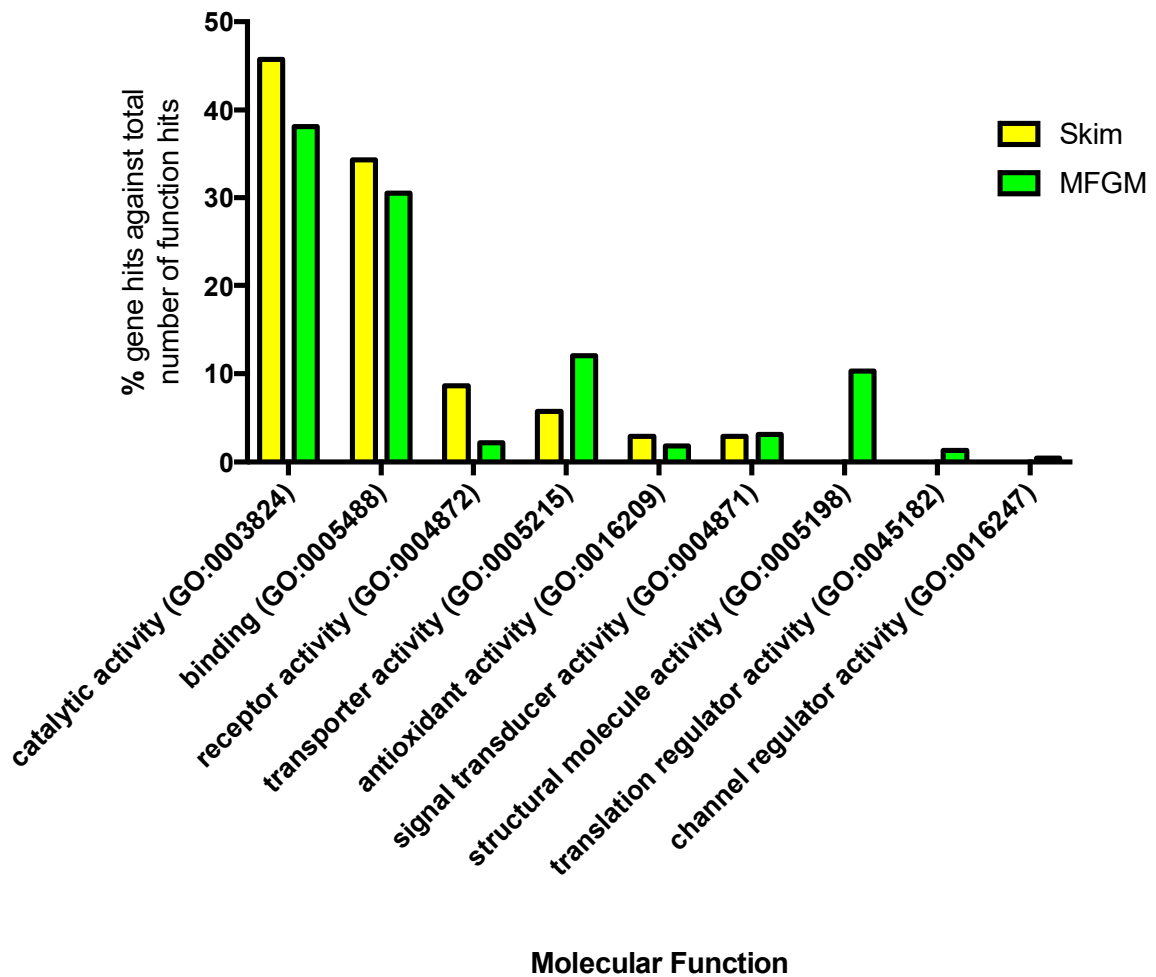


Figure 3.2 B. The gene ontology (GO) annotations of the proteins identified by LC-MS/MS analysis in both skim milk and milk fat globule membrane (MFGM) samples as according to their molecular function.

C)

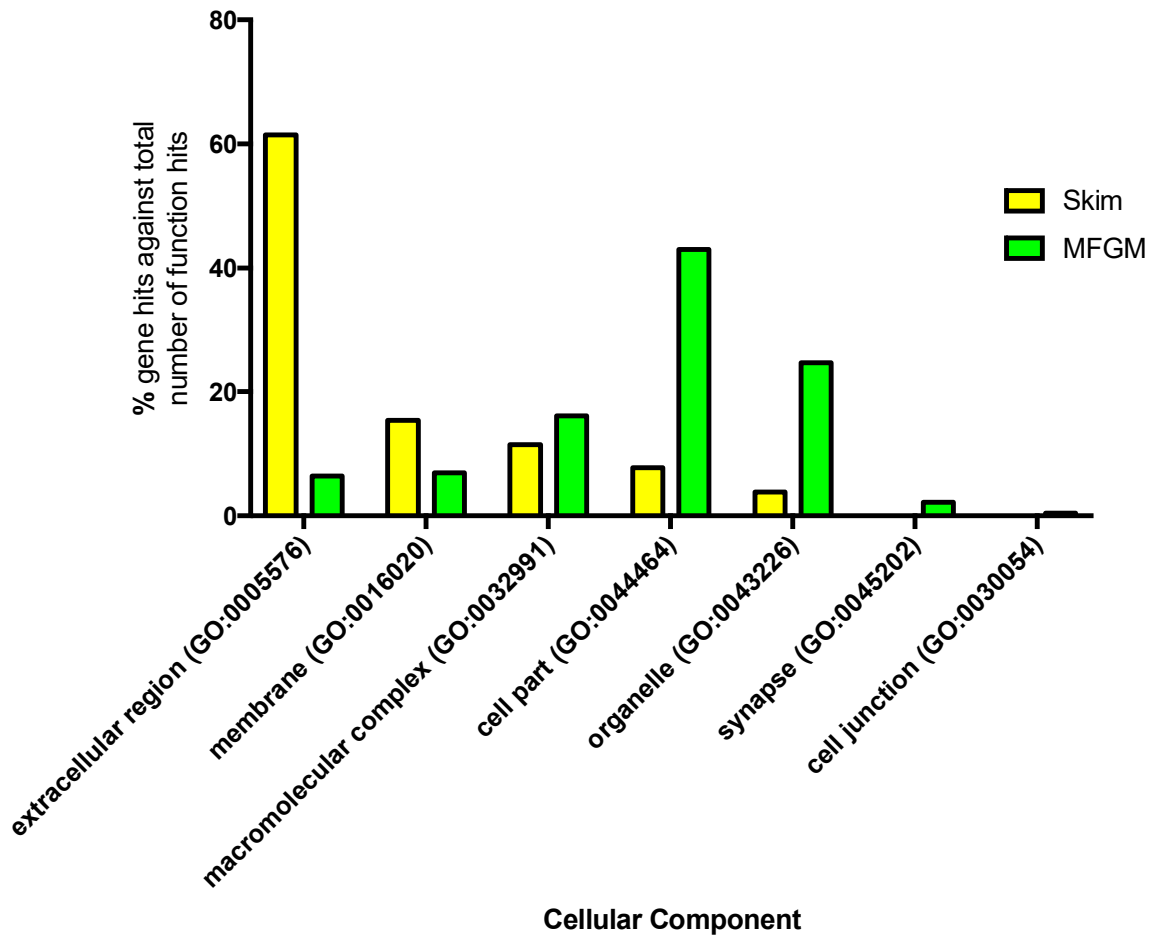


Figure 3.3 C. The gene ontology (GO) annotations of the proteins identified by LC-MS/MS analysis in both skim milk and milk fat globule membrane (MFGM) samples as according to their cell component.

