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Examination Of Bovine Rumen Fluid And Milk Fat Globule Membrane Proteome Dynamics

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EXAMINATION OF BOVINE RUMEN FLUID AND MILK FAT GLOBULE MEMBRANE PROTEOME DYNAMICS

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Abstract

Proteomic technology has been increasingly incorporated into agricultural research, as characterization of proteomes can provide valuable information for potential biomarkers of health and physiological status of an animal. As dairy cattle are a dominant production animal in the USA, their biofluids such as milk, blood, urine, and rumen fluid have been examined by proteomic analysis. The research outlined herein was performed to further characterize the dynamics of specific proteomes and relate them to dairy cattle physiology.

The first experiment evaluated the diurnal dynamicity of the rumen metaproteome in Holstein dairy cattle. Rumen fluid was collected from three mid to late lactation multiparous dairy cattle (207 ± 53.5 days in milk) at three time points relative to their first morning offering of a total mixed ration (TMR) (0 h, 4 h, and 6 h after feeding). Samples were processed and labeled using Tandem Mass tagging before being further fractionated with a high pH reversed-phase peptide fractionation kit. Samples were analyzed by LC-MS/MS and statistically analyzed for variations across hour of sampling using the MIXED procedure of SAS with orthogonal contrasts. A total of 242 proteins were characterized across 12 microbial species, with 35 proteins identified from a variety of 9 species affected by time of collection. Translation-related proteins were correlated positively with increasing hour of sampling while more specific metabolic proteins were negatively correlated with increasing hour of sampling. Results suggest that as nutrients become more readily available, microbes shift from conversion-focused biosynthetic routes to more encompassing DNA-driven pathways.

The second experiment aimed to characterize the milk fat globule membrane (MFGM) proteomes of colostrum and transition milk for comparison from multi- (n = 10) and primiparous (n = 10) Holstein dairy cattle. Samples were collected at four timepoints post-partum (milkings 1, 2, 4, and 14). After isolation of the protein lysates from the MFGM, proteins were labeled using Tandem Mass tagging and analyzed using LC-MS/MS techniques. Protein identification was completed using MASCOT and Sequest in Proteome Discoverer 2.2. Protein abundance values were scaled and analyzed using the MIXED procedure in SAS to determine the effect of parity, milking number, and parity x milking number, and the adaptive false-discovery rate (FDR)-adjusted P values were determined using the MULTTEST procedure of SAS. There were 104 proteins identified within the MFGM. Statistical analysis revealed that 44.2% of proteins were affected by parity, 70.2% by milking number, and 32.7% by the variable of parity x milking number. There was a two-fold difference in calcium sensing S100 proteins in cows differing in parity possibly due to the multiparous mammary gland being more adapted to the physiological demand of lactation or the lesser requirement of calcium in primiparous cows because of a lower production rate.

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Dedication Page

I dedicate this thesis to my mother for leading me by example and for my father who always reminds me of my strength.

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List of Abbreviations

α-CN	α -casein
AN	Accession number
αs1-CN	αs1-casein
s2-CN	αs2-casein
α-LA	α -lactalbumin
β-CN	β-casein
β-LG	β-lactoglobulin
κ-CN	κ-casein
γ-CN	γ-casein
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
CN	Casein
d	Day
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
DMSO	Dimethyl sulfoxide
DNA	Deoxynucleic acid
DTT	Dithiothreitol
GO	Gene ontology
h	Hour
HPLC	High performance liquid chromatography
IgG	Immunoglobulin G
iTRAQ	Isobaric tags for relative and absolute quantitation
LC-MS	Liquid chromatography mass spectrometry
MFGM	Milk fat globule membrane
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PBS	Phosphate-buffered saline
RF	Rumen fluid
RIPA	Radioimmunoprecipitation assay
SEM	Standard error mean
SER	Smooth endoplasmic reticulum
TCA	Trichloroacetic acid
TMR	Total mixed ration
VFA	Volatile fatty acid

Chapter 1: Literature Review

Proteomics, the large-scale study of proteins that aims to both quantify and characterize protein profiles, has been a growing field in recent decades and is becoming increasingly utilized in research to advance the dairy industry. Milk is a biofluid that has been a common focus of experimental investigation because of its importance in both calf and human health. Milk itself contains a diverse and complex protein composition and could also prove to contain useful protein biomarkers that could provide insight into the underworkings of the cow's physiology. Other biofluids such as urine, plasma, semen, and rumen fluid are also being increasingly characterized using proteomic approaches to reveal potential biomarkers of health state. While rumen proteomics is still a developing tool, it displays the potential to fill in gaps regarding our understanding of microbial metabolism in the rumen, as well as substrate production and availability for absorption and animal use. This literature review explores current research involving proteomics in the agricultural field with a specific focus on milk and rumen fluid and discusses the challenges that accompany this research due to the complexity and dynamicity of these biofluids.

1.1 Proteomics and the dairy industry

While genomics has been useful, it does not account for post-translational modifications (PTM's), or other factors that impact phenotype. Thus, while the genome may reflect an extensive set of genes that may be transcribed and translated to active proteins, this tool cannot determine the actual protein profile or shifts in this profile due

to outside environmental factors. Because of the dynamicity of the proteome, it offers the potential to reveal biomarkers in tissues or biofluids for targeted biological states of animals. Broadly, proteomic capabilities are rapidly advancing as technology, resources and understanding grows, and the use of proteomics has proven to be a useful tool in studying metabolism and identifying biomarkers because protein expression can serve as metabolic indicators of pathway or transport shifts. Methodology for proteomic analysis of various biofluids is still developing as protein isolation and purification from different locations of collection will present challenges. Efficient sample collection and preservation, removal of interfering compounds, and accurate representation of proteins lower in abundance that may be concealed by highly abundant proteins in mass spectrometry analysis are just some examples of these methodology challenges. As these methods improve, especially fractionation related methods, characterization of the different biofluid proteomes has expanded. In addition, public databases such as PANTHER (Mi et al., 2019), Unitprot (Chen et al., 2017), and BLAST (Camacho et al., 2008) provide bovine-related proteins but these bioinformatic databases are still somewhat limited in their identification capacity for proteins within many of these bovine biofluids, and infringe a limitation of knowledge and interpretation.

1.1.1 Proteomic techniques and methods

Proteomic technology has been utilized on a variety of bovine biofluids to gain a better understanding of the ruminant animal's biology at different physiological states. This kind of research has been employed to reveal homeostatic regulation, uncover the undercurrents of physiological state, and identify potential biomarkers that could advance

medical diagnosis. Figure 1.1 provides an abbreviated general overview of some published proteomic methods for the listed biofluids to illustrate some differences in approaches. There are naturally occurring proteases in these biofluids and in order to have a proteome representative of that at the time of sampling, a rapid freezing protocol such as immersion in a dry-ice ethanol bath (Tacoma et al., 2016), should be included in the workflow to minimize any degradation of proteins in the biofluid. Fractionation methods have proved of the utmost importance in milk samples in order to purify the proteins from the fraction, as well as separate proteins to isolate lower abundance proteins from highly abundant proteins that can conceal lower abundance proteins during analysis (Greenwood and Honan, 2019). These biofluids are complex substances and thus isolating a pure protein fraction can be challenging. It is common to use centrifugation techniques (Manza et al., 2015) to obtain a general protein containing pellet or phase which can often be further purified using an acetone precipitation (Crowell et al., 2013). From there, washing methods to purify the protein fraction (Yang et al., 2016) along with lysis to release proteins, has proved to be effective (Luccitt et al., 2008). Various other techniques such as acidification, desalting, and ultrafiltration have been used as depicted in Figure 1.1 (Xia et al., 2012; Bathla et al., 2015). Further workflow that includes labeling and subsequent mass spectrometry (MS) analysis can be completed once the protein fraction of interest is isolated and purified thus making the results and bioinformatic analysis more focused. Mass spectrometry and bioinformatic workflow that occurs after protein isolation is similar across biofluid studies. The use of databases will be specific to the species from which the biofluid or protein fraction has been isolated.

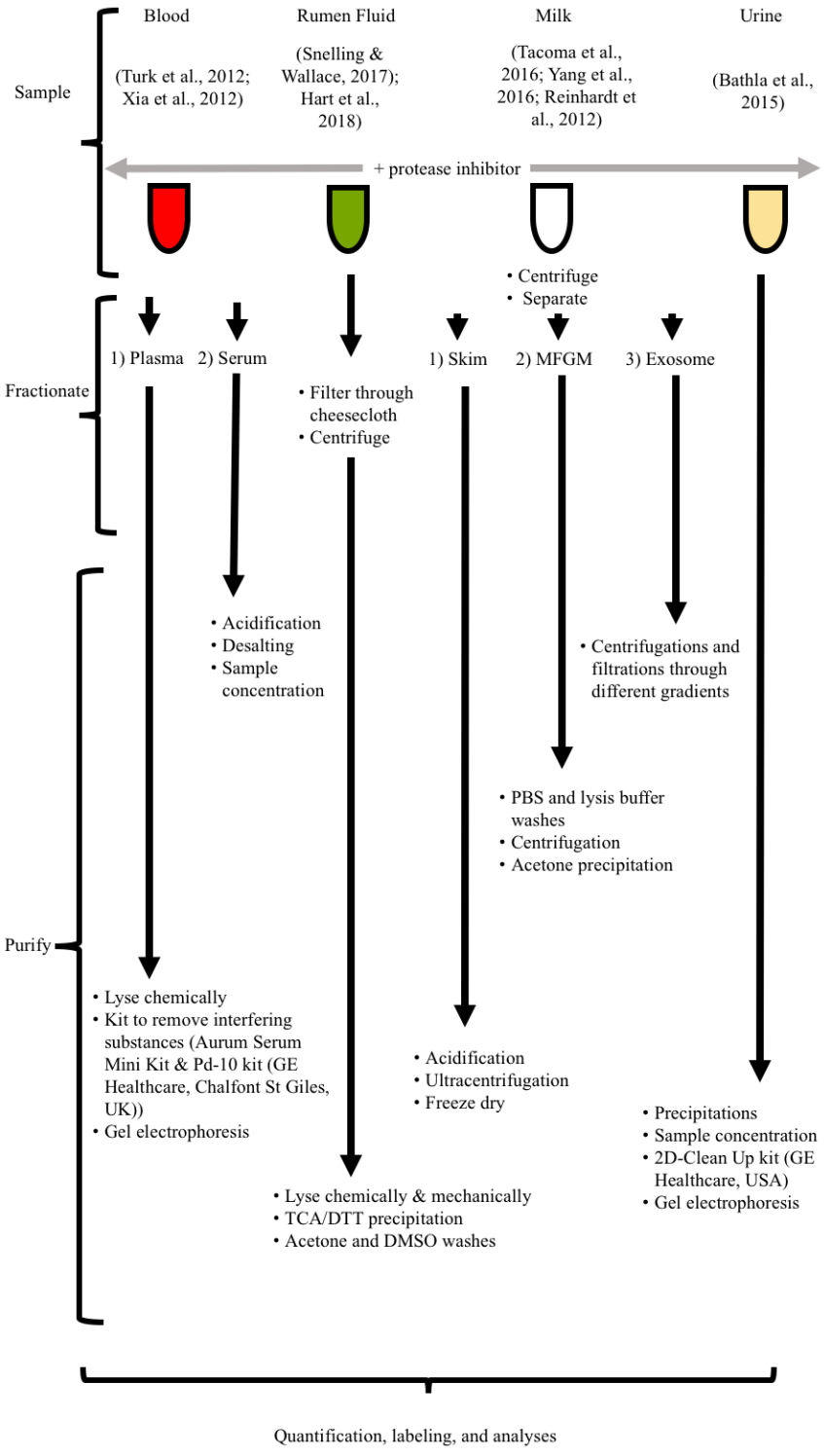

Figure 1.1. The general proteomic workflow adapted from published work for analysis of blood, rumen fluid, milk and urine.

1.1.2 Examples of proteomic analysis for bovine biofluid assessment to date

There has been research published investigating different proteomes of cattle including milk (O'Donnell et al., 2004; Reinhardt et al., 2013; Tacoma et al., 2016), blood and its components (Turk et al., 2012; Xia et al., 2012), rumen fluid (Snelling and Wallace, 2017; Hart et al., 2018), reproductive constituents (Zachut et al., 2016; Aslam et al., 2018), and urine (Bathla et al., 2015). Within agricultural reproduction, researchers (Zachut et al., 2016) have used proteomic methods to try and uncover potential biomarkers in the preovulatory follicular fluid of cows to increase reproductive efficiency. Zachut et al. (2016) identified 8 proteins of differential abundance in low fertility cows compared to high fertility cows which included SERPINA1 which is hypothesized to be a potential biomarker for polycystic ovary syndrome in humans (Ambekar et al., 2015). Milk proteomes have also been investigated to identify biomarkers to detect pregnancy in cows at fewer days of gestation than current detection tests (Johnston et al., 2018). Johnston et al. (2018) examined differences in the whey proteome from cows on d-21 of their estrous cycle and d-21 of pregnancy and found that there were four differentially expressed proteins and an additional 10 in extracellular vesicle enriched whey samples. Researchers highlighted these specific proteins as biomarker candidates: apolipoprotein B, perilipin, spermadhesin-1, the polymeric immunoglobulin receptor, sulfhydryl oxidase, mucin-1 and lymphocyte antigen 96.

Proteomic analysis of milk and/or blood can also serve as a potential diagnostic tool to elucidate systemic effects of diseases on the animal. Mastitis is a commonly occurring issue within dairy herds with both negative health and economic costs. In a trial

completed by Boehmer et al. (2008), *E. coli* –infected Holstein cows expressed several antimicrobial related proteins in the mastitic whey proteome that were differentially expressed in whey from non-infected cows. Researchers noted the increased abundance of serum albumin and serotransferrin in samples of milk collected from mastitic cows, supporting the idea that blood derived proteins leak into the mammary epithelial cells as this disease increases vascular permeability. However, the most promising shift was an increase in acute phase protein, α-1-acid glycoprotein, from mastitic cows, which also aligns with the idea that vascular leakage is occurring during mastitis. More recently, suggestion of milk biomarkers (cathelicidins), which are present in milk from cows with mastitis but are absent in milk from healthy cows, demonstrates the value and applicability of proteomics (Smolenski et al., 2011). Smolenski et al. (2011) confirmed that increasing concentration of cathelicidins was due to mastitis infection as it had a strong correlation with rising somatic cell count (SCC). In agreement to these findings, Reinhardt et al. (2013) found that there was an increase of cathelicidins in the whey fraction of milk but even more so in the MFGM portion. Apart from mastitis, metabolic disorders such as milk fever have also been studied through plasma proteomics. Xia et al. (2012) reported that blood coagulation (fibrinogen beta chain) and immune related proteins (IgG heavy-chain C-region) were lower in samples from cattle with milk fever compared to control cows. Additionally, the blood regulatory protein serpin peptidase inhibitor was higher in the plasma from cows with milk fever, highlighting the potential for blood-associated protein biomarkers of this disease. Identifying differential protein abundances due to disease provides a significant opportunity for noninvasive and accurate diagnostic measures to avoid elevated and unnecessary animal stress.

Parturition is a metabolically costly and physiologically stressful part of a dairy cow's life causing shifts in nutrient partitioning in order to accommodate lactation. Thus, the study of the subsequent mammary secretions after parturition and the different proteome fractions within the biofluids obtained post-partum have been a strong area of focus. Considering the importance of colostrum for calf nutrition, several studies have sought to characterize the protein profile, and incorporation of proteomic techniques has led to the identification of immune-associated bioactive proteins beyond immunoglobulins that may play an important role in calf health (Yamada et al., 2002). Aside from a calf health standpoint, the transition from colostrum to mature milk may also provide a more comprehensive understanding of the cow requirements and physiology. Given the metabolic disorders that often coincide with the delivery of a calf, further proteomic investigation of the milk proteome could provide insight to mitigate the detrimental effects of this demanding event by its ability to highlight nutrient and energy deficiencies experienced by the mammary gland.

The influence of diet on the milk proteome can potentially offer insight into diet-mediated metabolic shifts and what is being provided to the animal and subsequently the mammary gland, possibly influencing milk components that may influence the calf and consumer. For example, Li et al. (2014) investigated the effect of different processing methods of both corn grain and soybean meal on the milk proteome and found the difference in dietary supply of soybean meal altered specific proteins in the milk. β -casein, α-lactalbumin, and zinc-alpha-2-glycoprotein fragments were lower while αs_1-CN and αs_2-CN fragments were higher in the milk of cows fed heat treated soybean meal compared to those fed solvent extracted soybean meal. Additionally, inclusion of plants

with secondary compounds in ruminant diets has been used to explore limitations and abilities of rumen microbes through final outputs in the milk proteome (Scuderi et al., 2019). However, there is much need for speculation in these trials as there is limited evidence at how the rumen microbial community is processing the nutrients and what is being provided downstream for milk production. Rumen metaproteome analysis could, however, fill in these gaps.

Proteomic analysis of the rumen or rumen fluid is in its infancy due to the complexity of the microbial community, as feed-based compounds have impeded purification of samples. There have been two publications to date that have attempted characterizing the rumen metaproteome (Snelling and Wallace, 2017; Hart et al., 2018). Both of these studies found that dominating proteins stemmed from the bacterial phyla, *Bacteroidetes*. Hart et al. (2018) highlighted proteins that are essential to protein synthesis, such as elongation factor Tu and ribosomal proteins. Considering the reliance of the ruminant animal on microbial protein synthesis, gaining an understanding of the microbial protein metabolism is essential. Characterization in this regard may not accelerate the diagnostic field as rumen fluid is not typically an easily attainable biofluid. It can, however, bring a new level of understanding to metabolic processes and population dynamics which will be discussed further in this review.

Collectively, inclusion of proteomic techniques in the dairy industry have allowed for better characterization of the healthfulness of dairy products and is leading to a better understanding of animal physiology and biomarker development.

1.2 Current understanding of the bovine milk proteome

1.2.1 Milk protein fractions

One of the most extensively studied proteomes in the dairy industry has been milk. Milk proteins have been studied for over 60 years (O'Donnell et al., 2004), though, it was not until the early 2000's that the term "proteomics" began to be applied to milk; but it has been extensively studied since then using higher output approaches (Enza et al., 2005; Yamada et al., 2002). Milk is an attractive biofluid to be utilized for biomarker research because of its ease of collection. Whether cow-side or from a bulk tank sample, use of milk samples could improve our understanding of dairy cattle physiology.

