A Comprehensive Evaluation of Milk and the Milk Microbiome through Proteomic, Metabolomic, and Lipidomic Analyses to Identify and Characterize Subclinical and Clinical Mastitis in Early-Lactation Dairy Cows

A Dissertation
Presented in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
with a
Major in Animal Physiology
in the
College of Graduate Studies
University of Idaho
by
Haley Kathryn Peterson

Approved by:
Major Professor: Mark McGuire, Ph.D.
Committee Members: Amin Ahmadzadeh, Ph.D.; Gwinyai Chibisa, Ph.D.
Michelle McGuire, Ph.D.; Gordon Murdoch, Ph.D.
Department Administrator: Robert Collier, Ph.D.

December 2023

Abstract

Bovine mastitis is an inflammation of the mammary gland that negatively impacts milk production, animal welfare, and is a significant economic burden to the dairy industry. Many factors, including age, stage of lactation, housing, and milking procedures can affect incidence of mastitis on dairy farms worldwide. Mastitis typically occurs in the first few weeks postpartum and is generally caused by a bacterial infection. Mastitis can be clinical (CM; presence of clinical signs such as flakes or clots in the milk and/or redness or swelling of the mammary gland) or subclinical [SCM; milk somatic cell count (SCC) ≥200,000 cells/mL and no clinical signs]. Milk components, such as lactose, protein, sodium (Na), and potassium (K) concentrations; and the ratio of Na to K (Na/K) are distinctly altered during mastitis. The overall aim of the research presented in this dissertation was to comprehensively evaluate bovine mastitis utilizing different technologies, such as 16S rRNA full-length sequencing, proteomics, metabolomics, and lipidomics, on the same milk samples collected daily (over the first 22 d postpartum) from healthy cows and cows with naturally occurring CM or SCM on four commercial dairies in southern Idaho. The first objectives of this dissertation were to use SCC, concentrations of lactose, protein, Na and K, and Na/K to identify CM (in colostrum and milk) and SCM (in milk only) in individual mammary gland quarters of cows in early lactation and to test the diagnostic ability of components for mastitis. We hypothesized that components would accurately identify CM (in colostrum and milk) and SCM (in milk only) in individual mammary gland quarters. We found that diagnostic thresholds of SCC and K concentration could distinguish colostrum produced by quarters with CM from colostrum produced by quarters without CM. Furthermore, diagnostic thresholds of Na/K and Na and K concentrations were capable of accurately identifying milk produced by quarters with CM. Diagnostic thresholds of Na/K and lactose and Na concentrations accurately identified milk produced by quarters with SCM. These components in both colostrum and milk aid in the detection of CM and SCM in individual mammary gland quarters of cows in early lactation. The second objectives were to identify differences in milk bacterial communities among samples collected from quarters with CM, quarters with SCM, and healthy quarters between two timepoints: before the matched event (1-3 d prior to first observance of clinical signs) and during the matched event (first observance of clinical signs). The milk samples collected from quarters with SCM and from healthy quarters were matched, as closely as possible based on dairy, parity, and d postpartum, to milk samples collected from quarters with CM. We hypothesized bacterial communities would not be different among milk samples collected from quarters with CM, milk samples collected from quarters with SCM, and milk samples collected from healthy quarters before the matched event. We further hypothesized bacterial communities would be different among milk samples collected from quarters

with CM, milk samples collected from quarters with SCM, and milk samples collected from healthy quarters during the matched event. Finally, we hypothesized bacterial communities in milk collected from quarters with CM or SCM would exhibit distinct shifts between the two timepoints but that the bacterial communities in milk samples collected from healthy quarters would not exhibit such shifts. *Staphylococcus* was more abundant in milk samples collected from quarters with SCM relative to milk samples collected from healthy quarters and quarters with CM. An interesting aspect of this study was that *Bifidobacterium* was identified in high relative abundance across all milk samples. Accounting for environmental influences may provide important information to assist our understanding of mastitis and the milk microbiome. The third objective was to perform proteomic, metabolomic, and lipidomic analyses on milk samples collected from quarters with CM, milk samples collected from quarters with SCM, and milk samples collected from healthy quarters at two timepoints (before the matched event and during the matched event). A final objective was to identify specific proteins, metabolites, and lipids that may predict CM in early lactation, aiding in the management of mammary gland health. We hypothesized that proteins, metabolites, and lipids would be differentially abundant in milk collected from quarters with CM or SCM compared to milk collected from healthy quarters. Furthermore, we hypothesized that a few proteins, metabolites, and lipids would be early predictors of CM. We found differences in specific proteins, metabolites, and lipids in milk produced by quarters with CM or SCM compared to milk produced by healthy quarters among cows and within the same cow. Proteins and metabolites related to immune function were at higher levels in milk produced by quarters with CM or SCM relative to milk produced by healthy quarters. Milk produced by quarters with CM exhibited higher levels of lipids abundant in cell membranes and lower levels of main milk lipids relative to milk produced by healthy quarters. Several specific proteins (VPS37B subunit of ESCRT-I, transitional endoplasmic reticulum ATPase, fructose-bisphosphatase, complement C8 gamma chain, cartilage acidic protein 1, alpha-L-iduronidase, osteoclast stimulating factor 1, N-acetyl-alpha-glucosaminidase, ras-related protein Rab-18, nucleobindin 1, HRas proto-onco GTPase, ß-casein, NSF attachment protein gamma, filamin A, glycosylation-dependent cell adhesion molecule 1, transforming growth factor ß, MIA SH3 domain endoplasmic reticulum export factor 3, syntaxin-19, xanthine dehydrogenase/oxidase, and tartrate-resistant acid phosphatase type 5), metabolites (citric acid, L-leucine, L-tyrosine, palatinose, 3-indolelactic acid, L-isoleucine, L-lysine, and trans-3-hydroxyl-L-proline), and lipids (one sphingomyelin species, four phosphatidylcholine species, one triglyceride species, one phosphatidylinositol species, and one phosphatidylethanolamine species) were identified in milk via machine learning to accurately predict the occurrence of CM in individual mammary gland quarters

of cows in early lactation. Overall, this research will contribute to the advancement of bovine mastitis research and commercial milk production.

Acknowledgments

I would like to thank the following people for their important roles in this dissertation and my academic career:

My major professor, Dr. Mark McGuire, for his excellent mentorship, insight, encouragement, and considerable investment into my research career.

Drs. Amin Ahmadzadeh, Gwinyai Chibisa, Gordon Murdoch, and Shelley McGuire, for serving on my committee and for their professional advice, assistance, and support to this dissertation.

Dr. Janet Williams and Dr. Ryan Pace for their advice, encouragement, guidance, and research support and assistance.

The dairymen and employees of Swager, Bokma, and Peterson dairies for their willingness to collaborate on this project and allow us access to their dairy cows.

Taylor Kelley, Jonathan Ehlers, Makenzy Whittekiend, Emma Seamons, and Jayde Wilson for their tremendous help with sample collection.

Dr. Tom Metz, Dr. Sneha Couvillion, Carrie Nicora, Dr. Ernesto Nakayasu, and Dr. Bobbie-Jo Webb-Robertson of the Pacific Northwest National Laboratory for their assistance and expertise in the proteomic, metabolomic, and lipidomic analyses of this collaborative project.

Dan New and Maria Schneider at the Genomics and Bioinformatics Resources Core in the Institute for Interdisciplinary Data Sciences at the University of Idaho for their assistance and expertise in the full-length 16S rRNA gene sequencing component of this dissertation.