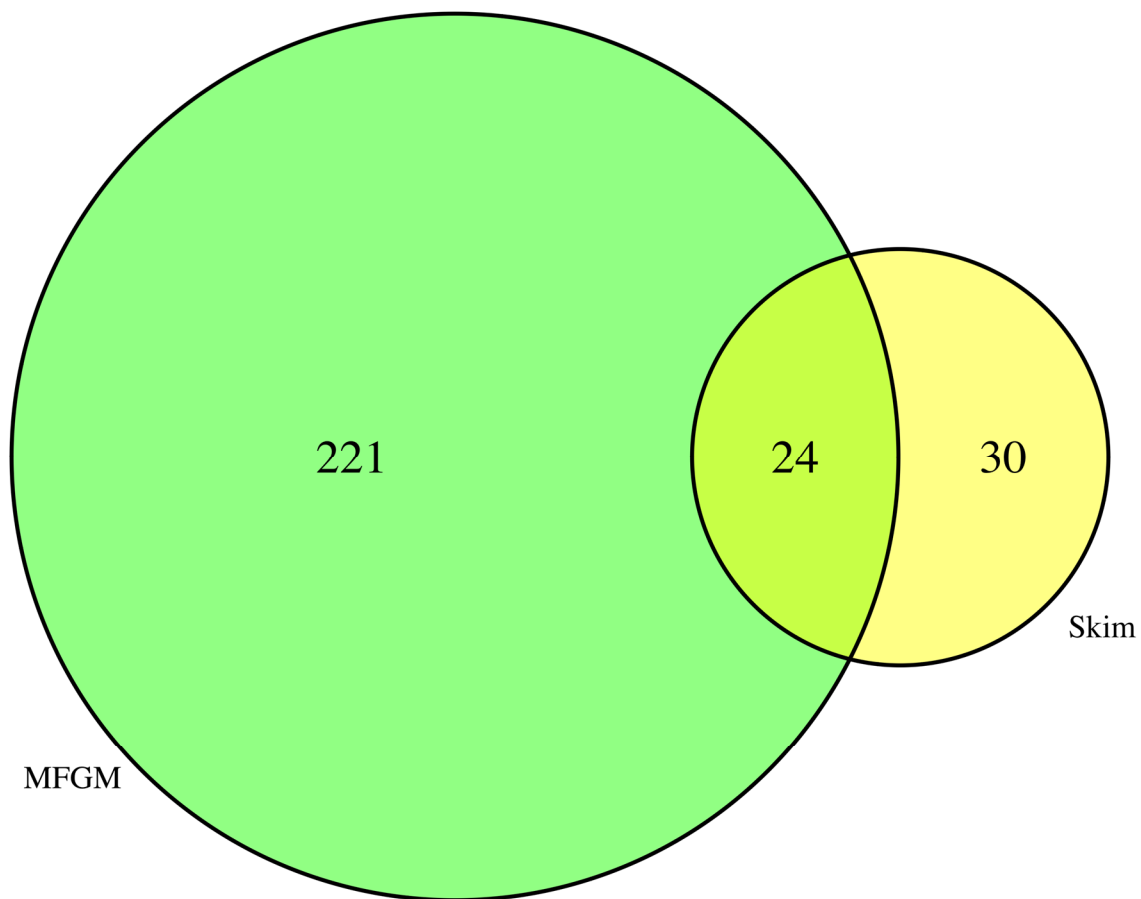


Figure 3.4. Venn diagram outlining the 24 conserved proteins between both the skim and MFGM fractions as identified by LC-MS/MS analysis.

A)

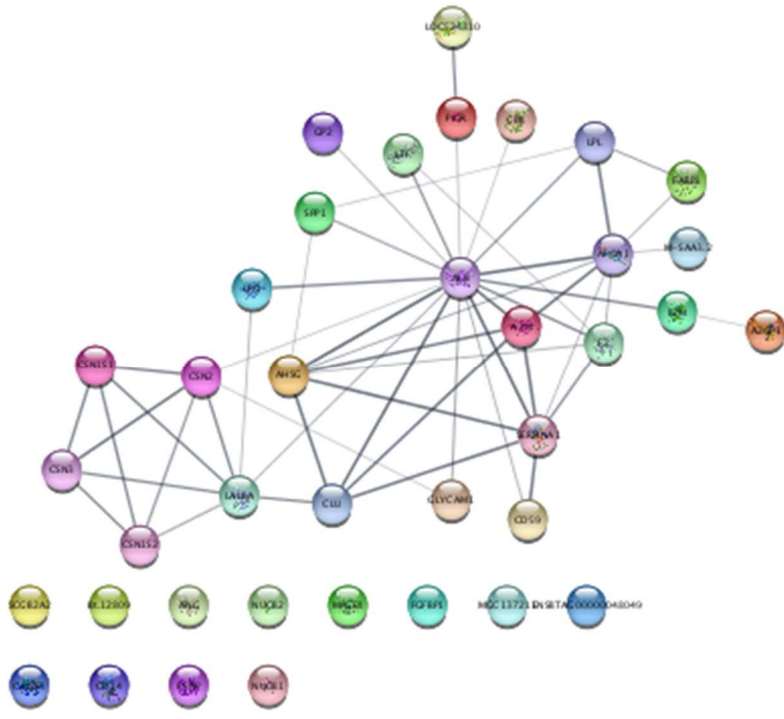


Figure 3.3 A. STRING network of the proteins identified in the skim milk protein fraction by LC-MS/MS analysis.

Figure 3.3 B. STRING network of the proteins identified in the milk fat globule membrane (MFGM) associated protein fraction by LC-MS/MS analysis.

C)



Figure 3.5 C. STRING network of the shared proteins identified in both fractions by LC-MS/MS analysis.

3.6 References

- Acamovic, T., and J. D. Brooker. 2005. Biochemistry of plant secondary metabolites and their effects in animals. *Proc. Nutr. Soc.* 64:403-412.
<https://dx.doi.org/10.1079/PNS2005449>
- Asano, M., and K. Komiyama. 2011. Polymeric immunoglobulin receptor. *J. Oral Sci.* 53:147-156
- Bourantas, K. L., E. C. Hatzimichael, A. C. Makis, A. Chaidos, E. D. Kapsali, S. Tsiara, and A. Mavridis. 1999. Serum beta-2-microglobulin, TNF-alpha and interleukins in myeloproliferative disorders. *Eur. J. Haematol.* 63:19-25
- Camacho, C., G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, and T. L. Madden. 2009. BLAST+: architecture and applications. *BMC Bioinformatics* 10:421. <https://dx.doi.org/10.1186/1471-2105-10-421>
- Cavaletto, M., M. G. Giuffrida, and A. Conti. 2009. Milk fat globule membrane components – A proteomic approach. *Adv. Exp. Med. Biol.* 606: 129-141.
https://doi.org/10.1007/978-0-387-74087-4_4
- Christian, M. P., C. Grainger, B. J. Sutherland, J. J. Mayes, M. C. Hannah, and B. Kefford. 1999. Managing diet quality for cheddar cheese manufacturing milk. 1. The influence of protein energy supplements. *J. Dairy Res.* 66:341-355.
- D'Amato, A., A. Bachi, E. Fasoli, E. Boschetti, G. Peltre, H. Senechal, and P. G. Righetti. 2009. In-depth exploration of cow's whey proteome via combinatorial peptide ligand libraries. *J Proteome Res.* 8:3925-3936.
<https://doi.org/10.1021/pr900221x>
- Emery, R. 1978. Feeding For Increased Milk Protein. *J. Dairy Sci.* 61:825-828.
[https://doi.org/10.3168/jds.S0022-0302\(78\)83656-X](https://doi.org/10.3168/jds.S0022-0302(78)83656-X)
- Greenwood, S. L., G. R. Edwards, and R. Harrison. 2012. Short communication: supplementing grape marc to cows fed a pasture-based diet as a method to alter nitrogen partitioning and excretion. *J. Dairy Sci.* 95:755-758.
<https://dx.doi.org/10.3168/jds.2011-4648>
- Jenkins, T. C. and M. A. McGuire. 2006. Major advances in nutrition: Impact on milk composition. *J. Dairy Sci.* 89:1302-1310.
[https://doi.org/10.3168/jds.S0022-0302\(06\)72198-1](https://doi.org/10.3168/jds.S0022-0302(06)72198-1)
- Juntwait, K. A., A. F. Brito, K. S. O'Connor, R. G. Smith, K. M. Aragona, and A. B. Pereira. 2016. Feeding Annual Forage Crops to Organic Dairy Cows During the

Spring and Summer Seasons in Northeast United States. Organic Agriculture Research Symposium, Jan. 20th, 2016. Pacific Grove, CA.

- Kalber, T., M. Kreuzer, and F. Leiber. 2013. Effect of feeding buckwheat and chicory silages on fatty acid profile and cheese-making properties of milk from dairy cows. *J. Dairy Res.* 80:81-88. <https://doi.org/10.1017/S0022029912000647>
- Korhonen, H. 2009. Milk-derived bioactive peptides: from science to applications. *J. Funct. Food* 1:177-187. <https://dx.doi.org/10.1016/j.jff.2009.01.007>
- Lee, M. R., V. J. Theobald, J. K. Tweed, A. L. Winters, and N. D. Scollan. 2009. Effect of feeding fresh or conditioned red clover on milk fatty acids and nitrogen utilization in lactating dairy cows. *J. Dairy Sci.* 92:1136-1147. <https://dx.doi.org/10.3168/jds.2008-1692>
- Li, S. S., J. S. Shen, D. X. Ren, and J. X. Liu. 2015. Effects of the processing methods of corn grain and soybean meal on milk protein expression profiles in dairy cows. *Animal* 9:267-274. <https://dx.doi.org/10.1017/S1751731114002225>
- Makkar, H. P. S. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small Ruminant Research* 49:241-256. [https://dx.doi.org/10.1016/S0921-4488\(03\)00142-1](https://dx.doi.org/10.1016/S0921-4488(03)00142-1)
- Mana, P., M. Goodyear, C. Bernard, R. Tomioka, M. Freire-Garabal, and D. Linares. 2004. Tolerance induction by molecular mimicry: prevention and suppression of experimental autoimmune encephalomyelitis with the milk protein butyrophilin. *Int. Immunol.* 16:489-499. <https://doi.org/10.1093/intimm/dxh049>
- Mi, H., X. Huang, A. Muruganujan, H. Tang, C. Mills, D. Kang, and P. D. Thomas. 2017. PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. *Nucleic Acids Res.* 45:D183-D189. <https://dx.doi.org/10.1093/nar/gkw1138>
- Mills, S., R. P. Ross, C. Hill, G. F. Fitzgerald, and C. Stanton. 2011. Milk intelligence: mining milk for bioactive substances associated with human health. *Int. Dairy J.* 21:377-401. <https://dx.doi.org/10.1016/j.idairyj.2010.12.011>
- Miller, L. A., J. M. Moorby, D. R. Davies, M. O. Humphreys, N. D. Scollan, J. C. MacRae, and M. K. Theodorou. 2001. Increased concentration of water-soluble carbohydrate in perennial ryegrass (*Lolium perenne*): Milk production from late-lactation dairy cows. *Grass Forage Sci.* 56:383-394. <https://dx.doi.org/10.1046/j.1365-2494.2001.00288.x>

