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Examination Of The Effects Breed And Nutrition Have On The Milk Protein Profile Produced By Lactating Dairy Cattle

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EXAMINATION OF THE EFFECTS BREED AND NUTRITION HAVE ON THE MILK PROTEIN PROFILE PRODUCED BY LACTATING DAIRY CATTLE

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ABSTRACT

Milk is a highly nutritious natural product and research over the last 10 years has proven that these milk proteins not only provide a rich source of amino acids to the consumer but also contains many bioactive proteins and peptides known to exert biological activity benefitting human health. In this research, proteomic methods were first used to characterize the low abundance proteome within the skim milk fraction produced by Holstein and Jersey dairy cows maintained under the same diet, management and environmental conditions. Milk samples were collected over a seven day period from six Holstein and six Jersey dairy cows. Samples were depleted of casein (CN) by acidification and ultracentrifugation followed by ProteoMiner treatment. Extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation followed by liquid chromatography mass spectrometry (LC-MS). Over 930 low abundance proteins were identified and label-free proteomic analysis allowed for semi-quantification of proteins. Gene ontology (GO) classified proteins into various cellular localization and function categories. Forty-three low abundance proteins were differentially expressed between the two dairy breeds. Some bioactive proteins with immunomodulatory activities were present at significantly different abundance between breeds such as lactotransferrin (P <0.01) and Complement C2 (P <0.01), whereas others like osteopontin (P = 0.17) and lactoperoxidase (P = 0.29) were present at similar levels. This work has identified the highest number of low abundance proteins within the whey fraction in bovine skim milk, providing a foundation for future research exploring the bovine milk proteome.

Nutrition is a significant animal factor that has potential to alter milk protein composition. Therefore in the second phase of this work, nutritional perturbances were used to alter the bovine milk proteome by feeding Holstein dairy cows different proportions of rumen degradable (RDP) and rumen undegradable protein (RUP) to alter whole-body nitrogen (N) metabolism. Six multiparous Holstein cows in mid-lactation were randomly assigned to one of two treatment groups. The experiment was conducted as a double-crossover design consisting of three 21-day periods. Within each period, treatment groups received diets with either 1) a high RDP:RUP ratio (control: 62.4:37.6 % of CP) or 2) a low RDP:RUP ratio (RUP: 51.3:48.7 % of CP). Both diets were isonitrogenous (CP = 18.5%) and isoenergetic (NE_L = 0.8 Mcal lbs^{-1}). Feeding a diet high in RUP decreased β-casein (P = 0.06), κ-casein (P =0.04) and total milk casein concentrations in milk (P <0.001). Milk urea nitrogen (MUN) and plasma urea nitrogen (PUN) were significantly higher in the RDP group (P = 0.04; P < 0.01, respectively). Over 590 low abundance proteins were identified and only three proteins were found to be differentially expressed between the two dietary groups. The high dietary crude protein (CP) inclusion may explain the lack of treatment effect since protein synthesis within the mammary gland (MG) may not be responsive to dietary changes when total CP levels is offered in excess. Additional feeding trials are needed to alter N utilization patterns within a dairy cow while maintaining isonitrogenous and isoenergetic diets and offering normal CP levels. Nutritional perturbances offer opportunities to selectively alter the bovine proteome, providing a tool to enhance the healthfulness of milk.

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α-CN	Alpha-casein
α_{s1}-CN	Alpha$_{s1}$-casein
α_{s2}-CN	Alpha$_{s2}$-casein
α-LA	Alpha-lactalbumin
β-CN	Beta-casein
β-LG	Beta-lactoglobulin
κ-CN	κ-casein
γ-CN	γ-casein
AA	Amino acid
ADF	Acid detergent fiber
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BHBA	Beta-hydroxybutyrate
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
CN	Casein
CID	Collision-induced dissociation
d	Day
DGAT1	Diglyceride O-acyltransferase 1
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxynucleic acid
DTT	Dithiothreitol

FA	Fatty acid
FID	Flame ionization detector
GE	Gel electrophoresis
GO	Gene ontology
GTP	Guanosine-5'-triphosphate
HPLC	High performance liquid chromatography
HS	Heat stress
IgG	Immunoglobulin G
iTRAQ	Isobaric tags for relative and absolute quantitation
LC-MS	Liquid chromatography mass spectrometry
LSM	Least square means
MCP	Microbial crude protein
MEC	Mammary epithelial cell
MFGM	Milk fat globule membrane
MG	Mammary gland
mRNA	Messenger ribosomal nucleic acid
mTOR	Mechanistic target of rapamycin
mTORC	Mechanistic target of rapamycin complex
MUN	Milk urea nitrogen
N	Nitrogen
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NE_L	Net energy of lactation
NEFA	Non-esterified fatty acid
NFC	Non fibrous carbohydrate
NPN	Non protein nitrogen
PBS	Phosphate-buffered saline
PUN	Plasma urea nitrogen

RDP	Rumen-degradable protein
RER	Rough endoplasmic reticulum
RI	Restricted intake
RNA	Ribonucleic acid
RUP	Rumen-undegradable protein
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error mean
tRNA	Transfer ribosomal nucleic acid
VFA	Volatile fatty acid

1 CHAPTER ONE: LITERATURE REVIEW

This review aims to provide background information to highlight the complex nature of the bovine milk proteome. In recent years, investigation into the low abundance protein profile in bovine milk has risen due to the presence of identified bioactive proteins known to have positive effects on human health. Low abundance proteins can be characterized as proteins present at low concentrations within the whey fraction in milk excluding beta-lactoglobulin (β-LG), alpha-lactalbumin (α-LA), bovine serum albumin (BSA), and immunoglobulins (O'Donnell et al., 2004; Yang et al., 2013; Vincent et al., 2016). The main focus of this thesis will be to expand the low abundance protein profile in bovine milk, with emphasis on the effects of breed and nutrition on milk protein composition.

1.1 Bovine mammary gland structure and function

The udder of a cow (*Bos Taurus*) is comprised of four, independently functioning mammary glands (MG), with a fore and rear quarter in each half. An extensive support system composed of ligaments and connective tissue holds the udder close to the body wall. The two halves are separated by a distinct septum known as the median suspensory ligament, functioning in conjunction with the lateral suspensory ligament to support the udder (Akers, 2002; Barber, 2007). The primary role of the MG is to synthesize and store milk components until removal of milk is initiated by suckling of offspring or by mechanical means (Barber, 2007). Milk is synthesized within specialized mammary epithelial cells (MEC) that are grouped in alveoli followed by secretion via a ductal system. Milk produced by the epithelial cells lining the mammary alveoli is stored in the

alveolar lumen. During milk ejection, milk is forced out of the alveoli by contraction of the myoepithelial cells (under endocrine hormonal control) surrounding the alveoli and milk flows through ductules into ducts draining the alveoli (Neville and Neifert, 1983; Akers, 2002). Bovine milk is predominately water (approximately 85%), a result from osmosis related to the synthesis of lactose. The MEC selectively extract nutrients from the blood and incorporate them into biosynthetic pathways for milk synthesis (Akers, 2002; Barber, 2007). Vitamins, minerals and some proteins are not synthesized within MEC and are transported across the alveolar lumen from plasma by receptor-mediated transport and incorporated into milk. The nutrients that ultimately end up in milk originate from the substrates present in plasma, which are products from the breakdown of dietary components and metabolites released from other body tissues. The MG is a highly specialized organ under strong hormonal control in conjunction with nutritional stimuli that trigger specific responses influencing milk yield and composition. Thus, there is potential for manipulation of MG function and secretion to produce higher milk yields and component composition.

1.2 Bovine milk composition

Bovine milk is composed of three main constituents that hold significant value to the producers, processors, and the consumers. These components are protein, fat and lactose. Generally, bovine milk contains 3.0−3.8% protein, 3.6−4.8% fat, 4.4−5.2% lactose, and 85−87% water (Kaufmann and Hagemeister, 1987). Other important constituents that are present at lower concentrations include amino acids (AA), vitamins, minerals, immunoglobulins, hormones, growth factors, cytokines, peptides, polyamines, enzymes, and other bioactive peptides (Haug et al., 2007). Glucose, AA, volatile fatty

acids (VFA) and minerals are substrates that act as building blocks for milk synthesis. The proportion of milk constituents present in bovine milk is dependent on several factors such as breed, stage of lactation, season, health and mammary inflammation, and nutrition (Christian et al., 1999; Heck et al., 2009; Boehmer et al., 2010a; Gustavsson et al., 2014; Yang et al., 2015).

1.2.1 Bovine milk protein composition

Milk proteins comprise 35-45% of total milk solids in bovine milk. Nitrogen (N) present in milk is distributed among casein (CN), whey proteins and non-protein N (NPN). The majority of milk proteins (80-95% depending on animal species) are synthesized within the MEC. These include CN (e.g., α_{s1}-, α_{s2}-, β-, and κ-CN) and whey proteins (α-LA and β-LG). A collection of other proteins are also known to enter milk via indirect pathways, either drawn from plasma and transported across the mammary epithelia or enter milk via the secretion of the milk fat globule membrane (MFGM) (Ballard and Morrow, 2013; Burgoyne and Duncan, 1998; Boisgard et al., 2001) (*Figure 1.1*).

Milk protein has a high biological value because it contains a rich source of AA to the consumer as well as containing bioactive proteins and peptides known to have positive effects on human health (Lönnerdal, 2003; Severin and Wenshui, 2005; Korhonen and Pihlanto, 2006). Bioactive components are defined as proteins and protein fragments that 'provide a source of nutrition to the consumer and can modulate physiological function influencing body functions and improving human health' (Sharma et. al., 2011; Ballard and Morrow, 2013). Most milk proteins are digested in the gut to provide AA to the neonate (and consumer), yet there are also a reduced number of

proteins that are resistant against proteolysis. For example, osteopontin, a highly glycosylated and phosphorylated milk protein is relatively resistant to proteolysis and can induce expression of specific proteins involved in the development of the immune system. Osteopontin has also been shown to form complexes with lactoferrin and lactoperoxidase and it has been hypothesized that osteopontin can also act as a transporter of these immunomodulating protein to their site of action together with protecting them from proteolysis (Christensen and Sørensen, 2016). Some proteins such as lactoferrin, and lactoperoxidase, are capable of exerting bioactivity directly in the gastrointestinal tract by enhancing absorption of other nutrients, being involved in humoral immune response and boosting intestine development (Lönnerdal, 2010; Sharma et al., 2011). Other bioactive peptides become active once they are released from the parent protein after proteolysis and can exert their activity by binding to extracellular receptors on the intestinal epithelial cells or are directly absorbed into the intestinal epithelial cells (Ricci-Cabello et al., 2012; Boutrou et al., 2013; Wada and Lönnerdal, 2014). A recent study characterized and quantified over 500 peptides from cleavage of whey and CN proteins in the jejunum in human subjects (Boutrou et al., 2013). These peptides were released from specific foci of each protein and frequent identification of a range of proteins derived from CN and whey protein digestion highlights that cleavage is not random and confirms that milk is the most important source of natural bioactive components. (Ricci-Cabello et al., 2012; Boutrou et al., 2013; Nongonierma and FitzGerald, 2015; Park and Nam, 2015). In order to understand protein synthesis within the bovine MG, it is necessary to unravel the bovine proteome and understand the site of protein synthesis within the mammary secretory cells, mammary stromal tissue or directly from blood.

1.2.1.1 Casein

CN proteins are phosphoproteins and represent approximately 80% of the protein in bovine milk. The CN proteins are comprised of α_{s1}-, α_{s2}-, β-, γ- and κ-CN, where γ-CN is a product from β-CN degradation. CN proteins exist as micelles held together by colloidal calcium phosphate, along with other salts such as magnesium, sodium, potassium, and citrate. The CN proteins have distinctive differences between the polypeptide chains and the number of phosphorylated serine residues (Swaisgood, 1992). β-CN is the most abundant CN protein, constituting up to 45% of the CN in bovine milk. α_{s1}-CN constitutes up to 40% of the CN fraction followed by α_{s2}-CN at 10% and the remaining 5% is κ-CN (Whitney et al., 1976; Donnelly et al., 1983). CN proteins present in bovine milk are synthesized by MEC from AA supplied by plasma. Biological functions associated with CN proteins include control of calcium and phosphate precipitation, suppression of amyloid fibril formation, and providing nutrition to the neonate (Holt et al., 2013; Berry et al., 2014). The function of κ-CN is associated with micelle stability (Shekar et al., 2006), while the specific function of β-CN is unclear. Homozygous β-CN mutant mice tend to secrete less milk protein, but overall maintain a normal lactation. The decrease in milk protein was found to be partially compensated by an increase in concentration of other proteins (Kumar et al., 1994), indicating that β-CN has no essential function.

1.2.1.2 Whey proteins

Whey proteins, unlike CN proteins, remain soluble at pH 4.6 and 20°C in the whey or serum fraction in milk. β-LG is the most abundant whey protein in bovine milk followed by α-LA. Both abundant whey proteins are synthesized from plasma AA within the MEC during lactation (Mahony and Fox, 2013). α-LA plays an important role in lactose synthesis within MEC whereas the function of β-LG is still unclear. There is accumulating evidence that β-LG may be involved in transport of numerous ligands as well as exhibiting its ability to bind to iron-siderophore complexes that are associated with immune-modulatory properties (Roth-Walter et al., 2014). Other constituents making up whey are bovine serum albumin (BSA), lactoferrin, and lactoperoxidase, together with other blood-borne minor components. While a few of the more abundant proteins, such as lactoferrin and lactoperoxidase are synthesized in the MECs, most other low abundance proteins originate from sources other than the MEC (Pepe et al., 2013).

1.2.1.2.1 Low abundance proteins

Within the whey fraction in bovine milk there is a group of proteins known as low abundance proteins that originate both from MECs and from other tissues via transcytotic and paracytotic pathways into the lumen of mammary alveoli. The transcellular pathway in MEC involves endocytosis and transport of macromolecules from the basolateral side to the apical membrane of the cell. Milk constituents such as immunoglobulins, hormones, proteins and growth factors tend to follow this route originating from either plasma or stromal tissue within the mammary gland. Similarly, paracellular transport involves the movement of macromolecules from plasma into milk but is dependent on the integrity of the tight junctions between MEC (Zhang et al., 2013) (*Figure 1.1*).

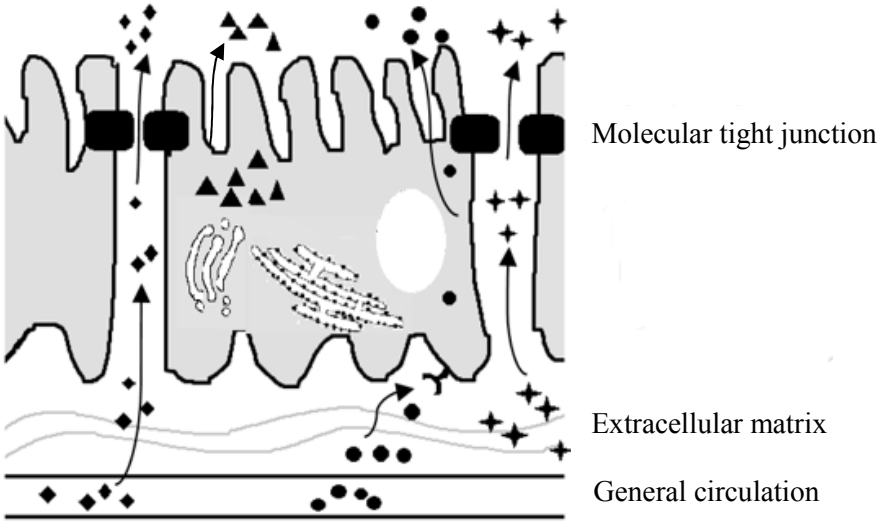


Figure 1.1. Secretion of milk proteins. High abundance proteins are synthesized within the MG (triangles), as well as proteins from plasma being transported via transcellular (diamonds and stars), or paracellular (circles) pathways (adapted from Zhang et al., 2013).

Though these low abundance proteins contribute little to the milk protein profile, there is evidence that despite their low concentrations, they have high potency with bioactivity ranging from inhibiting gastrointestinal disease such as necrotizing enterocolitis in preterm infants (Chatterton et al., 2013) to playing an important role in tumorigenesis and metastasis (Christensen et al., 2007). Therefore, milk is not only a source of nutrition for the infant but also provides bioactive factors that are directly modulating immune response development as well as boosting intestinal flora stromal colonization which in turn heightens immune maturation and immune response development (Calder et al., 2006).

Low abundance proteins present in the MFGM fraction originate from the apical plasma membrane, cytoplasm, and endoplasmic reticulum membrane in MECs. Hundreds of proteins have been identified from different cellular locations thus, MFGM proteins are considered to reflect changes in MEC function and metabolism (Lu et al., 2013; Lu et al., 2016). The main protein making up the MFGM fraction is the glycoprotein butyrophilin (representing 40% of total MFGM protein fraction) followed by xanthine oxidase, which comprises up to 13% of the total MFGM protein fraction (Spitsberg, 2005). In addition, cathelicidins were uniquely identified in bovine MFGM, known for their antibacterial properties by binding to bacterial lipopolysaccharides (Lu et al., 2016). Peptidoglycan recognition protein 1, another low abundance protein, was found in the bovine MFGM fraction, encompassing antibacterial activity. The presence of these proteins that are associated with the immune response reveals the importance of milk for protecting newborns (Lu et al., 2016).

1.2.2 Non-protein nitrogen

NPN contributes 2-6% of N to the total N in bovine milk. The main component of NPN is urea with other nitrogenous compounds contributing to the NPN fraction including orotic acid, creatinine, ammonia, and hippuric acid. Urea is a by-product from AA catabolism and diffuses into the general circulation (DePeters and Cant, 1992). Urea can diffuse freely across mammary cells so there is a high correlation between plasma and milk urea concentrations (Thomas, 1980). Therefore, analysis of milk urea N (MUN) can be used as a strategic management tool to evaluate the overall protein nutritional status of a group of cows.

1.3 Milk protein synthesis

Expression of milk protein genes begins in mid to late pregnancy and continues until involution is initiated. Moreover, the degree of expression of each individual gene varies as the mammal progresses through lactation. In general, protein synthesis within the MECs is a similar process to that seen in other tissue cell types where the proteins are synthesized from AA. The genetic code holding the blue prints for genes to be expressed and produce proteins is contained in the DNA located within the nucleus of the MECs. AA that are utilized by the MECs for protein synthesis are either synthesized within the body or are derived from the diet (Akers, 2002).

Protein synthesis can be divided into three stages, transcription involves the synthesis of messenger RNA where transcription factors promote the binding of RNA polymerase to its binding site on the DNA, known as the promotor. Following transcription, mRNA carries the encoded DNA message from the nucleus to the ribosome located either in the rough endoplasmic reticulum (RER) or in the cytoplasm. Translation is the process by which a protein is synthesized from the genetic code embodied in the mRNA strand (Bionaz et al., 2012). The translation of mRNA is performed by the ribosome, amino acyl-transfer ribosomal nucleic acids (tRNA) and many associated factors. The process can be divided into three phases, initiation, elongation, and termination. During initiation, methionyl-tRNA, several initiation factors, and the 40S ribosomal subunit associate and bind to mRNA. This complex migrates along the mRNA to the correct AUG initiation start codon followed by the binding of the 60S ribosomal subunit to form the functional 80S ribosome complex (Berry et al., 2014). The mRNA strand contains codons coding for specific AA, which corresponds to anticodons on the

tRNAs. Initiation of the peptide chain starts with a methionine. During subsequent elongation, AA are transported to the ribosome via tRNA molecules and tRNAs are placed in line to their corresponding codon on the mRNA strand. The AA are subsequently added to the growing peptide chain as the ribosome and mRNA move along relative to each other towards the carboxyl end of the mRNA strand. This process requires high energy compounds such as ATP, to attach new AA to the growing chain (Bionaz et al., 2012; Berry et al., 2014).