Graduate students Alex Gogel, Cassandra Partridge, and Sarah Boothman for research support and assistance.

Undergraduate students Katlyn Scarrow, Meghan Lewis, Léa Dussurget, Yvette Bonney, and Courtney Buchert for research assistance.

The University of Idaho Animal, Veterinary, and Food Sciences department faculty, staff, and students for support throughout my postgraduate education.

This project was supported by National Institutes of Health grant no. 1R01HD092297-01A1.

Dedication

This dissertation is dedicated to my mother, Kathryn, my father, Erik and my brother, Andrew. Thank you from the bottom of my heart for your steadfast love and unwavering support throughout this tremendous, exciting, occasionally difficult, but rewarding journey.

I never would have made it this far without each of you by my side.

Table of Contents

List of Tables

List of Figures

Statement of Contribution

Facilitated collaboration with dairymen: H.K.P.; milk sample collection: H.K.P., with assistance from J.E.W.; chapter two data analysis: H.K.P., with assistance from R.M.P. and J.E.W.; chapter two writing: H.K.P., M.A.M., J.E.W., R.M.P. and M.K.M.; chapter three laboratory analysis: H.K.P., with assistance from R.M.P. and J.E.W.; chapter three data analysis: H.K.P., R.M.P. and J.E.W.; chapter three writing: H.K.P., M.A.M., and M.K.M.; chapter four data analysis: S.P.C., C.D.N., E.S.N., T.O.M., B.M.W., H.K.P., R.M.P., and J.E.W.; chapter four writing: H.K.P., M.A.M., B.M.W., E.S.N., S.P.C., R.M.P., J.E.W. and M.K.M.

Chapter 1: Literature Review

Mastitis

Mastitis, or inflammation of the mammary gland, is a burdensome disease that affects many dairy cows. Mastitis tends to occur in the first few weeks postpartum, is often caused by bacterial infection, and generally leads to decreased milk production and compromised animal health (Pyörälä, 2008; Ballou, 2011). It is important to note that mastitis and intramammary infection (IMI) are not necessarily equivalent. Mastitis encompasses inflammation of the mammary gland, whether it results from an infection or not, whereas IMI necessitates the presence of a pathogen in the mammary gland (Andersen et al., 2010; Souza et al., 2020). These terms are commonly used interchangeably because of the inconsistency of their definitions. Milk somatic cell count (SCC), a common marker of inflammation, is typically used in the identification of mastitis. Milk SCC contains mostly white blood cells such as macrophages, lymphocytes, and neutrophils (Kehrli and Shuster, 1994; Alhussien and Dang, 2018; Malik et al., 2018). Somatic cell count is a valuable monitoring tool as somatic cells in milk give an impression of the inflammatory status of the mammary gland (Kehrli and Shuster, 1994; Schukken et al., 2003; Hagnestam-Nielsen et al., 2009; Alhussien and Dang, 2018). Eberhart et al. (1979) reported the factor that had the greatest effect on SCC was infection status and Djabri et al. (2002) noted that one infected quarter is usually enough to consider the entire mammary gland as infected. In milk produced by healthy quarters, macrophages are the main cell type but, in the event of an infection, they recruit polymorphonuclear neutrophils (PMN) from the blood via inflammatory chemoattractants such as lipopolysaccharide (LPS) and cytokines (Paape et al., 2003; Malik et al., 2018; Souza et al., 2020). Phagocytosis by PMN is the optimal defense against invading bacteria. However, PMN can also cause injury to mammary gland tissues and inhibit function by generating excessive reactive oxygen species (Paape et al., 2002; Paape et al., 2003) and releasing granular enzymes (serine proteases found in T cells; Tizard, 2013). Ultimately, infection outcome and disease severity are determined by number of circulating PMN, their efficiency at adhesion, migration, opsonization, and phagocytosis to eliminate the bacterial threat, and, finally, their ability to regress to pre-infection levels (Paape et al., 2002; Paape et al., 2003). The inflammatory response induces both local and systemic effects which can severely impact mammary gland function, milk production, and overall animal health. Local effects include leakage of milk components through compromised mammary epithelial cell barriers, increased nutrition and energy requirements to support recruited leukocytes and other inflammatory responses, and reduced ability of mammary epithelial cells to synthesize milk components, regardless of the substrate availability. Systemic effects encompass decreased dry matter intake, alterations in nutrient partitioning that negatively affect substrate

availability to mammary epithelial cells, and hyperthermia (Ballou, 2011). Inflammation is an important part of the immune response. However, the balance between pro- and anti-inflammatory mediators is critical in preventing uncontrollable disease.

Mastitis generally occurs in the first few weeks postpartum, possibly due to immunosuppression from physiological changes associated with parturition, colostrogenesis, and lactogenesis (Akers, 2002; Tizard, 2013). These rapid and dramatic changes around parturition may lead to minimized immune capacity, negative energy balance, hypocalcemia, systemic inflammation, and oxidative stress; all of which influence maternal health (Trevisi and Minuti, 2018; Bronzo et al., 2020; Horst et al., 2021). Problems of a metabolic and/or infectious nature can materialize when these issues become prolonged and exaggerated. Parturition is considered a stressful event as stress hormones such as glucocorticoids (like cortisol) and catecholamines (such as epinephrine and norepinephrine) are released during this time and negatively impact the immune system (Akers, 2002; Reece, 2004). This issue is further exacerbated as immunoglobulins and molecules, such as lysozyme and lactoferrin, that regulate the immune system are transferred to colostrum during colostrogenesis (Akers, 2002). Glucocorticoid concentrations increase to initiate lactogenesis (Aleri et al., 2016). The release of pro-inflammatory mediators such as interleukins-1 and -6 and tumor necrosis factor are inhibited by these stress hormones (Diez-Fraile et al., 2003). Populations of macrophages, lymphocytes, and PMN and their associated functions are also altered due to heightened glucocorticoid concentration (Aleri et al., 2016). Reduced effectiveness of PMN activities such as activation, migration, adherence, and phagocytosis may perpetuate increased susceptibility to intramammary infections (Pyörälä, 2008). Ultimately, cellular immunity may be selectively suppressed, and antibody-mediated immunity may be selectively promoted. This could lead to an imbalance of pro- and anti-inflammatory mediators and possibly result in immunosuppression.

Mastitis can be subclinical (SCM; milk SCC ≥200,000 cells/mL without clinical signs) or clinical (CM; presence of clinical signs) [Smith et al., 2001; International Dairy Federation (IDF) Bulletin 466, 2013]. Signs of CM include milk that contains blood, flakes, or clots and/or swelling, redness, or hardness of the mammary gland. Morrison et al. (2018) concluded mammary gland edema was associated with a greater incidence of CM in early lactation whereas other metabolic disorders were not. Clinical mastitis and SCM have different economic impacts as well. On average, a case of CM in early lactation can cost a producer $444 of which direct costs contribute $128 and indirect costs contribute $316 (Rollin et al., 2015). Direct costs include diagnostics, veterinary services, therapeutics, death loss, discarded milk, and labor. Indirect costs are associated with future reproductive and milk production losses, replacement costs, and premature culling. Economic loss at

the herd-level is far greater for SCM than for CM (Hagnestam-Nielsen et al., 2009). On average, SCM costs $110/case with an estimated 15 to 40 times greater incidence in most dairy herds compared to CM cases (Ott, 1999; Shaheen et al., 2016; Malik et al., 2018). The greater economic loss with SCM compared to CM is mainly attributed to decreased milk production by affected, yet generally undetectable, animals in the herd. Overall, the devastating economic losses associated with mastitis are attributable to both CM and SCM cases.