- Pereira, A. B. D., A. F. Brito, L. L. Townson, and D. H. Townson. 2013. Assessing the research and education needs of the organic dairy industry in the northeastern United States. *J. Dairy Sci.* 96:7340-7348. <https://doi.org/10.3168/jds.2013-6690>
- Ramirez-Restrepo, C. A., and T. N. Barry. 2005. Alternative temperate forages containing secondary compounds for improving sustainable productivity in grazing ruminants. *Anim. Feed Sci. Technol.* 120:179-201. <https://doi.org/10.1016/j.anifeedsci.2005.01.015>
- Shannon, P., A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski, and T. Ideker. 2003. Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13:2498-2504. <https://dx.doi.org/10.1101/gr.1239303>
- Spitsberg, V. L. 2005. Invited review: Bovine milk fat globule membrane as a potential nutraceutical. *J. Dairy Sci.* 88:2289-2294. [https://dx.doi.org/10.3168/jds.S0022-0302\(05\)72906-4](https://dx.doi.org/10.3168/jds.S0022-0302(05)72906-4)
- Stefflerl, A., A. Schubart, M. Storch, A. Amini, I. Mather, H. Lassmann, and C. Linington. 2000. Butyrophilin, a milk protein, modulates the encephalitogenic T cell response to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis. *J. Immunol.* 165:2859-2865.
- Stergiadis, S., C. Leifert, C. Seal, M. Eyre, M. K. Larsen, T. Slots, J. Nielsen, and G. Butler. 2015. A 2-year study on milk quality from three pasture-based dairy systems of contrasting production intensities in Wales. *J. of Agric. Sci.* 153:708-731.
- Susmel, P., and B. Stefanon. 1993. Aspects of lignin degradation by rumen microorganisms. *J. Biotechnol.* 30:141-148. [https://dx.doi.org/10.1016/0168-1656\(93\)90035-L](https://dx.doi.org/10.1016/0168-1656(93)90035-L)
- Szklarczyk, D., A. Franceschini, S. Wyder, K. Forslund, D. Heller, J. Huerta-Cepas, M. Simonovic, A. Roth, A. Santos, K. P. Tsafou, M. Kuhn, P. Bork, L. J. Jensen, and C. von Mering. 2015. STRING v10: protein–protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* 43(Database issue):D447-D452. <https://dx.doi.org/10.1093/nar/gku1003>
- Szklarczyk, D., J. H. Morris, H. Cook, M. Kuhn, S. Wyder, M. Simonovic, A. Santos, N. T. Doncheva, A. Roth, P. Bork, L. J. Jensen, and C. von Mering. 2017. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res.* 45:D362-D368. <https://dx.doi.org/10.1093/nar/gkw937>

- Tacoma, R., J. Fields, D. B. Ebenstein, Y.-W. Lam, and S. L. Greenwood. 2016. Characterization of the bovine milk proteome in early-lactation Holstein and Jersey breeds of dairy cows. *J. Proteomics* 130:200-210. <https://dx.doi.org/10.1016/j.jprot.2015.09.024>
- Tacoma, R., J. Fields, D. B. Ebenstein, Y. W. Lam, and S. L. Greenwood. 2017a. 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. *J. Dairy Sci.* 100:7246-7261. <https://doi.org/10.3168/jds.2017-12647>
- Tacoma, R., S. L. Gelsinger, Y. W. Lam, R. A. Scuderi, D. B. Ebenstein, A. J. Heinrichs, and S. L. Greenwood. 2017b. Exploration of the bovine colostrum proteome and effects of heat treatment time on colostrum protein profile. *J Dairy Sci. Inpress.* <https://doi.org/10.3168/jds.2017-13211>
- Team, R.C. 2015. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing.
- Tilman, D. and J. A. Downing. 1994. Biodiversity and Stability in Grasslands. *Nature* 367:363-365.
- Totty, V. K., S. L. Greenwood, R. H. Bryant, and G. R. Edwards. 2013. Nitrogen partitioning and milk production of dairy cows grazing simple and diverse pastures. *J. Dairy Sci.* 96:141-149. <https://dx.doi.org/10.3168/jds.2012-5504>
- Vanderghem, C., F. Francis, S. Danthine, C. Deroanne, M. Paquot, E. De Pauw, and C. Blecker. 2011. Study on the susceptibility of the bovine milk fat globule membrane proteins to enzymatic hydrolysis and organization of some of the proteins. *Int. Dairy J.* 21:312-318. <https://doi.org/10.1016/j.idairyj.2010.12.006>
- Waghorn, G. C., M. J. Ulyatt, A. John, and M. T. Fisher. 1987. The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *Lotus corniculatus* L. *Br. J. Nutr.* 57:115-126. <https://dx.doi.org/10.1079/BJN19870015>
- Yang, Y., N. Zheng, X. Zhao, Y. Zhang, R. Han, L. Ma, S. Zhao, S. Li, T. Guo, and J. Wang. 2015. Proteomic characterization and comparison of mammalian milk fat globule proteomes by iTRAQ analysis. *J. Proteomics* 116:34-43. <https://dx.doi.org/10.1016/j.jprot.2014.12.017>

- Ye, A., J. Cui, and H. Singh. 2011. Proteolysis of milk fat globule membrane proteins during in vitro gastric digestion of milk. *J. Dairy Sci.* 94:2762-2770. <https://doi.org/10.3168/jds.2010-4099>
- Zhang, L., A. D. van Dijk, and K. Hettinga. 2017. An interactomics overview of the human and bovine milk proteome over lactation. *Proteome Science* 15(1):1. <https://doi.org/10.1186/s12953-016-0110-0>

CHAPTER 4. GENERAL DISCUSSION AND CONCLUSIONS

4.1 General Discussion

The literature and projects presented in this thesis have outlined possible evidence linking diet to the proteomic composition of bovine milk, focusing on the investigation of dietary inclusion of GM or AFC and their impact on the milk proteome.

As described in Chapter 2, there were no changes in the metabolism or N status of the cows as a result from GM inclusion in the diet. Additionally, there was a relatively small inclusion of CT in the diet when compared to other studies, where N partitioning was altered (Greenwood et al., 2012). Despite this lack of change in N partitioning, about 12% of the low-abundance proteins identified in this study was differentially altered as a result of GM in the diet. Further bioinformatic analysis suggested an interaction-based relationship between many of the affected proteins; however, there was no significant pathway or mechanism identified that could help explain the findings observed.

Similarly, we investigated the impact of cows grazing AFC in pasture on the milk proteome in Chapter 3. By expanding our proteomic analysis to include the MFGM-associated protein fraction, we were hoping to identify more shifts in the proteome. Despite the additional characterization of the MFGM-associated protein fraction, the results from this second experiment showed very little change in the milk proteome from both skim milk and MFGM fractions in response to diet. The high-abundance protein, α -s1 CAS, was higher in cows that grazed AFC pastures. Additionally, the affected low-abundance proteins in the skim and MFGM fractions were all higher in relative-abundance in cows grazing AFC compared to cows that grazed traditional pasture which

did not contain alternative forage. Although the cows grazing AFC pasture had a higher α -s1 CAS content, as well as higher relative-fold abundances of beta-2-microglobulin and polymeric immunoglobulin receptor in the skim milk fraction, and lactoperoxidase in the MFGM fraction, the rest of the proteome was largely unchanged. Furthermore, it is unclear what role (if any) diet may have served in altering those proteins.

4.2 Dietary limitations and implications in this research

While measures were taken to eliminate confounding variables, there were some limitations that were encountered that make interpretation of the mechanistic drivers difficult to isolate and assess. In Chapter 2, the smaller inclusion of GM likely explains the lack of response in metabolic and N parameters. The CT concentration of the GM in the first experiment was 4.29 g CT per kg of GM, which equates to an approximate daily intake 6.44 g CT per cow. This is lower than what has been reported elsewhere in the literature (Greenwood et al., 2012), and is a probable explanation to the lack of response when cows consumed GM.