Once all the functional codons have been filled, one of three termination codons will terminate peptide elongation. The termination codons do not code for an AA thus elongation stops. The polypeptide that is synthesized is in its primary form and undergoes extensive conformational changes in the RER to develop tertiary and quaternary structure through folding and coiling of the chain, stabilization by hydrogen bonding, formation of salt linkages and sulphur bridges and association with other proteins (Bionaz et al., 2012; Berry et al., 2014). Synthesis of proteins that make up the CN fractions will exit the RER and translocate to the Golgi apparatus where they are phosphorylated. Phosphorylation of CN fractions creates binding sites for calcium, allowing for stabilization by calcium phosphate linkages and other ionic bonds before being packed into secretory vesicles with lactose molecules and transported to the apical membrane where they are secreted into the lumen of the alveolus (Akers, 2002; Berg, 2007; Hartwell et al., 2008).

1.3.1 Nutrient transport into mammary epithelial cells for milk protein synthesis

The MG extracts large amounts of AA from the circulation in order to meet the requirements for protein synthesis. The extraction of AA and peptides from the blood is

achieved by specific transporters embedded in the basolateral cellular membranes of alveolar cells (Shennan and Peaker, 2000). There are several types of AA transporters; neutral, cationic, and anionic, with many AA able to pass through several types of transporters. Some transporters are ion dependent (e.g., sodium, chloride, and potassium) or use the hydrogen gradient to drive transport (Akers, 2002). Most systems have been shown to be sodium dependent, using the trans-membrane sodium gradient to drive the AA uptake into the cell lumen. This is thought to occur when the transport molecule accepts both a sodium ion and an AA, generating an electrochemical gradient that concentrates the AA inside the cell. The sodium gradient is maintained by the sodium-phosphate adenosine triphosphotase in the plasma membrane (Baumrucker, 1985).

Nutrient uptake by mammary secretory cells is dependent on the regulation of nutrient transport into MEC. The absorption of AA into the MG is dependent on the capacity and activity of these transport systems which is controlled predominantly by hormonal regulation. Insulin, prolactin, growth hormone, glucocorticoids and thyroid hormone are key hormones known to play a role in nutrient uptake and milk protein synthesis. Measuring AA uptake by the bovine MG is obtained by the technique of arteriovenous sampling. This involves sampling blood at pre- and post-organ sites to quantitatively examine the net flux of nutrients per unit of time flowing into the MG (Baumrucker, 1985; Purdie et al., 2008). Using this method, factors associated with nutrient flux regulation by the MG can be investigated. AA influx into the bovine MG is heavily regulated and many factors come into play influencing what end products make it into the MEC. In short, AA uptake is dependent on several factors including AA requirements of MG, hormonal concentrations, arterial concentrations of AA, rate of

mammary blood flow, and rate of AA extraction from plasma (Mepham, 1980; Baumrucker, 1985; Fox and McSweeney, 1998).

1.3.2 Regulation of protein synthesis within the bovine mammary gland

The relative proportions of milk proteins present in bovine milk is under the control of nutritional and endocrine factors that influence protein metabolism in the MG (DePeters and Cant, 1992; Mackle et al., 2000). These include availability of AA and other nutrients required for protein synthesis, hormonal concentrations, rate of lactose synthesis, initiation rate of translation and transcription, rate of peptide chain elongation, and rate of post-translational modifications in the RER and Golgi apparatus (Thomas, 1983; Baumrucker, 1985; Toerien and Cant, 2007; Sancak and Sabatini, 2009; Burgos et al., 2010). Glucose and AAs are two essential nutrients required for protein synthesis.

Milk protein synthesis has a high requirement for energy indicating that dietary energy content plays an important role in the regulation of milk protein synthesis (Purdie et al., 2008; Hanigan et al., 2009; Bionaz et al., 2012). This has been observed in multiple studies where the milk protein yield changed in response to increased or decreased dietary energy intake (Macleod et al., 1983; Grieve et al., 1986; DePeters and Cant, 1992). Intracellular AMP:ATP ratios, a local indicator of cell energy status, and insulin, a hormone that reflects systemic energy status of the body, have been shown to regulate milk protein synthesis through the mechanistic target of rapamycin (mTOR) pathway (Hardie, 2004, Burgos et al., 2010). The mTOR kinase pathway integrates AA availability; cellular energy status and endocrine signals to regulate milk protein synthesis by altering phosphorylation stature and effecting activity of key regulatory

enzymes involved in transcription, mRNA initiation and elongation rates (Burgos et al., 2010; Arriola Apelo et al., 2014).

Recent work has demonstrated that some AA are key regulators in gene expression and AA transporters, modulating translation through downstream targets of the mTOR signaling pathway (Appuhamy et al., 2011a; Nan et al., 2014). Some AA alone can modify expression of target genes involved in milk protein synthesis. Specifically, leucine has been shown to increase β-LG expression rates in MEC (Moshel et al., 2006). The mTOR complex consists of two distinct functional units, mTORC1 and 2 (mTOR complex 1 and 2). mTORC1 plays a central role in nutrient signaling and cell growth and ongoing research continues to explore how specific AA activate mTORC1. Evidence suggests that AA do not directly activate mTORC1, rather they promote intracellular transport of mTORC1 to specialized compartments where activators are present, i.e., Rheb (Sancak and Sabatini, 2009; Burgos et al., 2010). AA are also thought to increase CN synthesis through transcriptional and post-transcriptional regulation. The JAK2-STAT5 signaling pathway is involved in regulation of gene transcription, while signaling through mTOR downstream is important for the regulation of translational processes of milk proteins (Nan et al., 2014).

1.4 Non-nutritional factors affecting milk protein composition in the bovine mammary gland

Generally, it was assumed that the milk protein composition stays relativity constant (Beever et al., 2001; Davis and Law, 1980). However, in recent years the development of proteomic technology has allowed scientists to expand the bovine proteome and accurately quantify changes in protein profiles present in milk. Hundreds

of low abundance proteins have been identified and many have been characterized with bioactivity benefitting human health (Lönnerdal, 2003, 2010; Gao et al., 2012; Korhonen, 2013). Many non-nutritional based factors are known to influence temporal expression and secretion of milk proteins including genetic variation, stage of lactation, season, and health (DePeters and Cant, 1992; Bohemer et al., 2008; Gustavsson et al., 2014; Zhang et al., 2015a). Considering the complex nature of bovine milk, proteomics is a tool that enables analysis of the entire bovine milk proteome, advancing our understanding of the mechanisms involved in MG function and milk protein synthesis. This type of research will provide opportunities to develop nutritional and on-farm management strategies to maximize the nutritious value of bovine milk and improve human health.

1.4.1 Genetic variation

Genetic variation is a major factor contributing to milk protein variation, with 55% of the variation observed in milk composition explained by genetics, and the remaining 45% explained by other external factors (Oltenacu and Algers, 2005). Proteomic characterization and comparison of mammalian MFGM proteomes were examined in a recent study using quantitative proteomic techniques using milk produced from multiple species, including two bovine breeds, Jersey and Holstein dairy cattle. Principal component analysis scored the two dairy breeds sharing similar MFGM proteomic patterns. However, there were also some distinct differences in the MFGM protein profile between the two breeds. The MFGM protein profile from milk produced by Jersey cows contained higher abundance of proteins involved in antimicrobial and angiogenic activities, whereas more MFGM proteins that are known to be involved in immune system modulatory processes were identified in milk produced from Holstein

dairy cattle. The MFGM proteins that were found at higher abundance in the Holstein and Jersey samples originated from MEC and their relative abundance is a consequence from intra- and extracellular factors affecting cellular protein synthesis machinery and regulating gene expression (Yang et al., 2015). Possible factors causing these breed differences in the MFGM protein profiles include differences in alveolar dynamics (Litwinczuk et al., 2011), feed conversion efficiency (Aikman et al., 2008), susceptibility to heat stress (West, 2003), and genetic variants existing for protein types (Gustavsson et al., 2014). These quantitative data provide insights into the composition of MFGM proteins and their potential physiological functions, and highlight that protein composition is significantly influenced by breed.

Variation in genotypes due to polymorphism variants are known to exist between cows and a recent study investigated the effects polymorphism has on milk production characteristics. Diglyceride O-acyltransferase 1 (DGAT1) is an enzyme involved in the synthesis of triacylglycerides in MEC. Advanced metabolomic and proteomic techniques were performed to determine the MFGM proteome of milk samples from cows with the *DGAT1* KK and AA genotypes. The proteins associated with the MFGM are considered representative of the physiological function of the secretory cells in the MG. Therefore, variation in the MFGM proteome may lead to better understanding of how this polymorphism affects milk protein synthesis. Using proteomics, 249 proteins were quantified from the MFGM fraction in milk, including a wide range of low abundance proteins. Stomatin, a major scaffolding component in lipid rafts, associated with lipid raft formation, cytoskeleton formation and iron transport across the cell membrane, was found at higher abundance in milk samples from *DGAT1* KK compared to samples from

DGAT1 AA cows (Lu et al., 2015). This difference found between genotypes highlights possible variation in mammary secretory cell membrane function, structure and organization providing direction for further understanding of how polymorphisms influence MEC function and regulation (Lu et al., 2015).

1.4.2 Stage of lactation

Changes in the milk protein profile over a lactation has recently been investigated by a number of research groups studying milk produced from humans and cattle. A comprehensive study using human participants used a quantitative proteomic approach and identified 1333 milk proteins with 615 being quantified (Zhang et al., 2013). Gene ontology (GO) analysis showed that low abundance milk proteins associated with acute inflammatory response were most abundant, and quantitative proteomic analysis showed levels decreasing over the course of lactation. The results suggest a higher degree of inflammatory response in early lactation reflecting the importance of immune-related proteins for protecting the neonate during early-lactation. Collectively, proteins associated with cellular detoxification were up-regulated during lactation suggesting that these proteins are involved in protection of the MG tissue and milk from harmful pathogenic species (Zhang et al., 2013). These results are in agreement with other studies (Liao et al., 2011; Senda et al., 2011). Similar results were found in a study investigating milk produced from four Holstein dairy cows. Two-hundred and twenty-nine low abundance proteins were identified including two-hundred and nineteen that were quantified (Zhang et al., 2015a). Immune-related proteins, such as complement components, lactoperoxidase, lactadherin, and several cell adhesion proteins rapidly decreased over the lactation period, whereas well known host-defense proteins, including

lactoferrin and osteopontin, increased as lactation advanced. The increase of these key immune-related proteins from early to mid-lactation highlights the protective function on the neonate as well as their importance in supporting the immune system maturation process in the neonate. The increase in lactoferrin in late lactation also suggests a protective role in the MG itself against pathogens due to its antimicrobial properties. In agreement with Zhang et al., (2013) where human milk was studied, proteins associated with milk synthesis and secretion decreased over lactation indicating physiological changes occurring in the bovine MG as the gland prepares for involution (Zhang et al., 2013; Zhang et al., 2015a).

1.4.3 Season

While evidence suggests that seasonal variation influences the bovine milk protein profile, modern proteomic technology has not yet been applied in this field. However, there has been some work directed towards measuring high abundance proteins during different seasons using reverse phase HPLC to quantify the relative proportions of the CN fractions, αs_1-, αs_2-, β- and κ-CN. The effect of seasonal variation on CN composition is relatively large, particularly the proportions of α_{S1}, α_{S2}- and κ-CN within total CN. With the exception of κ-CN, the proportion of α_{s1}-, α_{s2}-, and β-CN showed the lowest values during summer and highest values in the winter (7%, 9%, and 3% increase, respectively) (Barber, 2007). Similar results were observed in a more recent study where quantitative analysis using electrophorretically separated proteins allowed for high abundance proteins to be measured. The proportion of α- and β-CN in milk collected during the summer had reduced levels by 5.8% and 19.3% compared to samples collected in the winter, respectively. The seasonal effect on high abundance whey proteins showed

a significantly higher proportion of α-LA present in milk during the summer whereas, β-LG showed the lowest levels in summer and higher levels in the winter season. Additionally, immunoglobulin G (IgG) and lactoferrin were identified and quantified showing IgG content was 12.3% greater in summer than in winter, in contrast to lactoferrin that was found 11.1% higher in winter compared to summer (Bernabucci et al., 2015). The relative proportions of the CN fractions tend to follow the same pattern with reduced proportions of α-CN and β-CN during the summer months. The seasonal fluctuation in the CN fractions is suggested to be a result from reduced energy intake and increased maintenance costs during the summer months (Bernabucci et al., 2002; Barber, 2007; Bernabucci et al., 2015). However, more recently, an Australian research group hypothesized that other metabolic and physiological factors have a greater influence on milk protein concentration and composition when cows are under heat stress (HS). Two groups of cows, matched by body weight, were either imposed to HS on an *ad libitum* diet or had restricted intake (RI) based on the intake levels of their counterpart in the HS group. A change in plasma metabolite concentrations and milk CN composition was observed between HS and RI groups highlighting that both stressors influence protein synthesis in the MG differently. Cows with RI had elevated serum NEFA and small changes in plasma urea N (PUN), a typical response to negative energy balance indicating increased mobilization of adipose energy sources to meet whole-body energy requirements. In contrast, cows under HS had increased PUN and plasma creatinine concentrations and no change in NEFA. Elevated PUN and plasma creatinine levels indicate differential catabolism of tissues, favoring metabolism of muscle tissue and free AA as gluconeogenic precursors to meet energy requirements when the cow is under HS.

As a result, milk produced from cows under HS had greater changes in CN production and composition compared to milk produced from cows on RI levels. Conclusions from this study highlight that HS in cows induces changes in whole-body physiology, nutrient metabolism, and nutrient partitioning to the MG, causing a shift in differential tissue mobilization as an endogenous source of energy compared to cows on RI (Cowley et al., 2015).

However, results from another study showed contrasting results where the relative proportions of CN fractions remained constant throughout the year. Only one protein, α-LA, was identified to be affected by seasonal variation, and this was likely due to the seasonal variation that was observed in milk lactose (Heck et al., 2009). Caution is advised when interpreting the results presented in these studies due to the high number of external factors that could influence the bovine proteome. In particular, nutrition is highly variable between seasons as well as climate, different genetics between individuals, stage of lactation (for those studies on farms not based on a pastoral system), parity, age, and health (Barber, 2007; Heck et al., 2009).

1.4.4 Inflammation (mastitis)

It is well documented that dramatic changes in the milk protein profile occurs during coliform mastitis in response to toxins produced by gram-negative bacteria that damage MEC (Shuster et al., 1993; Auldist et al., 1995; Lee et al., 2006; Bohemer et al., 2008; Bohemer et al., 2010). Examination of low abundance whey proteins in response to bacterial infection was initially achieved using centrifugation followed by 2D-gel electrophoresis (GE) to separate proteins based on their isoelectric points and molecular weights (Bohemer et al., 2008). Specific protein spots were excised from the gel, digested

with trypsin and identified using mass spectrometry. Twenty-two low abundance proteins were identified and thirteen were unique to mastitic milk samples. Whey proteins identified in mastitic milk included serotransferrin, complement C3 and C4, apolipoproteins and several antimicrobial peptides that belonged to the cathelicidin family. Proteins that had a 3-fold or greater relative peptide count difference before and after the challenge were considered significant. The only acute phase protein found in both normal and mastitic milk was α-1-acid glycoprotein and was surprisingly found at higher abundance in only mastitic milk samples (Bohemer et al., 2008). Increased peptide counts of lactoferrin were detected after infection and doubled in concentration 48 hours after infusion with *E.coli*, but no relevance of fold changes were determined. BSA was the only protein with sufficient peptide counts above the cut-off criteria of fold change after infection (Bohemer et al., 2010). This increased flux of BSA is likely a result from extracellular fluid leaking through the damaged tight junctions between epithelial cells into the alveolar lumen (Auldist et al., 1995). The ultimate goal using proteomics for comparative analyses of bovine whey in healthy and infected cows is to better understand the physiological changes that occur in the MG in response to infection, while identifying biomarkers that could be used to predict the onset of mastitis in the early phases of infection. Development of more rigorous fractionation strategies to identify low abundance proteins with improved quantification techniques of low abundance proteins is a challenge that needs to be overcome before this goal can be achieved.

1.5 Ruminant digestion in relation to nutrient supply to the bovine mammary gland

Energy and protein substrates derived from feed degradation influences the physiological status and overall metabolism in an animal. The bovine is a classic ruminant animal with one stomach and four compartments (rumen, reticulum, omasum and abomasum). This digestive system enables ruminant animals to consume high roughage feedstuff with a high cellulose content. A number of breakdown products result from feed degradation and fermentation in the rumen and these are utilized by the animal as a source of nutrition. These nutrients are absorbed into the bloodstream and are available to the MG for milk synthesis. Generally, the nutrients released from digestion can be classified as either a source of energy or protein and they are essential for maintenance, growth, pregnancy, and lactation in dairy cows.

1.5.1 Substrates from dietary energy metabolism in the bovine

The main function of carbohydrates is to provide energy to the rumen microbes and host animal. Carbohydrate digestion by the rumen microbes provides the main energy substrates to tissues as VFA and glucose, where VFA alone provide up to 80% of the total energy required by the ruminant animal (Dijkstra, 1994; Sutton et al., 2003). Metabolism of carbohydrate digestion is largely influenced by diet, the physiological state of the animal along with DMI and rumen environmental conditions (Bannink et al., 2008; Saleem et al., 2012). The type of carbohydrates offered to the dairy cow influences the rumen microbiota thus, alter the fermentation patterns and metabolites released from digestion (Ørskov and Ryle, 1990; Van Soest, 1994). Bacterial digestion of carbohydrates can be divided into primary and secondary fermentation phases. Primary fermentation

involves intracellular metabolism of feed carbohydrates into pyruvate in which the major monosaccharides of cellulose, starch and sugar enter the glycolytic pathway converting to pyruvate. Secondary fermentation involves the conversion of pyruvate into VFA, by-products from microbial fermentation (Russell et al., 1992; Dijkstra, 1994). The VFA present at highest concentrations within the rumen include acetate, propionate, and butyrate. Others existing at lower concentrations are valeric, iso-butyric, and iso-valeric acid (Dryden, 2008).

Rapid absorption of VFA through the rumen epithelia transfers the nutrients into the portal blood stream. Butyrate is largely absorbed by the rumen epithelial cells and converted to β-hydroxybutyrate (BHBA) during the passage across the rumen wall. Acetate and BHBA are transported from the liver to the MG and other tissues and are precursors for lipogenesis and energy generation in the body (Sutton et al., 2003; Lascano and Heinrichs, 2009). Propionate, a gluconeogenic precursor, is transported to the liver where it is converted into glucose and released into the general circulation as an energy substrate or used to synthesize FA. The proportion and concentrations of VFA present in the rumen are closely related to dietary carbohydrate characteristics shifting the microbiota according to energy substrates available. For example, increased inclusion of starch in the diet is associated with increased propionate production, which has been linked to increased milk protein concentration (Thomas 1980, 1983; Beever et al., 2001; Raggio et al., 2006).

1.5.1.1 Substrates from dietary protein metabolism in the bovine

Digestion of dietary protein in a ruminant has two fates; it is either digested by the rumen microbes or bypasses the rumen and is digested in the abomasum. Dietary protein

available to the rumen microbes is degraded and utilized as a nutrient source with ammonia, AA and peptides produced as byproducts. Peptides and AA released from microbial digestion are incorporated into microbial crude protein (MCP), where the microbe population uses the modified protein for growth and reproduction. This class of protein is known as rumen-degradable protein (RDP). The other type of protein is rumen-undegradable protein (RUP), which bypasses the rumen and flows into the lower digestive tract where it undergoes digestion. Protein digested in the abomasum and small intestine consists of digestible-RUP and MCP. The products of protein digestion, including AA and small peptides are absorbed and metabolized by intestinal epithelial cells and potentially released into the general circulation to become available to body tissues, including the MG. MCP typically contributes up to 60% of the protein requirement for a lactating dairy cow and digestible-RUP represents the remainder. MCP is about 75% true protein and believed to have a true digestibility of 85% (NRC, 2001). Interestingly, regardless of dietary ingredients, the AA composition of MCP remains relatively consistent and is comparable to that in milk protein (Storm and Orskov, 1983; Brownlee, 1989; DePeters and Cant, 1992). This opens opportunity to alter the AA composition available for absorption in the small intestine by using dietary ingredients with digestible-RUP.