A variety of factors can increase the risk of mastitis in cows. History of mastitis, higher parity, milk production, and earlier stage of lactation are important risk factors (Zadoks et al., 2001; Williams, et al., 2012; Jamali et al., 2018). Zadoks et al. (2001) reported the rate of mastitis attributed to *Staphylococcus aureus* or *Streptococcus uberis* infection was diminished in lower-parity cows (≤2) compared to higher-parity cows (>2). Williams et al. (2012) found animals with elevated milk SCC (>200,000 cells/mL) in their first lactation were more likely (odds ratio of 3.11) to have days of elevated milk SCC in their second lactation. Any milk SCC patterns that appear in the first lactation will likely influence mammary gland health in later lactations. Syväjärvi et al. (1986) found older cows from herds with very high milk yields (>6,150 kg) were at a greater risk of CM (25% risk) compared to first-lactation cows from herds with very low milk yields (<4,870 kg; 2.2% risk). Using logistic regression models, Gröhn et al. (1990) reported individual cows with high levels of production (>7,060 kg) in the preceding lactation were at a greater risk for CM as well as teat injury and that, as milk production climbed, so did the risk for acute mastitis. The elevated risk of teat injury and/or chronic mastitis was linked to only the highest levels of production. Gröhn et al. (1995) also noted older cows were more at risk of contracting mastitis compared to younger cows. A review by Jamali et al. (2018) concluded higher levels of milk production and higher parity were notable risk factors for recurring CM (generally defined as additional cases of CM within the same lactation). Other important cow-specific risk factors include poor housing conditions, inadequate cleaning procedures, and improper milking procedures (Schukken et al., 1990; Taponen et al., 2016; Latorre et al., 2019). Dufour et al. (2011) reported lower incidence and prevalence of IMI caused by *S. aureus* when gloves were worn during milking, pre-milking teat disinfectant was used, and teat-end condition was adequately maintained. Clinical mastitis was positively associated with bedding materials such as manure solids and organic non-manure options (such as straw and sawdust) that have high levels of bacteria (Shaheen et al., 2016; Patel et al., 2019; Rowe et al., 2019). Breen et al. (2009) reported severe teat-end damage and extremely dirty mammary glands to be important cow-level risk factors for CM. Breed (such as Holstein-Friesian associated with lowest risk compared to Meuse-Rhine-Yssel) and season (like summer months associated with lowest risk compared to spring months) have also been found to increase the risk of CM in cows (Schukken et al., 1990; Shpigel et al., 1998;

Oliveira et al., 2015). Rear quarters are at a higher risk of infection likely due to their larger size and greater chance of injury compared to the front quarters (Barkema et al., 1997; Shpigel et al., 1998; Sumon et al., 2020). In summary, milk yield, age, stage of lactation, breed, season, and bedding materials are risk factors associated with mastitis incidence.

Directly measuring milk SCC is important for accurate characterization of mastitis but can be costly, time-consuming, and difficult to implement on-farm. Therefore, various indirect methods have been introduced and tested for accuracy. Pearson et al. (1971) compared the California Mastitis Test (CMT) with directly measured cell counts to see how well CMT indirectly measured SCC in quarter milk samples. They determined CMT scores corresponded well with actual SCC due to a high correlation coefficient of 0.88. Ward and Schultz (1972) estimated milk SCC using the filter-DNA method to investigate the relationship of SCC and the type of bacteria present in the infected gland. They also compared milk SCC of cows with a history of CM to milk SCC of cows without a history of CM. They reported the overall estimated mean SCC of milk collected from quarters that did not yield any bacteria was 310,000 cells/mL compared to milk collected from quarters that yielded bacteria (SCC range of 356,000-1,625,000 cells/mL). Regardless of bacterial presence, milk collected from cows without a history of CM had an estimated mean SCC of 414,000 cells/mL. Milk collected from cows with a history of CM had an estimated mean SCC of 1,120,000 cells/mL. Pearson and Greer (1974) conducted a follow-up study using CMT, but this time examined the relationship between CMT and different levels of bacterial isolation. They reported correlation coefficients of 0.71-0.88 depending upon the category of infection of which there were five denoting varying degrees of bacterial isolation (number of colonies) and CMT scores (from negative to CM). Wanasinghe and Frost (1979) used another indirect method of measuring milk SCC, the Wisconsin Mastitis Test (WMT), and bacterial culturing to examine infection prevalence between two herds. At the bulk-tank level, one herd had a low WMT score (<15 mm which is approximately equal to an SCC of 800,000 cells/mL) and the other herd had a high WMT score (>15 mm). They reported the herd with the higher WMT score of bulk tank milk had higher WMT scores for quarter milk samples. The herd with the higher WMT score also had a higher prevalence of infection compared to the herd with the lower WMT score of bulk tank milk and consequently lower WMT scores for quarter milk samples. Eberhart et al. (1982) reported a correlation coefficient of 0.77 between bulk tank SCC and infection prevalence. They also reported 0.59 as the coefficient of determination thus indicating bulk tank SCC was associated with infection prevalence. These studies documented fluctuations in milk SCC pertaining to age, lactation stage, and history of mastitis as well as described indirect methods to measure SCC.

Mastitis affects milk yield and milk components such as protein, lactose, fat, sodium (Na), and potassium (K). Rajala-Schultz et al. (1999) found that, depending upon parity and disease onset, milk loss over a full lactation can range from 110-552 kg. Pérez-Báez et al. (2019) reported cows with CM experienced reduced feed intake (expressed as dry matter intake as percentage of body weight) and reduced energy-corrected milk yield. Increased milk SCC is associated with decreased milk yield due to physical damage to milk epithelial cells stemming from the immune response to an infection (Kerli and Shuster, 1994). Mammary epithelial cells lose their capacity to synthesize and secrete milk components during mastitis, which can persist for the remainder of lactation (Ballou, 2011; Alhussien and Dang, 2018; Malik et al., 2018). Milk yield also decreases due to loss of milk lactose into circulation as it is the osmotic regulator in milk. Lactose leaks out of alveoli as tight junctions of the mammary epithelial cells are loosened by incoming white blood cells (Harmon, 1994; Auldist and Hubble, 1998). Not only does the damage to the tight junctions allow milk components to leak out but it allows for blood components, such as Na, to flow in. This results in above-normal concentrations of Na in milk (Harmon, 1994; Alhussien and Dang, 2018; Hughes and Watson, 2018). Elevated SCC and bacteria in milk contribute to heightened enzymatic activity that affects milk components such as protein. Murphy et al. (2016) describes the increased action of plasmin, an active protease in milk, derived from the increased activation of plasminogen (an inactive precursor of plasmin also found in milk). Enhanced plasminogen activation is related to somatic cells so increased SCC ultimately leads to digested milk proteins, such as α-caseins and β-casein. Whey proteins are not as susceptible to plasmin, so they remain largely undigested (Murphy et al., 2016). This results in a milk protein profile that contains a relatively higher concentration of whey proteins and a relatively lower concentration of casein proteins. However, the overall milk protein concentration remains unchanged (Harmon, 1994; Auldist and Hubble, 1998). Milk fat composition remains largely unaffected by changes in milk SCC (Lindmark-Månsson et al., 2006; Murphy et al., 2016; Alhussien and Dang, 2018). Murphy et al. (2016) reported that use of raw milk with an SCC >100,000 cells/mL resulted in reduced cheese yields while use of raw milk with an SCC >400,000 cells/mL produced cheese and other dairy products that had flavor and textural deficiencies (like rancidity and bitterness). These changes could also occur in high SCC milk that undergoes pasteurization and storage under refrigerated conditions. In summary, milk production, milk components, and, ultimately, dairy products for human consumption are all impacted by mastitis.