Another limitation with the CT content relates to the unpredictable binding properties. While CT are known to bind to proteins in a neutral environment and dissociate when the pH deviates (Waghorn et al., 1987), astringency has been shown to prevent dissociation of the CT-protein complex (Acamovic and Brooker, 2005). Alternatively, if the CT-protein complex can dissociate in the abomasum as a result from a decreased pH, it is unclear as to whether the CT remain un-bound as they pass through the remainder of the GIT. Hence, it is possible for proteins to re-associate with unbound CT after being hydrolyzed in the abomasum. Furthermore, free CT can alter the

permeability of the small intestine and decrease the absorption of peptides and amino acids in the GIT further inhibiting proper intestinal absorption (Acamovic and Brooker, 2005).

While the lower concentration of CT in the GM was a limitation in the first experiment for influencing the bovine milk proteome, it is important to reiterate that a low-level inclusion of GM still serves a useful purpose in the dairy industry relating to nutritional management. Our results support the use of GM as a supplement in lactating dairy cow rations since it did not negatively impact production, which has been observed elsewhere (Santos et al., 2014; Nudda et al., 2015). Furthermore, it is possible that other compounds present in GM could alter production, including anthocyanins (Yi et al., 2009); however, they were not measured in these experiments.

During the second experiment (Chapter 3), the AFC used were strip-tilled into the pasture to account for approximately 10% of the total pasture content. The relatively small inclusion of AFC could offer partial explanation for why there were only minimal changes observed in the proteomic composition of milk. However, despite the small inclusion, there were changes in milk fat percentage as well as α -s1 CAS content. The lignin content was higher in the AFC pasture, which is a known limitation for rumen fermentation and consequent animal metabolism (Susmel and Stefanon, 1993). Yet, it is not likely that lignin allowed for any of the shifts in milk content observed in this experiment, including higher milk fat and α -s1 CAS content. Another dietary-related limitation in this study relates to unavailable DMI data. It is important to make note of intake during any experiment involving dietary manipulations as intake per se affects the

outcome measures of an experiment. For example, it is possible that the cows grazing AFC pastures had an increased DMI when compared to animals on the CON pasture. If this were to hold true, then the observations made in this experiment could be a result of increased intake in the AFC group and not strictly due to the altered nutritive profile.

4.3 Methodology related limitations that could impact interpretation of the results

Methyl cellulose has been used to bind and precipitate CT from grape juices, wine, and grape homogenates; and hence, the methyl cellulose precipitation assay has been developed to determine CT content in GM (Sarneckis et al., 2006). Epicatechin standards in solution were used to generate a concentration curve for the calculation of CT concentrations; however, there are multiple different polymers of CT present in GM. While the use of epicatechin standards as part of the methyl cellulose precipitation assay has been well-established, it is possible that the use of a specific CT polymer yields a limitation in analyzing CT content.

Another methodology-related limitation includes the use of two different milk samples to examine the bovine milk proteome in Chapter 3. It cannot be guaranteed that the same proteins are present in one composited milk sample versus another composited milk sample, even if the milk is from the same animal on the sampling time points. Future studies examining both skim and MFGM fractions for low-abundance protein analysis should carefully take this into consideration during isolation procedures. Ideally, one composited milk sample should be used to subsequently fractionate and analyze these milk fractions.

4.4 Other indications of nitrogen fractions and flow for future directions

While the alteration of N metabolism through diet could be a sufficient method in manipulating the bovine milk proteome as aforementioned, recent studies have yet to validate this potential relationship. If such a relationship were to hold true, it could be determined by measuring N metabolism using the methods described in the first experiment with some modifications. While measuring N in the milk, plasma, and total 24-h urine and fecal samples are effective, it is also important to evaluate the outflow of microbial protein from the rumen as a result from altering N patterns. This can be achieved through measuring purine derivatives in urine. Nucleic acids leaving the rumen are absorbed in the small intestine and metabolized, with derivatives being excreted in urine as hypoxanthine, xanthine, allantoin, and uric acid (Chen and Gomes, 1995). Nucleic acids leaving the rumen are assumed to be of microbial origin due to the degradation of purines that otherwise occur in the rumen (Chen and Gomes, 1995). Purine derivatives can be measured in acidified urine samples and used in a calculation to provide an estimate of microbial protein synthesis. Using these calculations, future research could consider the supply of microbial protein leaving the rumen when evaluating the changes in the bovine milk proteome in response to dietary manipulation.

4.5 Limitations and opportunities in proteomic methodologies

The proteomic workflow applied to the present experiments involved extensive fractionation of the low-abundance skim milk fractions starting with calcium dichloride precipitation followed by ultra-centrifugation. Afterwards, ProteoMiner treatment was used to further enrich the samples for low-abundance proteins. While we were successful

in characterizing a fraction of low-abundance proteins, dynamic range was lost in these experiments likely due to the lack of a multi-step separation procedure. To better access the proteome of interest, mass spectrometry-based proteomics requires high resolution in samples as a result from separation; therefore, multi-dimensional or multiple separation techniques are necessary to help yield a higher dynamic range in samples (Twyman, 2014). It would be ideal to incorporate a separation step following calcium dichloride precipitation/ ultracentrifugation such as gel electrophoresis using SDS-PAGE, where proteins are separated based on mass. Multi-dimensional liquid chromatography can be employed as a method of separation as well, but this analysis is costly and time consuming in comparison to SDS-PAGE.

Following SDS-PAGE, the use of the ProteoMiner kit (Biorad; Hercules, CA) is effective in further separating proteins as part of a multi-step workflow (Tacoma et al., 2016). ProteoMiner utilizes combinatorial peptide ligands in bead form for protein binding in complex biological solutions (D'Amato et al., 2009). Each bead contains its own ligand for specific peptides, and the beads collectively provide a diverse library of combinatorial peptide ligands for different proteins to bind with high affinity; hence, allowing for proteins that are present in higher abundance to quickly saturate their specific beads with many of the same protein being left unbound (Righetti and Boschetti, 2008). The unbound proteins are then washed away, leaving behind a sample that contains less of the higher abundant proteins thereby enriching the low-abundance proteins. This process is accomplished in part by overloading the ProteoMiner columns which allows for oversaturation of the beads, as opposed to other similar affinity-based

chromatography methods (D'Amato et al., 2009). While ProteoMiner has been successfully utilized to enrich low-abundance proteins within a sample, the binding properties of the beads are not entirely predictable. For instance there are peptide ligands that also have very close similarity, some only having a single amino acid difference, and hence, one peptide ligand can have binding interactivity with multiple proteins (Righetti and Boschetti, 2008). Additionally, due to ProteoMiner's reliance on affinity-based binding, factors including protein quantity, concentration, and dynamic range can affect the number of proteins that are removed; all of which are expected to vary between biological samples (Fonslow et al., 2011).

While ProteoMiner offers advantages similar to affinity-based liquid chromatography through the use of a simpler kit-format, the consistency of the binding needs to be investigated and validated. If properly used as a second step in a multi-step fractionation workflow, ProteoMiner could be successfully implemented as an effective method to characterize changes in the low-abundance proteome. Future directions could focus towards developing combinational peptide ligand beads that are specific for biological samples such as milk. Perhaps each bead can be functionally developed to bind with high-abundance proteins using a very competitive affinity. Furthermore, downstream validation of such a technology could lead to the possibility of introducing specific concentrations of beads to proportionally represent the onset of high-abundance proteins. In bovine milk, it is well established that caseins represent 80% whereas 20% comprise the remaining whey proteins. Furthermore, the high-abundance proteins including α -LA, β -LGA, and β -LGB comprise 90% of the bovine milk proteome with the

remaining 10% being low-abundance proteins. It could be possible to apply combinatorial peptide ligand beads in concentrations that are specific to bovine milk allowing for a more selective approach in removing higher-abundance proteins; however, extensive development is needed to do so. An alternative to ProteoMiner could include more selective techniques such as charged-based chromatography separation (anionic and cationic), as well as using immunodepletion methods through the development of antibodies for capturing specific proteins. Furthermore, it would be best to implement a validation step such as western-blotting to confirm the identity of proteins as determined by LC-MS/MS.

Recently, the high-throughput analysis of proteins across multiple samples has been utilized in proteomic work flows through the incorporation of stable isotopes (Christoforou and Lilley, 2012). This process of isobaric labeling has become widely available in kit form allowing for researchers to increase analytical speed by multiplexing samples, and it has also allowed for more sample types to be analyzed in quantitative experiments as opposed to previous approaches that utilize stable isotope labeling with amino acids in cell culture (SILAC) (Christoforou and Lilley, 2012). Nevertheless, there are some significant issues with isobaric labeling using TMT or iTRAQ including missing values across complex samples, incomplete labeling, decreased accuracy and precision during MS analysis (Christoforou and Lilley, 2012). While the use of isobaric labeling has been applied in animal proteomics, including the bovine milk proteome elsewhere (Roncada et al., 2012; Yang et al., 2015; Tacoma et al., 2017b), it is possible to

see decreases in the dynamic range of the milk proteome due to the aforementioned limitations.