In bovine milk, protein comprises approximately 3.0-3.8% (Larson, 1985). For a proteomic assessment, milk proteins are commonly classified as either high or low abundance proteins or are categorized based on their milk fraction which could be part of the skim (whey or serum) portion, exosomes, or milk fat globule membrane (MFGM). High abundance proteins are comprised of the caseins (αs_1-, αs_2-, β-, γ- and κ-casein (CN)) as well as a few proteins from the whey fraction (α-lactalbumin and β-lactoglobulin). These CNs represent 80% of bovine milk protein, while whey constituents, including both the high abundance and low abundance whey proteins, comprise the remaining 20% of milk protein (Madureira et al., 2007). Low abundance proteins are present in various fractions of milk; within the MFGM, skim milk fraction, and exosomes. These fractions can be separated through basic centrifugation yielding a floating fat layer, a skim fluid portion, and a pelleted exosome whose density causes a congregation at the bottom of a centrifuged sample.

The exosome fraction is interesting because it composed of cellular debris of endocytic origin which may help elucidate cellular pathways and intracellular communications that are occurring in the mammary gland upon lactation (Mathivanan et al., 2010; Reinhardt et al., 2012). Reinhardt et al. (2012) identified 2,107 proteins in the exosome fraction of milk and noted a number of immunological functions that were encompassed in the proteome, hypothesizing that some of these proteins could serve as indicators for level of immunity of a cow. Some common proteins that are found within the exosome profile include rab family proteins, integrins, xanthine oxidase, butyrophilin, adipophilin and lactadherin (Samuel et al., 2017).

While the whey proteome is mostly comprised of α-LA and β-LG in terms of number of peptides per protein, the whey proteome has been expanded to over 900 characterized proteins (Tacoma et al., 2016). Predominantly, α-LA plays a key role in lactose synthesis in the mammary gland (Brew et al., 1968). Meanwhile, β-LG accounts for over 50% of the protein content in the whey fraction and is known for its contribution to neonate immunity and as a potential carrier for bioactive molecules (Świątek et al., 2019). The removal of α-LA and β-LG has been established for optimal analysis of the broader whey proteome, and removal of these dominant whey proteins has allowed for extensive characterization of the broader whey proteome. A small metanalysis of gene ontological classifications generated by Greenwood and Honan (2019) ranked catalytic activity as the dominant molecular function (39.4-46.8%) of the whey proteome, followed by proteins with roles in binding (33-51.5%). Proteins that fall within these categories can be somewhat representative of what is occurring in the mammary gland

during milk synthesis. Additionally, these proteins may trigger some level of response in the gastrointestinal tract of the consumer through bioactivity.

1.2.2 Milk fat globule membrane (MFGM) proteome

While the MFGM represents a small fraction of the milk protein profile (1-4% of milk protein (Cavaletto et al., 2008), this protein fraction is bioactive-rich and unique, as the synthesis of the MFGM is centered around the envelopment of over 400 different fatty acids for secretion in milk (Månsson, 2008), with triacylglycerides being synthesized on the surface of the smooth endoplasmic reticulum (SER) in mammary epithelial cells (Mather and Keenan, 1998). The released droplet travels through to the apical membrane of the secretory cell, and the secreted milk droplet released into the alveolar lumen possesses a tri-layer protective membrane comprised of proteins and polar lipids (Heid and Keenan, 2005). Between that initial dissociation from the SER and the final dissociation from the apical membrane into the alveolar lumen providing a phospholipid membrane, cytosol components have been known to also be incorporated while the droplet migrates through the cell (Huston and Patton, 1990). The membranes that enveloped the droplet as well at the cytosolic crescents all contribute to the MFGM proteome. The MFGM droplet has a unique profile, and consistently includes several proteins at higher abundances, including adipophilin, lactadherin, xanthine dehydrogenase/oxidase, and the most abundant MFGM protein that comprises up to 40% of this fraction, butyrophilin (Vanderghem et al., 2008; Mather, 2000; Reinhardt and Lippolis, 2006). Considering these are the major proteins of the MFGM, bioactivity should be highlighted. The effect of consumption of butyrophilin, a type 1

transmembrane glycoprotein, has been studied in humans due to its possible effect on multiple sclerosis (Rasmussen, 2009; Guggenmos et al., 2004). It also possesses immune related properties (Abeler-Dörner et al., 2012). Xanthine dehydrogenase/oxidase exhibits antimicrobial properties through its production of hydrogen peroxide and reactive oxygen species (Martin et al., 2004). Rasmussen (2009) outlined that lactadherin was originally recognized for its presence in the MFGM but is also present in other locations. Lactadherin has the ability to remove apoptotic cells (Rasmussen, 2009) and has been noted to prevent rotavirus infections (Kvistgaard et al., 2004).

Many of the studies have expanded to comparing the MFGM proteome across cattle breeds and mammalian species (Ji et al., 2017; Lu et al., 2016; Yang et al., 2016), identifying the effects of pasteurization (Ma et al., 2019), and characterizing shifts through the entire course of lactation, which will be expanded upon further in this review.

1.2.3.1 Milking number and parity influence on MFGM

Examination of transition and mature milk using proteomic techniques has also led to the identification of thousands of low abundance proteins in milk (Tacoma et al., 2016). Colostrum has long been of special interest due to its unique composition in order to provide a neonate with their first nutrients and initiate growth and development of an immune system. It has been theorized that the size of the milk fat globule (MFG) influences the membrane composition and in humans, the MFG is at its largest within the first 12 h postpartum (Michalski et al., 2005). Colostrum is generally known for the high levels of immune related proteins in order to complete passive immunity for the calf. Research on the MFGM in bovine colostrum is somewhat limited; however, there are

studies that compared colostrum to milk collected at a later date which will be further expanded upon.

Transition milk is often characterized as the secretions bridging the colostral phase to mature milk. Oftentimes, the milk is studied as a static point in time, either as colostrum or mature milk. There is a great potential to characterizing the proteome through time post-partum within the same study for the benefit of being able to compare profiles within the same cow rather than trying to compare across animals of similar physiological state in possibly different environments. Immunoglobulins are a primary focus of colostrum and transition milk. There is not extensive work published about specifically transition milk proteomics likely due to the loose definitions of what is considered mature milk. However, there is research available that compares colostrum to mature milk, whether it could be considered pre-mature milk or not. Reinhardt and Lippolis (2008) compared the MFGM proteome of colostrum and milk from 7 days post parturition and found that proteins that have been noted for their bioactive properties such as xanthine dehydrogenase, butyrophilin, and adipophilin were upregulated in the latter milk samples. There are other non-MFGM reports on how colostrum transitions into early lactation milk from a proteomics standpoint. Zhang et al. (2016) examined the milk whey proteome over the first 9 days of lactation. It was found that some immune-related proteins decreased up to 40-fold by day 9 post parturition. Xanthine dehydrogenase, which was mentioned previously as an important MFGM protein, was found in increase in abundance as time from parturition increased.

Mature milk itself could be considered more important from a health biomarker perspective, as well as a human consumer standpoint, as mature milk is the dairy substrate used for dairy foods, and is the milk being produced for the majority of a cow's lactation. An earlier proteomic study of the MFGM of mature milk from mid-lactation completed by Reinhardt and Lippolis (2008) found that 71% of the identified proteins were membrane associated, leaving the remaining to be derivative of the cytoplasm (24%) and other contaminants from the whey fraction (5%). At the time of this study, the analysis of MFGM protein functions yielded that 23% be associated with cell signaling, 23% membrane/protein trafficking, and 21% with unknown functions. In the 11 years since this study, proteomic technology has advanced and knowledge has expanded and with that, more understanding of the MFGM proteome.

Compared to milking number, the effect of parity on the MFGM or other milk fractions has not been extensively studied. Dairy operations commonly have a mix of primi- and multi-parous cattle within the herd thus making it a physiological variable that could have pertinent impacts for either herd assessment or milk quality. While several metabolic differences are known to be influenced by parity, multiparous cattle are known to have higher milk yields than primiparous cattle due to higher rates of cell differentiation in the mammary gland (Miller et al., 2006). Given that the MFGM is derived from secretory cells within the mammary gland, it could serve as an excellent biomarker to assess mammary gland functionality and could be have differential profiles across parity. Differences in the MFGM membrane across parity can be speculated based on other research but investigating the interaction of milking number with parity could provide insight into the dynamics and adaptability of the mammary gland of these cattle.

Literature reported gives evidence that the MFGM proteome is sensitive to milking number along with the other fractions of milk. Many MFGM studies have focused on comparing the MFGM through time or across different species or breeds. Compared to the extensive examination of the whey proteome, known physiological influences on the MFGM are limited. Factors such as lactation number and health status still need to be further examined. Resulting effects of milking number paired with lactation number can provide more insight into the underworkings of the mammary gland.

1.3 Rumen metaproteomics

The rumen microbiota includes a diverse array of microbes, each with different characteristics and pathway dynamics but collectively complete the majority of feed breakdown in cattle. This section will expand on microbial populations established through genomic and transcriptomic data and the opportunities of employing proteomic methodology to rumen samples.

1.3.1 Microbial population and influencing factors

Bacteria serve as the most dominant population with approximately 10^9 -10^{10} (per mL of digesta) present in the rumen and can represent 40-90% of the rumen microbial biomass (Nagaraja, 2016). Protozoa represent 0-60% of the rumen microbial biomass, fungi represent 10%, and archaea represent 2-4% (Nagaraja, 2016). However, this microbial community needs to first establish in the rumen before making significant contributions to the animal. Li et al. (2012) compared the rumen microbiota using

metagenomic tools between 14- and 42-day old calves. Results confirmed that rumen development leads to a complex microbiota with 170 genera of bacteria being represented in the older calves compared to the 45 genera found in the younger calves. Notably, *Bacteroidetes*, increased in abundance from 45.7% to 75.8% in 14-d to 42-d old calves. KEGG pathways were also generated based on predicted open reading frames in DNA sequences and it was observed that certain metabolic pathways decreased in older calves such as purine metabolism and biosynthesis of the amino acids Phenylalanine, Tyrosine, and Tryptophan. However, Li et al. (2012) noted that the functionality of the population did not change considerably as the calf aged even though microorganism diversity increased. Overall, researchers concluded that pre-ruminant calves had sufficient microbial populations to perform needed metabolic tasks despite significant fluctuations. There continues to be research in weaning strategies and post-weaning diets in order to effectively accommodate the developing rumen to positively influence future production (Khan et al., 2011; Roth et al., 2009).

Basic physiological and management differences besides age has shown to influence the rumen microbiome, including breed and diet. Utilizing 16S rRNA gene sequencing, Paz et al. (2016) concluded that Holstein dairy cattle had greater bacterial richness compared to Jersey cows based on alpha diversity metrics, but both shared the four main phyla of *Firmicutes, Bacteroidetes, Proteobacteria*, and *Fibrobacteres.* More recently, to expose possible gaps in gene sequence findings and functionality, Li et al. (2019) compared metagenomic and metatranscriptomic data across three beef cattle breeds, Angus, Charolais, and Kinsella composite hybrid. It was found that the metagenomes of the species were grouped closely together. However, when examining

the metatranscriptomes, there was less clustering, insinuating variation across species is more prevalent at the RNA versus DNA level. Obstacles in methods and results prevented researchers from drawing conclusions regarding the functionality differences in the breeds but the metatranscriptome results still provided valuable composition data.

A significant amount of research has been invested into the influence of diet on the rumen microbiome. For example, Thoetkiattikul et al. (2013) analyzed how different delivery of fiber to the animal would affect the rumen microbiome. While all diet groups of Holstein-Friesian dairy cattle shared the same three dominant phyla of Bacteroidetes, Firmicutes, and Proteobacteria, cows fed a high starch diet had a higher abundance of family groups *Prevotellaceae* and *Flavobacteriaceae*, polysaccharide degrading bacteria. In contract, fibrolytic bacterial family groups *Lachnospiraceae*, *Ruminococcaceae*, and *Fibrobacteraceae,* were most abundant in cows fed a higher fiber diet. Another study investigating feed management style effects on the rumen microbiome families by de Menezes et al. (2011) found that pasture-fed cows had a higher prevalence of *Veillonelaceae* while cows fed TMR expressed a higher abundance of *Fibrobacteraceae* but no differences were found in protozoal populations. Understanding rumen functionality will provide substantial benefits when designing feed management plans and formulating diets. These reported community analysis studies are significant and novel, yet many species have a variety of metabolic pathways. There may be some microbial populations that are not differing in abundance due to diet shifts, however, their flexibility may allow them to target different nutrients, upregulate different metabolic pathways, and yield products that go undetected in this style of research. The adaptability of these rumen microbes and their metabolic pathways will be discussed further.

1.3.2 Pathways of rumen microbes

Generally, cellulolytic bacteria (*Fibrobacter succinogenes, Ruminococcus flavefaciens,* and *Ruminococcus albus)* will dominantly yield acetic acid (Russell, 2002; Nagaraja, 2016). A few examples of bacteria that prefer starch as their energy source, considered amylolytic bacteria, include *Prevotella ruminicola* and *Succinomonas amylolytica,* both of which can produce both acetate and propionate upon fermentation (Russell, 2002; Nagaraja, 2016). A microbe species such as *Selenomonas ruminantium* is another starch preferring species but has the ability to produce acetate, propionate, and butyrate (Russell, 2002; Nagaraja, 2016). For the nutrition of the animal, notable metabolic pathways can be generalized into energy obtaining- and protein synthesis-associated pathways.

1.3.3 Characterization of the rumen metaproteome

Metaproteomics is the appropriate term to use when studying the rumen protein profile because the rumen serves more as an ecosystem to microbial species rather than representing one organism (Wilmes and Bond, 2004). Currently, published research on the rumen metaproteome is limited, with only two publications that provide valuable insight of the possibilities that would come with further investigating the rumen metaproteome. Snelling and Wallace (2017) presented research examining dairy cattle, beef steer, lamb, and reindeer rumen metaproteomes. The yielded results for dairy cattle was vastly limited due to the possible presence of humic compounds from feed, impeding the gel techniques by co-precipitating with proteins. Despite additional fractionation techniques, bovine rumen fluid, unlike rumen fluid from some of the other ruminants,

was difficult to interpret. This was perceived to be due to either the high concentrate diet that dairy cattle were receiving or the sampling method. Regardless of that initial limitation, protozoan structural proteins such as actin, α-tubulin, and β-tubulin, were the most notably abundant proteins as revealed through LC-MS/MS. Many of the identifiable bacterial proteins were derived from the three dominating phyla, Bacteroidetes, Firmicutes and Proteobacteria, and more than 50% of the proteins identified fell under the phyla of Bacteroidetes, specifically from the species *Prevotella*. Some of the proteins identified are known to be involved in carbohydrate metabolism such as glycolysis. These proteins included glyceraldehyde-3-phosphate dehydrogenase, phosphoenolpyruvate carboxykinase, phosphoglycerate kinase and triosephosphate isomerase. *Prevotella* is a prime example of a rumen microbe that can execute multiple different metabolic pathways. Evidence has shown *Prevotella* being involved in degradation of proteins and peptides (Wallace et al., 1997), fiber (Koike et al., 2003), starch, hemicellulose (Russell, 2002), and pectin (Marounek, M. and Dušková,1999). With this diverse substrate profile, this microbe produces both acetate and propionate as the product VFA. Being able to identify proteins being synthesized by a microbial species such as *Prevotella*, would prove valuable as metabolic pathways of use could be identified.

More recently, Hart et al. (2018) conducted a similar experiment examining dairy cattle rumen fluid to link established metagenomic methodology with newer metaproteomic technology. In contrast to Snelling and Wallace (2017), results were dominated by bacterial species specifically Bacteroidetes, Firmicutes, and Proteobacteria which was in agreement with the taxonomic data from 16S rRNA studies. Results were

primarily organized on the basis of abundant protein families within each of the three cows used for the study. Some proteins that were classified as most abundant across cows were elongation factor Tu, glyceraldehyde-3-phosphate dehydrogenase, nitrogen-fixing protein NifU, succinate dehydrogenase, rubrerythrin, and a variety of 30s and 50s ribosomal proteins. The authors did not link which specific species these proteins were derived from, but it still confirmed that the rumen is a dynamic ecosystem completing numerous tasks simultaneously. Research done by Hart et al. (2018) also highlighted that microbes may function in diversified niches across rumen microbe species. Glycolysis, gluconeogenesis, protein biosynthesis and electron transport dominated the functionality of the proteins identified. In line with findings by Snelling and Wallace (2017), Bacteroidetes was the dominating phylum though these proteins that fell into these functional classes were found across phyla.

1.4 Conclusions

Proteomic methodology is robust enough to capture the dynamicity of animal biofluids. Because of these specific results that it yields, it has the ability to provide significant insight into how the animal is physiologically adapting at different time points to different stressors whether they be driven by physiological, metabolic, or environmental conditions. In addition to providing insight into regulation by the animal, the information provided can also assist in better caring for these animals and in turn, contribute to creation of higher quality products for consumption by the calf or humans. The research reported herein focuses on the effect of time and parity on the MFGM proteome and effect of time on the rumen metaproteome.

1.4.1. Hypothesis

Combining protein isolation techniques with newer isobaric labeling methods was hypothesized to be a feasible approach to broaden the scope of rumen metaproteome characterization (Chapter 2). It was further hypothesized that there would be daily fluctuations in rumen microbial protein abundances due to feed intake-mediated nutrient availability in lactating dairy cattle. It is also hypothesized that MFGM proteome will be affected by days in milk and parity, and that inclusion of isobaric labeling proteomic techniques can highlight potential shifts in this proteome.

1.4.2 Objectives

The objectives of the research presented herein include using non-gel based fractionation methods and isobaric labeling techniques to further the characterization of the rumen metaproteome within Holstein dairy cattle and identify whether diurnal variations in these profiles could be elucidated using this approach. An additional aim was to use similar techniques to characterize the proteome of the MFGM over a time period rather than at a static point for a more comprehensive understanding of transition milk composition.

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Chapter 2: Characterization of daily patterns within the rumen metaproteome of Holstein dairy cattle

2.1 Abstract

Few studies have utilized proteomic techniques to progress our knowledge of protein-mediated pathways within the rumen microbial community. Additionally, none of these studies used these techniques to investigate any patterns or variations of these proteins within this community beyond single time point samples. It was hypothesized that there would be diurnal fluctuations of rumen microbial protein abundances due to feed intake-mediated nutrient availability. This study investigated the fluctuations of bovine rumen metaproteome utilizing three mid to late-lactation Holsteins. Rumen fluid was collected at three time points throughout the day for three days relative to their first morning feed offering (0h, 4h, and 6h). Samples were pooled within timepoint within cow across day, analyzed using LC-MS/MS techniques, and analyzed for variations across hour of sampling using the MIXED procedure of SAS with orthogonal contrasts to determine linear and quadratic effects. A total of 242 proteins were characterized across 12 microbial species, with 35 proteins identified from a variety of 9 species affected by time of collection. Translation-related proteins such as 50S and 30S ribosomal protein subunit variants and elongation factors were correlated positively with hour of sampling. Results suggest that as nutrients become more readily available, microbes shift from conversion-focused biosynthetic routes to more encompassing DNA-driven pathways.