Pathogens such as *Escherichia coli*, *S. aureus*, *Corynebacterium bovis*, *Klebsiella* spp., *Streptococcus dysgalactiae*, *S. uberis*, and non-*aureus* staphylococci (NAS) are thought to be responsible for most mastitis cases stemming from an infection (Heikkilä et al., 2018; Massé et al., 2019). A meta-analysis by Djabri et al. (2002) found that milk produced by healthy quarters had an average SCC of 70,000

cells/mL whereas milk produced by infected quarters had an average SCC between 110,000-150,000 cells/mL for minor pathogens and an average SCC >350,000 cells/mL for major pathogens. Major pathogens included *S. aureus*, *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, and coliforms (like *E. coli*) while minor pathogens included NAS and *C. bovis*. Heikkilä et al. (2018) reported milk loss resulting from mastitis by all pathogens studied (*S. aureus*, *E. coli*, *C. bovis*, *S. dysgalactiae*, *S. uberis*, and NAS). The extent of the loss was heavily influenced by the pathogen itself, whether it was SCM or CM, and when the mastitis occurred during lactation. *S. aureus* is a troublesome pathogen because it is highly contagious, has many genes that encode virulence factors (such as staphylococcus enterotoxins, Panton-Valentine leucocidin, hemolysins, and toxic shock syndrome toxin-1), and displays resistance to several antimicrobials (Ren et al., 2020). Different *S. aureus* genotypes dictate infection prevalence in cows and individual mammary gland quarters. Furthermore, multiple quarters within a cow are often infected (Fournier et al., 2008). Kümmel et al. (2016) tracked *S. aureus* from cow to dairy product and discovered certain subtypes, specifically those depicting characteristics of genotype B (*spa* type t2953, sequence type 8, and enterotoxin genes *sea*, *sed*, and *sej*), were able to infiltrate the manufacturing chain through contaminated milk. These *S. aureus* subtypes ended up in the saleable product, which demonstrates how toxins can potentially present a risk to human health. Genes encoding staphylococcal superantigens, which comprise several enterotoxins and toxic shock syndrome toxin 1, that were originally observed in *S. aureus* have more recently been found in NAS (Park et al., 2011a). Park et al. (2011b) detected several genes encoding staphylococcal superantigens, such as enterotoxin genes, in NAS isolated from milk collected from cows with IMI. The most frequent gene combination of *seb, seln,* and *selq* was found in 45 of 82 NAS isolates (Park et al., 2011). Non-*aureus* staphylococci collectively refer to a group of >50 species and subspecies of staphylococci that tend to cause many infections in early lactation, primiparous cows (Valckenier et al., 2019). Fox et al. (1995) reported NAS were responsible for the majority of IMI observed pre- and postpartum in both pregnant and nonpregnant heifers. Non-*aureus* staphylococci are the most pervasive agents of heifer mastitis with the highest incidence occurring at calving (Fox, 2009). *E. coli* is considered an opportunistic environmental pathogen that causes CM, which can sometimes be fatal, and is characterized by its potent endotoxin, LPS (Burvenich et al., 2003). Incidence of mastitis caused by *E. coli* is less of a concern than disease severity. Cow factors, such as stage of lactation, parity, and negative energy balance, rather than pathogenicity dictate the severity of *E. coli* mastitis. Acute signs of systemic disease occur around parturition whereas milder disease occurs in later lactation. Therefore, older cows in early lactation tend to experience more debilitating disease. *Klebsiella* spp. are prevalent in the dairy environment and are considered opportunistic pathogens because they can cause severe CM. Cows often exhibit acute clinical signs, suffer significant losses in

milk production, and, despite administration of antimicrobials, are very difficult to cure so they tend to be removed from the herd (Massé et al., 2019). Understanding the variations in disease severity and incidence caused by these pathogens is of utmost importance as they are responsible for most of the mastitis affecting dairy cattle worldwide.

Antibiotics are typically used to treat bovine mastitis; however, varying rates of success and the emergence of antimicrobial resistance have inspired the development and exploration of other treatment options. Cephalosporin tends to be the antibiotic of choice for treatment of mastitis for most dairy farms followed by lincosamide, and other β-lactam antibiotics (Oliver et al., 2011). Schukken et al. (2011) examined the effect of intramammary-infused ceftiofur hydrochloride, a third-generation cephalosporin, on CM caused by either *E. coli* or *Klebsiella* spp. They reported increased bacteriological cure (defined as the absence of the coliform species identified in the pretreatment sample in two posttreatment samples via bacterial culturing and strain typing) and greater clinical improvement in treated cows compared to untreated controls. There was also no difference in milk production or SCC between the two groups. Knowing the type of mastitis pathogen is important for determining treatment as some antibiotics are not effective against certain pathogens. Chronic *S. aureus* infections are very difficult, if not impossible, to treat with antibiotics (Oliver et al., 2011). Long-acting antibiotics such as cephapirin and dihydrostreptomycin are also used for prophylactic purposes in the form of dry cow therapy (DCT; Oliver et al., 2011). Dry cow therapy is administered at the beginning of the dry off period to all mammary gland quarters of all cows to treat existing infections and to prevent new infections. This traditional practice of blanket DCT has been very effective at decreasing incidence of mastitis but, with increasing concerns of antimicrobial resistance, a slightly different approach is gaining attention (Cameron et al., 2013). Selective DCT refers to identifying cows to receive treatment based on milk SCC and on-farm culture results to only treat those at the highest risk of mastitis but also reduce overall antibiotic use. Cameron et al. (2013) compared mastitis risk in cows that received selective DCT (based on a Petri-film culture method done on-farm) and cows that received blanket DCT. No differences were reported in terms of risk associated with cure of current infections, new infections over the dry period, new infections at calving, and CM occurrence in the first part of lactation between the two groups. Selective DCT holds promise to reduce antibiotic usage, but some research (Crispie et al., 2008; Piepers et al., 2016; Gao et al., 2020) is going even further to eliminate the need for antibiotics to treat mastitis.

Probiotics, vaccines, and immunomodulators have been, and are continuing to be, explored as novel treatments for mastitis. Crispie et al. (2008) and Klostermann et al. (2008) both reported intramammary infusion of a probiotic (a live culture of *Lactococcus lactis*) behaved similarly to a

commonly used antibiotic in the treatment of mastitis. Gao et al. (2020) observed decreased milk SCC and inflammatory activity (measured as milk concentrations of proinflammatory cytokines and enzymes) in cows with mastitis (SCC >500,000 cells/mL; CM or SCM not distinguished) supplemented with either yeast, lactic acid bacteria (LAB), or a mix of the two probiotics compared to unsupplemented cows with mastitis. The yeast and/or LAB supplements were mixed into the total mixed ration and fed to the cows. They reported no differences in milk SCC or inflammatory activity in the probiotic-supplemented cows compared to healthy controls (SCC <500,000 cells/mL and no clinical signs). This suggests yeast and LAB have potential as probiotic treatments. However, it is important to note that inflammation is usually present when milk SCC is >200,000 cells/mL so those cows denoted as healthy may have SCM and, therefore, would not provide an appropriate comparison. Yu et al. (2017) compared a teat disinfectant containing LAB to a commonly used teat disinfectant on cows with SCM (milk SCC >200,000 cells/mL and no clinical signs). They observed a slight decrease in SCC from cows treated with the LAB teat product compared to the cows treated with the commercial teat product. A review by Rainard and Foucras (2018) points out many issues and inconsistencies with probiotics research for managing mastitis. They conclude oral probiotics show little promise, intramammary probiotics should be regarded with caution, and teat end probiotics have the most positive outlook.