4.6 Conclusions

Characterizing the factors that influence the composition of the bovine milk proteome, including bioactive proteins and peptides, is an important step forward in interpreting the proteome. Nutrition is a known factor that can influence lactation, and a number of different studies have evaluated, and confirmed, a relationship between diet and the proteomic composition of bovine milk. Therefore, better understanding the complexity of the interactions within the bovine milk proteome can help scientists understand how factors such as diet can affect the composition, which in turn can potentially provide an application for manipulation through dietary intervention. Our experiments presented in this thesis do not fully support N partitioning as a mechanism for the changes observed; thereby, rejecting the original hypothesis. While the changes observed in proteomic composition of the high and low-abundance proteins in bovine milk were likely altered in response to diet, it appears that N metabolism was not directly related to the shifts. Further investigation is necessary in order to fully characterize the underlying mechanisms involved, including reevaluating the effects of N metabolism.

4.7 References

- Acamovic, T., and J. D. Brooker. 2005. Biochemistry of plant secondary metabolites and their effects in animals. *Proc. Nutr. Soc.* 64:403-412.
<https://dx.doi.org/10.1079/PNS2005449>
- Chen, X. B. and M. Gomes. 1995. Estimation of microbial protein supply to sheep and cattle based on urinary excretion of purine derivatives-an overview of the technical details. International Feed Resources Unit.
- Christoforou, A. L. and K. S. Lilley. 2012. Isobaric tagging approaches in quantitative proteomics: the ups and downs. *Anal. Bioanal. Chem.* 404:1029-1037.
- D'Amato, A., A. Bachi, E. Fasoli, E. Boschetti, G. Peltre, H. Senechal, and P. G. Righetti. 2009. In-depth exploration of cow's whey proteome via combinatorial peptide ligand libraries. *J Proteome Res.* 8:3925-3936.
<https://doi.org/10.1021/pr900221x>
- Fonslow, B. R., P. C. Carvalho, K. Academia, S. Freeby, T. Xu, A. Nakorchevsky, A. Paulus, and J. R. Yates III. 2011. Improvements in proteomic metrics of low abundance proteins through proteome equalization using ProteoMiner prior to MudPIT. *J. Proteome Res.* 10:3690-3700. <https://doi.org/10.1021/pr200304u>
- Greenwood, S. L., G. R. Edwards, and R. Harrison. 2012. Short communication: supplementing grape marc to cows fed a pasture-based diet as a method to alter nitrogen partitioning and excretion. *J. Dairy Sci.* 95:755-758.
<https://dx.doi.org/10.3168/jds.2011-4648>
- Righetti, P. G., and E. Boschetti. 2008. The ProteoMiner and the FortyNiners: searching for gold nuggets in the proteomic arena. *Mass Spectrom. Rev.* 27:596-608.
<https://doi.org/10.1002/mas.20178>
- Roncada, P., C. Piras, A. Soggiu, R. Turk, A. Urbani, and L. Bonizzi. 2012. Farm animal milk proteomics. *J Proteomics.* 75:4259-4274.
<https://doi.org/10.1016/j.jprot.2012.05.028>
- Nudda, A., F. Correddu, A. Marzano, G. Battacone, P. Nicolussi, P. Bonelli, and G. Pulina. 2015. Effects of diets containing grape seed, linseed, or both on milk production traits, liver and kidney activities, and immunity of lactating dairy ewes. *J. Dairy Sci.* 98:1157-1166. <https://dx.doi.org/10.3168/jds.2014-8659>
- Santos, N. W., G. T. D. Santos, D. C. Silva-Kazama, P. A. Grande, P. M. Pintro, F. E. de Marchi, C. C. Jobim, and H. V. Petit. 2014. Production, composition and antioxidants in milk of dairy cows fed diets containing soybean oil and grape

- residue silage. *Livestock Science* 159:37-45.
<https://dx.doi.org/10.1016/j.livsci.2013.11.015>
- Sarneckis, C. J., R. G. Dambergs, P. Jones, M. Mercurio, M. J. Herderich, and P. A. Smith. 2006. Quantification of condensed tannins by precipitation with methyl cellulose: development and validation of an optimised tool for grape and wine analysis. *Aust. J. Grape Wine Res.* 12:39-49.
<https://dx.doi.org/10.1111/j.1755-0238.2006.tb00042.x>
- Susmel, P., and B. Stefanon. 1993. Aspects of lignin degradation by rumen microorganisms. *J. Biotechnol.* 30:141-148.
[https://dx.doi.org/10.1016/0168-1656\(93\)90035-L](https://dx.doi.org/10.1016/0168-1656(93)90035-L)
- Tacoma, R., J. Fields, D. B. Ebenstein, Y.-W. Lam, and S. L. Greenwood. 2016. Characterization of the bovine milk proteome in early-lactation Holstein and Jersey breeds of dairy cows. *J. Proteomics* 130:200-210.
<https://dx.doi.org/10.1016/j.jprot.2015.09.024>
- Tacoma, R., S. L. Gelsinger, Y. W. Lam, R. A. Scuderi, D. B. Ebenstein, A. J. Heinrichs, and S. L. Greenwood. 2017b. Exploration of the bovine colostrum proteome and effects of heat treatment time on colostrum protein profile. *J Dairy Sci.* *In-press.*
<https://doi.org/10.3168/jds.2017-13211>
- Twyman, R. M. 2014. *Principles of Proteomics.* Pages 15-20, 23-54. Garland Science, New York, New York.
- Waghorn, G. C., M. J. Ulyatt, A. John, and M. T. Fisher. 1987. The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *Lotus corniculatus* L. *Br. J. Nutr.* 57:115-126.
<https://dx.doi.org/10.1079/BJN19870015>
- Yang, Y., N. Zheng, X. Zhao, Y. Zhang, R. Han, L. Ma, S. Zhao, S. Li, T. Guo, and J. Wang. 2015. Proteomic characterization and comparison of mammalian milk fat globule proteomes by iTRAQ analysis. *J. Proteomics* 116:34-43.
<https://dx.doi.org/10.1016/j.jprot.2014.12.017>
- Yi, C., J. Shi, J. Kramer, S. Xue, Y. M. Jiang, M. W. Zhang, Y. Ma, and J. Pohorly. 2009. Fatty acid composition and phenolic antioxidants of winemaking pomace powder. *Food Chem.* 114:570-576. <https://dx.doi.org/10.1016/j.foodchem.2008.09.103>

5. COMPREHENSIVE BIBLIOGRAPHY

- Acamovic, T., and J. D. Brooker. 2005. Biochemistry of plant secondary metabolites and their effects in animals. *Proc. Nutr. Soc.* 64:403-412.
<https://dx.doi.org/10.1079/PNS2005449>
- Arnett, H. A., and J. L. Viney. 2014. Immune modulation by butyrophilins. *Nat. Rev. Immunol.* 14:559-569. <http://dx.doi.org/10.1038/nri3715>
- Asano, M., and K. Komiyama. 2011. Polymeric immunoglobulin receptor. *J. Oral Sci.* 53:147-156
- Ben-Gera, I., and A. Kramer. 1969. The utilization of food industries wastes. *Adv. Food Res.* 17:77-152. [https://dx.doi.org/10.1016/S0065-2628\(08\)60309-2](https://dx.doi.org/10.1016/S0065-2628(08)60309-2)
- Bequette, B. J., F. R. C. Backwell, and L. A. Crompton. 1998. Current concepts of amino acid and protein metabolism in the mammary gland of the lactating ruminant. *J. Dairy Sci.* 81:2540-2559. [https://doi.org/10.3168/jds.S0022-0302\(98\)70147-X](https://doi.org/10.3168/jds.S0022-0302(98)70147-X)
- Bordin, G., F. C. Raposo, B. de la Calle, and A. R. Rodriguez. 2001. Identification and quantification of major bovine milk proteins by liquid chromatography. *J. Chromatogr. A.* 928:63-76. [https://dx.doi.org/10.1016/S0021-9673\(01\)01097-4](https://dx.doi.org/10.1016/S0021-9673(01)01097-4)
- Bourantas, K. L., E. C. Hatzimichael, A. C. Makis, A. Chaidos, E. D. Kapsali, S. Tsiara, and A. Mavridis. 1999. Serum beta-2-microglobulin, TNF-alpha and interleukins in myeloproliferative disorders. *Eur. J. Haematol.* 63:19-25
- Cabrita, A. R. J., J. Dewhurst, J. M. F. Abreu, and A. J. M. Fonseca. 2006. Evaluation of the effects of synchronising the availability of N and energy on rumen function and production responses of dairy cows – a review. *Anim. Res.* 55:1-24.
<https://doi.org/10.1051/animres:2005045>
- Camacho, C., G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, and T. L. Madden. 2009. BLAST+: architecture and applications. *BMC Bioinformatics* 10:421. <https://dx.doi.org/10.1186/1471-2105-10-421>
- Cavaletto, M., M. G. Giuffrida, and A. Conti. 2009. Milk fat globule membrane components – A proteomic approach. *Adv. Exp. Med. Biol.* 606: 129-141.
https://doi.org/10.1007/978-0-387-74087-4_4
- Cebo, C. 2012. Milk fat globule membrane proteomics: A 'snapshot' of mammary epithelial cell biology. *Food Technol. Biotechnol.* 50:306-314.