Ammonia, a by-product from RDP degradation, is primarily used as a source of N in the ruminant. As the rumen microbes deaminate AA, N in the form of ammonia, diffuses across the rumen wall where it is converted into urea in the liver. The degree of urea production is dependent on the amount of RDP but also the amount of available energy to the microbes (McDonald et al., 1998). If energy is limited or not in synchrony

with the supply of RDP, excess ammonia is converted to urea in the liver resulting in increased PUN levels along with increased excretion rates of N from the body. High N excretion rates indicate inefficient use of dietary protein as well as dietary energy since there is an energy cost associated with the conversion of ammonia to urea in the liver. Ensuring that the rumen microbes have an adequate supply of energy as well as providing sufficient RDP as a source of N, maximizes MCP production, meeting the protein requirements for milk protein production and maintaining rumen health.

1.6 Nutritional manipulation of the bovine milk protein profile

Nutritional perturbances offer the most effective means to rapidly alter the milk protein composition in lactating dairy cows. Attempts to increase milk protein levels via AA supplementation often result in a smaller than anticipated response, highlighting the lack of understanding of AA metabolism in the MG. By increasing dietary CP levels in the ration to a dairy cow, increases N supply for ruminal microbial synthesis and AA concentration in plasma (Walker et al., 2004). Provided there is sufficient energy available to the rumen microbes, increasing dietary protein increases total MCP and VFA production. Ultimately, this leads to increased substrate availably to the MG which drives milk protein synthesis (Dewhurst et al., 2000; Barber, 2007). The bioavailability of specific AA in the intestine can be altered by feeding protein sources that contain a higher RUP fraction (Santos et al., 1998). Results from responses to RUP supplementation are inconsistent (Thomas, 1983; Sutton, 1989) and positive results from when cows are fed high RUP diet is more likely due to increased dietary energy availability rather than a change in AA availability to the MG (Barber, 2007). Dietary energy influences milk protein synthesis patterns by increasing availability of energetic

precursors that are metabolized to produce intracellular energy transfer molecules (e.g., ATP, GTP, NADH, and NADPH), and increasing plasma insulin levels. Insulin is known to positively influence milk protein synthesis by influencing various physiological processes including regulation of expression of major milk proteins and increase nutrient availability to the MG (Menzies et al., 2009; Bionaz et al., 2012).

Research on the effect of nutrition on the bovine low abundance protein profile is limited and may be a significant animal management factor that has potential to alter milk protein composition (Kennelly et. al., 2005; Tripathi, 2014). Altering the nutrition by changing the proportion of RDP and RUP in the diet has been shown to affect milk protein composition. Christian et al. (1999) altered the proportions of bovine milk major proteins α_{s1}, β-, and γ-CN proteins by feeding a lupin-wheat based diet, a source of high RUP to lactating dairy cows compared to cows fed spring-pasture, which is high in RDP. This study highlights that the dietary protein composition fed to a dairy cow indirectly alters N partitioning within the animal, ultimately affecting the physiological function of MEC resulting in changes in the milk protein profile. More recently, changes in high abundance milk protein expression patterns in response to inclusion of different corn and soybean feedstuffs in the ration was investigated. Inclusion of heat-treated soybean meal caused distinctly reduced β-CN concentrations and zinc-α-2-glycoprotein fragments compared to diets that included solvent-extracted soybean meal, suggesting the importance for sufficient availability of RDP in secretion of specific milk proteins. Differential expression of α-LA and zinc-α-2-glycoprotein was also observed, highlighting the possibility that ruminal microbial protein synthesis could affect the milk protein profile produced (Li et al., 2014). Treatment differences observed in these trials

could be due to several factors including changes in total dietary energy or protein uptake by the animal, rumen microbial fermentation dynamics, animal N partitioning, or differences in diet carbohydrate and energetic fractions. All of these differences could alter substrate availability to the MG and influence physiological function in MEC resulting in changes in protein expression and protein synthesis rates.

1.7 Conclusion

Currently, there is substantial interest in milk proteins as health-promoting supplements with epidemiological studies showing consumption of dairy products is associated with lower risks of metabolic diseases and heart-related disorders. Dairy components such as low abundance whey proteins have been posited as the drivers of these beneficial effects and continuing assessment of the effects milk-derived proteins and peptides have on metabolic health is under intensive investigation (McGregor and Poppitt, 2013). Analysis of the bovine proteome has been in the spotlight for many years beginning in the late 1990's where immunoabsorbents were used to remove specific high abundance milk proteins followed by 2-DE to separate out the remaining proteins. Under thirty low abundance proteins were identified using microsequencing. Currently, with increasing developments in protein fractionation and mass spectrometry technology has enabled scientists to expand the number of identified low abundance proteins in milk. Over 930 low abundance proteins have been identified in the skim-milk fraction in bovine milk and further analysis of the exosomes and MFGM proteins has advanced our knowledge into unravelling the entire milk proteome. This review has outlined the composition of bovine milk and various factors that can influence the bovine milk

proteome. Milk protein synthesis within a MG is complex and milk production is a result from multiple interconnecting factors. Bovine breed is an important factor that highly influences the milk protein profile. Our hypothesis is when Jersey and Holstein breeds of dairy cow are held under the same nutritional and environmental conditions, that there will be significant differences in the bovine skim-milk low abundance proteome. The objective for this project is to characterize the skim milk protein fraction produced by Holstein and Jersey dairy cows maintained under the same nutritional, management and environmental conditions.

Nutrition is another important factor influencing the bovine proteome and it can be argued that nutritional manipulation is the most effective means to rapidly alter milk protein composition (Kennelly et. al., 2005; Tripathi, 2014). Limited research to date has focused on identifying methods to alter the bovine milk proteome. We hypothesize that changes in protein metabolism observed when cows are fed diets altering in RDP:RUP ratio, will result in changes in protein synthesis and secretion patterns within the MEC altering the skim milk protein proteome. Our objectives are to shift overall N metabolism in a lactating dairy cow by feeding diets either high in RDP or high in RUP and measure changes in plasma metabolites, N partitioning, and milk protein composition within the skim milk fraction produced by lactating Holstein dairy cows.

2 CHAPTER TWO: CHARACTERIZATION OF THE BOVINE MILK PROTEOME IN EARLY-LACTATION HOLSTEIN AND JERSEY BREEDS OF DAIRY COWS

2.1 Abstract

Milk is a highly nutritious natural product that provides not only a rich source of amino acids to the consumer but also hundreds of bioactive peptides and proteins known to elicit health-benefitting activities. Research examining the milk proteome has primarily focused on human milk and characterization of the bovine milk protein profile is not complete. We investigated the milk protein profile produced by Holstein and Jersey dairy cows maintained under the same diet, management and environmental conditions using proteomic approaches that optimize protein extraction and characterization of the low abundance proteins within the skim milk fraction of bovine milk. The extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation followed by liquid chromatography mass spectrometry (LC-MS). In total, 935 low abundance proteins were identified with a false discovery rate of <1%, and semi-quantified by the number of distinct peptides. Gene ontology (GO) classified all proteins identified into various cellular localization and function categories. A total of 43 low abundance proteins were differentially expressed between the two dairy breeds. Bioactive proteins involved in host-defense, including lactotransferrin (P <0.01) and complement C2 protein (P <0.01), were differentially expressed by the two breeds, whereas others such as osteopontin (P = 0.17) and lactoperoxidase (P = 0.29) were not. This work is the first to outline the protein profile produced by two important breeds of dairy cattle maintained under the same diet, environment and management conditions in order to

observe likely true breed differences. This research now allows us to better understand and contrast further research examining the bovine proteome that includes these different breeds.

2.2 Biological significance

Within the last decade, the amount of research characterizing the bovine milk proteome has increased due to growing interest in the bioactive proteins that are present in milk. Proteomic analysis of low abundance whey proteins has mainly focused on human breast milk; however, previous research has highlighted the presence of bioactive proteins in bovine milk. Recent publications outlining the cross-reactivity of bovine bioactive proteins on human biological function highlights the need for further investigation into the bovine milk proteome. The rationale behind this study is to characterize and compare the low abundance protein profile in the skim milk fraction produced from Holstein and Jersey breeds of dairy cattle, which are two major dairy cattle breeds in the USA. A combination of fractionation strategies were used to efficiently enrich the low abundance proteins from bovine skim milk for proteomics profiling. A total of 935 low abundance proteins were identified and compared between the two bovine breeds. The results from this study provide insight into breed differences and similarities in the milk proteome profile produced by two breeds of dairy cattle.

2.3 Introduction

Milk is a valuable, natural product that provides a matrix of essential nutrients, growth factors and immune protection to offspring. Within the last five years, there has

been a dramatic increase in the amount of published research focused on characterizing the milk proteome within different milk fractions, particularly in human milk (D'Alessandro et al., 2010; Liao et al., 2011a,b,c; Molinari et al., 2012; Roncada et al., 2013). Traditionally, milk proteins are categorized into three major groups: caseins, whey proteins and MFGM proteins (Liao et al., 2011c; Wada and Lönnerdal, 2014). By using a wide range of fractionation techniques, whey proteins can be separated from caseins and further processed to allow extraction and identification of the low-abundance protein fraction in milk. Bioactive proteins and peptides contained in, or derived from, the whey fraction are involved in a wide range of physiological activities, including antioxidant activity, immuno-stimulating functions, anti-inflammatory effects, and protection against pathogen-induced intestinal inflammation (Korhonen 2013; Lönnerdal, 2014; Raikos and Dassios, 2014; Wada and Lönnerdal, 2014). Many of the milk bioactive proteins and peptides are also known to exhibit multifunctional physiological properties (Korhonen, 2009). Thus, milk proteins are currently considered the most important source of bioactive peptides.

Cow's milk is an important nutrient for much of the human population, and studies have begun to characterize the bovine milk proteome, its bioactive profile, and the extent of cross-reactivity of bovine bioactive milk peptides on human biological function (Lönnerdal et al., 2011; Liao et al., 2012; Reznikov et al., 2014). In bovine milk, the CN (α_{S1}, α_{S2}, β and κ-CN) comprise approximately 80% of the total milk protein content, while whey proteins (primarily α-LA, β-LG and serum albumin) represent the remainder. However, these highly abundant whey and CN proteins are far outnumbered by low-abundance proteins within the whey fraction. Reindhardt et al. (2013), identified over 700

low-abundant whey proteins in skim bovine milk using quantitative proteomic techniques, including many with known immunological functions (Reinhardt et al., 2013). Lactoferrin is an important low-abundant protein involved in immune system development and is present in both human and bovine milk (Mills et al., 2011). Positive health benefits from lactoferrin in human breast milk are well documented (Arnold et al., 1980; Brock, 1980; Lönnerdal, 2014; Wada and Lönnerdal, 2014) and recent studies show similar responses when infants are fed formula supplemented with bovine lactoferrin (Lönnerdal, 2003, 2011; Weinberg, 2007; Liao et al., 2012; Korhonen, 2013). Osteopontin, another bioactive protein present in both human and bovine milk, is recognized for its involvement in intestinal and immunological development in infants (Jiang and Lönnerdal, 2013; Lönnerdal, 2014). Despite there being only 63% amino acid similarity between bovine and human osteopontin, osteopontin in bovine milk exerts effects on human intestinal cell proliferation *in vitro* similar to osteopontin in human breast milk infants (Jiang and Lönnerdal, 2013; Lönnerdal, 2014). The cross-reactive nature of milk bioactive proteins provides opportunity to use bovine milk derived bioactive proteins as potential ingredients for health promoting foodstuffs and biopharmaceuticals.

As with human breast milk, many external and genetic factors influence the composition of bovine milk. A recent study characterized and compared the MFGM proteins within several different species including two bovine breeds, the Jersey and Holstein breeds of dairy cattle (Yang et al., 2015). Using quantitative proteomic techniques, protein profiles were examined and principal component analysis scored the two breeds sharing similar proteomic patterns but also showing that each breed had

distinctive MFGM proteins that were present at different concentrations. The Jersey MFGM contained a higher abundance of proteins with antimicrobial and angiogenic activities, whereas the Holstein MFGM contained proteins involved in immune system modulatory processes including antioxidant, anti-apoptotic, anticancer, and host cell protection activities (Yang et al., 2015). Breed differences in alveolar dynamics (Litwinczuk, 2011) feed conversion efficiency (Blake et al., 1986; Aikman et al., 2008), susceptibility to heat stress (Sharma et al., 1983; West, 2003), and genetic variants existing for protein types (Gustavsson et al., 2014) could have contributed to the observed differences in MFGM protein profile. However, additional factors known to affect milk composition in cattle, including diet, cow health, parity, environment, management practices and stage of lactation (Christian et al., 1999; Auldist et al., 2004; Boehmer et al., 2010a,b), could have also contributed to the observed breed differences. While genetics are estimated to contribute 55% of the variation observed in milk composition between breeds, the remaining 45% is explained by differing management factors (Oltenacu and Algers, 2005). Feeding different breeds of cows the same diet while being maintained in the same environment under the same management practices allows for a more direct comparison of true breed differences in the milk proteome. We hypothesize that when Holstein and Jersey breeds of dairy cattle are fed the same balanced diet and maintained under the same management and environmental conditions, there will be significant differences in the low abundance milk protein profile contained within the skim fraction of milk from the two breeds of dairy cows. The objective of this study is to characterize and differentiate the low abundance protein profile within the

skim milk fraction produced by Holstein and Jersey dairy cows that are maintained under the same management practices and environmental conditions.

2.4 Methods and materials

2.4.1 Animals and diet

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont (Burlington, VT, USA). Six Jersey cows (80 ± 49 days in milk (DIM)) and six Holstein cows (75 ± 21 DIM) were housed in the same tie-stall barn at the Paul R. Miller Research and Educational Center (University of Vermont, Burlington, VT). All cows had free access to water and offered the same diet ad libitum (*Table 1*) for 7 days. All cows were fed individually for the duration of the trial. The diet consisted of a base forage ration that was fed twice daily (0600 and 1500 h) and a grain-based top-dress, which was thoroughly mixed into the offered forage three times a day at 0330, 1100, and 1800 h. Feed refusals from each animal were removed and weighed each morning. Feed and refusal samples were collected each morning before feeding for the duration of the 7-d trial and samples were stored at -20˚C until analysis. Samples were subsequently dried at 65˚C for 48 h to calculate daily dry matter intake for each animal. Additional fresh feed and grain samples were collected and composited across the 7-d collection period for wet chemistry analysis (DairyOne, Ithaca, NY, USA).

2.4.2 Measurements and sampling

Cows were milked twice daily at 0400 and 1600 h. Milk yield was recorded at each milking and milk samples were collected during the morning and afternoon milking throughout the 7-day experiment (Tru-Test WB Ezi-Test Meters, DHIA, Lancaster, PA,

USA). Milk samples for general milk component analysis were collected and preserved with bronopol and natamycin (D & F Control Systems, Inc., Broad Spectrum Microtabs, Norwood, MA, USA) and stored at 4˚C until commercial analysis (DHIA, Lancaster, PA, USA), which was performed within two days after collection. Milk subsamples collected for low abundance protein analysis were immediately frozen in a dry-ice ethanol bath after collection and stored at -80˚C until further analysis. Additional subsamples were chilled on ice immediately after collection and centrifuged at 4,000 × *g* for 10 min at 4˚C within 2 h of collection. The fat layer was removed and the skimmed milk samples were stored at -20˚C for high-abundance protein analysis.

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

2.4.3 Analysis of the high-abundance milk proteins

The skimmed milk samples were thawed overnight at 4°C for analysis of high abundance proteins by HPLC using a method adapted from Bordin et al., (2001). Thawed samples were vortexed to mix thoroughly and 500 µL of milk was transferred to a flint

glass culture tube. An equal volume of reducing buffer (6.0 M guanidine hydrochloride, 5.0 mM trisodium citrate dehydrate, 20.0 mM DTT) was added to the milk in each tube. The reducing buffer was prepared daily. The milk/buffer mixture was allowed to react at room temperature for 1 h and then transferred to a syringe and filtered through a 0.45 μm regenerated cellulose membrane syringe filter (Sartorious Stedim Biotech GmbH, 37070, Goettingen, Germany) into an HPLC autosampler vial. For each analysis, 4 μL of sample was injected into the HPLC (Shimadzu Corporation, Kyoto, Japan) for separation. The separations were performed on a C4 reversed-phase microbore analytical column (150 x 2.1mm, 300 Å pore diameter and 5 μm particle size, Ydac 214MS, Grace Davison, MD, USA). A two-component solvent system was used as the mobile phase for separations. Eluent A was composed of 10% (v/v) acetonitrile and 0.1% trifluoroacetic acid in ultrapure water and eluent B was composed of 90% acetonitrile and 0.1% trifluoroacetic acid in ultrapure water. The following solvent gradient program was used during the separations: linear gradient from 26.5 to 28.6% eluent B in 7 min (0.30% B min^{-1}), then from 28.6 to 30.6% B in 10 min (0.20% B min^{-1}), and from 30.6 to 33.5% B in 5.8 min (0.50% min^{-1}), followed by an isocratic elution at 33.5% B for 10 min, an increase from 33.5 to 36.1% B in 5.2 min (0.50% min^{-1}), an isocratic elution at 36.1% B for 10 min, an increase from 36.1 to 37% B in 5 min (0.18% min^{-1}), an isocratic elution at 37% B for 10 min, and a final increase to 41% B in 13 min (0.30% min^{-1}), at a flow rate of 0.25 mL min^{-1}. The column was maintained at 40°C and the autosampler was held at 15˚C. A detection wavelength of 214 nm was used and chromatographs were integrated using LCSolution software from Shimadzu (Kyoto, Japan).

2.4.4 Enrichment of the low abundance proteins

Milk samples collected for low abundance protein analysis were thawed overnight at 4˚C. To obtain a representative sample for each animal, aliquots were composited within animal across the week according to milk yield at each milking. A mammalian protease inhibitor cocktail (Protease Inhibitor Cocktail, Sigma, Milwaukee, WI, USA) was added at 0.24 mL inhibitor per gram of protein in the milk to a 50 mL aliquot sample which was then centrifuged at 4,000 × *g* for 15 min at 4˚C. The cream layer was then removed and skimmed samples were depleted of casein using a previously described method (Kunz and Lönnerdal, 1990). Briefly, addition of calcium chloride (60 mM) was mixed into the skimmed sample and the pH was adjusted to 4.3 using 30% acetic acid (Fisher Scientific, Fair Lawn, NJ, USA). Samples were then centrifuged at 189,000 x *g* at 4˚C for 70 min and the supernatant was collected and stored at -80˚C (Kunz and Lönnerdal, 1990; Molinari et al., 2012). Samples were lyophilized and reconstituted to 500 mg whey powder in 1 mL PBS. The samples were analyzed for their protein content using the bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA), using bovine serum albumin as the standard. The low-abundance minor proteins were enriched by the ProteoMiner Kit (BioRad Laboratories, Hercules, CA, USA) and 32 mg of whey protein was added to 100 µL of ProteoMiner beads. The whey samples were gently shaken with individual ProteoMiner columns for 2 h at room temperature and columns were washed thoroughly using HPLC grade water to remove excess proteins (Righetti and Boschetti, 2008; Bantscheff et al., 2014). The low abundance proteins were eluted off the beads by addition of 20 µl 4x Laemmli sample buffer (8% SDS, 40% glycerol, 250 mM Tris, pH 6.8, 400 mM DTT with trace amount of bromophenol blue). The mixture of the protein

solution with the beads was heated at 95˚C for 10 min and the supernatant was subjected to SDS-PAGE on a precast 8-16% polyacrylamide gel to separate proteins (BioRad, Hercules, CA, USA). Tris-glycine (pH 8.3) was used as the running buffer containing 0.1% SDS. Electrophoresis was performed for approximately 35 min at 200 V until the protein bands reached the bottom of the gel. Staining was performed using Coomassie Brilliant Blue (BioRad, Hercules, CA, USA) overnight. To visualize the protein bands, destaining with a solution of 10% acetic acid, 40% methanol, and 50% water was performed until the background was clear. The SDS-PAGE stained gels were scanned with a Gel Doc XR+ system (BioRad, Hercules, CA, USA).