Vaccines to protect against mastitis infections by various pathogens are being created and tested with a few even commercialized. Piepers et al. (2016) examined the efficacy of a polyvalent (now commercialized) vaccine against *S. aureus* in heifers and cows subject to an intramammary challenge. The inflammatory reaction was mild in vaccinated animals compared to unvaccinated controls, suggesting the vaccine elicited a more adept immune response that resulted in efficient clearance of the pathogens. Rainard et al. (2021) summarized effectiveness of commercial and experimental vaccines against certain mastitis pathogens and reported mixed results. Specifically, vaccines for coliform mastitis, streptococcal mastitis (such as *S. uberis, S. agalactiae,* and *S. dysgalactiae*), and *S. aureus* mastitis have been found to be mildly effective or not effective at all. The development of truly efficacious vaccines is advancing. However, there are still several questions to answer, inefficiencies to work out, and obstacles to overcome.

Nonetheless, mammalian secreted phospholipases A2 (sPLA2s) and GLP 810 (a patented immunomodulatory product consisting of lactic acid, glycopeptides, lysozyme, and 0.9% NaCl solution), show promise. Seroussi et al. (2018) tested the use of sPLA2s anti-inflammatory effects as treatment for cows infected with *S. dysgalactiae* or NAS. Infection of *S. dysgalactiae* was cleared up with one treatment of the sPLA2. However, no improvement was observed in infections by NAS.

Gulbe et al. (2020) examined the potential effects of GLP 810 on cows with SCM (milk SCC >400,000 cells/mL and no clinical signs). They reported intramammary infusion of GLP 810 increased the numbers of PMNs and some pro-inflammatory cytokines and decreased the number of macrophages and bacterial pathogens. They propose this compound could be used to enhance the immune response in cases of SCM. In summary, control of mastitis with probiotics, vaccines, and immunomodulators, though promising, is not yet viable, and further research is needed.

The Milk Microbiome

Table 1-1 highlights some of the wide variation of microbiomes in milk produced by healthy cows, cows with SCM, and cows with CM. Historically, milk was thought to be sterile. Any bacterial presence was attributed to contamination during collection or during cases of infection (like mastitis). Culture-dependent methods established presence of bacteria. They were used for isolation and characterization of known bacteria particularly related to mastitis. These methods have been used to isolate roughly 140 bacterial species or subspecies in milk from infected bovine mammary glands (Koskinen et al., 2010; Taponen et al., 2019). However, these methods fail to grow bacteria in 20-30% of CM cases in cows (Taponen et al., 2009). Culture-independent methods to identify bacteria in a sample are based on DNA, specifically the 16S rRNA gene present in all bacteria (Hugerth and Andersson, 2017). These methods are gradually being utilized to aid in identifying bacterial pathogens for every case of mastitis. Polymerase chain reaction (PCR) amplifies targeted regions (conserved regions and hypervariable regions) of the 16S rRNA gene. Results of sequencing of amplified PCR products, or amplicons, can be checked against databases of known sequences, thus allowing the identification of bacterial taxa or groups in a specific sample.

Bovine milk microbiome research indicates bacterial communities differ in milk collected from healthy cows compared to milk collected from cows with CM or SCM. Metzger et al. (2018) noted the milk microbiome of healthy quarters is richer (greater Chao richness) and more diverse (greater Shannon diversity) but less abundant compared to the milk microbiome of quarters with SCM. Derakhshani et al. (2018) hypothesized a reduction in commensal bacteria in the mammary gland may have a negative effect on mammary health and natural resistance to mastitis. Decreased diversity and changes in microbiome composition, or dysbiosis, may be associated with mastitis incidence (Derakhshani et al., 2018; Taponen et al., 2019; Andrews et al., 2019). Dahlberg et al. (2020) collected milk from healthy cows (SCC <100,000 cells/mL and no history of mastitis) and found differences in relative abundance of certain taxa between samples collected directly from the teat and samples collected from a quarter milking machine. Compared to milk samples collected from a quarter milking machine, *Dyella*, *Delftia*, and *Janthinobacterium* were in higher relative abundance in

milk collected directly from the teat. Compared to milk samples collected directly from the teat, *Clostridium XI*, *Stenotrophomonas*, and *Pseudomonas* were in higher relative abundance in milk collected from a quarter milking machine. Andrews et al. (2019) reported the microbiome of milk collected directly from the teat cistern (via a teat cannula) was less rich than the microbiome of teat skin samples collected by swabbing the end of the teat. There was no difference in richness between the microbiomes of milk collected from healthy cows (SCC <100,000 cells/mL and no clinical signs) and the microbiomes of milk collected from cows with SCM (SCC ≥200,000 cells/mL and no clinical signs) or with CM (SCC ≥200,000 cells/mL and clinical signs). However, milk samples collected from quarters with mastitis (either SCM or CM, defined above) had reduced evenness compared to milk samples collected from healthy quarters. The microbiomes of teat end samples from both healthy cows and cows with SCM or CM had similar richness and evenness. Milk microbiome and teat end microbiome composition overlapped the most in samples collected from cows with SCM or CM compared to samples collected from healthy cows. The most abundant taxon in the microbiomes of teat ends of healthy cows and cows with SCM or CM was *Staphylococcus*. The most abundant taxon in the microbiomes of milk collected from cows with SCM or CM was *Staphylococcus*. *Micrococcus* and *Acinetobacter* were the most abundant taxa in milk collected from healthy cows. The similarities between teat end microbiomes and teat cistern milk microbiomes suggest a connection that may be influenced by infection status or by proximity. Although great care was taken to reduce contamination, the possibility cannot be completely discounted.

Kuehn et al. (2013) collected milk samples from both a quarter with CM and a healthy quarter within a cow with CM (identified by animal care personnel). They reported differences in relative abundance of certain taxa. Milk collected from quarters with CM had higher relative abundances of *Sphingomonas, Stenotrophomonas,* and *Corynebacterium* compared to milk collected from healthy quarters within cows with CM. Milk collected from healthy quarters had higher relative abundances of *Ralstonia, Pseudomonas, Psychrobacter,* and *Bradyrhizobium* compared to milk collected from quarters with CM within cows with CM. Oikonomou et al. (2014) compared milk collected from healthy cows (culture negative with SCC <20,000 cells/mL) to milk collected from cows with CM (culture negative but exhibited signs of CM). Milk samples collected from healthy cows contained higher relative abundances of *Propionibacterium, Aeribacillus,* unclassified *Lachnospiraceae,* and unclassified *Ruminococcaceae* compared to milk collected from cows with CM. Milk produced by cows with CM had higher relative abundances of *Streptococcus,* unclassified *Bacteroidales, Porphyromonas, Fusobacterium,* and *Asticcacoulis* compared to milk produced by healthy cows. Hoque et al. (2019) also compared milk microbiomes of healthy cows and cows with CM (determined by CMT). They observed changes in relative abundances of certain taxa that are unique from those