2.2 Introduction

The pregastric rumen is the dominant site of microbial colonization and microbe-mediated fermentation within the ruminant digestive tract, and functionality of this chamber is a key factor that dictates the animal's efficiency of nutrient utilization and production (Jami et al., 2014; Jewell et al., 2015; Li et al., 2015; Shabat et al., 2016). Nutrient utilization, ruminant animal health, and environmental emissions are all affected by rumen ecology, hence there is strong interest within the ruminant livestock sector to understand the functionality of rumen microbes. Current knowledge of the rumen microbiome is cross-disciplinary and rapidly expanding, with novel research emerging that is focused on diversity analysis and community structures of the microbiota (Weimer et al., 2010; Bainbridge et al., 2016; Schären et al., 2018; Belanche et al., 2019), as well as metabolic pathway analysis and metatranscriptomics (Morvay et al., 2011; Wang et al., 2012; Comtet-Marre et al., 2018; Niu et al., 2018).

Despite advances in our understanding, there is still a gap in knowledge regarding the undercurrents and interplay of microbe-specific metabolic pathways because of their dynamicity, adaptability, and complexity. Utilizing a variety of approaches to characterize the rumen in terms of microbial ecology and pathway dynamics appears to be necessary. For instance, microbial diversity analysis has revealed that basal diet and diurnal rumen pH patterns can be somewhat independent of bacterial community profile (Palmonari et al., 2010), while more recently, Söllinger et al. (2018) paired metabolomics with quantitative metatranscriptomics to assess the diurnal fluctuations of individual rumen microorganisms and also reported a dissociation between the functional microbial transcripts and microbiome pathway products such as methane. Layered within these

challenges, the central dogma of translation appears to be disjointed, with microbial RNA not reflective of protein abundances within the rumen, possibly due to post-translational modifications along with other adaptations (Hart et al., 2018; Söllinger et al., 2018; Chen et al., 2019). In addition, issues such as a limitation in analytic capabilities to discern in vivo complexities and variations due to external drivers such as endogenous and management influences, have slowed progress and the application of knowledge to commercial systems.

Proteomic techniques are now integrated in livestock research, with published applications in milk (Reinhardt et al., 2013; Yang et al., 2013; Tacoma et al., 2016), urine (Bathla et al., 2015; Xu et al., 2015; Rawat et al., 2016), plasma (Sun et al., 2013; Kinkead et al., 2015), and reproductive fluid (Forde et al., 2014; Zachut et al., 2016). Proteomic characterization of the rumen metaproteome includes unique challenges due to the multitude of residing organisms, but undoubtedly would yield valuable data bearing in mind the reliance of animal production on protein-mediated pathways and microbial protein production. Only two known previous works have been published that apply proteomic techniques to investigate the protein profile of the rumen, both utilizing gel techniques which may limit the number of proteins that can be identified (Snelling and Wallace, 2017; Hart et al., 2018). Using these techniques, Snelling and Wallace (2017) were able to identify 50 unique proteins in rumen fluid samples collected from beef cattle and lambs; however, protein identification in rumen fluid samples collected from grazing dairy cattle was not achieved due to obstruction of protein bands on the gels presumably by plant-based humic compounds. More recently, Hart et al. (2018) used gel-based techniques to examine the rumen metaproteome, and included successful techniques to

partially separate interfering contaminants, including humic acid, from the protein extract. Both publications provided valuable solutions to methodological challenges and a first glimpse of microbe-specific proteins in the rumen. Combining these protein isolation techniques with newer isobaric labeling methods was hypothesized to be a feasible approach to broaden the scope of rumen metaproteome characterization. It was further hypothesized that there would be daily fluctuations in rumen microbial protein abundances due to feed intake-mediated nutrient availability in lactating dairy cattle. The objectives of this experiment were to use non-gel based fractionation methods and isobaric labeling techniques to further the characterization of the rumen metaproteome within Holstein dairy cattle and identify whether variations in these profiles could be elucidated using this approach.

2.3 Methods

2.3.1 Animals and maintenance

Samples were collected from three lactating Holstein dairy cows (207 ± 53.5 days in milk) housed at the Paul R. Miller Research Complex (The University of Vermont, Burlington, VT). Cows were fed their dietary ration (Table 2.1) *ad libitum*. Cows were offered a total mixed ration twice daily (0630 h and 1430 h) and a diet supplement (high grain pellet) four times daily (0645 h, 1045 h, 1730 h, and 2300 h). All feed refusals were discarded prior to morning (0630 h) feeding daily. Cows had *ad libitum* access to water. Animal use and samplings methods performed in this trial were approved by the Institutional Animal Care and Use Committee (Protocol #16-029).

2.3.2 Rumen sampling

As per sampling protocols previously described (Khafipour et al., 2011; Hook et al., 2012; Steele et al., 2012; Latham et al., 2018), rumen fluid (RF) samples were collected from cows at 0630 h (0 h), 1030 h (4 h), and 1230 h (6 h) on day 1, 3, and 5 of a 5-day protocol. The 0 h samples were collected after morning refusals were collected but immediately prior to initial TMR offerings.

As per methods outlined by Steele et al. (2012), digesta grab samples were collected at each timepoint via a rumen cannula from beneath the fiber mat within the ventral sac of the rumen. A minimum of three digesta grab samples were collected per cow at each sampling point for a single representative 15 mL sample per timepoint. Samples were immediately snap frozen in a dry-ice ethanol bath, transported on dry ice to the laboratory, and stored at -80 ℃ until processing.

2.3.3 Rumen sample processing

For processing, RF samples were thawed overnight at 4 ℃. Once thawed, samples were filtered through 4 layers of cheesecloth (Lion Services Inc., Charlotte, NC), and filtered samples were composited within cow within timepoint across day for a representative sample of 0 h, 4 h, and 6 h for each cow prior to freezing at -80 ℃. For lysis, the composited filtered samples were thawed on ice and centrifuged at 16,000 x *g* for 20 min at 4 ℃. The resulting supernatant was discarded, and the remaining pellets were retained.

The collected pellets were lysed using both chemical and mechanical lysis methods based on both Snelling and Wallace (2017) and Yu and Morrison (2004) with

modifications. Briefly, 1.5 mL of radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor (Pierce™ Protease Inhibitor Mini Tablets, Thermo Scientific) and a 5 mm stainless steel bead (Qiagen, Hilden, Germany) was added to each pelleted sample and samples were homogenized (TissueLyser II, Qiagen) through six repetitions of lysis at 30 Hz for 30 seconds with a 3-min incubation on ice between each repetition, similar to the BeadBeater protocol reported by Luccitt et al. (2008). A subsample of each sample homogenate was pipetted into a clean tube and precipitated overnight at 4 ℃ in a lysis solution (6 M TCA, 80 mM DTT) (3:1 protein extract to TCA/DTT) based on the protocol outlined by Snelling and Wallace (2017).

Following the overnight incubation, the samples were vortexed and centrifuged at 16,000 x *g* for 20 min at 4 ℃ and supernatants were discarded. The retained pellets were then washed four times as per methods of Snelling and Wallace (2017) with modifications by Song et al. (2012), where the retained pellets were washed in ice-cold 20% DMSO in acetone, incubated for 1 h at -20 ℃, and centrifuged at 10,000 x *g* for 5 min at 4 ℃. The supernatants were then discarded, and the pellets were again washed using the same protocol. This wash protocol was then repeated twice more using 100% ice-cold acetone. After the final wash, the collected pellets were air dried and resuspended in phosphate buffered saline. A new 5 mm stainless steel ball was added to each sample before samples were homogenized in the TissueLyser for 30 seconds at 30 Hz. A universal control (UC) sample was generated by combining equal volumes from each of the 9 samples. Samples were stored at -80 ℃ until protein quantification of samples was performed using the bicinchonic acid assay (BCA) kit (Pierce, Rockford, IL).

2.3.4 TMT isobaric labeling, high pH reversed-phase peptide fractionation and Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS).

Quantified samples (85 µg) were then labeled using TMT Isobaric Tags as per manufacturer instructions (Thermo Scientific, Rockford, IL). Equal volumes (75 µL) of each TMT-labeled sample was combined into a new tube and a 100 µL aliquot was vacuum dried to remove the triethyl ammonium bicarbonate (TEAB). The peptides were then fractionated using the high pH reversed-phase peptide fractionation kit (Thermo Scientific, Rockford, IL) as per kit instructions resulting in 8 fractions for LC-MS/MS per original sample. One-tenth of each of the fractionated samples was dried down and resuspended in 2.5% formic acid in water and 2.5% acetonitrile (CH_3CN). The LC-MS/MS analysis was carried out on the Q-Exactive Plus mass spectrometer coupled to an EASY-nLC 1200 (Thermo Scientific, Waltham, MA). Peptides were separated using a gradient of 2.5-35% CH_3CN/0.1% FA over 60 min, 35-100% CH_3CN/0.1% FA in 1 min and then 100% CH_3CN/0.1% FA for 4 min, followed by an immediate return to 2.5% CH_3CN/0.1% FA and a hold at 2.5% CH_3CN/0.1% FA. The nanospray and data acquisition methods were completed per Scuderi et al. (2019). Briefly, samples were loaded onto a 100 µm x 500 mm capillary column packed with Halo C18 (2.7 µm particle size, 90 nm pore size, Michrom Bioresources, CA) at a flow rate of 300 nL min^{-1}. The column end was laser pulled to a ~3 µm orifice and packed with minimal amounts of 5um Magic C18AQ before packing with the 3-µm particle size chromatographic materials. Peptides were introduced into the mass spectrometer via a nanospray ionization source with a spray voltage of 2.0 kV. Mass spectrometry data were acquired in a data-dependent "Top 10" acquisition mode with lock mass function activated (*m/z* 371.1012;

use lock masses: best; lock mass injection: full MS), in which a survey scan from *m/z* 350-1600 at 70, 000 resolution (AGC target $1e^6$; max IT 100 ms; profile mode) was followed by 10 higher-energy collisional dissociation (HCD) tandem mass spectrometry (MS/MS) scans on the most abundant ions at 35,000 resolution (AGC target $1e^5$; max IT 100 ms; profile mode). MS/MS scans were acquired with an isolation width of 1.2 *m/z* and a normalized collisional energy of 35%. Dynamic exclusion was enabled (peptide match: preferred; exclude isotopes: on; underfill ratio: 1%).

2.3.5 Data and statistical analysis

Product ion spectra were searched using SEQUEST and Mascot through Proteome Discoverer 2.2 (Thermo Scientific, Waltham, MA) against composite databases downloaded on Nov. 30, 2018 and Jan. 29, 2019, including *E. ruminantium* (UP000189857), *L. ruminis* (UP000001279), *T. bryantii* (UP000182360), *T. saccharophilum* (UP000003571), *F. succinogenes* (3AUP000000517), *M. barkeri3* (3AUP000033066), *M. elsdeniiDSM20460* (3AUP000010111), *O. formigenesHOxBLS* (3AUP000003973), *R. albusSY3* (3AUP000021369), *R. flavefaciens007c* (3AUP000019365), and *W. succinogenes* (3AUP000000422). All 9 raw files were searched against the database as one contiguous input file. Search parameters were as follows: (1) full trypsin enzymatic activity; (2) mass tolerance at 10 ppm and 0.02 Da for precursor ions and fragment ions, respectively; (3) dynamic modifications: oxidation on methionine (+15.995 Da); (4) dynamic TMT6plex modification on N-termini and lysine (+229.163 Da); and (5) static carbamidomethylation modification on cysteines (+57.021 Da). Percolator node was included in the workflow to limit the false positive (FP) rates

to less than 1% in the data set. The relative abundances of TMT labeled peptides were quantified with the Reporter Ions Quantifier node in the Consensus workflow and parameters were set as follows: (1) both unique and razor peptides were used for quantification; (2) Reject Quan Results with Missing Channels: False; (3) Apply Quan Value Corrections: False; (4) Co-Isolation Threshold: 50; (5) Average Reporter S/N Threshold = 10; (6) "Total Peptide Amount" was used for normalization and (7) Scaling Mode was set "on Control Average", so that the peptide abundances in the UC labels were set as 100 and the abundances in other channels were scaled accordingly. The scaled abundance values were used for subsequent statistical analyses. For any proteins identified that remained "uncharacterized", either PANTHER Classification System (Mi et al., 2017) was used to identify to protein name using the accession number, or the FASTA sequence was retrieved from UnitProt (Chen et al., 2017) and searched through BLAST (Camacho et al., 2008) and the top hit protein was selected as the protein ID. The IML procedure in SAS was used to determine appropriate weighting for orthogonal contrasts to determine linear and quadratic effects of time. The MIXED procedure of SAS was then utilized with time as a fixed effect, cow as a random effect, and protein as the random variable. Effect of time of sampling was deemed significant if $P < 0.05$.

2.4 Results

Metaproteomic analysis using the outlined protocol resulted in identification of 255 proteins across 12 microbial species. Using Proteome Discoverer 2.2, the proteins from the following microbial species and their strains were identified: [*Eubacterium*] *cellulosolvens* 6, *Eubacterium ruminantium*, *Fibrobacter succinogenes* (strain ATCC

19169 / S85), *Lactobacillus ruminis* (strain ATCC 27782 / RF3), *Megasphaera elsdenii* DSM 20460, *Methanosarcina barkeri* 3, *Oxalobacter formigenes* HOxBLS, *Ruminococcus albus* SY3, *Ruminococcus flavefaciens* 007c, *Treponema bryantii*, *Treponema saccharophilum* DSM 2985, and *Wolinella succinogenes* (strain ATCC 29543 / DSM 1740 / LMG 7466 / NCTC 11488 / FDC 602W). Of the 255 proteins identified across all species, 242 were quantified for downstream statistical analysis and bioinformatics (Table 2.2). Results were grouped and interpreted based on abundance pattern shifts relative to morning feeding (0630 h) with LOW0 representing proteins that demonstrated an increase in abundance either quadratically or linearly relative to initial sampling (0 h) and HIGH0 representing proteins that began with a higher abundance at 0 h and had either a linear or quadratic decrease in the latter sampling points at 4 h and 6 h.

Of the quantified proteins, there were 35 proteins (Table 2.3) across 9 microbial species that were affected by time of sampling, including 85.7% that responded linearly, while the remaining 14.3% followed a quadratic pattern of change.

2.4.1 Microbial proteins with an increase in abundance from 0h to 4h

There were 8 microbial species with proteins represented in this group (LOW0; Table 2.3): *E. cellulosolvens, E. ruminantium, M. elsdenii, M. barkeri, O. formigenes, R. albus, R. flavefaciens,* and *T. saccharophilum.* The majority (54.3%) of proteins that were affected by time of sampling had lower abundances at 0 h compared to 4 h. As represented in Figure 2.1, seven of the LOW0 proteins were different variants of the 50s ribosomal protein (L14 and L16, *R. flavefaciens*; L21 and L22, *E. ruminantium;* L33 and L7/12, *E. cellulosolvens*; and L6, *R. albus*), while shifts in abundance of individual 30s

ribosomal proteins (S11, S3, and S9) from *E. cellulosolvens, O. formigenes,* and *R. albus*, respectively, were also grouped into LOW0 based on their abundance patterns. Elongation factor proteins of *E. cellulosolvens* (elongation factor Tu) and *R. flavefaciens* (elongation factor G) were lower in abundance at 0 h compared to 4 h. Other proteins that were lower in abundance at 0 h compared to 4 h that were unique to a specific species were as follows: ATP-dependent Clp protease ATP-binding subunit ClpX (*E. cellulosolvens*), ATP-dependent zinc metalloprotease FtsH (*T. saccharophilum*), cell division protein FtsH (*M. barkeri*), DNA-directed RNA polymerase subunit alpha (*R. flavefaciens*), formate-tetrahydrofolate ligase (*M. elsdenii*), and translation initiation factor IF-2 (*R. albus*).

2.4.2 Microbial proteins with a decrease in abundance from 0h to 4h

There were 6 microbial species represented in HIGH0 group: *E. cellulosolvens*, *E. ruminantium*, *F. succinogenes*, *R. albus*, *R. flavefaciens*, and *T. saccharophilum*. No identified proteins from three of the species included in analysis, *M. elsdenii*, *M. barkeri*, or *O. formigenes*, were higher at 0 h compared to 4 h. The functional groupings of proteins that had higher abundances at 0 h compared to 4 h (45.7% of proteins affected by time of sampling) are listed in Figure 2.2. Cysteine synthase from both *T. saccharophilum* and *F. succinogenes*, as well as pyruvate, phosphate dikinase from both *E. cellulosolvens* and *R. flavefaciens*, were all higher in abundance at 0 h compared to 4 h. There was a single 50s ribosomal protein (from *E. ruminantium*) that exhibited a decrease in abundance from 0 h to 4 h. Other proteins that decreased in abundance from 0 h to 4 h included 3-hydroxyacyl-CoA dehydrogenase (*E. cellulosolvens*), GGGtGRT

protein and phosphoenolpyruvate carboxykinase (ATP) (*E. ruminantium*), DNA-binding protein HU (*F. succinogenes*), phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and triosephosphate isomerase (*R. albus*), twitching motility protein pilT and nitrogen-fixing protein NifU (*R. flavefaciens*), O-acetylhomoserine sulfhydrolase and monosaccharide ABC transporter substrate-binding protein (*T. saccharophilum*).

2.5 Discussion

Understanding microbial metabolism is a crucial step in the development of strategies to support and sustain maximal nutrient use efficiency in the rumen. Inclusion of proteomic techniques to articulate the underlying shifts in the rumen metaproteome is in its infancy, but gel-based protein fractionation from samples collected in static points of time highlight the breadth of protein identifications that can be achieved (Snelling and Wallace, 2017; Hart et al., 2018). Using a combination of rumen-specific fractionation protocols with isobaric labeling techniques, the research reported herein is the first to outline the potential dynamic diurnal range of the rumen microbial metaproteome. Compared to previous research, this trial was able to identify and characterize proteins from 12 microbial species. Snelling and Wallace (2017) were unable to characterize the metaproteome of grazing dairy cattle due to impeding compounds on gels; however, they were able to distinguish 50 unique proteins derived from other ruminants such as lambs and beef cattle. Hart et al. (2018) highlighted the 25 most common protein families found within each individual cow and discussed phyla dominance but did not delineate proteomes of specific microbes.