2.4.5 In-gel digestion

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

2.4.6 Protein identification by nano-scale LC/MS

LC-MS based protein identification was performed on a linear ion trap (LTQ)-Orbitrap Discovery mass spectrometer coupled to a Surveyor MS Pump Plus (Thermo Fisher Scientific, Waltham, MA, USA). Samples (5 µL) were loaded onto a 100 µm x 120 mm capillary column packed with MAGIC C18 (5 µm particle size, 20 nm pore size, Michrom Bioresources, CA, USA) at a flow rate of 500 nL min^{-1}. Peptides were separated using a gradient of 5-35% acetonitrile/ 0.1% formic acid over 98 min followed by 35-100% acetonitrile/ 0.1% formic acid in 1 min and then 100% acetonitrile for 10 min, followed by an immediate return to 2.5% CH3CN/0.1% formic acid and a hold at CH3CN/0.1% FA. Peptides were introduced into the linear ion trap via a nanospray ionization source and a laser pulled ~3 µm orifice with a spray voltage of 1.8 kV. Mass spectrometry data was acquired in a data-dependent "Top 10" acquisition mode with lock mass function activated (m/z 371.1012216635), in which a Orbitrap survey scan from m/z 360-1600 at 30, 000 (FWHM) resolution was paralleled by 10 collision-induced dissociation (CID) MS/MS scans of the most abundant ions in the LTQ. MS/MS scans were acquired with the following parameters: isolation width: 2 m/z, normalized collision energy: 35%, Activation Q: 0.250 and activation time = 30 ms. Review mode for FTMS master scans was enabled. Dynamic exclusion was enabled (repeat count: 2; repeat duration: 30 sec; exclusion list size: 180; exclusion duration: 60 sec). The minimum threshold was 500. Singly charged ions were excluded for MS/MS. Product ion spectra were searched using the SEQUEST search engine on Proteome Discoverer 1.4 (Thermo Fisher Scientific, Waltham, MA, USA) against a curated Bovine Uniprot (-Bos taurus database (24,206 entries) downloaded July 9, 2014-) with sequences in forward and

reverse orientations. The 15 raw files from each Holstein (6 samples) and the 15 raw files from each Jersey (6 samples) cows were searched as one contiguous input file and a single result file was generated for each (12 results files in total). Search Parameters were as follows: full trypsin enzymatic activity, two missed cleavages, and peptides between the MW of 350-5000; mass tolerance at 20 ppm for precursor ions and 0.8 Da for fragment ions, dynamic modifications on methionine (+15.9949 Da: oxidation) (4 maximum dynamic modifications allowed per peptide); and static modification on cysteine (+57.0215 Da: carbamidomethylation). The result files were then further analyzed by Scaffold 4.3 (Proteome Software, Portland, OR, USA) to compare the unique peptide counts and to identify GO functions of the identified proteins. Cross-correlation (Xcorr) significance filters were applied to limit the false positive rates to less than 1% in both data sets. The Xcorr values were as follows: (+1): 1.5, (+2): 2.2, (+3): 2.8, (+4): 3.5. Other filters applied were a minimum peptide cutoff of 2 as well as DeltaCN >0.1. Ultimately, the confidence parameters resulted in less than 1% false discovery.

2.4.7 Statistical analysis

Statistical analyses on daily DMI, plasma parameters, and milk composition data were performed using a linear mixed model of repeated measures ANOVA. The analyses were carried out with SAS software (9.4) (SAS Institute, Cary, NC, USA) and preliminary data screening revealed that all dependent variables were normally distributed. Data were analyzed by MIXED procedure of SAS with breed and day as fixed effects. All data were presented as least square means (LSM) ± standard error of means (SEM) and were considered to be significantly different at $P < 0.05$. The peptide count data was analyzed in SAS (9.4) using an independent sample t-test. The heat maps

were generated by cluster analysis using Ward's method in JMP (Pro 10) (SAS Institute, Cary,NC, USA).

2.5 Results

2.5.1 Diet and dry matter intake

The chemical composition of the diet is listed in *Table 2.1*. Daily DMI was significantly different between the two dairy breeds (*Table 2.2*; $P = 0.01$) where the Holstein cows consumed an average of 21.8 kg DM cow^{-1} day^{-1} and the Jersey cows consumed on average 17.3 kg DM cow^{-1} day^{-1}.

2.5.2 Milk composition and yield

Milk yield, as well as concentrations and yield of the individual major milk constituents, were significantly different between the two dairy breeds (*Table 2.2*). Holstein cows produced 49.9 kg milk per day containing 3.24% milk fat and 2.54% milk protein. The Jersey breed produced significantly less milk (34.6 kg milk per day) but had significantly higher percentages of milk fat (4.28%) and milk protein (3.24%). Despite milk from Jersey cows having higher concentrations of milk fat and protein in milk, Holstein cows produced significantly more total milk per day and more kg of milk fat and protein per day (*Table 2.2*). Optimum MUN concentrations are typically between 10-14 mg dl^{-1}. The MUN concentrations from Holstein (17.13 mg dL^{-1}) and Jersey (15.45 mg dL^{-1}) cattle in this trial were slightly above the optimum MUN concentrations (Jonker et al., 2002). These higher MUN concentrations are tightly correlated to PUN concentrations, and both support the observed high dietary protein and N intake.

2.5.3 Plasma

Blood plasma samples collected on day 0 and day 7 were used to monitor the energy and nitrogen status from each animal. Such biological tests are commonly used in the dairy industry to investigate the general health of animals and provide a diagnostic tool to identify problems in nutritional management, diet formulation or disease incidence (Borchardt and Staufenbiel, 2012; Kohn et al., 2005). Results from the blood data confirm that all twelve animals were in positive energy balance as highlighted by the normal ranges observed in the plasma glucose, NEFA and BHBA concentrations measurements of each cow (*Table 2.3*). Plasma NEFA, typically elevated in circumstances of under nutrition and adipose tissue mobilization, was not significantly different between the Holstein (0.17 mM) and Jersey (0.23 mM) breeds and was lower than the recommended maximum concentration threshold value of 0.33 mM in early lactation cows (Oetzel, 2007), indicating adequate dietary energy intake amongst both breeds of cows. Plasma BHBA was also within the normal physiological range for both Holsteins (0.31 mM) and Jersey (0.34 mM) dairy cows (Oetzel, 2007). The plasma glucose concentrations in both the Holstein (3.60 mM) and Jersey (3.59 mM) breeds indicated a high level of gluconeogenesis in both breeds, in agreement with the predicted rumen volatile fatty acid concentrations from our diet (Herbein et al., 1985). PUN was measured to assess nitrogen status within each animal. Mean PUN concentrations in Holstein cows (1.19 ± 0.03 mM) and Jersey cows (1.25 ± 0.03 mM) were above the normal PUN range of 0.66-0.88 mM (Oetzel, 2007); however, the mean PUN concentrations and variation measured in the present study were similar to those reported

previously in cows on high protein diets (Butler et al., 1996; Hammon et al., 2005) and was expected.

2.5.4 High-abundance milk proteins

Identification and quantification of high-abundance milk proteins was achieved using HPLC. The results were generally expected, with significant differences existing between the concentrations of casein and whey proteins produced by the two breeds as previously observed (*Table 2.4*) (Harding, 1995). Total casein and whey concentrations were significantly higher in Jersey milk (58.0 mg mL^{-1} and 5.3 mg mL^{-1}, respectively) compared to Holstein milk (46.4 mg mL^{-1} and 4.0 mg mL^{-1}, respectively). The most abundant casein protein, total α-casein, was found at significantly higher concentrations in Jersey milk (17.6 mg mL^{-1}) as compared to Holstein milk (14.0 mg mL^{-1}). Similarly, the most abundant whey protein, β-LG variant A, was present at much higher concentrations in Jersey milk (3.3 mg mL^{-1}) compared to milk from Holstein cows (1.8 mg mL^{-1}). β-LG variant B was present at similar concentrations in milk from the Holstein (1.3 mg mL^{-1}) and Jersey (0.96 mg mL^{-1}) breeds. This is most likely explained by high variation existing between individuals.

2.5.5 Low-abundance whey proteins

A total of 935 low-abundant proteins were identified in the skim milk fraction of the two breeds, including 43 proteins that were present at significantly different abundance (*Figure 2.1, Figure 2.2, Table 2.5*). Bioactive proteins that were found at significantly different abundance between the breeds in the skim milk samples include lactotransferrin ($P < 0.01$), a number of complement proteins (complement C2, $P < 0.01$; complement C1, $P < 0.01$; complement component 1, $P = 0.01$) and chitinase domain-

containing protein 1 ($P = 0.04$; *Tables 2.5 and 2.6*). However, the majority of the low abundance proteins within the skim milk fraction were found at relativity similar abundances between the two breeds (*Figure 2.1*). By using a range of extensive fractionation techniques including casein removal and ProteoMiner treatment, the highly abundant proteins were removed to allow for enrichment of the low abundance proteins within the skim milk fraction for subsequent proteomics analysis. *Appendix 1 and 2* contain the complete list of the low-abundance proteins identified in this study as well as total number of distinct sequenced peptides and GO functions for each protein. To ensure statistical validity, six animals were included in each group. A homoscedastic t-test was performed on the peptide count data of each protein for each breed generating *P*-values to compare abundance of the proteins between the two breeds. The relative abundance of a select few proteins was demonstrated using the SIEVE software, which plot the ion elution profile on the chromatographic time scale (extracted ion chromatograms) of the target identified peptides (*Appendix 3*). Identification of GO functions of the full protein profile highlights the similarities of the different breed proteomes (*Appendix 1*) while identification of the ontological functions of the differentially expressed proteins (*Table 2.5)* highlighted the lack of categorical specificity of these proteins.

2.6 Discussion

The aim of the present study was to investigate, characterize and compare the bovine milk proteome between Holstein and Jersey dairy cows. Over the past 30 years there has been growing interest to unravel the dynamic framework of the milk proteome, and there has also been development in the techniques used to fractionate and identify

these proteins (*Figure 2.3*). Murakami et al. (1998) used ultracentrifugation and immunoabsorption techniques on human colostrum and mature milk samples to remove the high abundance proteins. These researchers employed 2-DE protein separation and silver staining techniques, identifying 22 proteins in human colostrum and milk (Murakami et al., 1998). In 2002, Yamada et al., (2002) identified 29 low abundance proteins in bovine milk samples using immunoabsorption techniques and 2-DE to fractionate and separate the low abundance proteins, which were subsequently identified using microsequencing and mass spectrometry for protein identification (Yamada et al., 2002). Improvements made in LC-MS based proteomic technologies and high abundance protein precipitation techniques has since enabled the expansion of milk proteome characterization, resulting in the isolation and identification of over 150 low abundance milk proteins in human colostrum (Palmer et al., 2006). Further method development ensued, where pasteurized bovine skim milk was subjected to ultracentrifugation and the first use of peptide library treatment for protein separation was reported (D'Amato et al., 2009). In this research, ProteoMiner columns were used to deplete the pasteurized milk samples from high abundance proteins followed by SDS-PAGE for low abundance protein separation. Enrichment of the low abundance proteins resulted in the identification of 149 low abundance proteins, including 100 proteins that had not previously been reported in proteomic studies (D'Amato et al., 2009). The ProteoMiner technique had also been used to examine the human milk protein profile over a twelve-month lactation period, resulting in identification of 115 low abundance whey proteins; however, ultracentrifugation was not performed in this method which might explain the lower number of protein identifications (Liao et al., 2011). A combination of

ProteoMiner depletion, electrophoretic techniques, and isobaric tags for relative and absolute quantitation (iTRAQ) techniques were then used to identify over 400 low abundance whey proteins within skimmed human milk (Molinari et al., 2012). Proteomic methods continued to be developed by Gao et al., (2012) where ion-exchange and SDS-PAGE based protein fractionation methods were used to extend the human milk proteome to 976 low abundance proteins (Gao et al., 2012). Permutations of these methodologies were utilized to examine changes in the bovine milk MFGM, whey and exosome proteome induced by mammary bacterial infection. Over 740 low abundance whey proteins were identified using casein precipitation, ultracentrifugation, immunoglobulin depletion, acetone precipitation and iTRAQ labeling for protein identification and quantification (Reinhardt et al., 2013). In the research described herein, we used a combination of fractionation techniques followed by SDS-PAGE and mass spectrometry sequencing to comprehensively elucidate and compare the differential milk proteome produced by two breeds. Peptide counting approaches was applied to obtain statistically significant semi-quantitative data, rather than using isotopic tagging techniques, which can be used in future studies involving within-breed accurate quantification. We successfully identified a total of 935 low abundance whey proteins in bovine skim milk from two dairy cattle breeds (*Figure 2.1*). This allowed us to explore the diversity of the bovine milk proteome from two prominent North American dairy breeds maintained under the same diet, environment and management conditions in order to better assess true breed differences.

In agreement with past literature, total milk protein including all casein and whey fractions were all found at significantly different concentrations between the two breeds

(*Table 2.4*) (Harding, 1999). A significant proportion of the bioactive peptides found in human milk have been identified in bovine skim milk in the current study. Some key bioactive proteins identified in our study that are known to exert beneficial effects on human health include lactotransferrin, osteopontin, lactoperoxidase, and cathelicidin (Kussendrager and van Hooijdonk, 2000; Ramanathan et al., 2002; Lönnerdal et al., 2011; Jiang and Lönnerdal, 2013; Lönnerdal, 2014). However, contrary to our hypothesis, there were very few differences in the low abundance protein profile between breeds (*Figure 2.1*). A total of 43 identified proteins were found at difference abundances between breeds, 81% of which were higher in samples from Jersey cattle compared to Holstein cattle (*Figure 2.2*). Complements C1 and C2, ectonucleotide pyrophosphatase, and chitinase domain-containing protein 1 are described as having functions involved in the immune response and were all identified to exist at higher abundance in samples from Jersey cattle compared to Holstein cattle; however, whether they can elicit bioactivity upon consumption is yet unknown (Wheeler et al., 2012; Lu, 2013; Yang et al., 2015). Of the 8 proteins identified to be higher in Holstein cattle, the only one thus far known to possess bioactive properties was lactotransferrin, which is a well characterized multifunctional bioactive protein involved in many biological functions (Lönnerdal and Lyer, 1995; Ward et al., 2005; Buccigrossi et al., 2007). Osteopontin, lactoperoxidase, and growth factors including insulin-like growth factor and transforming growth factor-β are all important bioactive proteins known to affect human health (Korhonen, 2013; Lönnerdal, 2014), and these were all found to be at similar abundances in both breeds of cattle (*Appendix 1*). Overall, these results suggest that the bovine milk proteome, at least in skim milk fraction, is similar across breeds. This is valuable information, as it allows

for more direct comparison between research being performed using different breeds of dairy cattle, and can allow for general extrapolation and application of results seen in one breed to another. However, the knowledge this research also highlights is the need for caution of cross-breed comparisons, particularly if focusing on the select few proteins that were found to be differentially expressed between the two breeds in the current study.

2.7 Conclusion

The current research compares the skim milk proteome produced by the two dominant dairy cattle breeds in the USA and provides insight into what protein profiles these different breeds produce when maintained under the same environmental and nutritional conditions. Elucidation of the protein profile was accomplished using a combination of enrichment and fractionation techniques, followed by mass spectrometry-based proteomic analysis. The Holstein and Jersey proteomes were assessed using a peptide counting approach, while bioinformatic tools such as GO were used to understand biological functions in which these proteins are involved. Our findings expand the current knowledge on low abundance proteins present in bovine milk and offers insight for future research exploring the bovine milk proteome, providing an analytical platform for future use.

Table 2.1. Ingredient and chemical composition of diets

Ingredient (% of DM)	Diet
Corn silage	36.6
Haylage	18.3
Soybean meal	7.4
Canola meal solvent	4.9
Citrus pulp dry	8.5
Amino max	7.3
Corn grain ground fine	10.8
Vitamin-mineral mix[1]	5.9
Nutrient composition	
DM[2] (%)	59.6
NDF[3] (% of DM)	35.7
CP[4] (% of DM)	18.7
NFC[5] (% of DM)	34.9

[1]Vitamin-mineral mix contained (DM basis): 5.5% PGI amino enhancer, 2.3% Sodium Sesquinate , 2.6% Calcium carbonate, 1.3% salt, 1.4% PGI vitamin premix, 0.7% Magnesium, 0.05% Zinpro 40, 0.02% Rumensin, 0.31% Diamune trace mineral. [2]DM: Dry matter. [3]NDF: Neutral detergent fiber.[4]CP: Crude protein. [5]NFC: Non-fibrous carbohydrates.