- Chen, X. B. and M. Gomes. 1995. Estimation of microbial protein supply to sheep and cattle based on urinary excretion of purine derivatives-an overview of the technical details. International Feed Resources Unit.
- Christian, M. P., C. Grainger, B. J. Sutherland, J. J. Mayes, M. C. Hannah, and B. Kefford. 1999. Managing diet quality for cheddar cheese manufacturing milk. 1. The influence of protein energy supplements. *J. Dairy Res.* 66:341-355.
- Christoforou, A. L. and K. S. Lilley. 2012. Isobaric tagging approaches in quantitative proteomics: the ups and downs. *Anal. Bioanal. Chem.* 404:1029-1037.
- Clare, D. A., and H. E. Swaisgood. 2000. Bioactive milk peptides: a prospectus. *J. Dairy Sci.* 83:1187-1195. [https://doi.org/10.3168/jds.S0022-0302\(00\)74983-6](https://doi.org/10.3168/jds.S0022-0302(00)74983-6)
- Clark, J. H., T. H. Klusmeyer, and M. R. Cameron. 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. *J. Dairy Sci.* 75:2304-2323. [https://doi.org/10.3168/jds.S0022-0302\(92\)77992-2](https://doi.org/10.3168/jds.S0022-0302(92)77992-2)
- D'Amato, A., A. Bachi, E. Fasoli, E. Boschetti, G. Peltre, H. Senechal, and P. G. Righetti. 2009. In-depth exploration of cow's whey proteome via combinatorial peptide ligand libraries. *J Proteome Res.* 8:3925-3936. <https://doi.org/10.1021/pr900221x>
- DePeters, E. J., and J. P. Cant. 1992. Nutritional factors influencing the nitrogen composition of bovine-milk - a review. *J. Dairy Sci.* 75:2043-2070. [https://dx.doi.org/10.3168/jds.S0022-0302\(92\)77964-8](https://dx.doi.org/10.3168/jds.S0022-0302(92)77964-8)
- Dewettinck, K., R. Rombaut, N. Thienpont, T. T. Le, K. Messens, and J. Van Camp. 2008. Nutritional and technological aspects of milk fat globule membrane material. *Int. Dairy J.* 18:436-457. <https://doi.org/10.1016/j.idairyj.2007.10.014>
- Dewhurst, R., D. Davies, and R. Merry. 2000. Microbial protein supply from the rumen. *Anim. Feed Sci. Technol.* 85:1-21. [https://doi.org/10.1016/S0377-8401\(00\)00139-5](https://doi.org/10.1016/S0377-8401(00)00139-5)
- Emery, R. 1978. Feeding For Increased Milk Protein. *J. Dairy Sci.* 61:825-828. [https://doi.org/10.3168/jds.S0022-0302\(78\)83656-X](https://doi.org/10.3168/jds.S0022-0302(78)83656-X)
- Firkins, J. L. 1996. Maximizing microbial protein synthesis in the rumen. *J. Nutr.* 126:1347S-1354S.
- FitzGerald, R., and H. Meisel. 2003. Milk protein hydrolysates and bioactive peptides. Pages 675-698 in Volume 1: Proteins. *Advanced Dairy Chemistry*. 3rd ed. Part

B. P. F. Fox and P. L. H. McSweeney, ed. Kluwer Academic/ Plenum Publishers, New York, NY.

FitzGerald, R. J., B. A. Murray, and D. J. Walsh. 2004. Hypotensive peptides from milk proteins. *J. Nutr.* 134:980S-988S.

Fonslow, B. R., P. C. Carvalho, K. Academia, S. Freeby, T. Xu, A. Nakorchevsky, A. Paulus, and J. R. Yates III. 2011. Improvements in proteomic metrics of low abundance proteins through proteome equalization using ProteoMiner prior to MudPIT. *J. Proteome Res.* 10:3690-3700.
<https://doi.org/10.1021/pr200304u>

Gauthier, S. F., Y. Pouliot, and D. Saint-Sauveur. 2006. Immunomodulatory peptides obtained by the enzymatic hydrolysis of whey proteins. *Int. Dairy J.* 16:1315-1323. <https://doi.org/10.1016/j.idairyj.2006.06.014>

Gerling, C. 2011. Conversion Factors: From Vineyard to Bottle. Accessed Mar. 30th, 2017. <https://grapesandwine.cals.cornell.edu/newsletters/appellation-cornell/2011-newsletters/issue-8/conversion-factors-vineyard-bottle>

Gordon, F. 1977. The effect of protein content on the response of lactating cows to level of concentrate feeding. *Animal Science.* 25:181-191.
<https://doi.org/10.1017/S0003356100039404>

Greenwood, S. L., G. R. Edwards, and R. Harrison. 2012. Short communication: supplementing grape marc to cows fed a pasture-based diet as a method to alter nitrogen partitioning and excretion. *J. Dairy Sci.* 95:755-758.
<https://dx.doi.org/10.3168/jds.2011-4648>

Hagemeister, H., W. Luppig, and W. Kaufmann. 1981. Microbial protein synthesis and digestion in the high-yielding dairy cow. Page 31 in *Recent Developments in Ruminant Nutrition*. W. Haresign, and D. J. A. Cole. Butterworths, London, Engl.

He, J. A., and M. M. Giusti. 2010. Anthocyanins: natural colorants with health-promoting properties. *Annu. Rev. Food Sci. Technol.* 1:163-187.
<https://dx.doi.org/10.1146/annurev.food.080708.100754>

Hosoda, K., B. Eruden, H. Matsuyama, and S. Shioya. 2012. Effect of anthocyanin-rich corn silage on digestibility, milk production and plasma enzyme activities in lactating dairy cows. *J. Anim. Sci.* 83:453-459.
<http://dx.doi.org/10.1111/j.1740-0929.2011.00981.x>

- Jenkins, T. C. and M. A. McGuire. 2006. Major advances in nutrition: Impact on milk composition. *J. Dairy Sci.* 89:1302-1310.
[https://doi.org/10.3168/jds.S0022-0302\(06\)72198-1](https://doi.org/10.3168/jds.S0022-0302(06)72198-1)
- Jones, C. M., and A. J. Heinrichs. 2017. Milk components: understanding milk fat and protein variation in your dairy herd. Accessed Sept. 5, 2017.
<https://extension.psu.edu/milk-components-understanding-milk-fat-and-protein-variation-in-your-dairy-herd>
- Journet, M. and Y. Chilliard. 1985. Influence de l'alimentation sur la composition du lait. 1. Taux butyreux: facteurs généraux. *Bull. Tech. CRZV Theix, INRA* 60:13-23.
- Juntwait, K. A., A. F. Brito, K. S. O'Connor, R. G. Smith, K. M. Aragona, and A. B. Pereira. 2016. Feeding Annual Forage Crops to Organic Dairy Cows During the Spring and Summer Seasons in Northeast United States. *Organic Agriculture Research Symposium*, Jan. 20th, 2016. Pacific Grove, CA.
- Kalber, T., M. Kreuzer, and F. Leiber. 2013. Effect of feeding buckwheat and chicory silages on fatty acid profile and cheese-making properties of milk from dairy cows. *J. Dairy Res.* 80:81-88. <https://doi.org/10.1017/S0022029912000647>
- Keough, G. 2017. *New England Berries, Tree Fruit, and Grapes, 2016 Crop*. Pages 1-4. USDA, Concord, NH.
- Kilara, A., and D. Panyam. 2003. Peptides from milk proteins and their properties. *Crit. Rev. Food Sci. Nutr.* 43:607-633.
<http://dx.doi.org/10.1080/10408690390251138>
- Kim, K. H., Y. G. Oh, J. J. Choung, and D. G. Chamberlain. 1999. Effects of varying degrees of synchrony of energy and nitrogen release in the rumen on the synthesis of microbial protein in cattle consuming grass silage. *J. Sci. Food Agric.* 79:833-838.
- Kobayashi, K., S. Oyama, A. Numata, M. M. Rahman, and H. Kumura. 2013. Lipopolysaccharide disrupts the milk-blood barrier by modulating claudins in mammary alveolar tight junctions. *PloS One* 8: e62187.
<https://dx.doi.org/10.1371/journal.pone.0062187>
- Korhonen, H. 2009. Milk-derived bioactive peptides: from science to applications. *J. Funct. Food* 1:177-187. <https://dx.doi.org/10.1016/j.jff.2009.01.007>