In the present study, there were 41 variants of ribosomal proteins characterized within this research (14 30S and 27 50S) across 10 microbial species and is the most represented protein in this study. Approximately 20% of bacterial dry weight is made up of ribosomal proteins so it is not unexpected to see that these proteins are the majority of identified proteins (Russell, 2002). These ribosomal proteins are subunits within a larger bacterial ribosome that serves in mRNA translation to protein, and each subunit serves a specific function. The smaller 30s subunit decodes the mRNA strand while the larger 50s subunit assists in peptide bonding of the specified amino acids. Another protein involved heavily in the central dogma, elongation factor Tu, was also a widely represented protein being characterized within 8 microbial species. This protein contributes to the continuous process of sequentially adding amino acids to a peptide chain. Overall, nearly all LOW0 proteins are translation-related. Ribosomal proteins (50s and 30s) and their associated variants were the most commonly affected proteins across sampling time with 26.8% of identified ribosomal proteins being affected by time of sampling, and 90.9% of the affected ribosomal proteins being represented in LOW0. Elongation factors (G, *R. flavefaciens*; Tu, *E. cellulosolvens*) showed an increase in abundance relative to 0h which also placed them in the LOW0 group. While only expressed in one species, it is worth noting the increase in translation initiation factor IF-2 (*R. albus*) as it is an additional translation-related protein that expressed change. The large presence of proteins involved in protein-biosynthesis is in agreement with findings by Hart et al. (2018) who found elongation factor Tu to be the most highly abundant protein in their findings.

To interpret the results, the results were broadly partitioned into two conceptual categories, the first category being the above discussed general pathways of transcription

and translation. The second category includes proteins with more specific roles in metabolic pathways. While it can be difficult to draw conclusions regarding the substrate or environmental drivers causing an increase in proteins related to translational processing beyond simply surmising that protein synthesis is likely increasing, there were many proteins identified in the current study that are key players in many specific metabolic pathways, highlighting likely shifts in more specific rumen functionality. In contrast to proteins grouped in LOW0, in HIGH0 there was largely a lack of representation of 30s/50s ribosomal, elongation factors, and other translation-related proteins. Instead, there were shifts in proteins with more targeted functions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) demonstrated a linear increase in *R. albus* specifically, while the abundance of this protein in 7 other species was not affected by sampling period. GAPDH is multifunctional but commonly noted for its role in glycolysis. While this may suggest that *R. albus* was requiring more energy to perform needed functions, *E. cellulosolvens* and *R. flavefaciens* both displayed an identical pattern except with pyruvate, phosphate dikinase, a protein involved in gluconeogenesis. These results highlight the concept that rumen microbiota are independently reactive to their environment but yet are in synchrony with each other. Investigation of only the substrates or products within the rumen would not have likely yielded a comprehensive understanding of the individual microbial activities including the extent of synchronicity or similarity in protein profile across the different species within the rumen.

Cumulatively, the results reported herein suggest that in times of limited nutrient intake the microbes focus and prioritize their functions to conversion-focused biosynthetic routes, or low-energy requiring pathways, rather than replication, which has

an implication on nutritional outcomes due to feeding management strategies in dairy production systems. A first consideration from the results is that the identified protein shifts illustrate that feeding protocol- and bunk management- induced fasting periods may quickly limit potential productivity of microbes. While the cows were fed *ad libitum* in the current trial, it was apparent that the protein-mediated pathways were less diverse at the sampling point immediately prior to the offering of fresh feed in the morning (0 h) relative to after feeding (4 h). These results provide insight into the pathways and microbes more readily impacted by substrate-mediated suppression of microbial protein synthesis during non-eating periods (Martineau et al., 2011), and the impact that it may have on total microbial biomass production in the rumen and the consequent intestinal supply of microbial protein (Schwab and Broderick, 2017).

Another aspect of rumen function highlighted from the current results is that nutrient deprivation may expose the fundamental metabolic pathways of specific microbes. Identifying the proteins in HIGH0 may give insight into pathways that specific microbes deem vital. As highlighted by Weimer (2015), identifying and exploiting the roles of independent microbes or groups of microbes is a current challenge. The inclusion of proteomics in study methodology can more broadly highlight pathways affected by a treatment or physiologic state rather than focusing on a small set of parameters to directly or indirectly assess ruminal changes and can identify metabolic shifts that do not result in a change in microbial diversity. The use of dietary models in combination with these proteomic techniques is also proposed to be a feasible method in order to better identify the basic roles of the rumen microbes, and what protein-mediated pathways the different microbes divert to in different nutrient scenarios or feed management protocols.

This research is the first published work to report the rumen metaproteome beyond static points in time and demonstrates how proteomic technology can provide a meaningful contribution to the characterization of microbial activity and protein-mediated pathway dynamics. The trial reported herein demonstrates that the rumen protein profile is dynamic and appears to be sensitive to lower nutrient availability. Evidence of shifts in the ruminal protein profile to favor low energy and/or vital pathways after a period of low feed intake highlights possible mechanisms to either improve total microbial synthesis or target more specific protein-mediated pathways. Inclusion of proteomic technology to characterize the rumen metaproteome and the impact of diet, health, and environment on rumen functionality can provide a useful contribution and further advance our research to maximize production efficiency.

Table 2.1 General nutrient composition of the total diet

Nutrient or Parameter	Value
Dry Matter (DM), %	43.1
Crude Protein, % of DM	17.38
Acid Detergent Fiber, % of DM	16.54
Neutral Detergent Fiber, % of DM	30.48
Non-fiber Carbohydrates, % of DM	38.8
Crude Fat, % of DM	5.22
Ash, % of DM	8.16
Net Energy of Lactation, Mcal/kg	1.75

Table 2.2 Protein name and accession number of the 242 proteins that were characterized across 12 rumen microbial species in filtered (after thawing) rumen grab samples collected from Holstein dairy cattle at three timepoints relative to morning feeding.

Description	Accession Number	Species
2-dehydro-3-deoxygluconokinase	A0A1T4N496	E. ruminantium
2-ketoisovalerate ferredoxin reductase	W7UX66	R. flavefaciens 007c
2-oxoglutarate oxidoreductase	W7UDR0	R. flavefaciens 007c
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	C3X5K6	O. formigenes HOxBLS
3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	Q7MAS2	W. succinogenes DSM 1740
3-hydroxyacyl-CoA dehydrogenase	I5AR58	E. cellulosolvens 6
30S ribosomal protein S11	I5AVX2	E. cellulosolvens 6
30S ribosomal protein S12	I5AT56	E. cellulosolvens 6
	A0A011UYT2	R. albus SY3
30S ribosomal protein S19	I5AVU9	E. cellulosolvens 6
	G0VNH2	M. elsdenii DSM 20460
30S ribosomal protein S2	G2SND3	L. ruminis strain ATCC 27782
30S ribosomal protein S21	A0A1H5RWD6	E. ruminantium

30S ribosomal protein S3	C3X6H7	O. formigenes HOxBLS
	A0A1T4NKM7	E. ruminantium
30S ribosomal protein S5	H7ENC7	T. saccharophilum DSM 2985
	G0VNI5	M. elsdenii DSM 20460
	W7UV60	R. flavefaciens 007c
30S ribosomal protein S8	A0A011VVC5	R. albus SY3
30S ribosomal protein S9	A0A011WRM0	R. albus SY3
50S ribosomal protein L1	A0A1T4PP09	E. ruminantium
50S ribosomal protein L14	Q7M8E3	W. succinogenes DSM 1740
	W7UV65	R. flavefaciens 007c
50S ribosomal protein L15	W7UMC4	R. flavefaciens 007c
	C9RR15	F. succinogenes strain ATCC 19169
	C3X6J0	O. formigenes HOxBLS
50S ribosomal protein L16	A0A1T4NL93	E. ruminantium
	A0A011UF00	Ruminococcus albus SY3

	W7UWH6	R. flavefaciens 007c
50S ribosomal protein L18	I5AVW1	E. cellulosolvens 6
50S ribosomal protein L19	A0A011UI62	Ruminococcus albus SY3
50S ribosomal protein L2	I5AVU8	E. cellulosolvens 6
50S ribosomal protein L20	W7UGP4	R. flavefaciens 007c
50S ribosomal protein L21	A0A1T4M4Z7	E. ruminantium
50S ribosomal protein L22	A0A1T4NKS3	E. ruminantium
50S ribosomal protein L27	A0A1T4M509	E. ruminantium
50S ribosomal protein L3	A0A011V166	R. albus SY3
	I5AVU5	E. cellulosolvens 6
50S ribosomal protein L33	I5ATH8	E. cellulosolvens 6
50S ribosomal protein L4	A0A011VX58	R. albus SY3
50S ribosomal protein L5	A0A1T4NK00	E. ruminantium
	W7UMC8	R. flavefaciens 007c
50S ribosomal protein L6	A0A011V181	R. albus SY3
50S ribosomal protein L7/L12	I5ATH2	E. cellulosolvens 6
	G0VM61	M. elsdenii DSM 20460
	W7UH98	R. flavefaciens 007c
	H7ENF0	T. saccharophilum DSM 2985

6-phosphogluconate dehydrogenase, decarboxylating	G2SPQ0	L. ruminis strain ATCC 27782
60 kDa chaperonin	A0A011VWT5	R. albus SY3
	I5AUV5	E. cellulosolvens 6
	A0A1T4PC16	Eubacterium ruminantium
	G0VPW5	M. elsdenii DSM 20460
	W7UBJ0	R. flavefaciens 007c
ABC superfamily ATP binding cassette transporter, ABC protein	G2SMF4	L. ruminis strain ATCC 27782
ABC transporter substrate-binding protein	A0A011WR91	R. albus SY3
ABC-type glycerol-3-phosphate transport system, substrate-binding protein	A0A1H9JMR0	T. bryantii
ABC-type sugar transport system, periplasmic component	I5AUR0	E. cellulosolvens 6
Aconitate hydratase	A0A1H9I114	T. bryantii
Acyl-CoA dehydrogenase	A0A1T4JZ88	E. ruminantium
	G0VLZ7	M. elsdenii DSM 20460

Adenosylhomocysteinase	C3X5Q1	O. formigenes HOxBLS
AICAR transformylase/IMP cyclohydrolase PurH	I5AW15	E. cellulosolvens 6
Aldehyde-alcohol dehydrogenase	H7EPH6	T. saccharophilum DSM 2985
Alpha-1,4 glucan phosphorylase	I5AUH4	E. cellulosolvens 6
Argininosuccinate synthase	I5ASZ4	E. cellulosolvens 6
Asparagine--tRNA ligase	A0A1H9CBX1	T. bryantii
Aspartate-semialdehyde dehydrogenase O	A0A1T4MGX1	E. ruminantium
ATP synthase subunit alpha	Q7MA20	W. succinogenes DSM 1740
	A0A1T4KE00	E. ruminantium
ATP synthase subunit beta	A0A011WQV4	R. albus SY3
	A0A1T4LDC1	E. ruminantium
	C3X5Y9	O. formigenes HOxBLS
ATP-dependent Clp protease ATP-binding subunit ClpX	I5ASS5	E. cellulosolvens 6
ATP-dependent zinc metalloprotease FtsH	H7EMZ4	T. saccharophilum DSM 2985

ATPase component of ABC-type sugar transporter	I5AUV7	E. cellulosolvens 6
Branched-chain amino acid aminotransferase	A0A1T4PNX1	E. ruminantium
Carbohydrate ABC transporter ATP-binding protein, CUT1 family	A0A1T4L550	E. ruminantium
	H7EH79	T. saccharophilum DSM 2985
Cell division protein FtsH	A0A0E3SMD8	M. barkeri 3
Chaperone protein ClpB	I5ATK3	E. cellulosolvens 6
	C3X252	O. formigenes HOxBLS
Chaperone protein DnaK	G0VQE6	M. elsdenii DSM 20460
	W7UMY7	R. flavefaciens 007c
	I5AUT4	E. cellulosolvens 6
	A0A011UWY5	R. albus SY3
Chaperone protein HtpG	G0VLS1	M. elsdenii DSM 20460
Cobalamin biosynthesis protein CobW	A0A011V1V0	R. albus SY3
Cold shock protein	I5ASJ1	E. cellulosolvens 6

Cysteine synthase	C9RQ00	F. succinogenes strain ATCC 19169
	A0A1H9EB54	T. bryantii
	H7ELA1	T. saccharophilum DSM 2985
DNA-binding protein HU	C9RNK1	F. succinogenes strain ATCC 19169
DNA-directed RNA polymerase subunit alpha	I5AVX4	E. cellulosolvens 6
	A0A011WQD4	R. albus SY3
	A0A1T4NIV2	E. ruminantium
	W7UFQ9	R. flavefaciens 007c
DNA-directed RNA polymerase subunit beta	W7UVJ6	R. flavefaciens 007c
	A0A1T4PHL8	E. ruminantium
	G0VNF9	M. elsdenii DSM 20460
	W7UEJ2	R. flavefaciens 007c
dTDP-glucose 4,6-dehydratase	C9RNE2	F. succinogenes strain ATCC 19169
DUF323 domain-containing protein	H7EM78	T. saccharophilum DSM 2985
Electron transfer flavoprotein alpha subunit apoprotein	A0A1T4JZ74	E. ruminantium

Electron transfer flavoprotein beta subunit	A0A1T4JZ78	E. ruminantium
Elongation factor G	I5AT54	E. cellulosolvens 6
	A0A011WMW4	R. albus SY3
	W7UWA8	R. flavefaciens 007c
Elongation factor Ts	A0A1T4MF54	E. ruminantium
Elongation factor Tu	P42475	F. succinogenes strain ATCC 19169
	G0VND3	M. elsdenii DSM 20460
	I5AT53	E. cellulosolvens 6
	A0A1T4PHQ9	E. ruminantium
	C3X764	O. formigenes HOxBLS
	W7UFF4	R. flavefaciens 007c
	H7ENE5	T. saccharophilum DSM 2985
	P42482	W. succinogenes DSM 1740
Enolase	I5AQF9	E. cellulosolvens 6
	G0VM74	M. elsdenii DSM 20460

	A0A1H9FLF5	T. bryantii
	A0A011VXI1	R. albus SY3
Enoyl-CoA hydratase/carnithine racemase	I5AR59	E. cellulosolvens 6
Flagellar filament outer layer protein FlaA	A0A1H9AGZ2	T. bryantii
	H7EP63	T. saccharophilum DSM 2985
Flagellar secretion chaperone FliS	A0A1H9ERU0	T. bryantii
Flagellin	A0A1H9IAR9	T. bryantii
	H7ENV1	T. saccharophilum DSM 2985
Formate C-acetyltransferase	C9RPI5	F. succinogenes strain ATCC 19169
Formate--tetrahydrofolate ligase	G0VS15	M. elsdenii DSM 20460
Fructose-bisphosphate aldolase	A0A011W108	R. albus SY3
	H7ENS7	T. saccharophilum DSM 2985
Fructose-bisphosphate aldolase, class II	C9RP04	F. succinogenes strain ATCC 19169
GGGtGRT protein	A0A1T4L946	E. ruminantium
	A0A1H9I1I4	T. bryantii

Glucose-1-phosphate thymidylyltransferase	C9RNE3	F. succinogenes strain ATCC 19169
Glucose-6-phosphate isomerase	A0A1T4KM91	E. ruminantium
	C9RQH1	F. succinogenes strain ATCC 19169
	W7UPL1	R. flavefaciens 007c
	H7ELR2	T. saccharophilum DSM 2985
Glutamate dehydrogenase	A0A1T4NZG1	E. ruminantium
	G0VS03	MEgasphaera elsdenii DSM 20460
	C3X4W9	O. formigenes HOxBLS
	H7EN97	T. saccharophilum DSM 2985
	I5ATV2	E. cellulosolvens 6
	C9RNS6	F. succinogenes strain ATCC 19169
	G2SRL5	L. ruminis strain ATCC 27782
	A0A011UG76	R. albus SY3
	A0A011VXT9	R. albus SY3

	W7V3C2	R. flavefaciens 007c
	A0A1H9E6W0	T. bryantii
Glyceraldehyde-3-phosphate dehydrogenase	A0A1T4MD15	E. ruminantium
	C9RJ72	F. succinogenes strain ATCC 19169
	W7ULJ1	R. flavefaciens 007c
	I5AS92	E. cellulosolvens 6
	G2SMN7	L. ruminis strain ATCC 27782
	G0VNR2	M. elsdenii DSM 20460
	A0A011V4S8	Ruminococcus albus SY3
	A0A1H9FDW5	Treponema bryantii
Glycogen synthase	A0A011VTH6	R. albus SY3
Glycosyl transferase	A0A011V1F0	R. albus SY3
GTP-binding protein TypA	G0VQL9	Megasphaera elsdenii DSM 20460
Heat shock protein Hsp20	W7V253	Ruminococcus flavefaciens 007c
HMGL-like protein	G0VR43	M. elsdenii DSM 20460

Hypothetical protein	I5AUG9	E. cellulosolvens 6
	A0A1H9G5S8	T. bryantii
	I5AQF7	E. cellulosolvens 6
	I5AQF8	E. cellulosolvens 6
	G0VRR0	M. elsdenii DSM 20460
	A0A011WUN5	R. albus SY3
Hypothetical protein RASY3_18340	A0A011VSH7	R. albus SY3
Inosine-5'-monophosphate dehydrogenase	G2SRM0	L. ruminis strain ATCC 27782
Isocitrate dehydrogenase [NADP]	H7ELM0	T. saccharophilum DSM 2985
Ketol-acid reductoisomerase (NADP(+))	A0A1T4LAN1	E. ruminantium
	A0A011W1L5	R. albus SY3
	H7EMT9	T. saccharophilum DSM 2985
L-fucose isomerase	A0A1H9H211	T. bryantii
Lactaldehyde reductase	H7EJE5	T. saccharophilum DSM 2985
Major cold shock protein CspA	G0VQA6	M. elsdenii DSM 20460