Table 2.2. Daily dry matter intake (DMI), milk yield and milk components by Holstein and Jersey dairy cows

	Breed		SE[1]	*P* value
	Holstein	Jersey		
DMI (kg day^{-1})	21.78	17.34	1.02	0.01
Milk yield (kg day^{-1})	49.90	34.61	1.45	<0.01
Milk component yields (kg day^{-1})				
Fat	1.56	1.48	0.04	ns
Protein	1.24	1.11	0.03	<0.01
Milk components (%)				
Fat	3.24	4.28	0.12	<0.01
Protein	2.54	3.24	0.12	<0.01
Somatic cell count (×1000)	54.31	139.05	57.02	ns
Milk urea nitrogen (mg dL^{-1})	15.45	17.13	0.86	ns

[1]SE: Standard error

Table 2.3. Plasma metabolite concentrations of Holstein and Jersey dairy cows

	Breed		SE[1]	*P* value
	Holstein	Jersey		
Glucose (mmol L^{-1})	3.60	3.59	0.13	ns
BHBA (mmol L^{-1})	0.31	0.34	0.03	ns
PUN (mmol L^{-1})	1.19	1.25	0.03	ns
NEFA (mmol L^{-1})	0.17	0.23	0.03	ns

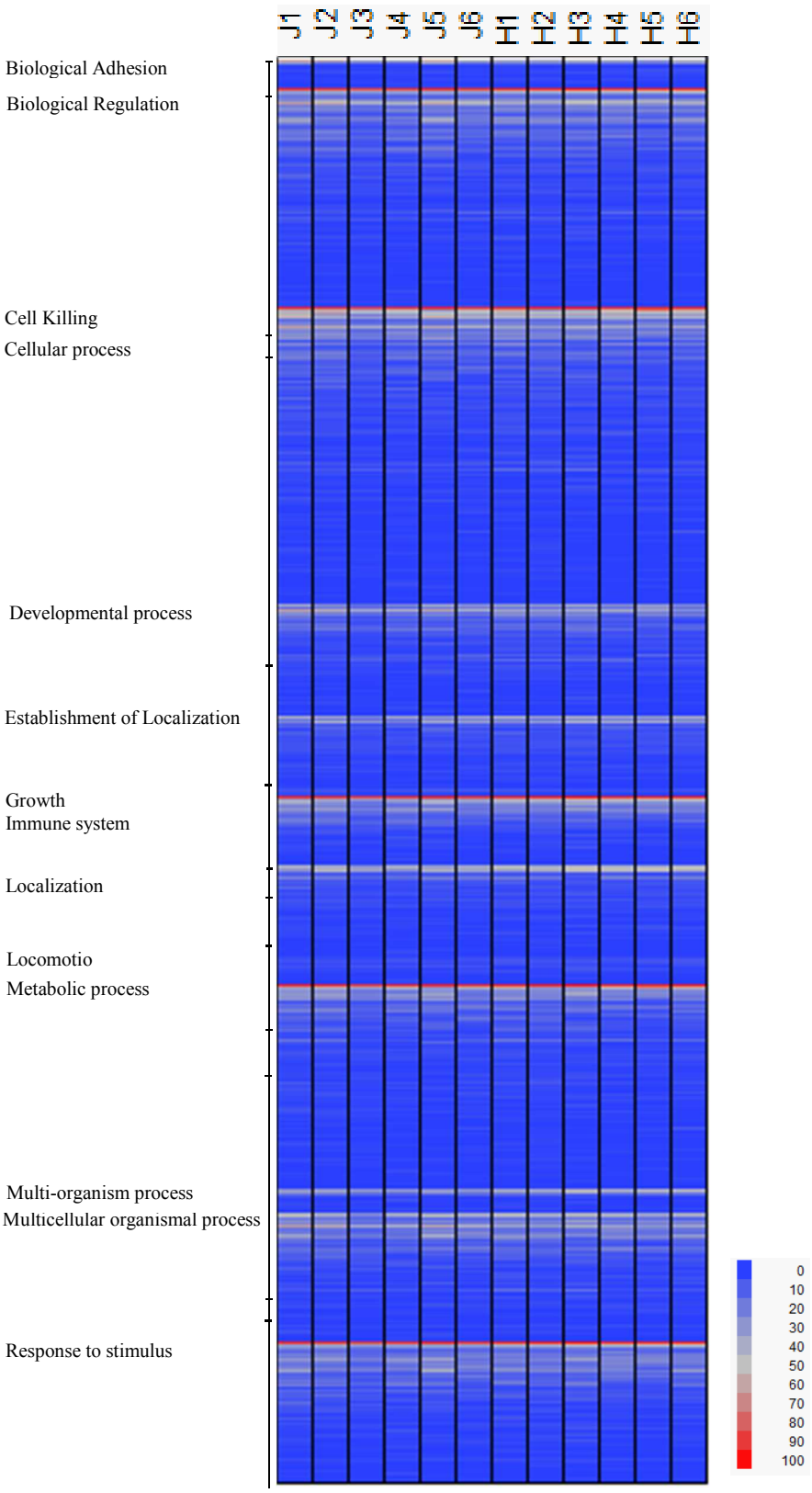
[1]SE: Standard error

Table 2.4. High abundance milk protein concentration from Holstein and Jersey dairy cows

	Breed		SE[1]	*P* value
	Holstein	Jersey		
CN (mg mL^{-1})				
β-CN	13.4	15.9	0.36	<0.01
κ-CN	4.93	6.82	0.19	<0.01
Total α-CN	13.9	17.6	0.44	<0.01
α-s1	12.7	16.1	0.40	<0.01
α-s2	1.30	1.57	0.67	0.01
Total CN	46.3	58.03	1.39	<0.01
Whey (mg mL^{-1})				
α-LA[2]	0.93	1.05	0.02	<0.01
β-LgA[3]	1.81	3.31	0.29	<0.01
β-LGB[4]	1.28	0.96	0.16	ns
Total α-LA, β-LGA, β-LGB	4.02	5.32	0.16	<0.01

[1]SE: Standard error. [2]α-LA: α-Lactalbumin. [3]β-LGA: β-Lactoglobulin variant A. [4]β-LGB: β-Lactoglobulin variant B.

Figure 2.1. Heat map presentation of spectral counting data. Cluster analysis was performed in JMP (Pro 10) using the ward method where the differentially expressed low abundant proteins were assembled into their GO functional groups. Clustering was based on peptide counts abundance in Holstein and Jersey cow milk.


J1
J2
J3
J4
J5
J6
H1
H2
H3
H4
H5
H6
Biological Adhesion
Biological Regulation
Cell Killing
Cellular process
Developmental process
Establishment of Localization
Growth
Immune system
Localization
Locomotio
Metabolic process
Multi-organism process
Multicellular organismal process
Response to stimulus
0
10
20
30
40
50
60
70
80
90
100

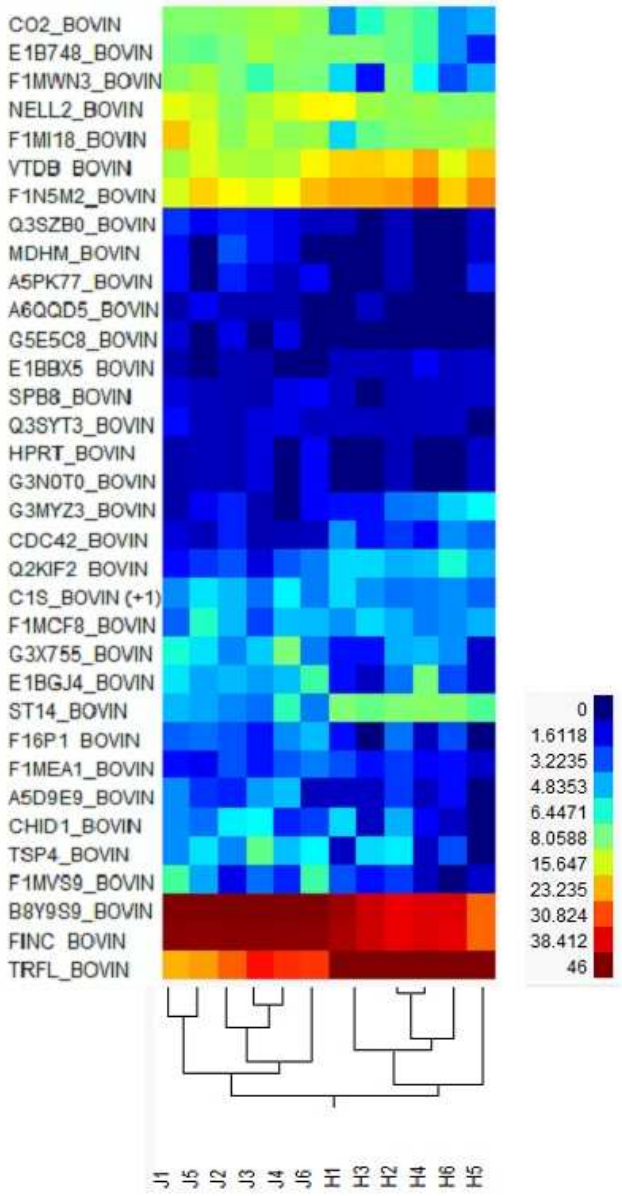


Figure 2.2. Hierarchical clustering of the 43 significantly differentially expressed protein within bovine skim milk between Holstein and Jersey cattle. Analysis was performed in JMP (Pro 10) using multivariate clustering. Bar color represents peptide count data where ark red represents higher expression and blue color represents lower expression.

Table 2.5. Gene Ontology functions associated with the low abundant proteins present at significantly different peptide counts in bovine milk between Holstein and Jersey dairy cattle breeds

	Complement C2	Nidogen-1 precursor	Glucuronosyl-transferase I	Embryo-specific Fibronectin 1 transcript variant	Fibronectin
Accession Number	CO2-BOVIN	F1MWN3-BOVIN	Q3SZB0-BOVIN	B8Y9S9-BOVIN	FINC-BOVIN
MW (kDa)	83	136	37	262	272
Response to stimulus	complement activation, classical pathway			wound healing	wound healing
Multi-organism process					
Metabolic process	complement activation, classical pathway				
Immune system process	complement activation, classical pathway				
Developmental process				Angiogenesis	Angiogenesis
Cellular process	complement activation, classical pathway			cell adhesion	cell adhesion
Biological regulation	complement activation, classical pathway			regulation of cell shape	regulation of cell shape

	Leucine-rich alpha-2-glycoprotein 1	Lactotransferrin	Stromal cell-derived factor 2-like protein 1	Fructose-1,6-bisphosphatase 1	Vitamin D-binding protein O
Accession Number	Q2KIF2-BOVIN	TRFL-BOVIN	SDF2L-BOVIN	F16P1-BOVIN	VTDB-BOVIN
MW (kDa)	38	78	24	37	53
Response to stimulus		antifungal humoral response			
Multi-organism process		antibacterial humoral response			
Metabolic process				gluconeogenesis	
Immune system process		antibacterial humoral response			
Developmental process		bone morphogenesis			
Cellular process		cellular iron ion homeostasis		protein homotetramerization	
Biological regulation		positive regulation of NF-kappaB transcription factor activity		negative regulation of cell growth	

	Complement C1s subcomponent	Protein kinase C-binding protein NELL2	Thrombospondin-4	Plexin-B2 precursor	Afamin precursor	Collectin-43
Accession Number	C1S_BOVIN (+1)	NELL2-BOVIN	TSP4-BOVIN	G3X755-BOVIN	G3MYZ3-BOVIN	CL43-BOVIN
MW (kDa)	77	91	106	205	70	34
Response to stimulus	complement activation, classical pathway		behavioral response to pain			
Multi-organism process						
Metabolic process	complement activation, classical pathway					
Immune system process	complement activation, classical pathway					
Developmental process			myoblast migration			
Cellular process	complement activation, classical pathway		myoblast migration			
Biological regulation	complement activation, classical pathway		positive regulation of cell division			

	Cell division control protein 42 homolog	Hypoxia up-regulated protein 1 isoform X1	Aspartate aminotransferase	Vitamin D-binding protein	Complement component 1, r subcomponent
Accession Number	CDC42-BOVIN	E1B748-BOVIN	AATC-BOVIN	F1N5M2-BOVIN	A5D9E9-BOVIN
MW (kDa)	21	112	46	53	80
Response to stimulus			cellular response to insulin stimulus		
Multi-organism process					
Metabolic process			glutamate catabolic process to 2-oxoglutarate		
Immune system process					
Developmental process	nervous system development				
Cellular process	small GTPase mediated signal transduction		oxaloacetate metabolic process		
Biological regulation	regulation of filopodium formation		fatty acid homeostasis		

	Suppressor of tumorigenicity 14 protein homolog	Mannan-binding lectin serine protease 1	Ectonucleotide pyrophosphatase	Alpha-mannosidase 2x	SLC27A6 protein	Alpha-2-macroglobulin
Accession Number	ST14-BOVIN	F1MVS9-BOVIN	ENPP2-BOVIN	E1BGJ4-BOVIN	A6QQD5-BOVIN	F1MI18-BOVIN
MW (kDa)	95	81	102	130	72	166
Response to stimulus			chemotaxis			
Multi-organism process						
Metabolic process			phosphatidylcholine catabolic process			
Immune system process			immune response			
Developmental process	keratinocyte differentiation					
Cellular process	keratinocyte differentiation		phosphatidylcholine catabolic process			
Biological regulation			regulation of angiogenesis			negative regulation of complement activation, lectin pathway

	Transmembrane protein 132A	Integrin alpha-6 precursor	IGL@ protein	Transaldolase	Aldehyde oxidase isoform X5	Serpin B8
Accession Number	F1MEA1-BOVIN	E1BFQ6-BOVIN	F1MCF8-BOVIN	TALDO-BOVIN	E1BBX5-BOVIN	SPB8-BOVIN
MW (kDa)	111	119	25	38	149	42
Response to stimulus	response to cytokine stimulus					
Multi-organism process		interspecies interaction between				
Metabolic process				pentose-phosphate shunt	oxidation reduction	
Immune system process						
Developmental process		blood vessel development				
Cellular process	signal transduction	cell adhesion		pentose-phosphate shunt		
Biological regulation	signal transduction	integrin-mediated signaling pathway				regulation of proteolysis

	Malate dehydrogenase	Coagulation factor X	Cartilage oligomeric matrix protein	Chitinase domain-containing protein 1	SERPINA 11 protein	complement factor H-related 1-like isoform 2
Accession Number	MDHM-BOVIN	FA10-BOVIN	COMP-BOVIN	CHID1-BOVIN	A5PK77-BOVIN	F1MW79-BOVIN
MW (kDa)	36	55	82	45	47	31
Response to stimulus		blood coagulation		inflammatory response		
Multi-organism process						
Metabolic process	tricarboxylic acid cycle			chitin catabolic process		
Immune system process				innate immune response		
Developmental process			apoptosis			
Cellular process	internal protein amino acid acetylation		cell adhesion	chitin catabolic process		
Biological regulation		positive regulation of protein kinase B signaling cascade				

	Hypoxanthine-guanine phosphoribosyltransferase	hypoxanthine-guanine phosphoribosyltransferase isoformX2	Transaldolase	Complement component 1, r subcomponent
Accession Number	HPRT-BOVIN	G3N0T0-BOVIN	G5E5C8-BOVIN	Q3SYT3-BOVIN
MW (kDa)	24	25	38	45
Response to stimulus				
Multi-organism process				
Metabolic process	GMP catabolic process		pentose-phosphate shunt	
Immune system process				
Developmental process				
Cellular process	protein homotetramerization		pentose-phosphate shunt	
Biological regulation	positive regulation of dopamine metabolic process			

Table 2.6. Peptide count data of the low abundant proteins present at significanctly different abundance in bovine milk between Holstein and Jersey dairy cattle breeds

Accession Number	CO2_BOVI	F1MWN3BOVIN	Q3SZB0BOVIN	B8Y9S9BOVIN	FINCBOVIN	Q2KIF2BOVIN	TRFLBOVIN	SDF2LBOVIN	F16P1BOVIN	VTDBBOVIN
H1	4	5	1	40	39	5	42	2	2	19
H2	8	9	1	38	38	5	46	1	4	20
H3	6	2	0	41	41	5	47	1	0	21
H4	7	6	0	39	39	5	46		1	24
H5	4	4	1	30	30	4	49		0	22
H6	4	3	0	42	41	6	50		3	17
Av. H	5.5	4.8	0.5	38	3.0	5.0	46	1.3	1.7	20
J1	13	15	4	63	62	3	32	0	5	18
J2	13	10	3	58	57	4	37	0	4	17
J3	16	9	3	59	58	2	46	0	3	17
J4	15	11	2	51	50	4	37		5	16
J5	10	14	2	48	47	3	26	0	4	17
J6	10	8	1	42	41	4	30		5	17
Av. J	12	11	2.5	53	52	3.3	34	0.0	4.3	17
p-value	0.000	0.002	0.002	0.002	0.003	0.003	0.003	0.005	0.005	0.007

Accession Number	C1S BOVIN	NELL2 BOVIN	TSP4 BOVIN	G3X755 BOVIN	G3MYZ3 BOVIN	CL43 BOVIN	CDC42 BOVIN	E1B748 BOVIN	AATC BOVIN	F1N5M2 BOVIN	A5D9E9 BOVIN
H1	5	16	1	2	2	1	4	8	0	22	1
H2	4	11	6	5	4	2	3	8	1	25	3
H3	4	12	5	2	2	2	2	8	0	24	1
H4	4	12	1	5	4	0	2	7		29	1
H5	3	9	0	1	5	0	3	2		27	0
H6	4	9	3	4	5	1	4	4	0	22	2
Av. H	4.0	11	2.7	3.2	3.7	1.0	3.0	6.2	0.3	24	1.3
J1	6	23	6	9	1	5	2	11	2	22	6
J2	6	12	5	5	3	2	3	10	2	22	3
J3	5	18	10	7	1	2	1	17	1	21	6
J4	7	18	6	10	0	3	1	12	1	20	6
J5	6	16	6	6	2		1	8		22	3
J6	4	17	6	4	2	3	1	9	1	21	1
Av. J	5.7	17	6.5	6.8	1.5	3.0	1.5	11	1.4	21	4.2
p-value	0.007	0.009	0.011	0.011	0.012	0.012	0.013	0.013	0.014	0.014	0.015

Accession Number	ST14 BOVIN	F1MVS9 BOVIN	ENPP2 BOVIN	E1BGJ4 BOVIN	A6QQD5 BOVIN	F1MI18 BOVIN	F1MEA1 BOVIN	E1BFQ6 BOVIN	F1MCF8 BOVIN	TALDO BOVIN	E1BBX5 BOVIN
H1	8	3	0	2	0	5	3		4	0	1
H2	9	3	0	4	0	10	3	0	5	0	1
H3	7	2		1	1	7	2	1	5	0	1
H4	10	1	0	8	0	11	2	0	4	0	2
H5	6	1	0	1	0	12	1	0	4	0	1
H6	9	0	0	3	0	11	2	0	4	0	1
Av. H	8.2	1.7	0.0	3.2	0.2	9.3	2.2	0.2	4.3	0.0	1.2
J1	7	10	7	8	1	30	3	2	5	2	1
J2	5	2	3	6	1	13	4	1	6	2	1
J3	5	5	6	6	1	19	3	1	4		1
J4	8	3	4	6	1	13	4	0	6	1	0
J5	5	5	0	5	2	17	2	2	7	0	0
J6	4	7	1	7	0	12	4	1	5	0	0
Av. J	5.7	5.3	3.5	6.3	1.0	17	3.3	1.2	5.5	1.0	0.5
p-value	0.016	0.016	0.020	0.021	0.022	0.023	0.028	0.033	0.035	0.035	0.038

Accession Number	SPB8 BOVIN	MDHM BOVIN	FA10 BOVIN	COMP BOVIN	CHID1 BOVIN	A5PK77 BOVIN	F1MW7 9BOVIN	HPRT BOVIN	G3N0T0 BOVIN	G5E5C8 BOVIN	Q3SYT3 BOVIN
H1	1	0	0		5	0	0	0	0	0	1
H2	1	1	0	1	5	1	1	1	1	0	1
H3	0	0	0	0	1	0	1	0	0	0	1
H4	1	0	0		2	0		0	0	0	1
H5	1	1	0	0	0	2		1	1	0	0
H6	1	0	0	1	1	0	1	0	0	0	1
Av. H	0.8	0.3	0.0	0.5	2.3	0.5	0.8	0.3	0.3	0.0	0.8
J1	2	3	1	1	6	3	1	1	1	2	3
J2	1	4	0	1	7	3	2	1	1	2	1
J3	1	3	0	1	8	2	1	2	2	0	2
J4	2	2		1	3	1	2	0	0	2	2
J5	1	0	1	2	4	0	2	1	1	0	1
J6	2	0	2	2	3	2		2	2	0	1
Av. J	1.5	2.0	0.8	1.3	5.2	1.8	1.6	1.2	1.2	1.0	1.7
p-value	0.038	0.042	0.042	0.044	0.045	0.046	0.047	0.049	0.049	0.049	0.049

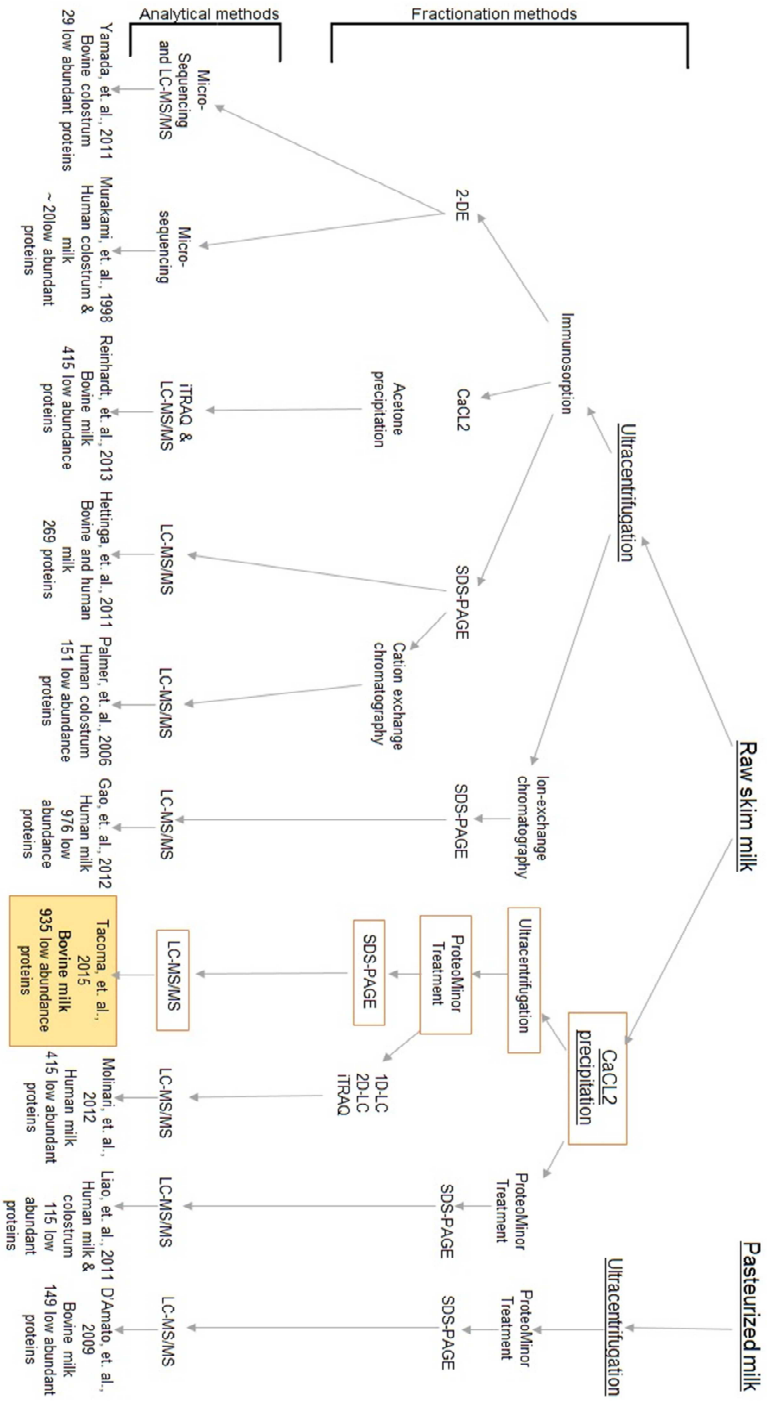


Figure 2.3. Summary of methods and results from proteomic studies analyzing the low abundant whey protein profile in milk.