reported by Kuehn et al. (2013) and Oikonomou et al. (2014). Milk collected from cows with CM had higher relative abundances of *Acinetobacter, Campylobacter, Pantoea,* and *Klebsiella* compared to milk collected from healthy cows. Milk collected from healthy cows had higher relative abundances of *Acinetobacter, Pseudomonas, Micromonospora,* and *Eubacterium* compared to milk collected from cows with CM. There are some concerns with this study as *Acinetobacter* was the most abundant taxon in milk collected from both CM (60.1%) and healthy cows (52.9%), which differs from most other studies. Furthermore, the same *Acinetobacter* strain, *Acinetobacter johnsonii* XBB1, was reported as the most abundant strain in milk collected from both cows with CM (39.0%) and healthy (31.2%) cows. Very few DNA extraction details were provided, and the references cited for their protocol do not offer clarification. The discrepancy in taxa identification of PathoScope and MG-RAST is large. For those taxa that were identified by both methods, differences in relative abundance are notable. Pang et al. (2018) noted differences in milk microbiomes of healthy cows (SCC <100,000 cells/mL and no inflammation of the mammary gland) and cows with SCM (SCC >500,000 cells/mL and no inflammation of the mammary gland) on two different dairies (A and B). The authors' definition of SCM was contradictory as inflammation would most certainly be present at that level of milk SCC. The intention may have been to denote that no clinical signs were observed, but that is not clear. On dairy A, milk produced by healthy cows had higher relative abundances of *Acinetobacter* and *Listeria* compared to milk produced by cows with SCM. Milk produced by cows with SCM had higher relative abundances of *Halomonas, Klebsiella,* and *Escherichia-Shigella* compared to milk produced by healthy cows. On dairy B, milk from healthy cows had higher relative abundances of *Halomonas* and *Atopostipes* compared to milk produced by cows with SCM. Milk produced by cows with SCM had higher relative abundances of *Streptococcus* and *Corynebacterium* compared to milk produced by healthy cows. Tong et al. (2019) characterized microbiomes in milk collected from healthy cows and milk collected from cows with SCM likely caused by *S. agalactiae* (determined by culture dependent analyses). Very few sampling details were provided so it is not possible to know if samples were collected aseptically, which could have an impact on their results. Milk microbiomes of cows with SCM had higher relative abundances of *Streptococcus*, *Romboutsia*, and *Turicibacter* compared to milk microbiomes of healthy cows. Milk microbiomes of healthy cows had higher relative abundances of *Acinetobacter*, *Stenotrophomonas*, and *Microbacterium* compared to milk microbiomes of cows with SCM. Currently, substantial variation exists among reported microbiomes of milk produced by healthy cows and those of milk produced by cows with mastitis.

Sources that may contribute to the variation seen among reported milk microbiomes include geographical location, environmental conditions, and farm hygiene practices (Taponen et al., 2019). Sample collection methods could also influence the characterization of bovine milk microbiomes

(Dahlberg et al., 2020). No differences in milk microbiota were found between milk samples collected from healthy cows (no history of CM and SCC <100,000 cells/mL) collected directly from the teat (through hand-stripping, teat canal cannula, or trans-teat wall needle aspirate) either before or after milking. However, microbiome differences in milk collected directly from the teat compared to milk collected from a quarter milking machine were observed suggesting contamination of milk by bacteria within a milking machine. Wang et al. (2020a) reported differences in milk microbiomes among healthy cows (SCC <100,000 cells/mL, no clinical signs, and negative CMT results), cows with SCM (SCC 500,000-800,000 cells/mL, no clinical signs, and weakly positive CMT results), and cows with CM (SCC >1,000,000 cells/mL, clinical signs, and strongly positive CMT results). They observed three phyla as the most abundant in each health group: Proteobacteria (relative abundance of 73.5% in milk collected from healthy cows; relative abundance of 67.5% in milk collected from cows with SCM and relative abundance of 68.6% in milk collected from cows with CM), Firmicutes (relative abundance of 16.4% in milk collected from healthy cows; relative abundance of 13.9% in milk collected from cows with SCM and relative abundance of 25.6% in milk collected from cows with CM), and Actinobacteria (relative abundance of 7.0% in milk collected from healthy cows; relative abundance of 8.7% in milk collected from cows with SCM and relative abundance of 2.9% in milk collected from cows with CM). Their results indicate no differences among milk microbiomes of healthy cows, cows with SCM, and cows with CM, which contradict results from the above-mentioned studies and others (Oikonomou et al., 2012; Hoque et al., 2020). Few sampling details are provided (such as the use of an automated milking system, samples collected in sterile centrifuge tubes) so it is hard to discern whether these results represent the milk samples themselves or the milking equipment.

Some studies (Oikonomou et al., 2012; Bhanderi et al., 2014; Hoque et al., 2020) applied metagenomic analyses to characterize milk microbiomes of healthy cows and cows with SCM or CM. Oikonomou et al. (2012) used metagenomic pyrosequencing of the bacterial 16S rRNA gene to identify bacterial communities in milk collected from healthy cows and from cows with mastitis (either SCM or CM). No details on mastitis determination were provided, nor were SCM and CM segregated. A higher prevalence of *Fusobacterium* and *Streptococcus* in milk collected from cows with mastitis was found compared to milk collected from healthy cows. Milk collected from healthy cows had a higher prevalence of *Propionibacterium* and unclassified *Ruminococcaceae* compared to milk collected from cows with mastitis. Bhanderi et al. (2014) also used metagenomic analysis to characterize milk microbiomes of cows with SCM (determined according to guidelines of IDF Bulletin 215, 1987). *Staphylococcus, Ralstonia, Serratia, Pseudomonas,* and *Bacillus* were identified as the top taxa in milk collected from cows with SCM; however, no bacteria in milk collected from

healthy cows were included for comparison. Sampling details were also not provided. Hoque et al. (2020) employed whole metagenome sequencing to characterize milk microbiomes of healthy cows, cows with SCM, cows with CM, and cows with recurring CM (defined as either a persistent case of CM by a mastitis pathogen or a reinfection after bacteriological cure of CM) using CMT to identify different states of mastitis. Findings included higher relative abundances of *Pseudomonas* and *Escherichia* in milk collected from cows with CM, higher relative abundances of *Pseudomonas* and *Aeromonas* in milk collected from cows with recurrent CM, higher relative abundances of *Lactococcus* and *Chryseobacterium* in milk collected from cows with SCM, and higher relative abundances of *Acinetobacter* and *Pseudomonas* in milk collected from healthy cows.

Milk Multi-omics: Metabolomic, Proteomic, and Lipidomic Analyses

Metabolomics, or the study of metabolites in an environment, can monitor the metabolic status of lactating dairy cows by identifying and quantifying metabolites present in milk (Zandkarimi et al., 2018). **Table 2-1** provides a summary of the reported metabolite differences in milk produced by cows with SCM or CM compared to milk produced by healthy cows. Chromatography and mass spectrometry techniques such as gas chromatography time-of-flight mass spectrometry (GC-TOF-MS), liquid chromatography mass spectrometry (LC-MS), ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS), and proton nuclear magnetic resonance (^{1}H-NMR) are used to quantitatively measure metabolites ranging from organic acids and their derivatives, vitamins and cofactors, nucleotides and their derivatives, alcohols, conjugated bile acids, carbohydrates, free and acylated carnitines, free fatty acids, and amino acid metabolites (Xi et al., 2016; Zandkarimi, et al., 2018; Huang et al., 2019; Tong et al., 2019). Sundekilde et al. (2012) employed ^{1}H-NMR to identify and quantify metabolites in milk collected from cows with varying SCC. Cows were divided into two categories: low milk SCC (<14,000 cells/mL) and high milk SCC (>720,000 cells/mL). Such extremes of milk SCC were specifically chosen by the authors to increase their likelihood of detecting differences should they exist. Milk collected from the high SCC group had increased butyrate, acetate, lactate, β-hydroxybutyrate (BHBA), and isoleucine and decreased fumarate and hippurate compared to milk collected from the low SCC group. Thomas et al. (2016b) performed an intramammary challenge with a strain of *S. uberis* to induce mastitis in one quarter of healthy dairy cows (the quarter had to be culture-negative) and used LC-MS to measure changes in milk metabolites. They observed increases in bile acids, leucine, and lactate and decreases in hippurate and lactose over the course of the post-challenge period. Xi et al. (2016) utilized UPLC-Q-TOF-MS to measure metabolites in milk collected from cows with CM (SCC >500,000 cells/mL with clinical signs of inflammation), from cows with SCM (SCC 100,000-500,000 cells/mL and no clinical