- Larson, B. L., H. L. Heary, Jr., and J. E. Devery. 1980. Immunoglobulin production and transport by the mammary gland. *J Dairy Sci* 63:665-671. [https://doi.org/10.3168/jds.S0022-0302\(80\)82988-2](https://doi.org/10.3168/jds.S0022-0302(80)82988-2)
- Lascano, G. J., G. I. Zanton, A. J. Heinrichs, and W. P. Weiss. 2010. Technical note: a noninvasive urine collection device for female cattle: modification of the urine cup collection method. *J. Dairy Sci.* 93:2691-2694. <https://dx.doi.org/10.3168/jds.2009-3027>
- Le, A., L. D. Barton, J. T. Sanders, and Q. Zhang. 2011. Exploration of bovine milk proteome in colostrum and mature whey using an Ion-Exchange approach. *J. Proteome Res.* 10:692-704. <https://dx.doi.org/10.1021/pr100884z>
- Lee, M. R., V. J. Theobald, J. K. Tweed, A. L. Winters, and N. D. Scollan. 2009. Effect of feeding fresh or conditioned red clover on milk fatty acids and nitrogen utilization in lactating dairy cows. *J. Dairy Sci.* 92:1136-1147. <https://dx.doi.org/10.3168/jds.2008-1692>
- Li, S. S., J. S. Shen, D. X. Ren, and J. X. Liu. 2015. Effects of the processing methods of corn grain and soybean meal on milk protein expression profiles in dairy cows. *Animal* 9:267-274. <https://dx.doi.org/10.1017/S1751731114002225>
- Lonnerdal, B. 2013. Bioactive proteins in breast milk. *J. Paediatr. Child Health.* 49(S1):1-7. <https://dx.doi.org/10.1111/jpc.12104>
- Lopez-Exposito, I., and I. Recio. 2008. Protective effect of milk peptides: antibacterial and antitumor properties. *Adv. Exp. Med. Biol.* 606:271-293.
- López-Fandiño, R., J. Otte, and J. Van Camp. 2006. Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. *Int. Dairy J.* 16:1277-1293. <https://doi.org/10.1016/j.idairyj.2006.06.004>
- Makkar, H. P. S. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. *Small Ruminant Research* 49:241-256. [https://dx.doi.org/10.1016/S0921-4488\(03\)00142-1](https://dx.doi.org/10.1016/S0921-4488(03)00142-1)
- Mana, P., M. Goodyear, C. Bernard, R. Tomioka, M. Freire-Garabal, and D. Linares. 2004. Tolerance induction by molecular mimicry: prevention and suppression of experimental autoimmune encephalomyelitis with the milk protein butyrophilin. *Int. Immunol.* 16:489-499. <https://doi.org/10.1093/intimm/dxh049>

- Manso, T., B. Gallardo, A. Salva, C. Guerra-Rivas, A. R. Mantecon, P. Lavin, and M. A. de la Fuente. 2016. Influence of dietary grape pomace combined with linseed oil on fatty acid profile and milk composition. *J. Dairy Sci.* 99:1111-1120. <https://dx.doi.org/10.3168/jds.2015-9981>
- McAllan, A., J. Cockburn, A. Williams, and R. Smith. 1988. The degradation of different protein supplements in the rumen of steers and the effects of these supplements on carbohydrate digestion. *Br. J. Nutr.* 60:669-682. <https://doi.org/10.1079/BJN19880136>
- Mercurio, M. D., R. G. Damberg, M. J. Herderich, and P. A. Smith. 2007. High throughput analysis of red wine and grape phenolics-adaptation and validation of methyl cellulose precipitable tannin assay and modified somers color assay to a rapid 96 well plate format. *J. Agric. Food Chem.* 55:4651-4657. <https://dx.doi.org/10.1021/jf063674n>
- Mi, H., X. Huang, A. Muruganujan, H. Tang, C. Mills, D. Kang, and P. D. Thomas. 2017. PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. *Nucleic Acids Res.* 45:D183-D189. <https://dx.doi.org/10.1093/nar/gkw1138>
- Mills, S., R. P. Ross, C. Hill, G. F. Fitzgerald, and C. Stanton. 2011. Milk intelligence: mining milk for bioactive substances associated with human health. *Int. Dairy J.* 21:377-401. <https://dx.doi.org/10.1016/j.idairyj.2010.12.011>
- Miller, L. A., J. M. Moorby, D. R. Davies, M. O. Humphreys, N. D. Scollan, J. C. MacRae, and M. K. Theodorou. 2001. Increased concentration of water-soluble carbohydrate in perennial ryegrass (*Lolium perenne*): Milk production from late-lactation dairy cows. *Grass Forage Sci.* 56:383-394. <https://dx.doi.org/10.1046/j.1365-2494.2001.00288.x>
- Moate, P. J., S. R. Williams, V. A. Torok, M. C. Hannah, B. E. Ribaux, M. H. Tavendale, R. J. Eckard, J. L. Jacobs, M. J. Auld, and W. J. Wales. 2014. Grape marc reduces methane emissions when fed to dairy cows. *J. Dairy Sci.* 97:5073-5087. <https://doi.org/10.3168/jds.2013-7588>
- Molenaar, A. J., D. P. Harris, G. H. Rajan, M. L. Pearson, M. R. Callaghan, L. Sommer, V. C. Farr, K. E. Oden, M. C. Miles, R. S. Petrova, L. L. Good, K. Singh, R. D. McLaren, C. G. Prosser, K. S. Kim, R. J. Wieliczko, M. H. Dines, K. M. Johannessen, M. R. Grigor, S. R. Davis, and K. Stelwagen. 2009. The acute-phase protein serum amyloid A3 is expressed in the bovine mammary gland and plays a role in host defence. *Biomarkers* 14:26-37. <http://dx.doi.org/10.1080/13547500902730714>

- Molinari, C. E., Y. S. Casadio, B. T. Hartmann, A. Livk, S. Bringans, P. G. Arthur, and P. E. Hartmann. 2012. Proteome mapping of human skim milk proteins in term and preterm Milk. *J. Proteome Res.* 11:1696-1714. <https://dx.doi.org/10.1021/pr2008797>
- Monks, J., and M. C. Neville. 2004. Albumin transcytosis across the epithelium of the lactating mouse mammary gland. *J. Physiol. London* 560:267-280. <https://dx.doi.org/10.1113/jphysiol.2004.068403>
- Nissen, A., E. Bendixen, K. L. Ingvarstsen, and C. M. Rontvedt. 2013. Expanding the bovine milk proteome through extensive fractionation. *J. Dairy Sci.* 96:7854-7866. <https://dx.doi.org/10.3168/jds.2013-7106>
- Nudda, A., F. Correddu, A. Marzano, G. Battacone, P. Nicolussi, P. Bonelli, and G. Pulina. 2015. Effects of diets containing grape seed, linseed, or both on milk production traits, liver and kidney activities, and immunity of lactating dairy ewes. *J. Dairy Sci.* 98:1157-1166. <https://dx.doi.org/10.3168/jds.2014-8659>
- O'Riordan, N., M. Kane, L. Joshi, and R. M. Hickey. 2014. Structural and functional characteristics of bovine milk protein glycosylation. *Glycobiology* 24:220-236. <https://doi.org/10.1093/glycob/cwt162>
- Pereira, P. C. 2014. Milk nutritional composition and its role in human health. *J. Nut.* 30:619-627. <https://doi.org/10.1016/j.nut.2013.10.011>
- Pereira, A. B. D., A. F. Brito, L. L. Townson, and D. H. Townson. 2013. Assessing the research and education needs of the organic dairy industry in the northeastern United States. *J. Dairy Sci.* 96:7340-7348. <https://doi.org/10.3168/jds.2013-6690>
- Pihlanto, A. 2006. Antioxidative peptides derived from milk proteins. *Int. Dairy J.* 16:1306-1314. <https://doi.org/10.1016/j.idairyj.2006.06.005>
- Ramirez-Restrepo, C. A., and T. N. Barry. 2005. Alternative temperate forages containing secondary compounds for improving sustainable productivity in grazing ruminants. *Anim. Feed Sci. Technol.* 120:179-201. <https://doi.org/10.1016/j.anifeedsci.2005.01.015>
- Reinhardt, T. A., and J. D. Lippolis. 2006. Bovine milk fat globule membrane proteome. *J Dairy Res.* 73:406-416. <https://doi.org/10.1017/S0022029906001889>
- Righetti, P. G., and E. Boschetti. 2008. The ProteoMiner and the FortyNiners: searching for gold nuggets in the proteomic arena. *Mass Spectrom. Rev.* 27:596-608. <https://doi.org/10.1002/mas.20178>