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3 CHAPTER THREE: EFFECT OF DIETARY RUMEN DEGRADABLE PROTEIN: RUMEN UNDEGRADABLE PROTEIN RATIO ON THE BOVINE MILK PROTEOME PRODUCED BY MID-LACTATION HOLSTEIN DAIRY COWS

3.1 Abstract

Bioactive proteins and peptides in milk contribute to the healthfulness of milk; however, little is known about the profile of these bioactive compounds in bovine milk or whether the bovine milk proteome can be affected by the diet profile fed to lactating dairy cattle. The objective of this study was to determine if the proportion of dietary RDP:RUP could alter the milk proteome produced by mid-lactation Holstein dairy cows. Six multiparous Holstein cows in mid-lactation were blocked by DIM, milk yield, and DMI then randomly assigned to one of two treatment groups. The experiment was conducted as a double-crossover design consisting of three 21-day periods. Within each period, treatment groups received diets with either 1) a high RDP: RUP ratio (RDP treatment: 62.4:37.6 % of CP) or 2) a low RDP: RUP ratio (RUP treatment: 51.3:48.7 % of CP). Both diets were isonitrogenous and isoenergetic (CP: 18.5%, NE_L: 1.8 mCal kg^{-1} DM). Milk samples were collected at morning and afternoon milking at the beginning of the trial and at the end of each period, while rumen fluid samples, blood samples, and 24 h urine and fecal samples were collected at the end of each period. No treatment differences were observed in DMI, milk yield or milk composition. Feeding a diet high in RUP decreased β-casein ($P = 0.06$), κ-casein ($P = 0.04$), and total milk casein concentrations ($P < 0.01$) in milk. MUN and PUN concentrations were higher in the RDP group but plasma glucose, BHBA, and NEFA concentrations were not different between

groups. Nitrogen secreted in milk, urine and feces was not different between treatment groups. VFA analysis showed no differences in acetic, propionic, or butyric acid between groups. A combination of enrichment and fractionation techniques resulted in identification of 595 by mass spectrometry analysis. Few differences in the low abundance milk protein profile between dietary groups were found suggesting that milk protein synthesis mechanisms within the MG is not responsive to nutritional manipulation when total dietary CP levels exceed the nutrient requirement for the animal. Further investigations into dietary perturbances that are known to significantly alter nitrogen utilization patterns are needed in order to manipulate the milk low abundance protein profile using diet while at the same time having normal CP inclusion levels and maintaining a similar nutrient composition of both diets.

3.2 Introduction

It is well established that milk plays an important role in neonatal nutrition as a complete and wholesome part of their diet; however, it is now known that milk proteins are a source of bioactive compounds that have physiological importance. These bioactive substances play a role in human health, modulating physiological functions by various binding interactions with target cells and organs inducing physiological responses. Various functional properties associated with bioactive proteins and peptides include antimicrobial, antihypertensive, opioid, immunomodulatory, mineral binding and antioxidative activities (Lönnerdal, 2003; Severin and Wenshui, 2005; Korhonen and Pihlanto, 2006; Sharma et al., 2011; Park and Nam, 2015). Investigation of human breast milk has identified several bioactive proteins and peptides that can influence infant

health, particularly gut physiology and motility (Chatterton et al., 2013). Milk proteins present in bovine milk have also been identified to have bioactivity and cross-reactivity with human cells (Buccigrossi et al., 2007; Lönnerdal et al., 2011; Raikos and Dassios, 2014). Understanding secretion profiles of bovine milk proteins as well as mechanisms to manipulate this protein profile are important steps in further enhancing the healthfulness of bovine milk products.

The profile of proteins in bovine milk is influenced by animal factors such as breed, mastitis, and stage of lactation (Boehmer et al., 2010b; Reinhardt et al., 2013; Gustavsson et al., 2014; Tacoma et al., 2016). Altering the milk protein profile and bioactive properties of the milk by manipulating the diet of the dairy cow offers a promising approach to naturally enhance the healthfulness of milk products. Research examining the relationship between nutrition and the bovine milk protein profile is limited and nutrition is a significant management factor that has potential to alter milk protein composition (Kennelly et. al., 2005; Tripathi, 2014). Christian et al. (1999) altered the proportions of high abundance bovine milk proteins by feeding a lupin-wheat based diet, a high RUP source, to lactating dairy cows compared to cows fed spring-pasture, a high RDP source. Cows offered the lupin-wheat based diet had higher concentrations of αs_1-CN, αs_2-CN, and γ-CN in the milk compared to cows on the high pasture diet, while concentrations of β- and κ-CN were present at higher concentrations in milk from cows fed spring-pasture compared to cows on the lupin-based diet. More recently, a study was published outlining changes in high abundance milk protein expression patterns in response to inclusion of different corn and soybean feedstuffs in the ration. While the type of corn included in the diet did not influence the milk protein profile, inclusion of heat-treated soybean meal

resulted in a decrease in β-CN and zinc-α-2-glycoprotein fragments indicating the importance for sufficient availability of RDP in secretion of specific milk proteins. These authors also reported differential expression of α-LA and zinc-α-2-glycoprotein due to diet, suggesting that ruminal microbial protein synthesis could affect the milk protein profile (Li et al., 2014).

Possible changes in total dietary energy or protein uptake by the animal, rumen microbial fermentation dynamics, or animal N partitioning could explain the treatment differences observed in these trials; however, the differences in diet carbohydrate and energetic fractions in the previous research may also contribute to the changes observed. All of these differences possibly leads to changes in substrate availability to the MG and altered physiological function in mammary epithelial cells resulting in changes in protein expression and protein synthesis rates (Christian et al., 1999). The research outlined by Christian et al. (1999) and Li et al. (2014) include diets with different RDP:RUP ratios amongst other differences between diets, acting as cofounding factors when interpreting the results. The goal of our study was to create two isonitrogenous and isoenergetic dairy rations with at least a 10% difference in the RDP: RUP ratio. We hypothesize that altering the dietary protein fraction ratio will cause a shift in rumen microbial synthesis patterns resulting in changes in VFA production as well as amino acid (AA) availability in the small intestine. By inducing changes of the AA profile available for absorption in small intestine could lead to altered nutrient availability to the MG, resulting in a shift in mammary secretory cell gene expression patterns and milk protein composition.

3.3 Materials and Methods

3.3.1 Experimental design

Six multiparous mid-lactation Holstein dairy cows were blocked by DIM (80 ± 20 DIM) and milk yield (59.1 ± 28.3 kg) then were randomly divided into two experimental groups in a double-crossover design. Each period lasted 21 d and consisted of 16 d for diet adaptation and the last 5 d for sample collection. Cows were maintained in the same tie-stall facility with sawdust bedding at the Paul R. Miller Research and Educational Center (University of Vermont, Burlington, VT). All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

3.3.2 Diet and feeding

All animals had free access to water throughout the trial and were fed to target 10% refusals. Cows were fed the same partial mixed ration once daily (0600 h) and a pelleted top-dress that was mixed thoroughly into the ration thrice daily (0330, 1100, and 1800 h) that was formulated to contain either 1) a higher RDP: RUP ratio (RDP diet), or 2) a high RUP: RDP ratio (RUP diet). The treatment groups switched between the RDP or RUP topdress after each period. Diets were formulated to be isonitrogenous and isoenergetic (*Table 3.1*). Feed samples were collected thrice weekly and stored at -20˚C. Feed samples were later composited within feedstuff over each period throughout the experiment and analyzed by wet chemistry (DairyOne, Ithaca, NY). Daily feed refusals from each animal were collected each morning before feeding for the duration of the trial. These samples were stored at -20˚C until analysis and subsequently dried at 65˚C for 48 h to calculate individual daily DMI.

3.3.3 Milk production and milk sample collection

Cows were milked twice daily (0700 and 1600 h). Milk yield was recorded daily and milk samples were collected on d 1 as baseline samples and on d 16-19 at the end of each experimental period from the morning and afternoon milking. Milk samples were collected and preserved with bronopol and natamycin (D & F Control Systems, Inc., Broad Spectrum Microtabs) and stored at 4˚C. Samples were analyzed commercially (DHIA, Lancaster, PA) within three days after collection for general milk composition. Additional milk samples collected for analysis of high abundance proteins were immediately put on ice and skimmed within 2 h of collection at 4000 × *g* for 10 min at 4˚C. The fat layer was removed and the skimmed milk samples were stored at -20˚C until further analysis. Subsamples collected for low abundance protein analysis were immediately frozen in a dry-ice ethanol bath after collection and stored at -80˚. Milk samples for high abundance analysis were analyzed individually, whereas milk samples for low abundance protein analysis were composited during the last week of each period by individual animal based on milk weights recorded at each milking.

3.3.4 Blood collection

Blood samples were collected from the coccygeal artery or vein of each cow into heparinized and EDTA-coated tubes (Becton Dickinson and Company Franklin Lakes, N.J.) after milking (0800 and 1900 h) on d 0, 17, 19, and 21 of each period. Samples were placed on ice immediately after collection and plasma was isolated within 2 h of blood collection by centrifugation at 3000 × *g* for 15 min at 4˚C. Plasma was transferred into polypropylene tubes and frozen at -20˚C until analysis. Plasma samples were later thawed and aliquoted into 0.5 mL centrifuge tubes and plasma concentrations of BHBA

(Sigma, Saint Louis, MO, USA), PUN (Teco Diagnostics, Anaheim, CA), plasma glucose (Sigma, Milwaukee, WI, USA), and NEFA (ZenBio, Inc., Research Triangle Park, NC) were determined using commercially available kits. Samples were analyzed according to manufacturer's instructions and all coefficients of variance were < 5%.

3.3.5 Rumen fluid collection

Rumen fluid samples were collected by oesophageal intubation, which was performed at 0100 h on d 0, 19, and 21 of each period to determine rumen VFA profiles. Rumen fluid samples were centrifuged at 14,000 × *g* for 20 min at 8°C and the supernatant was filtered through a 25 mm hardened ashless filter (Whatman 540). The extracted supernatant was mixed with equal parts of an internal standard (50 μM mL^{-1} trimethyl acetic acid in 0.06 M oxalic acid). The samples were analyzed as per methods similar to those previously described by Dann et al., (2008). N was used as the carrier gas at a flow rate of 15 mL min^{-1}, where the other gases were purified air at 300 mL min^{-1} and hydrogen gas at 30 mL min^{-1} to the flame ionization detector. The oven temperature was held at 175°C for 25 min and the injector and detector temperature were held at 200˚C. Star Chromatography software (v. 6) was used to analyze peaks based on the flame ionization detector response. Peaks were identified using individual VFA standards (Supelco, Sigma-Aldrich, St. Louis, MO) and molar proportions were calculated using molecular weights and sample volume.

3.3.6 Urine and fecal collection

Urine and fecal samples were collected for 24 h on d 0 and 19 of each period. Urine and feces was collected using buckets and weights of each event were recorded before the sample was thoroughly mixed and a subsample collected. Four drops of

sulfuric acid was added immediately to each urine subsample to acidify the sample to a pH < 4. Fecal and acidified urine subsamples were placed on ice after collection and stored at -20˚C until further analysis. All urine and fecal samples were thawed overnight at 4˚C, and composited within animal based on the volume of each event in proportion to their total daily urine and fecal weights. The composite fecal samples were dried for 48 h at 65˚C and ground. Composite urine and dried fecal samples were then submitted for N analysis (University of Vermont Agricultural and Environmental Testing Lab, University of Vermont, Burlington, VT). Briefly, total carbon and N of dried fecal samples were determined by combustion, with thermal conductivity detection using a FlashEA NC Soil Analyzer (Thermo Electron Corp., Milan, Italy; CE Elantech, Inc, Lakewood, NJ, USA). Fecal samples were analyzed in triplicate and if the Relative Standard Deviation of the replicates was greater than 10%, samples were reanalyzed three times The instrument was calibrated for each run, using a reference sample of known composition, at four points and a blank. A second reference sample was analyzed as a QC Check Sample every 10-12 samples, and it was considered normal if within 10% of the expected value.

Prior to analysis, urine samples were dehydrated using Chromosorb. Dissolved ammonium-N in urine samples was determined on an automated colorimetric analyzer (Flow Injection Analysis, QuikChem 8000, Hach Company, Loveland, CO). Ammonia was heated with salicylate and hypochlorite in an alkaline phosphate buffer to produce an emerald green color (absorbing at 660 nm), the color was then intensified by the addition of sodium nitroprusside (Lachat QuikChem Method 10-107-06-2-O). Urine samples were diluted with deionized water if necessary to bring them within the instrument calibration range. The estimated N balance of each cow was determined through the following

calculation: N retained = N content of feed (g d^{-1}; g CP intake d^{-1} /6.25) – (urine N output (g d^{-1}) + fecal N output (g d^{-1}) + milk N output (g d^{-1})). The milk N content was assumed to be milk protein/6.38.

3.3.7 Analysis of the high abundance milk proteins

The skim milk samples stored for high abundance milk protein determination were thawed at 4°C overnight and mixed thoroughly by vortexing and then sonication at 33W for 15 min at less than 25˚C with ice (Bransonic Model 220, Branson Ultrasonics, Danbury, CT). Following sonication, a 0.5 mL aliquot of milk was pipetted into a borosilicate test tube. An equal amount of reducing buffer (6.0 M guanidine hydrochloride, 5.0 mM trisodium citrate dehydrate, 20.0 mM dithiolthreitol) was then added to the sample before incubation at room temperature for 1 h. The milk/buffer mixture was allowed to react for at least 1 h at room temperature, a further weighed volume of the buffer without the dithiolthreitol reducing agent was added, and the sample transferred to a syringe and passed through a 0.45 μm regenerated cellulose membrane syringe filter (Sartorius, Goettingen, Germany) into an HPLC autosampler vial.

Samples were analyzed using a Shimadzu (Kyoto, Japan) HPLC with the following solvent gradient protocol, outlined by Bordin et al. (2001) with minor modifications: linear gradient from 26.5 to 28% eluent B in 2.5 min (0.60% B min^{-1}), an isocratic elution at 28.0% B for 4 min then from 28.6 to 30.6% B in 3.4 min (0.70% B min^{-1}), and from 30.6 to 33.5% B in 2.9 min (1.0% B min^{-1}), followed by an isocratic elution at 33.5% B for 3 min, an increase from 33.5 to 36.1% B in 2.6 min (1.0% B min^{-1}), an isocratic elution at 36.1% B for 5 min, an increase from 36.1 to 37% B in 1.5 min (0.6% B min^{-1}), an isocratic elution

at 37% B for 2 min, and a final increase to 41% B in 6.5 min (0.60% B min^{-1}), for a total run time of 42 min at a flow rate of 0.50 mL min^{-1}.

For each analysis, 4 μL of sample were injected into the HPLC. Chromatograms were obtained at 214 nm and individual protein peaks were identified by comparison to injections of standard protein solutions prepared in our lab from purchased isolated proteins (Sigma, Saint Louis, MO, USA), and integrated using Shimadzu LCsolution software (version 1.22, 2006) to determine the area under the peak. For quantification of total α-CN, as well as β-CN, κ-CN α-LA, and β-LG, standard curves were directly determined by injecting known concentrations of the standard protein solutions. The constituent α_{s1}- and α_{s2}- CN proteins are not readily available as isolates, therefore, quantification of α_{s1}-CN and α_{s2}-casein was performed by interpolating the results from the total α-CN standard curve for semi-quantitative comparisons between experimental groups.

3.3.8 Low abundance protein isolation, digestion and identification

Milk samples collected for low abundance protein analysis were thawed overnight at 4˚C. To obtain a representative sample, milk samples from the morning and afternoon milking were composited within cow from d 16-19 within each period according to milk weights at each milking. The resulting 50 mL composite samples were subjected to fractionation and proteomic techniques as previously described (Tacoma et. al., 2016).

Samples were then analyzed by LC-MS/MS on a LTQ Mass Spectrometer (Thermos Fisher Scientific), 5 uL of the digest was loaded onto a 100 μm x 120 mm capillary column packed with MAGIC C18 (5 μm particle size, 20 nm pore size, Michrom Bioresources, CA) at a flow rate of 500 nL min^{-1}. Peptides were separated by a gradient of 5-35% acetonitrile/ 0.1% formic acid over 98 min, 40-100% acetonitrile/0.1%

formic acid in 1 min, and 100% acetonitrile for 10 min, followed by an immediate return to 2.5% CH3CN/0.1% formic acid and a hold at CH3CN/0.1% formic acid. Peptides were introduced into the linear ion trap via a nanospray ionization source and a laser pulled ~3 μm orifice with a spray voltage of 1.8 kV. Mass spectrometry data was acquired in a data-dependent "Top 10" acquisition mode, in which a survey scan from m/z 400-1600 is followed by 10 collision-induced dissociation (CID) MS/MS scans of the most abundant ions. Tandem mass spectrometry (MS/MS) scans were acquired with the following parameters: isolation width: 2 m/z, normalized collision energy: 35%, Activation Q: 0.250 and activation time = 30 ms. Dynamic exclusion was enabled (repeat count: 2; repeat duration: 30 sec; exclusion list size: 180; exclusion duration: 60 sec). The minimum threshold was 500. Product ion spectra were searched using the SEQUEST search engine on Proteome Discoverer 1.4 (Thermo Fisher Scientific, Waltham, MA, USA) against a curated Bovine Uniprot (Bos taurus database (24,206 entries) downloaded July 9, 2014). The 15 raw files from each sample (6 samples per period: 24 samples total) were searched as one contiguous input file and a single result file was generated for each sample. The database was indexed to allow for full trypsin enzymatic activity, two missed cleavages, and peptides between the MW of 350-5000. Search parameters set the mass tolerance at 20 ppm for precursor ions and 0.8 Da for fragment ions. Search Parameters were as follows: full trypsin enzymatic activity, two missed cleavages, and peptides between the MW of 350-5000; mass tolerance at 2 Da for precursor ions and 0.8 Da for fragment ions. dynamic modifications on methionine (+15.9949 Da: oxidation) (4 maximum dynamic modifications allowed per peptide); and static modification on cysteine (+57.0215 Da: carbamidomethylation) The result files

were then further analyzed by Scaffold 4.3 (Proteome Software, Portland, OR, USA) to compare the unique peptide counts and to identify GO functions of the identified proteins. Cross-correlation (Xcorr) significance and minimum peptide cutoff filters were applied to limit the false positive rates to less than 1% in the data sets.