Malate dehydrogenase (Oxaloacetate-decarboxylating)	A0A1T4NHD5	E. ruminantium
Malic enzyme	I5ATE5	E. cellulosolvens 6
Meso-diaminopimelate D-dehydrogenase	A0A011WMJ4	R. albus SY3
Methyl-accepting chemotaxis protein	I5AV92	E. cellulosolvens 6
Monosaccharide ABC transporter substrate-binding protein	H7EKI7	T. saccharophilum DSM 2985
Monosaccharide ABC transporter substrate-binding protein, CUT2 family	A0A1H9HZH4	T. bryantii
NADH dehydrogenase	W7UHX7	R. flavefaciens 007c
	A0A011VU56	R. albus SY3
NADPH-dependent glutamate synthase, homotetrameric	I5AQS1	E. cellulosolvens 6
NifU homolog involved in Fe-S cluster formation	A0A1T4L931	E. ruminantium
Nitrogen-fixing protein NifU	W7UL50	R. flavefaciens 007c
O-acetylhomoserine sulfhydrolase	H7ELT2	T. saccharophilum DSM 2985
Ornithine carbamoyltransferase	A0A011VT28	R. albus SY3

Phosphoenolpyruvate carboxykinase (ATP)	I5ASX4	E. cellulosolvens 6
	A0A1T4PPS8	E. ruminantium
	A0A011VTU0	R. albus SY3
Phosphoenolpyruvate carboxykinase [GTP]	C9RPJ7	F. succinogenes strain ATCC 19169
Phosphoglucosamine mutase	A0A1T4L0Z0	E. ruminantium
Phosphoglycerate kinase	A0A1T4MDF3	E. ruminantium
	A0A011UG26	R. albus SY3
	I5AS91	E. cellulosolvens 6
	A0A1H9JUX3	T. bryantii
Phosphomannomutase	I5AUJ3	E. cellulosolvens 6
Phosphoribosylaminoimidazole-succinocarboxamide synthase	W7UFK6	R. flavefaciens 007c
Phosphoribosylformylglycinamidine synthase	A0A1H9JNY3	T. bryantii
Phosphoserine aminotransferase	A0A011VWI9	R. albus SY3
Polyribonucleotide nucleotidyltransferase	I5AW29	E. cellulosolvens 6
Protein RecA	A0A011WM91	R. albus SY3
Protein translocase subunit SecY	W7UMJ0	R. flavefaciens 007c

PTS family mannose/fructose/sorbose porter component IID	G2SMJ5	L. ruminis strain ATCC 27782
PTS system, mannose-specific IIAB component	G2SMJ7	L. ruminis strain ATCC 27782
Putative cellobiose/cellodextrin phosphorylase	C9RP13	F. succinogenes strain ATCC 19169
Pyruvate-flavodoxin oxidoreductase	I5AT31	E. cellulosolvens 6
	A0A011UEZ1	Ruminococcus albus SY3
	W7UTZ9	R. flavefaciens 007c
Pyruvate, phosphate dikinase	I5AS89	E. cellulosolvens 6
	A0A011V1T4	R. albus SY3
	W7UDV4	R. flavefaciens 007c
	A0A1H9GSI3	T. bryantii
	A0A0E3WX55	M. barkeri 3
Reverse rubrerythrin-1	A0A011UF85	R. albus SY3
	W7UES7	R. flavefaciens 007c
Ribonuclease Y	G2SNA9	L. ruminis strain ATCC 27782
Rubrerythrin	A0A1T4PX41	E. ruminantium

	C9RLG2	F. succinogenes strain ATCC 19169
	H7EN05	T. saccharophilum DSM 2985
S-adenosylmethionine synthase	I5ATU9	E. cellulosolvens 6
Serine hydroxymethyltransferase	W7UY96	R. flavefaciens 007c
Serine--tRNA ligase	G2SQV0	L. ruminis strain ATCC 27782
Short-chain dehydrogenase/reductase SDR	H7EIC9	T. saccharophilum DSM 2985
Simple sugar transport system substrate-binding protein	A0A1T4L1U1	E. ruminantium
Succinate dehydrogenase or fumarate reductase, flavoprotein subunit	C9RLR4	F. succinogenes strain ATCC 19169
Sugar ABC transporter ATP-binding protein	A0A011VW90	R. albus SY3
	W7UW32	R. flavefaciens 007c
Sugar ABC transporter substrate-binding protein	A0A011VVQ2	R. albus SY3
Thioredoxin reductase	I5AT60	E. cellulosolvens 6
Transcription elongation factor GreA	A0A1H5VX84	E. ruminantium

Translation initiation factor IF-2	A0A011V0D3	R. albus SY3
Triosephosphate isomerase	I5AS90	E. cellulosolvens 6
	A0A011V0N9	R. albus SY3
Tryptophan synthase	W7UG72	R. flavefaciens 007c
Twitching motility protein pilT	W7UV77	R. flavefaciens 007c
V-type ATP synthase alpha chain	I5AVR6	E. cellulosolvens 6
	A0A0E3SMH9	M. barkeri 3
V-type ATP synthase beta chain	I5AVR7	E. cellulosolvens 6

Table 2.3 Scaled abundance values of the 35 proteins within strained rumen grab samples collected from Holstein dairy cows that were affected by time of sampling relative to morning feeding (0 h, 4 h, or 6 h after feeding), depicted as a hybridized heatmap with intensity of blue increasing as scaled protein abundance increases above 100.0 and intensity of red increasing as scaled protein abundance decreases below 100.0.

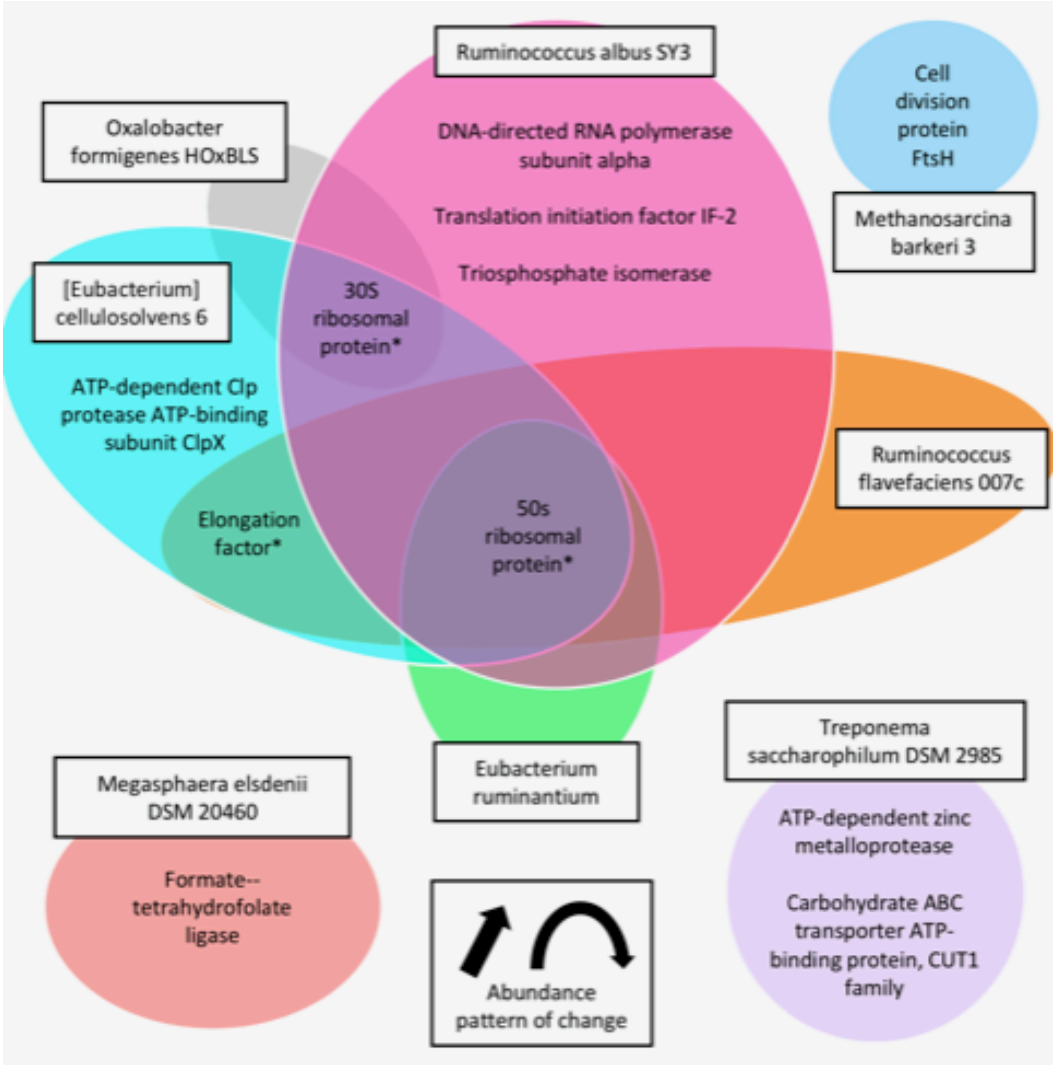
Accession Number	Description	Species	0 h	4 h	6 h	Standard Error	*P* value linear	*P* value quadratic
I5ATH2	50S ribosomal protein L7/L12	[Eubacterium] cellulosolvens 6	85.20	102.60	100.93	1.93	0.001	0.028
I5ATH8	50S ribosomal protein L33	[Eubacterium] cellulosolvens 6	86.27	110.40	105.83	3.49	0.004	0.044
A0A1T4NKS3	50S ribosomal protein L22	Eubacterium ruminantium	87.03	104.43	99.93	3.99	0.037	0.127
A0A011WRM0	30S ribosomal protein S9	Ruminococcus albus SY3	88.03	106.13	105.50	5.25	0.041	0.362
A0A011V181	50S ribosomal protein L6	Ruminococcus albus SY3	90.57	103.17	105.60	3.29	0.014	0.553
W7UV65	50S ribosomal protein L14	Ruminococcus flavefaciens 007c	91.20	101.47	102.63	1.02	0.0001	0.083
A0A011V0D3	Translation initiation factor IF-2	Ruminococcus albus SY3	91.33	96.33	98.767	2.172	0.049	0.987
G0VS15	Formate--tetrahydrofolate ligase	Megasphaera elsdenii DSM 20460	92.07	102.03	98.87	1.56	0. 011	0.032

W7UWA8	Elongation factor G	Ruminococcus flavefaciens 007c	93.00	98.73	104.40	1.39	0.001	0.323
A0A1T4M4Z7	50S ribosomal protein L21	Eubacterium ruminantium	93.47	106.30	102.77	2.56	0.024	0.083
I5AT53	Elongation factor Tu	[Eubacterium] cellulosolvens 6	93.83	101.00	103.53	1.34	0.002	0.690
I5ASS5	ATP-dependent Clp protease ATP-binding subunit ClpX	[Eubacterium] cellulosolvens 6	93.90	102.27	100.33	1.97	0.037	0.148
A0A011WQD4	DNA-directed RNA polymerase subunit alpha	Ruminococcus albus SY3	95.97	96.10	102.47	1.00	0.007	0.015
A0A0E3SMD8	Cell division protein FtsH	Methanosarcina barkeri 3	96.33	101.93	98.77	1.25	0.107	0.043
W7UWH6	50S ribosomal protein L16	Ruminococcus flavefaciens 007c	97.10	105.20	100.87	1.42	0.045	0.020
I5AVX2	30S ribosomal protein S11	[Eubacterium] cellulosolvens 6	98.20	103.80	102.80	1.26	0.026	0.158
A0A011UG26	Phosphoglycerate kinase	Ruminococcus albus SY3	99.37	93.63	99.87	1.89	0.771	0.042
H7EH79	Carbohydrate ABC transporter ATP-binding protein, CUT1 family	Treponema saccharophilum DSM 2985	100.37	104.50	97.20	1.94	0.522	0.041

A0A1T4L946	GGGtGRT protein	Eubacterium ruminantium	101.70	97.80	97.07	1.19	0.027	0.603
A0A1T4M509	50S ribosomal protein L27 O	Eubacterium ruminantium	102.23	98.07	91.17	1.39	0.002	0.113
W7UDV4	Pyruvate, phosphate dikinase	Ruminococcus flavefaciens 007c	102.77	95.17	99.23	0.92	0.011	0.004
H7EKI7	Monosaccharide ABC transporter substrate-binding protein	Treponema saccharophilum DSM 2985	103.90	98.10	97.47	0.66	0.0003	0.115
I5AS89	Pyruvate, phosphate dikinase	[Eubacterium] cellulosolvens 6	104.17	97.77	99.00	1.27	0.016	0.110
H7EMZ4	ATP-dependent zinc metalloprotease FtsH	Treponema saccharophilum DSM 2985	104.20	108.30	103.03	1.31	0.949	0.024
A0A011V0N9	Triosephosphate isomerase	Ruminococcus albus SY3	104.27	90.43	98.67	2.76	0.089	0.026
I5AR58	3-hydroxyacyl-CoA dehydrogenase	[Eubacterium] cellulosolvens 6	104.33	93.30	94.17	2.96	0.036	0.293
A0A011V4S8	Glyceraldehyde-3-phosphate dehydrogenase	Ruminococcus albus SY3	104.60	96.03	98.90	1.91	0.044	0.092
C3X6H7	30S ribosomal protein S3	Oxalobacter formigenes HOxBLS	106.43	122.87	106.53	5.33	0.643	0.049

W7UL50	Nitrogen-fixing protein NifU	Ruminococcus flavefaciens 007c	106.67	94.47	98.70	2.58	0.039	0.077
W7UV77	Twitching motility protein pilT	Ruminococcus flavefaciens 007c	107.67	86.27	101.70	4.94	0.207	0.030
C9RQ00	Cysteine synthase	Fibrobacter succinogenes (strain ATCC 19169 / S85)	108.17	95.63	95.00	2.94	0.014	0.345
A0A1T4PPS8	Phosphoenolpyruvate carboxykinase (ATP)	Eubacterium ruminantium	108.77	102.17	99.70	2.40	0.033	0.859
H7ELT2	O-acetylhomoserine sulfhydrolase	Treponema saccharophilum DSM 2984	109.47	96.37	99.43	2.79	0.026	0.115
H7ELA1	Cysteine synthase	Treponema saccharophilum DSM 2985	110.53	104.80	99.60	2.75	0.032	0.666
C9RNK1	DNA-binding protein HU	Fibrobacter succinogenes (strain ATCC 19169 / S85)	118.97	89.53	89.30	3.15	0.0003	0.049

Figure 2.1 Diagrammatical representation of the groups of proteins within rumen microbial species that were higher in abundance in samples collected at 4 h after feeding relative to initial sampling at feeding (0 h).



*Indicates that there are subunit or other types of variations not written out. See Table 2.2 for details.

Figure 2.2 Diagrammatical representation of the groups of proteins within rumen microbial species that were lower in abundance in samples collected at 4 h after feeding relative to initial sampling at feeding (0 h).

*Indicates that there are subunit or other types of variations not written out. See Table 2.2 for details.

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Chapter 3: Comparative analysis of colostrum and transition milk fat globule membrane proteomes from primi- and multi-parous Holstein dairy cows

3.1 Abstract

Changes in milk nutritive profiles are often a topic of focus to address the needs of the developing neonate; however, the study of milk composition, specifically proteomic components, can also provide valuable information regarding the mammary gland physiology. The objectives of this research were to characterize the milk fat globule membrane (MFGM) proteomes of colostrum and transition milk through sample collections at four timepoints post-partum (milkings 1, 2, 4, and 14) and compare these proteomes between multi- (n = 10) and primi- (n = 10) parous Holstein dairy cattle. After isolation of the protein lysates from the MFGM, proteins were labeled using Tandem Mass tagging isobaric-labeling and analyzed using LC-MS/MS techniques. Protein identification was completed using MASCOT and Sequest in Proteome Discoverer 2.2, and the scaled abundance values were analyzed using the MIXED procedure in SAS to determine the effects of parity, milking number, and milking number x parity (MN x P), and the adaptive false-discovery rate (FDR)-adjusted P values were determined using the MULTTEST procedure. Bioinformatic analysis of the proteomes was completed using a combination of PANTHER, Blast, and Uniprot. There were 104 proteins identified within the MFGM. Statistical analysis revealed that 44.2% of proteins were affected by parity, 70.2% by milking number, and 32.7% by parity x milking number. The research reported herein illustrates the patterns of MFGM protein abundance from cows differing in parity, further elucidating how the difference in mammary secretory cells can influence milk composition, specifically the MFGM proteome.

3.2 Introduction

The milk fat globule membrane (MFGM) proteome, though only constituting 1-4% of milk protein (Cavaletto et al., 2008), is diverse in its profile. Changes in the MFGM proteome during early lactation, particularly in the transition from colostrum to mature milk, may be important from a calf feeding standpoint (Ontsouka et al., 2003; Demmelmair et al., 2017). Currently, immunoglobulin concentrations are the main focus of colostrum quality, but our rapidly expanding knowledge of the milk proteome has led to a greater understanding of other potentially bioactive proteins in colostrum that could play an important role in calf health (Jørgensen et al., 2010). In 2008, Reinhardt et al. (2008) analyzed changes in the MFGM proteome in first-milking colostrum and milk seven days post-partum. Proteins related to lipid transport synthesis and bioactive proteins xanthine dehydrogenase, butyrophilin, adipophilin were upregulated in the later post-partum milk compared to colostrum. Many of the recognized proteins have the ability to contribute to overall host immune defenses (Murgiano et al., 2009; Affolter et al., 2010). While this membrane that encapsulates fat droplets partly protects contents from immediate enzymatic digestion (Le at al., 2012), it also contributes to the overall biologically active profile of milk (Rasmussen, 2009). Additionally, understanding this proteome has enhanced our understanding of lactation physiology because of its representation of secretory cells in the mammary gland (Lopez et al., 2011), and applications of MFGM protein use as a biomarker could enhance our diagnostic capabilities in the field to assess mammary health and functionality.

In general, parity is not considered a major factor for differences in milk component percentages, though it is well established that milk production is lower in

primiparous (PP) cows. In addition to the continued mammary development in PP animals during their first cycle of lactogenesis (Knight and Peaker, 1982; Lang et al., 2012), PP heifers also continue to have energetic requirements for gain (Freetly et al., 2006). These additional development-related considerations may be important when analyzing the MFGM proteome of cows of different maturity, as secretory mechanisms have reliance on cellular metabolism. Research focused on colostrum, transition, and mature milk has been extensive for the different milk fractions (Reinhardt et al., 2008; Le et al., 2010; Tacoma et al., 2017). However, the impact of milking number postpartum on mammary protein-mediated cellular activity and the MFGM proteome is still somewhat speculative.

For this research, it is hypothesized that the MFGM proteome will be affected by parity, milking number, and milking number × parity because of the reported changes in milk composition over time and what is known comparatively about mammary gland development in cows that differ in parity such as rate of cell differentiation. The objectives of this research were to characterize dynamic shifts in the MFGM proteome of both PP and MP cows from colostrum to transition and mature milk to gain a better understanding of the physiological shifts of the mammary gland through both maturity and postpartum acclimation.