signs), and from healthy cows (SCC <100,000 cells/mL and no clinical signs). They detected decreased 4-hydroxyphenyllactate, citrate, glucose, L-carnitine, D-glycerol-1-phosphate, hippurate, and sn-glycero-3-phosphocholine in milk collected from cows with CM compared to milk collected from healthy cows. They also found decreased L-carnitine, D-glycerol-1-phosphate, *cis*-aconitate, and benzoic acid in milk collected from cows with SCM compared to milk collected from healthy cows. Leu-Leu and arginine were increased in milk collected from cows with mastitis (SCM or CM) compared to milk collected from healthy cows. Milk collected from cows with SCM had higher amounts of *cis*-aconitate, oxoglutarate, 4-hydroxyphenyllactate, and lactose compared to milk collected from cows with CM. The oligopeptides Phe-Pro-Ile, Val-Phe-Val-Tyr, Leu-Ala, and Asn-Arg-Ala-Ile were increased in milk collected from cows with CM compared to milk collected from cows with SCM. Johnzon et al. (2018) experimentally induced mastitis in healthy dairy cows (milk SCC <100,000 cells/mL and no signs of mastitis) using LPS purified from *E. coli* and measured milk metabolites by ^{1}H-NMR. They reported a decrease in lactose as the only significant metabolite change in milk post-infusion. Tong et al. (2019) measured milk metabolites in milk collected from cows with SCM (culture positive for *S. agalactiae*) and cows that were healthy (not culture positive for *S. agalactiae*) via GC-TOF-MS. They did not explicitly state whether healthy cows were culture-negative, just that *S. agalactiae* was not detected. The metabolites phenylpyruvic acid, the ratio of xanthine:guanine, glycerol, homogentisic acid: 4-Hydroxyphenylpyruvic acid (4-HPPA), and uridine were significantly elevated in milk collected from cows with SCM compared to milk collected from cows that were free from *S. agalactiae* infection. Luangwilai et al. (2020) used ^{1}H-NMR to measure metabolites in milk collected from cows with CM, cows with SCM, and healthy cows (determined via CMT). Milk collected from cows with CM had increased lactate, acetate, hippurate, valerate, benzoate, formate, BHBA, histidine, leucine, isoleucine, alanine, phenylalanine, valine, threonine, and *N*-acetylamino acid compared to milk collected from healthy cows. Leucine, isoleucine, histidine, hippurate, valerate, and *N*-acetylglucosamine were increased in milk collected from cows with SCM compared to milk collected from healthy cows. Interestingly, hippurate was increased in milk collected from cows with CM and SCM in this study as opposed to other studies (Sundekilde et al., 2012; Thomas et al., 2016a; Xi et al., 2016) where it was decreased in milk collected from cows with CM and SCM (Table 2-1). Wang et al. (2020) quantitatively measured metabolites in milk collected from cows with CM (SCC >1,000,000 cells/mL and clinical signs of inflammation; CMT strongly positive), from cows with SCM (SCC 500,000-800,000 cells/mL and no clinical signs; CMT weakly positive), and from cows that were healthy (SCC <100,000 cells/mL and no clinical signs; CMT negative) via LC-MS. Milk collected from cows with CM had increased 5-methyl-tetrahydrofolate (THF) and testosterone glucuronide and decreased urea, creatine, L-arginine phosphate, melibiose,

flavin mononucleotide (FMNH), brassidic acid, orotic acid, xanthine, thiamine, and 5-aminoimidazole ribonucleotide (AIR) compared to milk collected from healthy cows. Milk collected from cows with SCM had decreased thiamine, AIR, xanthine, orotic acid, melibiose, L-arginine phosphate, and uridine compared to milk collected from healthy cows. Finally, they observed milk collected from cows with CM had increased testosterone glucuronide and THF and decreased orotic acid, FMNH, and AIR compared to milk collected from cows with SCM.

Proteomics refers to the study of all the proteins in an environment. **Table 3-1** summarizes the reported protein differences in milk produced by cows with mastitis (SCM or CM) compared to milk produced by healthy cows. Chromatography and mass spectrometry are used to quantitatively measure the proteins found in milk of lactating dairy cows. A commonly used proteomic method is liquid chromatography tandem mass spectrometry (LC-MS/MS) but other methods such as selected reaction monitoring mass spectrometry (SRM) and shotgun tandem mass tag (TMT) are used as well. Many milk proteins, including caseins, β-lactoglobulin, α-lactalbumin, apolipoproteins, cathelicidins, transferrin, lactoferrin, and bovine serum albumin (BSA) are measurable via proteomics (Boehmer et al., 2009; Danielsen et al., 2010; Brink et al., 2019; Turk et al., 2021). Boehmer et al. (2009) utilized LC-MS/MS to identify proteins in milk collected from healthy cows (SCC <200,000 cells/mL) challenged with *E. coli* in the right front quarter. They found transferrin, fibrinogen, cathelicidin-1, apolipoprotein I, and peptidoglycan recognition receptor protein (PGRP) present in the milk after challenge that were not present pre-challenge. They also noted BSA, lactoferrin, and transferrin were increased in post-challenge milk compared to pre-challenge milk. Danielsen et al. (2010) used LC-MS/MS to measure proteins in milk collected from healthy cows (SCC <120,000 cells/mL and culture negative) challenged with LPS. Apolipoproteins (I, II, and IV), caseins (α-, β-, and κ-caseins), complement proteins C3 and C4, calgranulin B, and growth-related α protein were all upregulated in post-challenge milk compared to pre-challenge milk. Thomas et al. (2016a) measured high abundance proteins and acute phase proteins in milk collected from cows challenged with a specific strain of *S. uberis*. They reported decreased β-lactoglobulin, α-lactalbumin, and caseins and increased lactoferrin, albumin, and immunoglobulin G (IgG) in milk post-challenge. Increased mammary associated serum amyloid A3 (MSAA3), C-reactive protein (CRP), and haptoglobin were also found in milk post-challenge. Mudaliar et al. (2016) employed LC-MS/MS to identify proteins in milk collected from cows challenged with a specific *S. uberis* strain. Post-challenge milk contained upregulated SAA, haptoglobin, PGRP, cathelicidin proteins, LPS-binding protein, and histidine-rich glycoprotein and downregulated mucin-1, cystatin-B, dystroglycan, α-lactalbumin, and myozenin-1 compared to pre-challenge milk. Kusebauch et al. (2018) utilized SRM to quantify proteins in milk collected from healthy cows (SCC <100,000 cells/mL). Three cows were challenged with LPS to mimic *E. coli*