3.3.9 Statistical analysis

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

The spectral abundance from the low abundance protein data was performed using a generalized linear mixed model for repeated measurers ANOVA. Data were analyzed by GLIMMIX procedure of SAS with baseline values used as covariates and day as repeated effect. A poisson distribution was assumed because all dependent variables were count data. All data were presented as LSM ± SEM and were considered to be significantly different at $P < 0.05$. Trends were recognized at $P < 0.10$.

3.4 Results

3.4.1 Diet and dry matter intake

Total crude protein (% of DM) was similar in both diets but a 13% difference (% of CP) in the RDP: RUP ratio between diets was achieved while maintaining similar neutral detergent fiber (NDF), NFC and NE_L content (% of DM; *Table 3.1*). DMI was not different between the two treatment groups (*Table 3.2*).

3.4.2 Milk yield and general composition

Total milk yield as well as concentrations and yields of the individual milk constituents were not different between the two treatment groups (*Table* 3.2). Similarly, somatic cell count was not significant between groups. MUN was higher ($P = 0.04$) in milk samples analyzed from the RDP group (15.7 mg dL^{-1}) compared to those from the RUP group (14.6 mg dL^{-1}).

3.4.3 Plasma metabolites

Plasma glucose, BHBA and NEFA concentrations did not differ between treatment groups (*Table 3.3*). PUN concentrations were higher ($P = 0.01$) from cows fed the RDP diet (1.02 mmol L^{-1}) compared to samples from cows fed the RUP diet (0.98 mmol L^{-1}). There was a significant period effect on the concentrations of plasma glucose ($P \leq 0.01$), BHBA ($P \leq 0.01$), and PUN ($P \leq 0.01$).

3.4.4 Rumen volatile fatty acids

Rumen propionate concentrations tended to be higher in cows that were fed the RUP diet ($P = 0.06$; *Table 3.4*); however, this response was primarily due to an outlier measured in the second period. For the remaining discussion, this trend was not included in the data interpretation.

3.4.5 Nitrogen partitioning

Nitrogen balance data is presented in *Table 3.5* as total N excreted as well as proportion of N intake. Nitrogen intake was similar between the RDP and RUP diets (718 g d^{-1} and 717 g d^{-1}, respectively (P = 0.93)). Total fecal, urine and milk N output (g d^{-1} or g g^{-1} N intake) was not different between treatment groups. There was no effect of diet on calculated N retention. Urine N excretion was affected by both the period and the period x treatment interaction.

3.4.6 High abundance milk proteins

Concentrations of both κ-CN (P = 0.04) and total CN (P <0.01) was lower in milk samples from the RUP group (5.39 mg mL^{-1}, 36.3 mg mL^{-1}, respectively) compared to the RDP group (5.61 mg mL^{-1}, 37.8 mg mL^{-1}, respectively). Total α-CN (P = 0.06) concentration tended to be higher in milk samples collected from the RUP group (16.3 mg mL^{-1}) compared to the RDP group (15.7 mg mL^{-1}). There was a period effect on total CN, β-CN, κ-CN and total α-CN concentrations over the experimental period. No difference was found between treatment groups in the skim milk whey fraction (*Table 3.6*).

3.4.7 Low abundance milk proteins

Analysis of the skim milk samples from both dietary groups resulted in identification of 595 low abundance proteins. Four of these low abundance proteins were present at different abundance between treatment groups; α-mannosidase (P = 0.01), transforming growth factor (P = 0.01), α-2-macroglobulin (P = 0.01), and embryo-specific fibronectin (P = 0.05; *Table 3.7*). A number of low abundance proteins were also

identified to be significantly influence by time where their abundance decreased over the course of the trial.

3.5 Discussion

3.5.1 Effect of diet RDP: RUP ratio on N partitioning and rumen VFA concentrations

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

The concentrations of VFA did not change in response to diet in the current experiment and could be affected by factors including individual animal variation and

sampling procedure, the carbohydrate fractions were similar across the diets and would support this lack of difference observed in VFA profile and concentration. However, total rumen or whole tract VFA production was not measured and could have been altered by the higher N availability for MCP production in the rumen of cows fed the higher RDP diet (Hoover and Stokes, 1991; Reis and Combs, 2000; Reynal and Broderick, 2005).

3.5.2 Milk proteins affected by diet RDP: RUP ratio

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

Cows on the RUP diet also had lower individual β-CN and κ-CN concentrations in the skim milk fraction compared to those on the RDP diet. These results suggest that, at least in part, the results observed by Christian et al. (1999) and Li et al. (2014) are due to changes in ruminal protein availability and consequent animal N partitioning. The mechanisms of action could be due to specific AA availability to the MG, which is known to affect total protein secretion in the milk (DePeters and Cant, 1992; Rius et al., 2010). However, the diets used in the current study were predicted to satisfy all AA requirements and without the observation of increased total milk protein output, it would indicate an additional requirement of specific AA above the current estimated AA requirements for synthesis of specific CN isoforms. Though this prospect is feasible,

further investigation of mammary AA supply during differential RDP: RUP feeding with focus on its relationship to mammary function and CN isoform secretion is needed in order to address this mechanistic hypothesis.

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

Over 590 low abundance proteins were identified using a combination of fractionation and enrichment techniques. Gene ontological (GO) analysis revealed that 83% of the low abundance proteins with identified GO functions were involved in cellular processes such as protein folding and stabilization, signal transduction, cell adhesion, complement activation pathways, and glycolytic and catabolic processes. Additionally, 73% of low abundance proteins identified with known GO functions involved in binding processes were predominately proteins involved in metal-ion binding such as calcium, copper, magnesium, manages and zinc as well as ATP- and GTP-binding. Twenty-five percent of the low abundance proteins identified with known GO functions were involved in immune system regulation and these proteins were involved in activation of the complement proteins, the innate immune response and antibacterial

activities. Many of the low abundance proteins have multifunctional properties contributing to the complex regulation of cellular metabolism.

Only four low abundance proteins were affected by dietary treatment: α-mannosidase, transforming growth factor, α-2-macroglobin, and embryo-specific fibronectin by peptide count analysis. The abundances of these proteins were all higher in milk samples from cows fed the RDP diet compared to samples from cows fed the higher RUP diet. Alpha-mannosidase is a common cellular protein located in the cytoplasm and is involved in glycoprotein synthesis. This protein is not mammary-specific and has been previously identified in milk (D'Amato, et al., 2009; Le, et al., 2010). Transforming growth factor-beta, a major cytokine present in both human breast milk and bovine milk is an important protein particularly in infant immune development, preventing intestinal, inflammation and modulating intestinal epithelial proliferation (Peroni, et al., 2009; Penttila, 2010; Pieters, et al., 2015). α-2-macroglobin originates from both the MG and from the liver, where transcellular pathways are likely to be the route of passage from the plasma into the alveolar space (Boisgard et al., 2001;Westwood et al., 2001; Yamada et al., 2002). This protein is recognized as a plasma proteinase inhibitor, predominately secreted by the liver into plasma and is involved in the innate immune response (Armstrong and Quigley, 1999). Embryo-specific fibronectin, another common cellular protein present in milk is known to be involved in cell growth and differentiation (D'Alessandro, et al., 2010). The four low abundance proteins identified to exist at higher abundance in milk from cows on the RDP diet are characterized generally as common non-mammary specific cellular proteins and there does not appear to be clear pathways or common regulation patterns between these proteins. It is unclear how the expression of

these low abundance proteins are influenced by the diet and more research is needed in this field to understand the physiological and metabolic factors affecting milk protein expression within the MG.

3.5.3 Effect of days in milk on milk protein profile

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

Eleven low abundance proteins were influenced by DIM where eight of the proteins lowered in abundance with increasing DIM and one protein increased in abundance with increasing lactation stage. Another two proteins were found to decrease in number when transitioning into period two but increased in abundance by the third period. The observation that the abundance of fibronectin, glucose-regulated protein, and inter-α-trypsin inhibitor, decreased with increasing DIM is supported by previous research (Zhang et al., 2015a, 2016). Proteins involved in host defense functions, such as fibronectin, sulfhydryl oxidase, and inter-α-trypsin inhibitor, may become less prevalent as the MG undergoes involution, and stressors from lactation as well as inflammation in the MG subsides (Zhang et al., 2015a,b). Interestingly, significant decreases in protein abundance were found for C4b-binding protein, a protein known to inhibit complement C4. Complement C4 is a protein involved in the complement activation system,

recognizing surface pathogens and protecting the neonate from inflammation after milk consumption. The decreased abundance of C4b-binding protein in later lactation may suggest its role in milk as supporting the acute inflammatory response via the complement pathways (Zhang, et al., 2013).

Conversely, the abundance of protein kinase C-binding protein, an active protein involved in cell differentiation, increased with increasing DIM. This protein originates from MEC and the increased abundance in milk is likely also due to alveolar involution (Masso-Welch at al., 1998).

3.6 Conclusion

Nutritional manipulation of the dairy cow's diet to alter milk composition offers a promising approach to naturally enhance the milk profile and could provide an opportunity for future development into functional foods directed towards increased healthfulness of milk. While altering the RDP: RUP ratio of the diet induced few differences in the low abundance milk protein profile, the impact of this diet alteration on the CN profile produced by the cattle demonstrates the potential to influence specific mammary-derived milk proteins. Further investigation into the mechanisms of this interaction are needed to more accurately predict the effect of diet changes on the milk protein profile. Future studies could begin by gaining a deeper understanding on how the MG responds to changes in plasma AA composition along with examining the regulatory mechanisms behind AA transport across the mammary epithelia and how a change in AA availability to the MG influences protein synthetic pathways.

Table 3.1. Ingredient and nutrient composition of the diets

	Treatment	
Ingredient, % of DM	RDP	RUP
Corn silage	48.5	48.5
Haylage	6.4	6.4
Soybean meal	13.1	5.6
Molasses cane	1.4	1.1
Corn grain	14.2	14.0
Citrus pulp dry	1.1	1.2
Canola meal	3.6	-
Wheat midds	0.7	-
Wheat red dog	1.4	-
Berga fat	0.9	-
Corn distillers	-	3.5
Vitamins/minerals	2.9	2.7
Amino max	4.8	13.9
Urea	0.4	0.14
Amino enhancer	-	1.4
Nutrient composition, % DM		
DM[1], %	57.1	57.0
CP[2]	18.5	18.5
RDP[3]	11.7	9.0
RUP[4]	6.8	9.5
ADF[5]	20.3	21.7
NDF[6]	30.8	32.7
NFC[7]	40.1	39.1
NE_L[8] (Mcal/lbs)	0.8	0.8

[1]DM: Dry matter. [2]CP: Crude protein. [3]RDP: Rumen-degradable protein. [4]RUP: Rumen-undegradable protein. [5]ADF: Acid detergent fiber. [6]NDF: Neutral detergent fiber. [7]NFC: Non-fibrous carbohydrates. [8]NE_L: Net energy of lactation.

Table 3.2. Daily dry matter intake (DMI), milk yield and milk components of Holstein dairy cattle fed diets with either a high RDP: RUP ratio (RDP) or low RDP: RUP (RUP) ratio

	Treatment		[1]SE	*P* value	
	RDP	RUP		Treatment	Period
DMI (kg d^{-1})	24.2	24.2	0.7	ns	ns
Milk yield (kg d^{-1})	58.3	58.7	3.5	ns	0.05
Milk components yield (kg d^{-1})					
Fat	1.56	1.48	0.04	ns	ns
Protein	1.71	1.72	0.07	ns	ns
Milk components (%)					
Fat	3.48	3.43	0.22	ns	ns
Protein	2.96	2.93	0.13	ns	ns
Somatic cell count (×1000)	55.5	78.2	23.7	ns	ns
Milk urea nitrogen (mg dL^{-1})	15.7	14.6	0.86	0.04	<0.01

[1]SE = standard error of the difference.

Table 3.3. Plasma metabolites from Holstein dairy cattle fed diets with either a high RDP: RUP ratio (RDP) or low RDP: RUP (RUP) ratio

	Treatment		[1]SE	*P* value	
	RDP	RUP		Treatment	Period
Glucose (mmol L^{-1})	3.51	3.52	0.04	ns	ns
BHBA (mmol L^{-1})	0.26	0.27	0.01	ns	<0.01
PUN (mmol L^{-1})	1.02	0.98	0.01	0.01	<0.01
NEFA (mmol L^{-1})	0.10	0.09	0.01	ns	ns

[1]SE = standard error of the difference.

Table 3.4. Rumen volatile fatty acids from Holstein dairy cattle fed diets with either a high RDP: RUP ratio (RDP) or low RDP: RUP (RUP) ratio

	Treatment		[1]SE	*P* value	
	RDP	RUP		Treatment	Period
Acetate (mmol L^{-1})	19.9	21.9	0.86	ns	ns
Acetate (% of total)	59.3	65.4	5.10	ns	ns
Butyrate (mmol L^{-1})	3.34	3.91	0.24	ns	ns
Butyrate (% of total)	10.9	10.8	0.85	ns	ns
Propionate (mmol L^{-1})	6.96	8.05	0.45	0.06	ns
Propionate (% of total)	19.7	18.1	3.50	ns	ns
Acetate: Propionate ratio	2.85	2.76	0.06	ns	ns
Total VFA (mmol L^{-1})	35.1	33.9	2.18	ns	ns

[1]SE = standard error of the difference.

Table 3.5. N partitioning in plasma, urine, feces, and milk from Holstein dairy cattle fed diets with either a high RDP: RUP ratio (RDP) or low RDP: RUP ratio

	Treatment		[1]SE	*P* value	
	RDP	RUP		Treatment	Period
N intake (g d^{-1})					
Forage	193	193	5.99	ns	ns
Concentrate	524	523	16.2	ns	ns
Total	718	717	22.2	ns	ns
N output (g d^{-1})				ns	ns
Feces	196	210	10.4	ns	ns
Proportion of N intake	0.27	0.28	0.009	ns	ns
Urine	253	237	9.31	ns	<0.01
Proportion of N intake	0.35	0.32	0.01	ns	<0.01
Milk	249	257	9.31	ns	ns
Proportion of N intake	0.35	0.34	0.03	ns	ns
Retention (g d^{-1})	38.8	43.2	29.7	ns	0.02

[1]SE = standard error of the difference.

Table 3.6. High abundance milk proteins from Holstein dairy cattle fed diets with either a high RDP: RUP ratio (RDP) or low RDP: RUP (RUP) ratio

	Diet		[1]SE	*P* value	
	RDP	RUP		Treatment	Period
CN (mg mL^{-1})					
β-CN	16.3	15.8	0.29	0.06	0.03
κ-CN	5.61	5.39	0.08	0.04	0.01
Total α-CN	15.7	16.3	0.33	0.06	ns
α-s_1	13.7	13.3	0.45	ns	ns
α-s_2	1.93	1.93	0.07	ns	ns
Total CN	37.8	36.3	0.48	<0.01	0.04
Whey (mg mL^{-1})					
α-LA[2]	1.48	1.50	0.09	ns	ns
β-LGA[3]	2.52	2.50	0.19	ns	ns
β-LGB[4]	1.60	1.64	0.11	ns	ns
Total α-LA, β-LGA, β-LGB	5.50	5.75	0.37	ns	ns

[1]SE = standard error of the difference. [2]α-LA: α-Lactalbumin. [3]β-LGA: β-Lactoglobulin variant A. [4]β-LGB: β-Lactoglobulin variant B.

Table 3.7. Average peptide counts of low abundance milk proteins significantly affected by feeding either a high RDP: RUP ratio (RDP) or low RDP: RUP (RUP) ratio to Holstein dairy cattle

Protein	Diet[1]		
	RDP	RUP	*P*-value
α-mannosidase	3.81 (1.76)	0.80 (0.47)	0.01
Transforming growth factor-beta	3.81 (1.76)	0.80 (0.47)	0.01
α-2-macroglobulin	23.9 (2.40)	15.8 (1.80)	0.02
Embryo-specific fibronectin	12.9 (1.70)	9.21 (1.34)	0.05

Diet[1] = standard error values are presented in parenthesis adjacent to means.

Table 3.8. Average peptide counts of low abundance milk proteins significantly affected by period (time) during the experiment

Protein	Period[1]			*P*-value
	1	2	3	
Sulfhydryl oxidase	20.6 (2.31)	16.6 (2.00)	11.9 (1.64)	0.01
Inter-α-trypsin inhibitor	12.2 (4.70)	1.02 (0.56)	1.41 (0.72)	0.01
Lipopolysaccharide-binding protein	5.63 (0.99)	2.31 (0.62)	2.31 (0.66)	0.03
α-2-macroglobulin	5.63 (0.99)	2.31 (0.62)	2.31 (0.66)	0.03
Fibronectin	21.01 (2.41)	16.7 (2.06)	12.3 (1.70)	0.03
Laminin	8.77 (1.21)	5.80 (0.98)	4.73 (0.89)	0.04
Glucose-regulated protein	8.77 (1.21)	5.80 (0.98)	4.74 (0.89)	0.04
Prothrombin	1.52 (0.63)	4.62 (1.14)	4.21 (1.09)	0.06
Protein kinase C-binding protein	3.89 (0.81)	1.97 (0.63)	1.61 (0.52)	0.06
C4b-binding protein	7.15 (1.41)	2.75 (0.76)	4.29 (1.01)	0.08
Apolipoprotein A-I	9.81 (1.84)	4.64 (1.08)	6.99 (1.46)	0.09

Period[1] = standard error values are presented in parenthesis adjacent to means.

3.7 References

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4 GENERAL DISCUSSION

Factors known to influence milk protein composition range from environmental conditions, such as season, to animal and management factors including stage of lactation, breed, inflammation and nutrition. Examination of the milk protein composition in different breeds has previously been reported but studies have predominately focused on the high abundance proteins such as CN, α-LA, and β-LG (Auldist et al., 2004; Gustavsson et al., 2014). Investigation into the low abundance protein profile has been explored in the human milk proteome (Liao et al., 2011; Molinari et al., 2012; Roncada et al., 2013) but little is known about the low abundance proteome in bovine milk. Low abundance proteins are potent, multifunctional molecules capable of exerting biological functions beneficial to human health (Schack et al., 2009; D'Alessandro et al., 2011; Lönnerdal, 2014). Given the strong correlation between nutrition and health, characterization of these bioactive proteins in bovine milk is essential to advance research that improves human health (Mills et al., 2011; Berry et al., 2014).