3.3 Materials and Methods

3.3.1 Animal and sample collection

For this trial, 10 PP and 10 MP (parity = 3.1 ± 0.43) healthy Holstein dairy cattle housed at Breevliet Farms Ltd. (Alberta, Canada) were included. All cows were milked

twice daily (0500 h and 1600 h). Milk samples were collected from cattle using continuous in-line samplers and snap frozen at four timepoints postpartum: 1) at the first milking postpartum (**M1**: 5.3 ± 0.73 h postpartum), 2) the second milking postpartum (**M2**), 3) the fourth milking postpartum (**M4**), and 4) the fourteenth milking postpartum (**M14**). All collected milk samples were stored at -80°C, shipped on dry ice to the University of Vermont (Burlington, VT), and stored at -80°C until proteomic analysis.

3.3.2 MFGM proteomic analysis

Samples were thawed overnight at 4°C, and a protease inhibitor (Protease Inhibitor Cocktail, Sigma Aldrich, St. Louis, MO) was added at 0.24 mL/mg of protein. The milk samples were centrifuged at 4,000 x *g* at 4°C and the cream layer was collected in a new 15 mL tube, and this separation step was repeated. The cream layer was stored at -80°C.

Methodology was established by Yang et al. (2016) with modifications. Briefly, up to 10 volumes of phosphate buffered saline (PBS) was pipetted into each thawed sample and vortexed. All samples were incubated for 20 min at 37°C, centrifuged at 4,000 x *g* for 30 min, and PBS was aspirated. Samples were washed with PBS as per this method twice more.

After washing with PBS, the cream was transferred into a clean 50 mL round-bottom Nalgene tube (United States Plastic Corp., Lima, OH). Five volumes of lysis buffer (50mM Tris-HCl at pH 7.4, 4% SDS (w/v) solution) was added to each tube and vortexed. These samples were incubated at room temperature (21 °C) for 1 h with periodic vortexing every 10-15 min and then subsequently incubated at 95°C for 5 min.

Samples were then centrifuged at 12,000 x *g* for 15 min and the resulting fat layer was removed. These centrifugation and collection steps were repeated. The aqueous phase was collected aspirated into a fresh 15mL tube. An aliquot was then combined with acetone at a 1:6 ratio (sample: acetone) and incubated at -20°C for 20 h. Samples were then centrifuged at 14,000 x *g* for 20 min at 4°C and the subsequent supernatant was discarded. Radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Rockford, IL) was used to resuspend the pellet before storage at -80°C.

3.3.3 Bicinchoninic acid assay and isobaric TMT labeling

Processed samples were thawed on ice and a universal control (UC) was generated by creating a pooled sample before protein quantification. Protein amount was determined using a bicinchonic assay (BCA) kit (Pierce Biotechnology, Rockford, IL). Samples were then subjected to isobaric labeling using Tandem Mass Tag™ (TMT™) 10plex Isobaric Labeling Kits (Pierce Biotechnology, Rockford, IL). All LC-MS/MS analysis was completed at The Vermont Genetics Network Core Proteomics Facility (Burlington, VT).

3.3.4 Liquid chromatography-tandem mass spectrometry (LC-MS/MS*)*

The labeled samples were resuspended in 2.5% acetonitrile (CH_3CN) and 2.5% formic acid (FA) in water for subsequent LC-MS/MS based peptide identification and quantification. Analyses were performed on the Q-Exactive mass spectrometer coupled to an EASY-nLC ULTRA (Thermo Scientific, Waltham, MA). Samples were loaded onto a 100 μm x 500 mm capillary column packed with Halo C18 (2.7 μm particle size,

90 nm pore size, Michrom Bioresources, CA) at a flow rate of 300 nL/min. Program settings and parameters for LC-MS/MS analysis was performed as outlined by Scuderi et al. (2019).

3.3.5 Statistical and bioinformatic analysis

The scaled abundance values resulting from the LC-MS/MS protocol were used for statistical analysis. The effects of milking number, parity, and milking number × parity using repeated measures in the MIXED procedure of SAS (Version 9.4) and multiple testing was corrected using the MULTTEST procedure to determine the adaptive false-discovery rate (FDR) adjusted treatment effects. Proteins that were categorized as "uncharacterized" were identified either through PANTHER version 14.1 (Mi et al., 2019) through their accession number or through BLAST (Camacho et al., 2008) after obtaining their FASTA sequence through UniProt (Chen et al., 2017). Gene ontology (GO) information, specifically the biological processes of identified proteins, was obtained through use of the PANTHER database (Mi et al., 2019).

3.4 Results

3.4.1 The characterized proteome

A total of 104 proteins were characterized across all cows within the MFGM, with 78 (75%) being affected by milking number, parity, and/or milking number × parity (Table 3.1). There were 27 proteins that were affected by all of the variables (Figure 3.1), some of which included cathelicidin-1-like (A0A0N4STN1), IGK protein (F1MH40), protein S100-A8 (P28782), -A9 (F1MHS5), -A12 (P79105), immunoglobulin

J chain (Q3SYR8), leukocyte elastase inhibitor (Q1JPB0), lactotransferrin (P24627), and complement C3 (G3X7A5). The total identified proteome was diverse in its biological processes, as determined through PANTHER (Figure 3.2). Cellular process was the dominant biological process (26%), followed by localization (14%), metabolic process (13%), response to stimulus (12%), biological regulation (10%), and immune system process (10%). Proteins participating in biological adhesion, cellular component organization, developmental process, multicellular organismal process, and reproduction collectively represented 16% of the biological process profile.

3.4.2 Proteins affected by parity

Parity affected 46 proteins, including immune-related proteins such as lactotransferrin (Table 3.1). Three proteins affected by parity alone included monocyte differentiation antigen CD14 (A6QNL0), keratin 1 (G3N0V2), and annexin (F6QVC9). Both monocyte differentiation antigen CD14 (A6QNL0) and keratin 1 (G3N0V2) were higher in abundances from MP cattle. In contrast, annexin (F6QVC9) was affected differently and was lower in abundance in samples from MP cattle compared to PP cattle.

3.4.3 Proteins affected by milking number

Milking number affected 73 of the identified 104 proteins (70.2%) and was the most influential variable (Table 3.1). There were 25 proteins affected only by milking number, representing 23.1% of total proteins identified and 32.1% of the proteins affected by milking number. Affected proteins included lactadherin (F1MXX6),

hemopexin (Q3SZV7), β-1,4-galactosyltransferase 1 (P08037), glycosylation-dependent cell adhesion molecule 1 (P80195), and perilipin (F1N1N6).

3.4.4 Proteins affected by milking number and parity interaction

A total of 34 total proteins (32.7% of identified proteins) were affected by the interaction of milking number × parity. Proteins affected by this interaction included vitamin D-binding protein (F1N5M2), lactotransferrin (P24627), complement C3 (G3X7A5), clusterin (P17697), and gelsolin (F1N1I6). The biological processes of the proteins affected by the interaction of parity and milking number were diverse and similar in profile to the biological processes of the greater proteome characterized in this experiment. The dominant categories again were cellular process (26%), metabolic process (15%), immune system process (14%), and response to stimulus (14%). The cellular process grouping (Figure 3.2) included 24 proteins that represented the following subcategories: cell cycle (15%), cell death (2%), cellular component organization (24%), cellular homeostasis (2%), cellular metabolic processes (7%), processes involved in reproduction in multicellular organisms (2%), cellular response to stimulus (22%) execution phase of apoptosis (2%), secretion by cell (2%), and signal transduction (20%).

3.5 Discussion

3.5.1 Characterization of the MFGM proteome

Milk fat globule membrane proteins reportedly comprise approximately 22% of the droplets in bovine milk (Fong et al., 2007), and the proteins within this proteome are derived from membranes of the secretory cell that have accumulated during droplet

migration (Lopez, 2011). Identification of the proteins within this fraction appear to vary, likely due to differing protein isolation and fractionation methodologies, and research examining the MFGM has identified anywhere from 100 to over 400 proteins (examples include Reinhardt et al., 2008; Ji et al., 2017; Nguyen et al., 2017). Despite the variation in protein number identification, characterization of the MFGM proteome has been relatively consistent in terms of the identified profile and proportions of the dominant proteins. Butyrophilin can represent up to 40% of the protein weight within the MFGM proteome (Lopez, 2011; Mondy and Kennan, 1993). In the MFGM proteome isolated in the current study, butyrophilin subfamily 1 member A1 (E1BHI7 and P18892) contributed a large proportion of identified peptides. However, the abundance of this protein was affected by parity and milking number (E1BHI7) and milking number alone (P18892), neither being affected by the interaction of MN × P. Xanthine dehydrogenase/oxidase (F1MUT3) has also been reported to be a common protein within the MFGM (Ji et al., 2017) and in the current study, the abundance of this protein was not affected by parity, milking number, or their interaction.

3.5.2 Bioactivity and implications for calf health

A number of the above highlighted proteins have been reported to be biologically active when ingested and may be important from a calf feeding perspective (Tripathi and Vashishtha, 2006). Characterization of the colostrum proteome could be important to further enhance the healthfulness of colostrum for the pre-weaned calf. Previous research has characterized the colostral proteome in the skim milk fraction (Tacoma et al., 2017), exosome (Yang et al., 2017), and the MFGM (Yang et al., 2016), as was focused on in

the current study. Immunoglobulins have been considered the gold standard to assess colostrum quality and it is recommended that at least 100 g of immunoglobulin G (IgG) be delivered to the neonate immediately postpartum (Dander et al., 2016). Though when considering the comprehensive proteome, there have been over 900 proteins identified in the skim milk fraction of milk, which should not be overlooked when considering the quality of colostrum (Tacoma et al., 2017). For example, butyrophilin is consistently highly represented protein in the MFGM, and while not named as an immunoglobulin, it still belongs to the immunoglobulin family (Jiménez-Flores et al., 2009). With the immune function being beneficial from a calf standpoint, from a consumer standpoint, butyrophilin has been studied for its potential in suppressing multiple sclerosis in humans (Mana et al., 2004). Similarly, xanthine dehydrogenase and xanthine oxidase are the two interconvertible forms of xanthine oxidoreductase which has been speculated to possess antimicrobial properties due to the reduction of oxygen to reactive oxygen species (ROS) and hydrogen peroxide (Martin et al., 2004). Though none of the imposed variables (MN, P, or MN × P) affected xanthine dehydrogenase/oxidase, having a quality supply of antimicrobial in milk for calf consumption could pair commendably with immunoglobulins while the calf builds a sufficient immune system. This begs the question as to whether there are additional proteins that should be measured as indicators for colostrum quality. However, in these results immunoglobin related proteins exhibited similar patterns to other proteins associated with triggering immune responses thus drawing attention to the fact that the MFGM may not be necessarily the appropriate fraction of milk to use for biomarker analysis.

3.5.3 Use of MFGM to understand parity impact on mammary gland

The effect of parity on milk fatty acid composition (Samková et al., 2018) and total protein (Nudda et al., 2003; Yoon et al., 2003; Miller et al., 2006) is well documented. However, there are no known publications that describe how parity affects the MFGM proteome. In this experiment, parity affected 46 proteins (Table 3.1) and 3 of these proteins (monocyte differentiation antigen CD14, keratin 1, and annexin) were not affected by MN or MN × P. Multiparous cows are known to have a higher milk yield than PP cattle, normally due to higher rates of cell differentiation in the MP mammary gland compared to that of PP cows (Miller et al., 2006). Research by Miller et al. (2006) compared the mammary gland DNA concentration between MP and PP cows and concluded that PP mammary glands had less DNA content in the mammary gland suggesting that the lower milk production could be due to the reduced number of secretory cells. With the knowledge that the MFGM is largely derived from secretory cells, our findings align well with this previous work. It was consistently observed that protein abundances were higher in MP cows. However, by M14, there was little difference in protein abundances between the parities. It seems as though in MP cattle, the MFGM protein content may contribute to a higher level of bioactivity in colostrum. This could be important from a management prospective when storing colostrum for future calvings and prioritizing early milk secretions from MP cattle over PP cattle. Additionally, it is interesting that in the research by Miller et al. (2006), total milk protein and fat content did not vary across parity. The lower quantity of cells in the PP mammary gland likely leads to a limited contribution to the membrane surrounding the fat globules. This could also draw attention to the mechanism of fat droplet transport within the

secretory cells. Lopez et al. (2011) reviewed that mammary glands with higher metabolic activity secrete smaller droplets thus yielding higher amounts of membrane material encapsulating the droplets. Therefore, while secreted fat content may not vary across parity, secretory mechanisms have a high chance of providing more or less of the membrane components to the neonate.

3.5.4 Transition from colostrum to mature milk effect

The proteomics field itself has reflected the considerable amount of research that is devoted to colostrum, transition, and mature milk (Le et al., 2010; Reinhardt et al., 2008; Tacoma et al., 2016; Tacoma et al., 2017). In the research presented herein, milking number was the dominant variable affecting 70.2% of identified proteins (Table 3.1). In addition, 25 of these proteins were not affected by any other examined variable including the known bioactive protein mentioned previously, lactadherin (F1MXX6). Reinhardt et al. (2008) explored how milk collected at parturition and seven days post-partum would influence the proteome of the MFGM. Parity of cows from the trial completed by Reinhardt et al. (2008) was not noted; however, when reflecting on the results of milking number serving as the sole variable, similar results were observed in their research with 32.6% of identified MFGM proteins reflecting a change in days in milk. Immune-related proteins such as lactotransferrin (P24627) and lactadherin (F1MXX6) were both reported as affected proteins. In comparison to Reinhardt et al. (2008)'s research, we further observed shifts in abundance of immune associated proteins vitamin D-binding protein (F1N5M2), perilipin (F1N1N6), and hemopexin (Q3SZV7). A

change in abundance of immune-related proteins is expected based on already well-established knowledge of the purpose of colostrum.

3.5.5 Abundance patterns of proteins affected by parity × milking number

The hybridized heatmap (Table 3.1) illustrates patterns of protein abundances of PP and MP cattle as milking number postpartum increases. The majority of proteins had a lower abundance in PP cattle compared to MP cattle within the early milkings. However, there are a few exceptions where PP cattle exhibited a higher abundance compared to MP cattle including apolipoprotein A-I (P15497), apolipoprotein C-III (P19035), serum amyloid A protein (Q8SQ28), and clusterin (P17697). By M14, the difference in protein abundances across parity were less apparent.

Proteins S100-A8, -A9, and -A12 were all affected by MN×P and followed similar trends (Figure 3.4). Variants of S100 proteins serve as calcium sensors (Zimmer et al., 2013). There is an increase in intracellular ionized calcium concentration at the onset of lactation (Kimura et al., 2016), and this, paired with the demand of calcium for milk production, increases the need for calcium after parturition. When examining the trends in abundance of these calcium sensing proteins, they are relatively higher in abundance at the first two milkings. After the first two milkings, the abundance sharply drops in PP cattle. Conversely in MP cattle, the abundance stays consistent until the 4th milking before dropping in abundance. In MP cows, the abundances of S100 protein abundances were at least two-fold higher than PP cattle. Based on the previous discussion on secretory cells numbers and DNA content in the mammary gland, it could be assumed that the PP mammary gland does not have the capacity to keep up the production of this

class of proteins compared to MP cows. With the MP cattle's established mammary system, they are more physiologically adapted and have a higher capacity to synthesize needed proteins. However, the lower abundance could be related to the fact that first-calf heifers have a lower production rate thus not requiring the same high levels of calcium. Both arguments further support the evidence that parity has the ability to affect production at a cellular level, an in particular the proteome of the MFGM.

3.6 Conclusions

The research reported herein highlights the dynamicity of the lactating mammary gland within the first week postpartum, and how the patterns of change of the MFGM proteome, which seem representative of known changes in mammary function and cellular activity, are impacted by parity. While there was not one specific pattern of change that was observed amongst all proteins across parity and milking number, it was typical to see that at the initial milking, PP cattle exhibited a lower protein abundance comparatively but by M14 protein abundances were comparable across parity. The elevated abundance of known bioactive MFGM-associated proteins in samples from MP cattle could be important from a calf feeding standpoint and selection of colostrum donors for calf feeding.

Table 3.1 Scaled abundance values of the proteins identified within the milk fat globule membrane (MFGM) collected from primiparous (PP) and multiparous (MP) Holsteins at four milkings (M1, M2, M4, M14) after calving, depicted as a hybridized heat map of the estimate values of protein abundances with red illustrating a lower relative abundance and blue representing a higher abundance paired with the appropriate standard error (SE values).