- Rode, L., D. Weakley, and L. Satter. 1985. Effect of forage amount and particle size in diets of lactating dairy cows on site of digestion and microbial protein synthesis. *Can. J. of Anim. Sci.* 65:101-111. <https://doi.org/10.4141/cjas85-011>
- Roncada, P., C. Piras, A. Soggiu, R. Turk, A. Urbani, and L. Bonizzi. 2012. Farm animal milk proteomics. *J Proteomics.* 75:4259-4274. <https://doi.org/10.1016/j.jprot.2012.05.028>
- Rooke, J. A., N. H. Lee, and D. G. Armstrong. 1987. The effects of intraruminal infusions of urea, casein, glucose syrup and a mixture of casein and glucose syrup on nitrogen digestion in the rumen of cattle receiving grass-silage diets. *Br. J. Nutr.* 57:89-98. <https://doi.org/10.1079/BJN19870012>
- Santos, N. W., G. T. D. Santos, D. C. Silva-Kazama, P. A. Grande, P. M. Pintro, F. E. de Marchi, C. C. Jobim, and H. V. Petit. 2014. Production, composition and antioxidants in milk of dairy cows fed diets containing soybean oil and grape residue silage. *Livestock Science* 159:37-45. <https://dx.doi.org/10.1016/j.livsci.2013.11.015>
- Shannon, P., A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski, and T. Ideker. 2003. Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13:2498-2504. <https://dx.doi.org/10.1101/gr.1239303>
- Shennan, D. B., and M. Peaker. 2000. Transport of milk constituents by the mammary gland. *Physiol. Rev.* 80:925-951.
- Shingfield, K. J., Y. Chilliard, V. Toivonen, P. Kairenius, and D. I. Givens. 2008. Trans fatty acids and bioactive lipids in ruminant milk. *Adv. Exp. Med. Biol.* 606:3-65.
- Sniffen, C. and P. Robinson. 1987. Microbial growth and flow as influenced by dietary manipulations. *J. Dairy Sci.* 70:425-441. [https://doi.org/10.3168/jds.S0022-0302\(87\)80027-9](https://doi.org/10.3168/jds.S0022-0302(87)80027-9)
- Sok, M., D. R. Ouellet, J. L. Firkins, D. Pellerin, and H. Lapierre. 2017. Amino acid composition of rumen bacteria and protozoa in cattle. *J. Dairy Sci.* 100:5241-5249. <https://doi.org/10.3168/jds.2016-12447>
- Spitsberg, V. L. 2005. Invited review: Bovine milk fat globule membrane as a potential nutraceutical. *J. Dairy Sci.* 88:2289-2294. [https://dx.doi.org/10.3168/jds.S0022-0302\(05\)72906-4](https://dx.doi.org/10.3168/jds.S0022-0302(05)72906-4)

- Stefflerl, A., A. Schubart, M. Storch, A. Amini, I. Mather, H. Lassmann, and C. Linington. 2000. Butyrophilin, a milk protein, modulates the encephalitogenic T cell response to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis. *J. Immunol.* 165:2859-2865.
- Stergiadis, S., C. Leifert, C. Seal, M. Eyre, M. K. Larsen, T. Slots, J. Nielsen, and G. Butler. 2015. A 2-year study on milk quality from three pasture-based dairy systems of contrasting production intensities in Wales. *J. of Agric. Sci.* 153:708-731.
- Susmel, P., and B. Stefanon. 1993. Aspects of lignin degradation by rumen microorganisms. *J. Biotechnol.* 30:141-148.
[https://dx.doi.org/10.1016/0168-1656\(93\)90035-L](https://dx.doi.org/10.1016/0168-1656(93)90035-L)
- Sutton, J. D. 1989. Altering milk composition by feeding. *J. Dairy Sci.* 72:2801-2814.
[https://dx.doi.org/10.3168/jds.S0022-0302\(89\)79426-1](https://dx.doi.org/10.3168/jds.S0022-0302(89)79426-1)
- Szklarczyk, D., A. Franceschini, S. Wyder, K. Forslund, D. Heller, J. Huerta-Cepas, M. Simonovic, A. Roth, A. Santos, K. P. Tsafou, M. Kuhn, P. Bork, L. J. Jensen, and C. von Mering. 2015. STRING v10: protein–protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* 43(Database issue):D447-D452. <https://dx.doi.org/10.1093/nar/gku1003>
- Szklarczyk, D., J. H. Morris, H. Cook, M. Kuhn, S. Wyder, M. Simonovic, A. Santos, N. T. Doncheva, A. Roth, P. Bork, L. J. Jensen, and C. von Mering. 2017. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res.* 45:D362-D368.
<https://dx.doi.org/10.1093/nar/gkw937>
- Tacoma, R., J. Fields, D. B. Ebenstein, Y.-W. Lam, and S. L. Greenwood. 2016. Characterization of the bovine milk proteome in early-lactation Holstein and Jersey breeds of dairy cows. *J. Proteomics* 130:200-210.
<https://dx.doi.org/10.1016/j.jprot.2015.09.024>
- Tacoma, R., J. Fields, D. B. Ebenstein, Y. W. Lam, and S. L. Greenwood. 2017a. 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. *J. Dairy Sci.* 100:7246-7261.
<https://doi.org/10.3168/jds.2017-12647>
- Tacoma, R., S. L. Gelsinger, Y. W. Lam, R. A. Scuderi, D. B. Ebenstein, A. J. Heinrichs, and S. L. Greenwood. 2017b. Exploration of the bovine colostrum proteome and

- effects of heat treatment time on colostrum protein profile. *J Dairy Sci. Inpress*. <https://doi.org/10.3168/jds.2017-13211>
- Team, R.C. 2015. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing.
- Thomas, P., and P. Martin. 1988. The influence of nutrient balance on milk yield and composition. Page 97. *Nutrition and Lactation in the dairy cow*. P. C. Garnsworthy. Butterworths, London, Engl.
- Tilman, D. and J. A. Downing. 1994. Biodiversity and Stability in Grasslands. *Nature* 367:363-365.
- Totty, V. K., S. L. Greenwood, R. H. Bryant, and G. R. Edwards. 2013. Nitrogen partitioning and milk production of dairy cows grazing simple and diverse pastures. *J. Dairy Sci.* 96:141-149. <https://dx.doi.org/10.3168/jds.2012-5504>
- Tsiplakou, E., and G. Zervas. 2008. The effect of dietary inclusion of olive tree leaves and grape marc on the content of conjugated linoleic acid and vaccenic acid in the milk of dairy sheep and goats. *J. Dairy Res.* 75:270-278. <https://dx.doi.org/10.1017/S0022029908003270>
- Twyman, R. M. 2014. *Principles of Proteomics*. Pages 15-20, 23-54. Garland Science, New York, New York.
- USDA. 2010. *National Organic Program: Access to Pasture (Livestock)*. Pages 7153-7195. Vol. 75. Office of the Federal Register, National Archives and Records Administration, Washington DC.
- US Department of Agriculture. 2015. *Noncitrus fruit and nuts 2014 preliminary summary*. ISSN 1948-2698.
- USDA. 2017. *Noncitrus Fruits and Nuts 2016 Summary*. Pages ISSN: 1948-2698.
- Vanderghem, C., F. Francis, S. Danthine, C. Deroanne, M. Paquot, E. De Pauw, and C. Blecker. 2011. Study on the susceptibility of the bovine milk fat globule membrane proteins to enzymatic hydrolysis and organization of some of the proteins. *Int. Dairy J.* 21:312-318. <https://doi.org/10.1016/j.idairyj.2010.12.006>
- Van Soest, P. J. 1994. *Nutritional Ecology of the Ruminant*. Second Edition. Pages 258-259. 2nd ed. Cornell University Press.
- Waghorn, G. 2008. Beneficial and detrimental effects of dietary condensed tannins for

- sustainable sheep and goat production-progress and challenges. *Anim. Feed Sci. Technol.* 147:116-139. <https://dx.doi.org/10.1016/j.anifeedsci.2007.09.013>
- Waghorn, G. C., M. J. Ulyatt, A. John, and M. T. Fisher. 1987. The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *Lotus corniculatus* L. *Br. J. Nutr.* 57:115-126. <https://dx.doi.org/10.1079/BJN19870015>
- Woodward, S. L., G. G. Waghorn, K. A. Watkins, and M. A. Bryant. 2009. Feeding birdsfoot trefoil (*Lotus corniculatus*) reduces the environmental impacts of dairy farming. *Proc. N.Z. Soc. Anim. Prod.* 69:179-183.
- Yamamoto, N., M. Ejiri, and S. Mizuno. 2003. Biogenic peptides and their potential use. *Curr. Pharm. Des.* 9:1345-1355. <https://doi.org/10.2174/1381612033454801>
- Yang, W., K. Beauchemin, and L. Rode. 2001. Effects of grain processing, forage to concentrate ratio, and forage particle size on rumen pH and digestion by dairy cows. *J. Dairy Sci.* 84:2203-2216. [https://doi.org/10.3168/jds.S0022-0302\(01\)74667-X](https://doi.org/10.3168/jds.S0022-0302(01)74667-X)
- Yang, Y., N. Zheng, X. Zhao, Y. Zhang, R. Han, L. Ma, S. Zhao, S. Li, T. Guo, and J. Wang. 2015. Proteomic characterization and comparison of mammalian milk fat globule proteomes by iTRAQ analysis. *J. Proteomics* 116:34-43. <https://dx.doi.org/10.1016/j.jprot.2014.12.017>
- Ye, A., J. Cui, and H. Singh. 2011. Proteolysis of milk fat globule membrane proteins during in vitro gastric digestion of milk. *J. Dairy Sci.* 94:2762-2770. <https://doi.org/10.3168/jds.2010-4099>
- Yi, C., J. Shi, J. Kramer, S. Xue, Y. M. Jiang, M. W. Zhang, Y. Ma, and J. Pohorly. 2009. Fatty acid composition and phenolic antioxidants of winemaking pomace powder. *Food Chem.* 114:570-576. <https://dx.doi.org/10.1016/j.foodchem.2008.09.103>