Bovine breed is a significant factor affecting the milk protein profile and influences the nutritional value of milk. After our recent work was published (Tacoma et al., 2016), a study compared three proteomic extraction procedures using milk samples from Holstein and Jersey cows (Vincent et al., 2016). The three methods involved fractionation techniques that were different to the ones used in our research. One hundred and eighty six proteins were identified over the three different methods performed and results showed few differences in the protein profile between breeds. Dark bands corresponding to the high abundance proteins were present on the SDS-PAGE images which would mask the low abundance proteins and possibly explain why less than 200

proteins were identified. Extensive fractionation and enrichment techniques are required to isolate the low abundance proteome in milk to allow for clean separation necessary for optimal mass spectroscopic identification. Acidification of milk to precipitate out CN, followed by ultracentrifugation, ProteoMiner enrichment, and protein separation using SDS-PAGE has resulted in the highest identification of low abundance proteins in bovine milk thus far (Tacoma et al., 2016). Further development in fractionation techniques and proteomic analysis will provide increasing opportunities to unravel the milk proteome and advance our understanding of the mechanisms involved in protein expression within the MEC. Identification and characterization of regulatory mechanisms involved in milk protein synthesis will provide opportunities to improve milk production and conversion efficiency of AA into milk protein as well as provide insight to naturally enhance the healthfulness of milk products.

Dietary manipulation, an animal management tool on farm, can influence the secretion of proteins in bovine milk and is considered to have the most rapid and influential effect on milk protein composition (Kennelly et. al., 2005; Tripathi, 2014). Few studies have investigated how nutrition influences milk composition driving our research to explore the feasibility of altering the bovine milk protein profile using dietary perturbances. Nutritional factors show promise to influence the milk proteome because the substrates available to the MG for protein synthesis are derived from preformed sources from the diet and the body. Shifting the forage:concentrate ratio, or changing the amount and source of dietary protein alters energy and N metabolism in a ruminant animal leading to modifications in substrate availability to the MG (DePeters and Cant, 1992; Jenkins and McGuire, 2006). Currently there is lack of knowledge about how

various nutritional components influence milk protein composition limiting our ability to formulate diets that are biologically beneficial and economical. In the present study (Chapter 3), two diets that were isonitrogenous and isoenergetic were fed to two groups of cows and formulated with either a high RDP or high RUP proportion. Few differences were detected in the low abundance profile between diets likely due to surplus dietary protein inclusion in the diets, outbalancing the differences in protein supply from the RDP and RUP sources. Limitations associated with methodological procedures and analysis may also have masked possible changes in the low abundance protein profile and will be discussed later. Although few differences were identified in the whey proteome, changes in the CN profile were detected and this confirms the potential to influence synthesis of specific mammary-derived milk proteins. Casein proteins originate from MEC indicating that changes in mammary cell metabolism were likely to have occurred in response to dietary protein source. Changes in MEC protein synthesis patterns may be a result from outside influences including a change in gene expression due to altered hormonal regulation or from changes in substrate availability in the general circulation. Investigation into the mechanisms involved in milk protein synthesis, such as hormonal regulation (predominately by insulin and prolactin), AA transport into MEC via specific AA transporters, and dietary energy-induced pathways will be needed to understand how nutritional perturbances could be used to enhance the nutritional value of bovine milk.

4.1 Limitations of the experimental approach

4.1.1 Laboratory methodology limitations

Recently, the *Bos Taurus* genome was sequenced and this has rapidly led to new development in the bovine milk-omics field (Bovine Genome Sequencing and Analysis Consortium, 2009). Identification of all proteins in bovine milk has long been a challenge due to its complex nature containing a diverse array of proteins present at different abundances, ranging from 9 orders of magnitude (from hundred mg ml^{-1} for IgG to few pg for some growth factors) (Altomare et al., 2016). Experimental work has been performed to unravel the bovine proteome (Yamada et al., 2002; Reinhardt and Lippolis, 2008; D'Alessandro et al., 2010; Mills et al., 2011; Senda et al., 2011; Yang et al., 2013; Lu et al., 2013; Yang et al., 2015), and development of fractionation techniques to enrich the detection of low abundance proteins offers opportunities to expand the bovine proteome. In this research, (Chapter 2 and 3) removal of high abundance proteins was achieved by acidification and precipitation of CN, followed by ultracentrifugation and ProteoMiner treatment. These procedures likely contribute to variation in the results where some low abundance proteins remain bound to CN and/or ProteoMiner beads, obstructing identification of the entire low abundance bovine proteome. Modifications in individual protein structure from the multiple freeze and thaw cycles involved in our methodology presents a potential drawback limiting identification of the entire bovine proteome. Additionally, the variation associated with each procedure poses a concern since the risk for contamination (demonstrated by the high number of keratin proteins identified in each sample), and potential loss of proteins between method procedures are important factors to take into account when developing milk proteomic methodologies

and interpreting high out-put data. An overview of the protein profile unfolded in both human and bovine milk using different fractionation and proteomic techniques has been illustrated and shows how successful each method is at unravelling the milk proteome (Tacoma et al., 2016).

ProteoMiner treatment is an alternative method to immunoprecipitation and uses a complex hexapeptide bead library where a binding partner exists for every protein in the sample. Because bead number limits binding capacity, high abundance proteins quickly saturate their hexapeptide ligand partner and excess protein is washed away. Low abundance proteins, on the other hand, also have unique binding partners, and will concentrate with their specific ligands. This reduces the dynamic range of total protein concentrations and maintains relative concentrations of all proteins within the original sample. This technology was designed to identify proteins in human plasma and we adapted the method to analyze proteins in bovine milk samples (Bantscheff et al., 2014). The randomness associated with the volume of beads added to a whey sample poses a source of variation between samples since the exact number and functional property per bead added to each sample varies. Future considerations may involve testing the same milk samples repeatedly after using ProteoMiner beads to assess the reproducibility of each sample to account for both biological and technical variability. Additionally, the amount of starting material and homogeneity of the samples needs to be examined further for compatibility in future studies. Following ProteoMiner treatment, visual results of the protein samples stained and separated on the gels highlighted a discrepancy in band patterns between each sample despite assurances that the same amount of whey protein was added to the ProteoMiner beads using the BCA assay. This leads to question the

effect ProteoMiner treatment has on each protein and whether the interactions are consistent between samples.

4.1.2 Animal-based experimental limitations

The first experiment investigating how breed effect the bovine proteome involved six cows from each breed. Considering the significance genetic variation influences the milk proteome between individuals may explain the small number of low abundance proteins found at different abundances between breeds. Stage of lactation also significantly influences the milk protein composition (Senda et al., 2011; Zhang et al., 2015a), and the relatively large variation in DIM between animals may have reduced potential for significance. The second trial involved six cows in a double-cross over design examining how nutrition effects the bovine proteome. The number of cows per treatment group was a major limitation in this study. A double-cross over design was specifically used to increase power and account for possible confounding effects such as DIM. Few significant dietary treatment effects were observed and the number of cows per group is likely to have influenced these results due to high variation in the bovine milk proteome known to exist between individuals.

Diet formulation was an important component to master to ensure that both groups were fed diets that were isonitrogenous and isoenergetic. Other experimental work has been performed and reviewed internationally using nutritional manipulation to alter the bovine milk protein composition (Christian et al., 1999; Mackle et al., 1999; Li et al., 2014). The challenge was to take this information and formulate diets with different RDP:RUP ratios to alter N utilization patterns within the cow, while maintaining the same overall chemical composition profile between diets. The final diets had a 13%

difference as % CP in the RDP:RUP ratio with crude protein at 18.5% DM. Excess protein inclusion in the diet may explain why few differences in the expression of low abundance proteins were detected between groups, as high dietary CP inclusion may have masked the effect of the ratio difference in dietary protein. The challenge for future research is to formulate diets known to significantly alter N utilization patterns within the cow and at the same time supplying adequate dietary protein in diets that have the same chemical composition.

4.2 Future perspectives

4.2.1 Laboratory Methodology

Several hundred low abundance proteins were detected in our milk samples and relative abundance of proteins based on peptide counts allowed for semi-quantification of our results. However, the results obtained from analysis of low abundance proteins from the present studies dose pose concern over proteomic method accuracy where identical methodologies were used in both studies and a three-hundred protein count difference was detected between the two studies. This large difference in the number of proteins identified in each study may be due to the two different mass spectrometers that were used to analyze samples from each study. In the breed study, the LTQ Orbitrap Discovery system was used in contrast, to the nutrition study where the LTQ XL system was used to analyze the protein samples. Technically, both instruments show similar sensitivity levels within the specified molecular mass range of 350-5000, suggesting that this variable is not responsible for the protein count difference detected between experiments (Thermo Electron Corporation, 2006; Thermo Fisher Scientific., 2007). Further investigation into

factors that may have caused this large difference in protein count between studies could begin by re-analyzing the samples and comparing the accuracy and reproducibility of the results obtained from MS data.

Many research groups have used similar proteomic methods where relative quantification was used to measure changes in the proteome (Boehmer et al., 2008, 2010a; Liao, et al., 2011; Smolenski et al., 2014; Guerrero et al., 2015), but future studies should consider using isobaric labeling technology to achieve more accurate qualitative results (Reinhardt et al., 2008; Lu et al., 2013; Zhang et al., 2015a,b, 2016). Isobaric labeling is a mass spectrometry technique using isobaric labels to tag specific peptide fragments and quantifying peptide relative abundances in samples by comparing fragment patterns produced from mass spectrometric analysis (Rauniyar and Yates, 2014). The lack of treatment effect detected in the nutrition study may indicate that the proteomic techniques used in this study were not sensitive enough to detect changes in protein abundances between samples. Isobaric labelling is a tool that could be used in the future to accurately detect changes in the proteome.

Further investigation into the bovine milk proteome should also modify proteomic techniques to account for common post-translational modifications (e.g., glycosylation) in milk proteins since the current methods disregard N-deglycosylation processes. Carbohydrate groups bound to proteins play essential roles in the function of cellular components and processes, important features to be identified to understand the biological functions of milk proteins (Barile and Lange, 2012; Nwosu et al., 2012; O'Riordan et al., 2014). Furthermore, as studies continue to investigate the biological functions of specific bioactive proteins, accurate identification and quantification of

bioactive proteins is essential. Western-blot analysis is the gold standard to quantify proteins and has been used to validate peptide count data of selected proteins to ensure similar results are obtained with semiquantitative methods (Liao et al., 2011). However, this analysis is often restricted by availability of antibodies for proteins of interest. Development of antibodies to specific bioactive proteins present in milk will become increasingly important as research continues to demonstrate health benefits associated with specific milk proteins and consumer demands for bioactive protein-enriched foods increase.

Following mass spectrometry analysis, identified milk proteins are functionally characterized by computational database programs used to assign proteins with specific functions based on previous research. However, if the function of a protein is unknown, a predicted function is assigned. These predictions largely rely on bioinformatic programs such as Scaffold and Mascot to analyze the protein sequence and estimate three-dimensional structure comparisons to optimize alignment of known protein sequences to unknown proteins based on their AA sequences (Zhang et al., 2013; Berry et al., 2014). This form of analysis of assigning function to a protein based on its sequence and structure similarity has led to misannotations of protein function (Mills et al., 2015). Only a small portion of peptides can accurately be analyzed based on functional and structural features of a homologue, because often only some of the characteristics are shared between proteins. Data interpretation is dependent on knowledge of proteins which is tightly associated with genomics, transcriptomics and metabolomics data. More comprehensive research integrating functional protein annotation and omics data alongside development of reliable computational methods will become increasingly

important as the number of proteins identified continues to increase (Almeida et al., 2015; Dallas et al., 2015). Using functional genomics in combination with proteomic methods to fully characterize protein gene expression and regulatory properties of milk proteins will help to better understand cellular regulatory processes involved in milk synthesis (Berry et al., 2014).

4.2.2 Feeding strategies

A diverse range of ingredients can be used when formulating diets to alter N utilization in a dairy cow. Alternative forages grown on commercial dairy farms are becoming increasingly popular to incorporate into their feeding systems due to rising feed and land prices. Red clover feed plays a fundamental role on organic dairy farms providing a high quality feed and providing a unique N fixing ability recycling N back into the soil. In respect to ruminant nutrition, red clover has a distinctive characteristic in that it contains polyphenol oxidases, an enzyme that inhibits protein degradation in the rumen (Van Ranst et al., 2011). This results in an increased proportion of degradable RUP in the diet leading to physiological changes in protein metabolism in the dairy cow (Lee et al. 2009a, b). Birdsfoot trefoil, another common alternative forage used in North America dairy systems contains unique compounds known as condensed tannins. Condensed tannins function similarly to polyphenol oxidases in that they bind to soluble protein in the rumen, protecting it from proteolysis and release the proteins once the complex reaches the acidic abomasum. (Christensen, 2015). Physiological changes in protein metabolism observed when red clover or birdsfoot trefoil are consumed by the ruminant animal is likely to lead to changes in protein synthesis and secretion within the mammary epithelial cells altering the milk protein profile. In this way, altering rumen N

metabolism could be an ideal method to create functional foods enriched with bioactive proteins as well as improve animal production and soil fertility on farm.

Another approach to alter N utilization in a lactating dairy cow is to choose fermentable energy and protein sources that 'synchronize' ruminal fermentation, maximizing the efficiency of microbial protein synthesis and minimizing production of rumen ammonia (Reynolds et al., 2013). Fermentable carbohydrates are those that are readily available to the rumen microbes to breakdown. Increasing the supply of fermentable carbohydrates can improve N efficiency by decreasing deamination of dietary proteins, leading to reduced production of ruminal NH_3 and enhanced microbial utilization of AA, in addition with increasing microbial capture of excess NH_3 (Hristov et al., 2005). A change in N partitioning was observed with increased starch supply and adequate rumen available protein resulting in increased microbial protein synthesis altering the proportion of available AA in the lower digestive tract (Reynolds et al., 2013; Cantalapiedra-Hijar et al., 2014). Increasing the proportion of fermentable starch in the diet of a lactating dairy cow results in a shift in the rumen microbiota community structure, favoring propionate-producing bacteria (Belanche et. al., 2012). Propionate is a gluconeogenic precursor and is converted into glucose in the liver. Protein synthesis has a high requirement for energy signifying that dietary energy is involved in the regulation of milk protein synthesis (Purdie et al, 2008; Bionaz et al., 2012). Increasing dietary starch has been associated with greater milk protein content and production (DePeters and Cant, 1992). However, examination into the changes in milk protein composition in response to increased fermentable starch has yet to be studied. An increase in plasma glucose concentrations in response to increased dietary starch supply influences metabolic

pathways in CN synthesis (Rius et al., 2010; Arndt et al., 2015). Further research investigating how nutritional manipulation could be used to alter the bovine proteome may consider feeding brassicas that are vary in organic matter digestibility. For example, fodder beet is nutritionally dense brassica fed to lactating dairy with high organic matter digestibility, 9 % higher compared with kale (Jenkinson, 2013). A potential comparative study examining how milk protein expression is influenced by forage-based diets known to optimize N synchronization and maximize MCP provides an opportunity to measure the effects dietary energy has on mammary protein expression and advance understanding of long-term, diet-mediated effects on the bovine milk proteome.

Processing of a feed also can alter N partitioning in a lactating dairy cow by manipulating structural characteristics of the feed and changing the degree of protein digestibility within a rumen. Corn is an integral feed used in the dairy industry and can be fed to dairy cows in many different forms. Corn gluten feed is a byproduct from manufacturing of corn starch or syrup and corn distillers grain is the product from ethanol extraction. Corn gluten feed is considered to contain a high proportion of rumen digestible protein (RDP = 55%; RUP = 45% of % CP), whereas corn distillers grain typically contains a higher proportion of rumen undegradable protein (RUP = 65%; RDP = 35%, of CP). By formulating isonitrogenous and isoenergetic diets using these two feeds as the main sources of protein could provide an opportunity to alter N utilization patterns with a dairy cow. Similarly, varying alfalfa silage to corn silage ratios is an alternative method to alter N partitioning in a ruminant. Both feeds are major components in dairy cow rations in the USA, where AS typically has higher CP and lower starch content compared to CS (Arndt et al., 2015). Altering the proportions of each silage

between two diets results in changes in N partitioning patterns within a dairy cow. The diet containing high inclusion of corn silage is likely to maximize N synchronization within the rumen, maximizing microbial synthesis and shifting the rumen microbiome to produce more propionate, a gluconeogenic precursor. An increase in propionate concentration favors greater production of glucose by splanchnic tissue thereby, increasing plasma glucose levels (Santos, 2002). Together with altered substrate composition available to the MG and a change in hormonal regulation is likely to influence gene expression and protein synthesis activity of MEC. Insight into variables that impact the activity of MEC will provide the knowledge needed to understand how nutrition can be manipulated to strategically alter the bovine proteome.

4.2.3 Milk pasteurization

Bioactive proteins increase the nutritional value of milk however, future research needs to investigate the effect pasteurization has on the bovine proteome. Milk is pasteurized in the dairy industry to remove pathogenic microorganisms and prolong shelf life. Few studies highlight a decrease in a number of well-known bioactive proteins alongside a decrease in digestibility and reduced activity of specific milk proteins (Korhonen et al., 1998; Akinbi et al., 2010), whereas others suggest pasteurization has positive effects on the biological characteristics of milk (Wada and Lönnerdal, 2014, 2015). Quantitative proteomic techniques comparing the proteome in heat-treated and raw bovine milk is important research to be explored in order to understand how milk processing alters the bovine proteome and ultimately determine the protein profile available to the consumer. Many bioactive proteins present in mature milk are also present in colostrum at higher concentrations and in different proportions (Korhonen et

al., 1998). Heat treatment of colostrum is a method used on some dairy farms to reduce bacterial contamination and it has also been shown to enhance absorption efficiency of IgG present in colostrum. (Elizondo-Salazar and Heinrichs, 2009; Gelsinger et al., 2015). Hundreds of proteins and peptides are present in bovine colostrum, many associated with immunomodulatory functions (Szwajkowska et al., 2011) including immunoglobulin proteins that are considered to influence immune development in neonatal calves (Weaver et al., 2000). This type of research can lead to identification of the entire low abundance proteome in colostrum, provide insight into differences in protein fractions of colostrum before and after heat-treatment and highlight specific bioactive proteins that are directly involved in calf health and development. In addition, examination of the protein profile in colostrum provides further insight into the functional roles MEC are involved in during early post-partum lactation.

4.3 Conclusions

It is well recognized that milk proteins possess a high nutritive value and have diverse functional properties with potential to benefit human health. However, little is known about the regulatory mechanisms involved in the synthesis of these proteins within the MG. Longitudinal studies on the regulation of milk protein biosynthesis, gene expression and factors that influence milk protein synthesis within the MG will be essential to understanding of the synthesis of these key bioactive proteins.

A number of factors are known to influence the bovine proteome, breed and nutrition included. The effect of breed on the bovine milk proteome has been previously studied but limited to the composition of high abundance proteins and MFGM proteins.

The first study in this thesis involved investigation into the low abundance bovine skim-milk proteome between Holstein and Jersey dairy cows. Results confirmed significant differences in the high abundance protein profile between the two breeds but little differences in the low abundance profile were detected. However, the few low abundance proteins that were identified to exist at different abundance is important information to consider, particularly in future studies when making cross-breed comparisons of milk proteomes (Chapter 2). The results from this trial provided the foundation for the following study investigating the effects nutrition may have on the bovine proteome. Two diets were formulated, both isonitrogenous and isoenergetic, where each diet contained either a high RDP or high RUP ratio of % CP. A difference in RDP:RUP ratio was used in an attempt to alter whole-body N utilization patterns, in the rumen and post-nutrient absorption. By altering the partitioning of N within a ruminant provides an opportunity to examine how various metabolites influence the bovine milk protein composition, and more indirectly, protein synthesis regulation mechanisms within the MG. Results from this trial highlighted the potential to use dietary strategies to influence specific mammary-derived milk proteins as demonstrated by the change in the milk CN composition in response to dietary treatment. Exploration into the regulatory mechanisms of this interaction are needed to further understand the factors influencing milk protein synthesis within the MG in order to predict dietary effects on the milk protein profile.

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