		M1			M2			M4			M14			*P*-value		
AN[1]	Protein	PP	MP	SE[2]	PP	MP	SE	PP	MP	SE	PP	MP	SE	P[3]	MN[4]	MN×P
G3MXB5	Immunoglobulin IgA heavy chain constant region, partial	61.6	159.2	18.8	68.2	197.3	19.5	16.6	122.9	11.9	15.1	37.0	6.7	<.0001	<.0001	<.0001
P81265	Polymeric immunoglobulin receptor	79.8	146.0	17.5	75.4	169.6	19.0	30.7	131.1	9.3	32.1	49.1	6.9	<.0001	<.0001	<.0001
Q3SYR8	Immunoglobulin J chain	89.6	159.0	12.4	85.5	138.0	11.1	40.1	112.6	6.0	37.4	48.4	6.7	<.0001	<.0001	<.0001
F1MH40	IGK protein	92.3	210.8	38.1	76.4	122.3	13.0	27.4	102.1	11.1	27.3	31.5	4.4	0.0001	<.0001	0.0001
G3N2D7	Immunoglobulin light chain, partial	89.7	120.4	12.7	71.7	94.4	9.6	43.9	108.3	9.2	49.1	42.4	5.9	0.0001	<.0001	0.0002
F1MCF8	IGL@ protein	92.0	201.6	48.9	72.4	100.5	13.9	21.4	90.6	10.5	18.6	23.6	3.8	0.0053	<.0001	0.0007

Q0VCN9	Folate receptor 2 (Fetal)	139.8	160.9	15.6	124.3	119.6	19.4	59.0	97.3	6.1	47.1	39.5	4.3	0.2050	<.0001	0.0007
F1N5M2	Vitamin D-binding protein	118.1	176.1	24.3	83.4	109.8	15.8	42.8	93.1	9.2	44.0	36.0	4.9	0.0048	<.0001	0.0008
F1MHS5	Protein S100-A9	68.0	162.5	37.6	69.6	149.9	23.0	38.6	161.0	23.0	30.8	33.2	7.5	<.0001	<.0001	0.0011
P24627	Lactotransferrin	54.0	182.0	45.9	53.0	100.2	13.8	41.2	109.8	12.0	42.7	44.7	6.1	0.0008	0.0003	0.0014
Q1JPB0	Leukocyte elastase inhibitor	85.0	180.7	32.8	67.2	118.8	14.3	49.2	122.8	12.2	47.3	46.1	8.7	0.0002	<.0001	0.0024
G5E5V1	Immunoglobulin iota chain-like, partial (TPA)	95.1	175.5	29.6	75.2	112.8	11.4	29.6	100.6	12.6	28.9	28.8	7.0	0.0003	<.0001	0.0027
P28782	Protein S100-A8	70.0	151.7	33.8	66.7	160.1	22.9	37.0	153.9	24.4	24.8	37.0	6.0	<.0001	<.0001	0.0031
P19035	Apolipoprotein C-III	185.1	104.9	21.7	167.0	117.9	26.0	64.7	90.1	7.6	45.2	46.0	5.0	0.0410	<.0001	0.0054
F1N514	CD5 antigen-like precursor	122.2	184.4	19.8	102.0	129.6	17.7	44.3	80.6	6.8	32.1	33.4	4.4	0.0018	<.0001	0.0079
A8YXY3	Selenoprotein F	119.9	117.7	11.5	133.6	105.3	13.9	95.2	125.3	6.2	119.7	101.5	11.6	0.5553	0.7037	0.0089
P60712	Actin, cytoplasmic 1	95.6	157.6	26.9	90.0	128.8	14.7	59.6	121.1	10.4	57.2	60.3	7.2	0.0008	<.0001	0.0105

G5E5T5	Immunoglobulin M heavy chain secretory form	117.7	174.6	19.5	104.9	129.4	18.7	47.2	87.9	7.2	45.0	46.6	5.2	0.0030	<.0001	0.0111
A8DC37	Fc-gamma-RII-D	133.6	175.8	20.3	175.1	120.9	25.8	64.1	105.1	10.7	46.8	42.8	4.6	0.6090	<.0001	0.0111
G5E513	IgM heavy chain constant region, secretory form, partial	122.3	179.5	20.3	104.6	133.3	20.7	43.4	86.9	7.3	34.0	38.9	4.7	0.0025	<.0001	0.0113
P79105	Protein S100-A12	81.4	151.4	22.8	82.0	160.8	20.7	35.3	151.3	24.7	21.4	39.2	7.1	<.0001	<.0001	0.0123
P13753	BOLA class I histocompatibility antigen, alpha chain BL3-7	85.2	129.9	14.1	131.4	124.2	31.6	69.6	132.0	11.1	77.0	73.2	9.8	0.0756	0.0103	0.0142
F1N726	Glycoprotein 2	69.7	126.1	17.4	90.0	174.4	19.4	38.6	150.6	16.5	39.1	63.2	10.9	<.0001	<.0001	0.0156
Q8SQ28	Serum amyloid A protein	146.6	101.0	16.4	137.4	129.5	32.4	81.2	137.2	16.1	54.4	80.7	13.7	0.6285	0.0010	0.0185
P01888	Beta-2-microglobulin	56.9	163.2	65.7	74.5	111.5	24.1	39.9	140.3	23.2	36.5	34.2	7.9	0.0241	0.0004	0.0198

Q32PA1	CD59 molecule (CD59 blood group)	134.8	109.6	9.4	117.8	107.1	15.5	85.9	130.4	13.5	100.5	123.1	20.3	0.4715	0.6332	0.0256
G3X7A5	Complement C3	96.4	150.4	24.8	91.3	90.8	12.5	68.2	136.2	17.7	75.1	67.1	9.2	0.0217	0.0124	0.0343
P17697	Clusterin	153.1	144.0	15.8	171.3	108.2	19.5	61.0	89.7	10.5	34.8	39.3	5.4	0.3227	<.0001	0.0357
F1MLW8	Immunoglobulin lambda-1 light chain-like isoform X5	101.6	166.3	28.6	75.1	93.3	14.7	32.9	105.8	23.5	38.7	31.7	5.2	0.0108	<.0001	0.0359
P15497	Apolipoprotein A-I	160.1	110.2	14.5	173.4	108.5	24.2	109.3	123.4	10.6	79.4	67.9	7.6	0.0129	<.0001	0.0390
A0A0A0MP90	Histone H2A	58.5	125.4	37.0	65.4	147.1	22.0	42.7	163.6	34.3	24.5	36.9	8.8	0.0006	<.0001	0.0396
F1N1I6	Gelsolin	103.1	146.0	18.6	95.1	120.3	12.9	75.4	115.0	8.7	69.0	62.0	8.4	0.0070	<.0001	0.0426
A0A0N4STN1	Cathelicidin-1-like	76.7	155.5	20.6	73.3	127.8	14.9	54.9	137.6	10.3	53.9	73.4	12.1	<.0001	0.0043	0.0448
F1MUD2	Histone H2B	40.2	136.8	52.1	63.6	140.8	19.9	36.8	171.9	37.4	24.6	41.1	9.5	0.0011	<.0001	0.0463
F1MGC2	Non-secretory ribonuclease	83.2	90.7	11.6	115.2	69.5	18.3	168.3	131.7	30.0	178.1	103.3	18.8	0.0130	0.0013	0.0516

	isoform X1															
G3MWV5	Histone cluster 1 H1 family member e	40.4	139.4	58.5	66.5	121.9	19.9	29.9	143.8	33.2	16.6	27.1	4.7	0.0065	<.0001	0.0528
A0A140T881	Apolipoprotein E	93.8	139.7	16.3	107.9	113.5	14.3	78.7	123.0	8.1	77.4	79.6	9.5	0.0071	0.0079	0.0672
E1BGN3	Histone H3	40.7	125.5	38.4	63.5	130.8	18.0	47.2	157.4	32.0	28.3	44.9	8.6	0.0005	<.0001	0.0674
P81644	Apolipoprotein A-II	145.3	94.9	18.5	145.3	111.2	21.1	92.3	112.1	10.1	93.5	76.8	12.4	0.0785	0.0410	0.0685
P10152	Angiogenin-1	72.7	95.2	12.9	80.6	75.5	16.5	98.3	119.2	18.6	257.7	92.4	49.2	0.1173	0.0298	0.0694
P62803	Histone H4	51.3	144.8	50.6	78.5	132.7	17.4	43.7	172.7	33.5	28.2	44.5	9.1	0.0018	<.0001	0.0739
P02663	Alpha-S2-casein	54.8	90.6	18.8	95.6	58.4	18.5	165.1	136.7	34.9	188.2	113.6	25.4	0.1485	0.0006	0.0761
G5E604	TPA: immunoglobulin iota chain-like	119.9	152.0	23.4	86.5	105.2	17.4	38.8	94.0	17.1	37.7	31.5	5.8	0.0435	<.0001	0.0790
E1BF48	CD177 molecule	53.5	136.2	38.2	69.3	146.9	20.8	48.9	145.2	26.5	22.5	43.1	8.3	0.0003	<.0001	0.0799
P48616	Vimentin	86.2	139.0	26.4	98.1	118.4	14.9	68.3	141.3	18.9	50.4	53.1	7.8	0.0053	<.0001	0.0813
Q29S21	Keratin, type II cytoskeletal 7	103.2	120.1	13.0	150.0	113.7	17.8	90.0	114.3	10.8	80.9	67.7	7.5	0.8184	<.0001	0.0824

P56425	Cathelicidin-7	61.8	123.6	24.0	86.4	144.1	29.7	75.6	165.8	22.6	40.7	54.3	8.1	0.0008	<.0001	0.0868
P62992	Ubiquitin-40S ribosomal protein S27a	118.3	139.7	15.8	99.2	126.3	16.0	105.2	122.5	15.7	105.5	74.9	11.0	0.4032	0.0338	0.0876
Q3SZV7	Hemopexin	114.3	155.3	24.5	100.1	99.8	14.8	60.5	118.2	17.1	57.9	45.3	11.7	0.0904	<.0001	0.0882
Q17QG8	Histone H2A	43.6	119.3	38.8	76.4	134.9	21.8	43.5	149.6	30.0	20.8	38.4	8.0	0.0012	<.0001	0.1220
G3N0V0	Secreted immunoglobulin gamma2 heavy chain constant region, partial	93.4	180.9	52.6	65.7	70.1	14.5	24.7	137.3	37.7	32.1	28.0	7.5	0.0373	0.0005	0.1239
F1MMW8	Serum amyloid A protein	123.4	108.9	15.5	113.4	123.9	26.5	80.1	137.0	14.8	63.4	75.3	11.5	0.2064	0.0016	0.1304
P08728	Keratin, type I cytoskeletal 19	105.7	128.0	14.9	146.7	122.4	18.5	68.7	113.3	11.6	45.9	56.8	6.5	0.1673	<.0001	0.1325
F1MX83	Protein S100	97.0	172.1	26.8	109.1	130.7	13.3	61.7	129.1	12.0	48.7	74.4	9.0	0.0002	<.0001	0.1413
P00711	Alpha-lactalbumin	38.3	71.4	21.3	30.6	30.8	7.2	72.4	233.4	93.4	210.6	44.6	69.1	0.8663	0.0331	0.1491

P08037	Beta-1,4-galactosyltransferase 1	135.6	119.0	18.8	172.8	91.3	24.5	88.2	96.5	19.6	54.4	53.8	8.4	0.0935	<.0001	0.1588
A6QNL0	Monocyte differentiation antigen CD14	76.3	124.0	10.7	96.5	131.4	12.3	99.7	149.8	15.7	116.3	98.4	18.8	0.0074	0.3035	0.1751
P80195	Glycosylation-dependent cell adhesion molecule 1	61.0	51.7	8.8	75.4	59.1	13.2	131.6	196.6	23.8	250.7	272.0	53.6	0.4818	<.0001	0.1861
F1MHH9	Low affinity immunoglobulin gamma Fc region receptor II	138.5	153.9	32.4	126.9	98.5	22.6	64.0	97.6	13.5	46.8	43.4	5.4	0.7731	<.0001	0.2498
F1MLZ1	Cytochrome b reductase 1	197.0	89.3	35.7	113.0	78.8	22.7	84.1	71.6	12.1	45.0	33.6	7.1	0.0105	<.0001	0.2858
P02662	Alpha-S1-casein	80.0	73.6	17.2	130.9	45.6	25.9	155.2	105.0	34.6	190.2	134.9	37.3	0.0222	0.0163	0.3293
G3N0V2	Keratin 1	97.5	129.2	14.9	85.7	114.5	12.6	87.6	116.3	8.9	84.8	83.0	10.2	0.0112	0.1021	0.3482
F1MXX6	Lactadherin	144.2	116.9	15.5	139.4	98.1	18.1	89.0	96.8	11.9	79.5	71.6	12.6	0.1041	0.0010	0.3649

F1N4Y5	Non-classical MHC class I antigen precursor	131.7	131.2	14.2	147.5	113.4	21.4	84.0	105.5	11.1	69.9	66.9	7.7	0.6953	<.0001	0.3688
F6QVC9	Annexin	116.2	111.5	13.8	103.2	79.2	14.6	148.1	94.2	15.9	177.7	122.9	32.5	0.0218	0.0665	0.3807
Q8WML4	Mucin-1	58.1	68.0	5.7	86.3	74.5	8.8	168.8	140.6	23.0	211.4	229.2	41.6	0.860	<.0001	0.382
F1N650	Annexin	52.9	142.2	41.7	56.5	115.7	21.1	83.5	151.1	27.2	37.6	63.4	10.7	0.0027	0.0044	0.4805
P62261	14-3-3 protein epsilon	129.2	114.7	12.1	126.8	107.0	15.8	92.6	106.0	11.5	96.4	79.2	13.4	0.3132	0.0397	0.5011
Q2KII3	Hepatitis A virus cellular receptor 1 N-terminal domain containing protein	125.3	129.3	13.1	127.3	106.6	18.7	99.4	112.2	10.7	71.1	65.5	5.5	0.7986	<.0001	0.6234
Q3SYS6	Calcineurin B homologous protein 1	90.9	123.1	16.2	98.8	115.2	10.6	128.1	144.3	11.8	135.1	126.4	16.2	0.1589	0.0376	0.6495
E1BHI7	Butyrophilin subfamily 1 member A1	99.3	76.5	13.4	122.9	79.5	20.1	155.1	122.6	30.8	257.2	153.6	47.7	0.0234	0.0041	0.6796

F1N1N6	Perilipin	89.0	77.1	7.8	105.0	97.3	13.6	112.7	134.2	19.3	194.6	216.0	42.1	0.7379	0.0002	0.6803
P10790	Fatty acid-binding protein, heart	71.5	65.5	7.0	89.6	67.6	10.6	157.6	131.4	20.6	260.7	211.1	46.2	0.1636	<.0001	0.7104
P18892	Butyrophilin subfamily 1 member A1	92.9	81.8	7.0	109.1	99.5	13.2	122.3	138.4	21.8	166.9	175.1	33.6	0.9519	0.0008	0.8435
Q2KIS4	Dehydrogenase/reductase (SDR family) member 1	99.3	88.1	7.7	108.2	98.5	14.3	128.3	138.6	16.1	122.0	110.0	16.6	0.5763	0.0149	0.8571
B2D1N9	ATP-binding cassette sub-family G member 2	87.9	80.8	5.8	90.1	77.9	10.4	127.9	122.2	18.5	222.7	255.8	47.5	0.9127	<.0001	0.9240
Q0IIG8	Ras-related protein Rab-18	100.2	95.7	6.7	107.9	98.0	13.0	124.6	128.7	16.4	173.0	149.9	30.1	0.5300	0.0083	0.9379

F1N6D4	Sodium-dependent phosphate transport protein 2B	98.8	91.5	7.8	100.9	91.5	13.5	131.1	121.2	17.5	181.2	168.8	33.6	0.5030	0.0032	0.9991

[1]AN: Accession number
[2]SE: Standard error
[3]P: Parity
[4]MN: Milking number

Figure 3.1 Venn diagram of the number of proteins that were affected by milking number (MN), parity (P), and milking number × parity (MN×P) within the milk fat globule membrane (MFGM) collected from primiparous (PP) and multiparous (MP) Holstein dairy cattle at four time points (M1, M2, M4, M14) post calving.

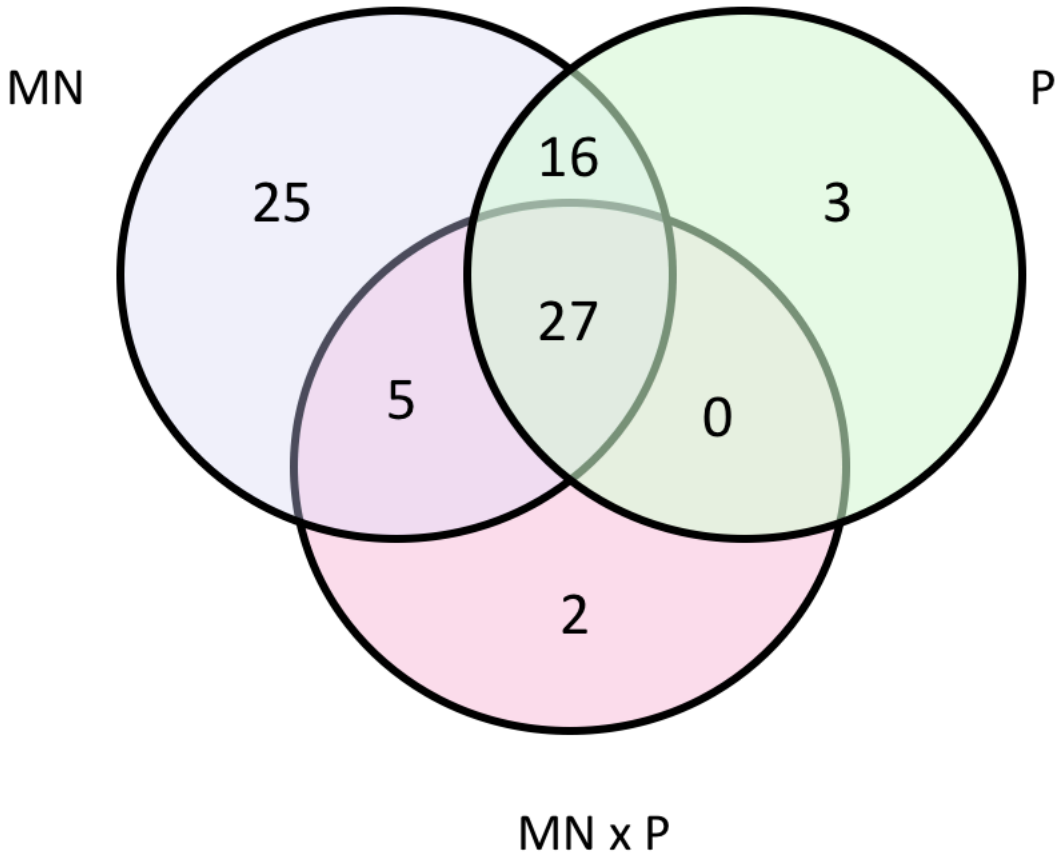
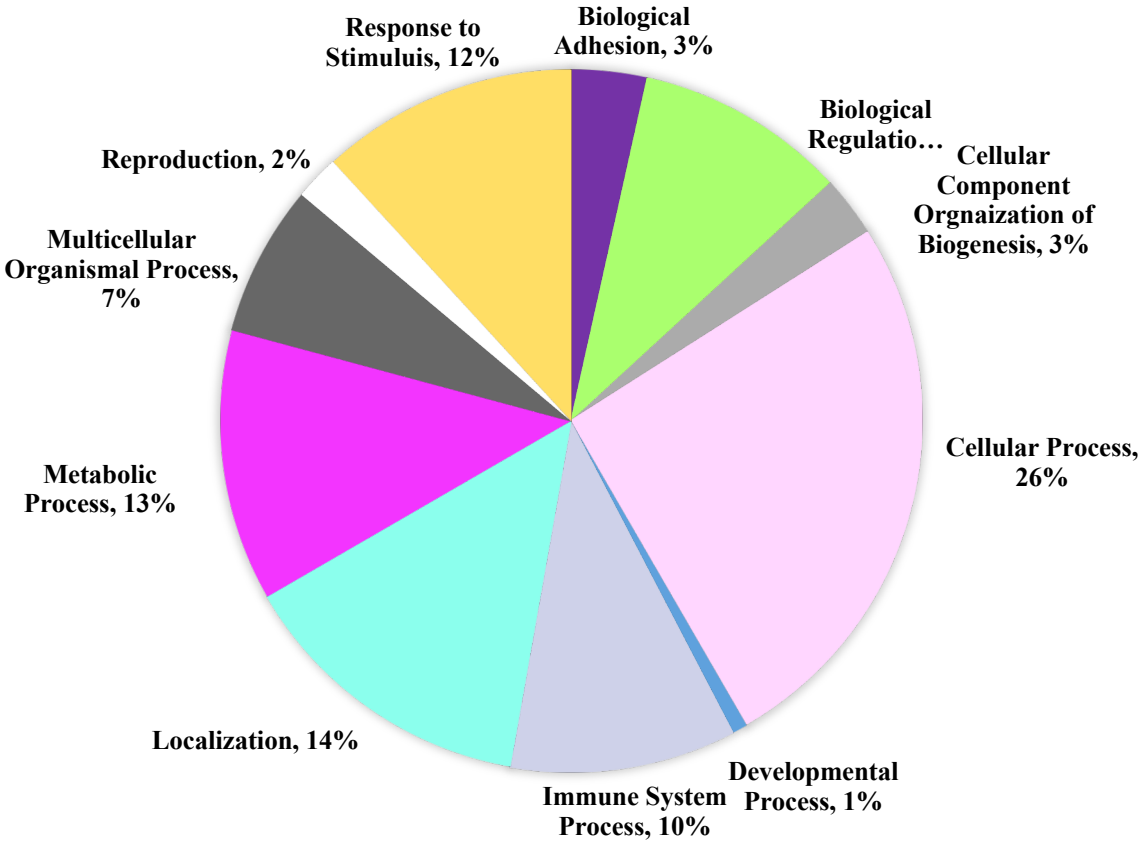


Figure 3.2 Pie chart representing the biological processes of all characterized proteins MFGM proteins collected from primiparous (PP) and multiparous (MP) Holstein dairy cattle at four time points (first milking (M1), second milking (M2), fourth milking (M4), and fourteenth milking (M14)) post calving. Categories were determined by PANTHER database and chart was generated in Excel.



This chart excludes F1MHH9 and G3N2D7 due to limitations in the PANTHER database

Figure 3.3 Bar chart highlighting the gene ontology (GO) of MFGM proteins characterized across four time points post calving (first milking (M1), second milking (M2), fourth milking (M4), and fourteenth milking (M14)) from primiparous (PP) and multiparous (MP) Holstein dairy cattle that comprise the cellular process component of biological processes (26%).

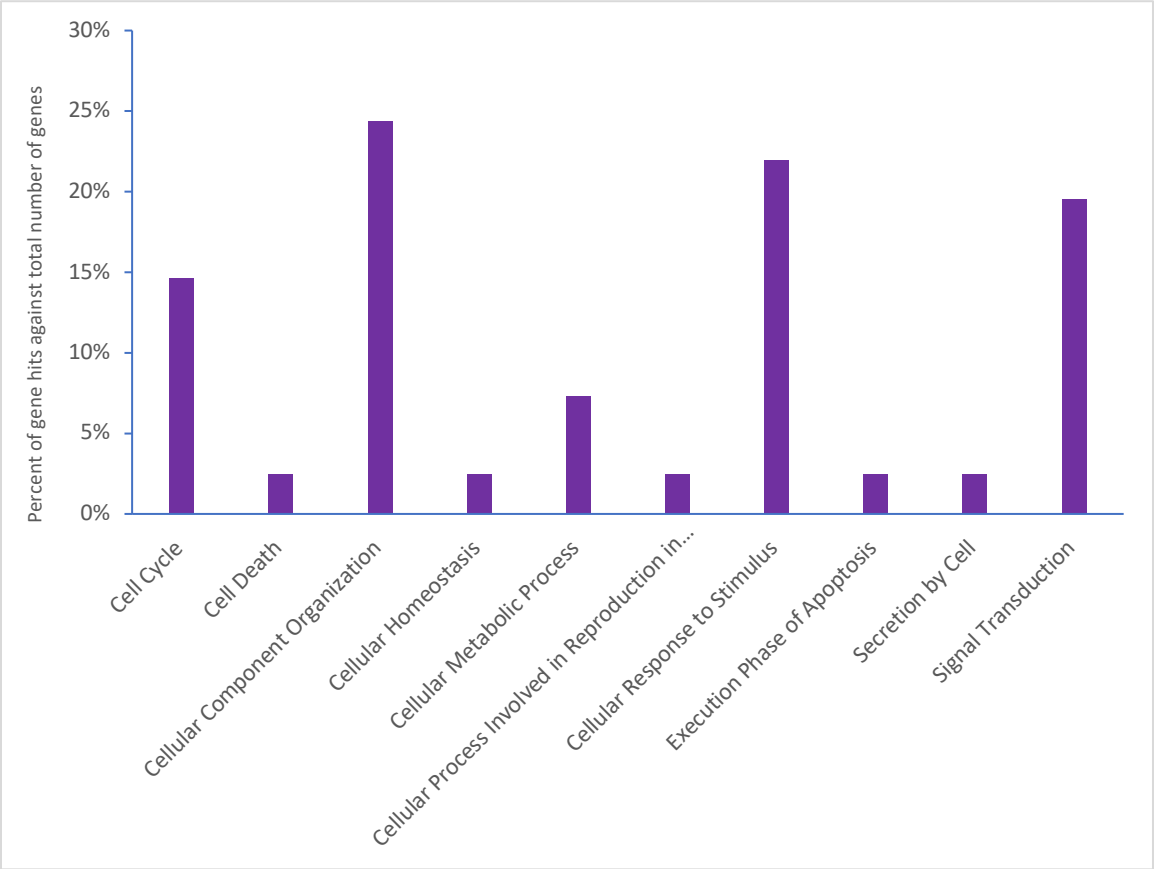
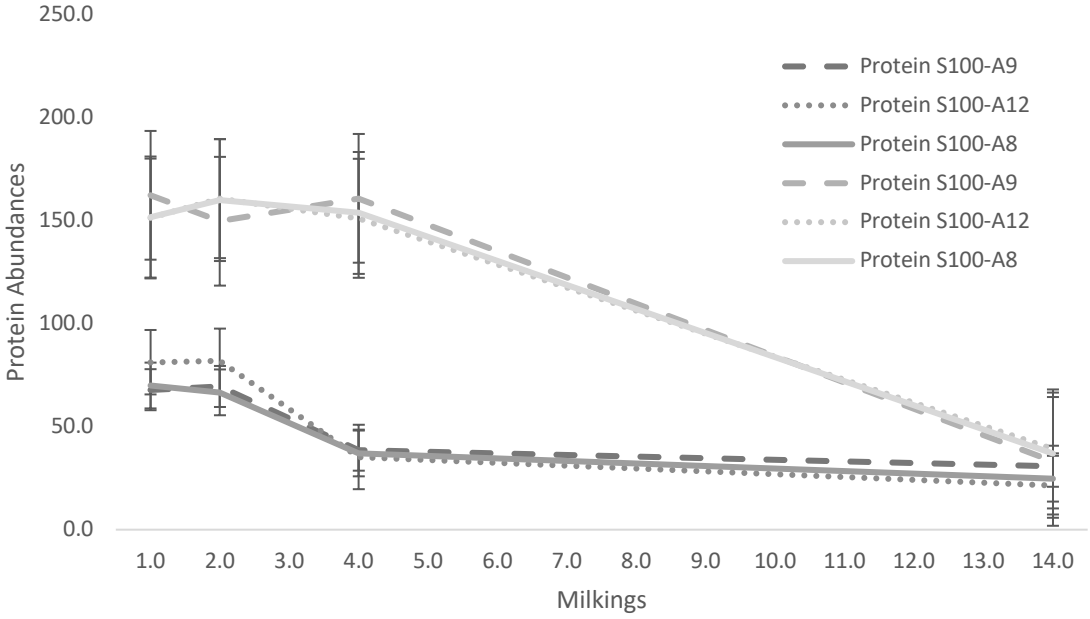


Figure 3.4 A line graph representing scaled (relative to a universal control set at 100) abundances of S100 proteins affected by the interaction between milking number and parity (MN×P) from the MFGM identified by PANTHER in colostrum and transition milk samples collected from primiparous (PP) and multiparous (MP) cows at four time points (first milking (M1), second milking (M2), fourth milking (M4), and fourteenth milking (M14)). Standard errors also depicted.



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Chapter 4: General Discussion and Conclusions

4.1 General Discussion

Despite a large breadth of biofluids being explored in dairy systems using proteome analysis, all results have a common goal of improving agricultural production whether that be in management or production through a greater physiological understanding. Aspects of management that could be refined due to a higher understanding of animal responses to the different management systems stemming from proteomic results include feed composition, scheduling of feed offerings, and environmental variables. As for production, an increase in quantity and components would increase margins for management to help business efficiency and profitability. Profiling results may be too intricate to translate to a production level at this time, but if it continues in this direction, proteomics could have a profound effect on the dairy industry.

The research presented in Chapter 2 expands current knowledge of the rumen metaproteome by identifying over 200 proteins derived from 13 rumen microbial species using novel proteomic workflows. We observed a change in 35 proteins based on sampling time (0 h, 4 h, 6 h after morning feeding). Upon further scrutiny, proteins that are related to the central dogma such as transcription and translation processes, were abundant at sampling times after feed was offered. However, at the initial sampling period after cows had gone through a period of not receiving fresh feed, proteins involved in more specific metabolic processes were more highly abundant. These results draw attention to microbial metabolic fluctuations due to feed and nutrient availability. Assessing whether these microbial communities have adapted to a feeding schedule and shift to low energy pathways when needed or are reflecting their preferred pathways

could provide insight into nutrient utilization preferences by rumen microbial populations. Understanding this more will lead to more opportunity for metabolic manipulation.

Chapter 3 presents the research analyzing the MFGM proteome in cows with physiological differences as time from parturition increased and assessed whether the MFGM proteome was different from primiparous versus multiparous cows. The physiological difference of parity (P) affected 44.2% of identified proteins, milking number (MN) affected 70.2%, and the interaction of the two (MN × P) affected 32.7%. Previous publications have established how stage of lactation influence milk components. What is novel about this research is that this is the first known investigation of the effect of parity on the MFGM proteome. Understanding that the MFGM is derived from secretory cells of the mammary gland, uncovering differences and examining trends can show how the mammary gland develops and adapts through their lifetime as a dairy cow. Comparing the differences in the MFGM proteome may also illustrate deficiencies of the animal and contribute to learning how to better accommodate the lactating cow in this physiologically stressful time of their life.

4.2 Limitations and improvements in rumen metaproteome study

The understanding of rumen microbial dynamics is limited, and while proteomic analysis could contribute a considerable amount, there are only two known publications regarding the rumen metaproteome. The research reported in Chapter 2 expands upon this knowledge but also draws attention to more limitations that are involved with rumen metaproteome studies. The following section offers some insight into limitations of the

experiment presented in this thesis, as well as potential considerations to improve future research.

4.2.1 Considerations for on-farm experimental design

Examination of the rumen metaproteome outlined in Chapter 2 examined 3 time point samplings within the first 6 hours of initial feed offerings. To characterize the full diurnal variations of the metaproteome, more sampling points are needed. To sample the rumen each hour could provide the most comprehensive outlook of the fluctuations that take place throughout a 24-h period. This would, however, disturb the rumination times of the cows as they would need to be standing at each sampling period. This would be especially disruptive during nighttime sampling. Nighttime samples are typically representative of fasting for the animal and the rumen microorganisms because new feed is rarely offered during this period, and feed availability decreases until the following morning when cows are again fed and feed intake is stimulated. Feeding protocols also fluctuate across farms and the research proposed did not change the feeding schedules for the trial. There were two offerings of a mixed silage feed to keep a steady supply of feed in front of the cow with four separate offerings of a grain concentrate mix throughout the day. Each feeding system is unique which can yield different results in the proteomic analysis. In the future, it would be suggested to adapt cows to a total mix ration (TMR) so the feed is homogenous at each offering. This could avoid major diurnal shifts in the rumen metabolism hence establishing a more consistent measurement. These measured diurnal shifts would remove nutrition supply as a variable and could focus more on the

effect of evening fasting, possible health issues, breed variations, and differences across lactation stages.

When considering nutrition, which could be the most insightful when it comes to the rumen metaproteome, thoughtful experimental design could also provide the opportunity to study the effect of supplementation on rumen metabolism. Analyzing the rumen metaproteome upon the induction of a feed manipulation trial may be able to provide the most direct insight into how pathways are altered. Feed manipulation trials are important when it comes to reaching the highest production and production efficiency for a cow, reducing environmental effects, and possibly incorporating byproducts from the human food chain that would otherwise be wasted. Further baseline studies will be needed to fully compare the effect of nutritional manipulation on the animal.

4.2.2 Lab and analysis considerations

Samples from the rumen can be challenging to work with because much of the feed is only partially digested, and the presence of many plant compounds still persists at this stage of digestion. This leads to interfering compounds within the samples that take additional filtering and washing. While these filtration steps are important to remove feed particulates that contain compounds, which have proved to be an obstacle in previous studies, it is an important consideration that inclusion of more filtration protocols in the workflow may diminish certain populations of the rumen organisms, specifically larger protozoal populations, thus overlooking the metabolic activity and contributions of an important member of the rumen microbial community.

One of the most challenging aspects during the in-lab protocol was resuspending the microbial pellet into buffer for quantification and LC-MS/MS analysis. In order to accomplish this, mechanical lysis was utilized to homogenize the pellet. While time of homogenization was kept to a minimum, one round of 30 s, heating of the samples can cause additional degradation of proteins limiting interpretation because it may not be representative of the proteome at that time. This can be more of an issue when comparing across studies, but since all samples were treated equally within this research, they are assumed to be comparable to each other. It was assumed that resuspension was a challenge because of the stringent precipitating agents used beforehand to obtain proteins in the solution to collect in pellet form. If there are other alternative and effective agents other than TCA, it may prevent the difficulty of dissolving and the need for homogenization. In the future, it may be considered to decrease the percentage of TCA if its presence proves to be pivotal in the protocol.

There is still much development needed in order to completely represent a metaproteome through current technology. The experiment outlined in Chapter 2 reported identification of proteins from 13 rumen microbial species which does not encompass all rumen microbial species, as McSweeney and Mackle (2012) have reported that rumen bacterial species numbers including species strains can reach 7,000 and archaeal species 1,500. Only identifying proteins from 13 microbial species is assumed to be a database limitation and not because of lab methodology as the group of microbes identified, as well as their proteins, are diverse. If it were to be seen that strains of only a few microbial species were identified, there may be a key flaw in the protocol that was removing

various species. Each of the 13 species identified were an individual strain of a different species.

A key part of the fractionation workflow included the high pH reversed-phase peptide fractionation kit (Thermo Scientific, Rockford, IL) which was a new addition to our lab protocols. This likely reduced the incidence of highly abundant proteins concealing lower abundance proteins through overlap in MS curves, which may explain our high quantity of proteins identified compared to previous research. The inhibition of low abundance protein identification has been a previous issue when working with milk due to the presence of a select few high abundance proteins, and should be considered in further proteomic workflows of any biofluid.

4.3 Limitations and improvements in MFGM proteome study

The study of milk proteomics is additionally important from a consumer standpoint. The MFGM is especially under characterized and the potential importance of this fraction to consumer and calf health is vastly overlooked at present. In the case of our research, we focused on colostrum and transition milk which brings more attention to understanding the physiology of the cow and healthful attributes of milk-feeding to calf health. The most important findings from our research related to the physiological impact of parity on the MFGM proteome.

4.3.1 Lab and analysis

Obtaining the proteins from the MFGM incorporates two major isolation procedures. The first being separation of the fat fraction from the whey portion of the

milk, which contains high abundance proteins such as casein isoforms (αs_1-, αs_2-, β-, γ- and κ-CN), α-LA, and β-LG. Careful centrifugation is essential but it is difficult to wash the fat portion or any skim proteins and the presence of these proteins will conceal lower abundance proteins during LC-MS/MS analysis. Also having these proteins present for analysis can give false representations of the MFGM proteome. In our study, despite two rounds of centrifugations, some of these aforementioned proteins were still present in our final results. There will need to be an additional step in order to fully remove these proteins, perhaps a filtered centrifugation. The second isolation procedure is separating the membrane proteins from the fat fraction. This is done by washing with PBS and lysis buffer with additional centrifugation and an overnight acetone precipitation. It is known that these steps are efficient because otherwise fat present would impede quantification readings. There is, however, a step in this workflow that includes heating the samples in a 95°C water bath. As mentioned previously, heating samples should be avoided as it can result in protein degradation. Finding a different technique to disrupt the membrane may prove to be more beneficial for subsequent results.

4.4 Future perspectives

Little research has been completed on connecting proteomes in various biofluids or even tissues to the greater animal physiology. The original design included a comparison of the rumen metaproteome to the milk proteome in the first experiment. However, with research being so limited in rumen metaproteomics, it was not known how successful the ability to correlate the two would be. After developing our own rumen metaproteomics protocols, over 200 proteins were able to be identified, and that number

will grow substantially as database entries grows. With this increase in identification capabilities, the comparison of those two would be a great prospect in understanding what proteins are contributing downstream to milk production. This could grow into a health and biomarker aspect. A metabolic disease with no specific and accurate diagnosis such as sub-acute ruminal acidosis, has proven to be detrimental to animal health and productivity. If it could be characterized how the metabolic shifts affect the downstream milk proteome, biomarkers could be accurately established and may expand beyond general inflammatory markers.

Inclusion of proteomic techniques to link the different platforms, including potentially the blood proteome, and potentially the tissue proteome, could be useful for progression of any biomarker development. As of right now, when analyzing and characterizing the MFGM, many of the conclusions are hypotheses of what may be happening in the mammary gland. Being able to complete proteomic analysis on both mammary tissue and the MFGM, more solidified conclusions could be drawn about exact contributions being made at a cellular level. This will further elucidate contributions to the MFGM and identify reliable colostrum quality indicators or oncoming mammary infections that can be prevented. The cellular makeup of the mammary gland could also contain indicators of animals that are predisposed to mammary complications such as mastitis.

4.5 Conclusions

Proteomics has gained traction in dairy cattle research as it has provided valuable information on the animal's physiology. However, current results have not been more

exploratory to date, and have not been concrete or uniform enough across studies to influence any management decisions or develop diagnostic tests geared towards proteins that have been shown to change in abundance due to disease. Further proteomic database expansions will be necessary for future studies as well as additional fractionation protocols that do not skew the isolated protein fraction being used for analysis. With this development, it will be novel to connect proteomes within the same study for further physiological understanding and considerations for management.

Chapter 5: Comprehensive Bibliography

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