mastitis and three cows were challenged with peptidoglycans (PGN) to mimic *S. aureus* mastitis. Haptoglobin, cathepsin C, α-1 antitrypsin, α-2 macroglobulin (A2M), galectin 3, galectin 1, interleukin-8 (IL-8), vanin-1, calgranulin B, cluster of differentiation 14 (CD 14), and SAA3 were increased in post-challenge milk from both LPS- and PGN-challenged cows compared to pre-challenge milk. The protein increases in LPS-challenged milk were more pronounced than the protein increases in PGN-challenged milk. Turk et al. (2021) quantitatively measured proteins in milk collected from cows with CM (exhibiting clinical signs), from cows with SCM (SCC >400,000 cells/mL without clinical signs and CMT positive), and from healthy cows (SCC <400,000 cells/mL without clinical signs and CMT negative) using shotgun TMT. Haptoglobin, serpins B1 and B3, β-defensin, and SAA increased in milk collected from cows with mastitis (both SCM and CM) compared to milk collected from healthy cows. Immunoglobulin heavy chain, cathelicidin 3, A2M, and pregnancy zone protein increased and mucin-1, ATP-binding transporter, and butyrophilin decreased in milk collected from cows with mastitis (greater increases/decreases in CM compared to SCM) compared to milk collected from healthy cows. Furthermore, they noted milk collected from cows with CM had increased calponin, apolipoproteins I and II, variant 19 of A2M, and cathelicidin 1 and had decreased α_{s2}-casein, κ-casein, β-lactoglobulin, and thrombospondin compared to milk collected from healthy cows.

Lipidomics refers to the study of all the lipids in an environment. **Table 4-1** summarizes the reported lipid differences in milk produced by cows with SCM or CM compared to milk produced by healthy cows. Liquid chromatography-quadrupole time-of-flight mass spectrometry, ultra-high-performance LC-Q-TOF-MS (UHPLC-Q-TOF-MS), and LC-MS/MS are some of the methods used to quantitatively measure lipids in bovine milk. In addition to free fatty acids, several subclasses of lipids, including triglycerides (TG), diglycerides (DG), triacylglycerols (TAG), sphingomyelin (SM), phospholipids (PL), and ceramides (Cer) have been identified and quantified (Mavangira et al., 2015; Li et al., 2017; Cecillani et al., 2021). Oxylipids, or products of oxidized polyunsaturated fatty acids (PUFA), produced by reactive oxygen species (ROS) during oxidative stress are also measured by lipidomic methods (Mavangira et al., 2015; Mavangira and Sordillo, 2018). Li et al. (2017) used UPLC-Q-Exactive Orbitrap MS to measure lipids in commercial bovine milk. They detected three Cer species, two phosphatidylglycerol (PG) species, and two phosphatidylinositol (PI) species. Tsiafoulis et al. (2019) used ^{1}H-NMR metabolomics to identify lipids in both raw (bulk-tank) and retail milk from both organic and conventional practices. They found increased linoleic acid (LA), α-linolenic acid (ALA), C18:2 *cis*-9, *trans*-11 conjugated linoleic acid (CLA), and total unsaturated fatty acids (UFA) and decreased caproleic acid in organic milk (both raw and retail) as compared to conventional milk (both raw and retail). The authors note that ^{1}H-NMR metabolomics is not as

sensitive and has lower classification capabilities compared to traditional lipidomic methods such as LC-MS. However, they determined it was an appropriate analysis for the aim of their investigation. Wang et al. (2020b) utilized UHPLC-Q-TOF-MS to identify lipids in bovine milk purchased from a farm. It is not clear whether the milk was raw or commercial. They identified a TG species, two phosphatidylcholine (PC) species, and a SM species. Li et al. (2020) employed UHPLC-Q-TOF-MS to identify lipids in raw bovine colostrum (1-5 d postpartum) and milk (2-3 mo postpartum). They reported several lipid subclasses, TG, DG, SM, phosphatidylethanolamine (PE), PC, Cer, hexosylceramide (HexCer), phosphatidylserine (PS), PI, dihexosylceramide (Hex2Cer), cardiolipin (CL), PG, and PA were higher in colostrum compared to milk. Mitina et al. (2020) utilized LC-Q-TOF-MS to identify and quantify lipids in milk collected from seven different mammalian species: pig, goat, yak, macaque (two species), human, and cow. Only four cows were used, and no other details were provided (such as age, lactation stage, health status, etc.). Milk TAG profiles were the only results given for each species. The authors noted they focused on the high-abundance TAGs because of the small sample size. They reported long monounsaturated fatty acids (MUFA) were most abundant in milk collected from cows as compared to milks collected from the other species. In comparing cow milk to human milk, they reported cow milk was higher saturated fatty acids (SFA) whereas human milk was higher in PUFA. Mavangira et al. (2015) utilized LC-MS/MS to quantify targeted fatty acids and oxylipids in milk collected from cows with coliform mastitis (culture positive for *E. coli* and clinical signs) and milk collected from healthy cows (culture negative with SCC <250,000 cells/mL). They found LA, arachidonic acid (ArA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and oleic acid were increased in milk collected from cows with coliform mastitis compared to milk collected from healthy cows. Several oxylipids including 6-Keto$PGF_{1\alpha}$, PGE_2, 13-hydroxyoctadecadienoic acids (HODE), 9-$HODE^3$, 14,15-dihydroxyeicosatetraenoic acid (DiHETE), 20-hydroxyeicosatetraenoic acids ($HETE^3$), 11-$HETE^3$, and 9-$HETE^3$ were increased in milk collected from cows with coliform mastitis compared to milk collected from healthy cows. Ceciliani et al. (2021) employed LC-Q-TOF-MS to identify lipids present in milk collected from cows with SCM caused by NAS and milk collected from healthy cows (SCC <100,000 cells/mL and culture negative). The SCM cows were culture positive for NAS and split into two groups: milk SCC >200,000 cells/mL (high SCC quarters) and milk SCC <200,000 cells/mL (low SCC quarters). They reported a decreased TAG species and increased Cer species and HexCer species in milk collected from cows with SCM compared to milk collected from healthy cows. No differences in milk lipid profiles were found between the high SCC group and the low SCC group.

To date, most bovine milk microbiome studies (like Oikonomou et al., 2012; Pang et al., 2018; Tong et al., 2019; Hoque et al., 2020) have been cross-sectional, thus characterizing milk microbiomes of healthy cows, milk microbiomes of cows with SCM, and milk microbiomes of cows with CM at one time point. Furthermore, several metabolites, proteins, and lipids are altered (increased/decreased or absent/present) in milk collected from cows with mastitis (SCM or CM) compared to milk collected from healthy cows. However, differences in methods and definitions of CM, SCM, and healthy across studies create uncertainty in the interpretation of multiomic results. Implementing longitudinal microbiome studies along with metabolomic, proteomic, and lipidomic analyses could prove to be beneficial as information gleaned from such studies combined with results from previously conducted research may make it possible to predict mastitis (SCM or CM) before it occurs. Observing shifts in bacterial communities and the host response (like changes in milk SCC, Na/K, metabolites, proteins, lipids) shortly before and during the actual mastitis event could play an important role in prevention protocols and better inform treatment strategies.

References

Akers, R.M., 2002. Pages 125-128 in Lactation and the mammary gland. First ed. Blackwell Publishing Professional, Iowa, US.

Aleri, J.W., B.C. Hine, M.F. Pyman, P.D. Mansell, W.J. Wales, B. Mallard, and A.D. Fisher. 2016. Periparturient immunosuppression and strategies to improve dairy cow health during the periparturient period. Res Vet Sci. 108:8-17.

Alhussien, M.N. and A.K. Dang. 2018. Milk somatic cells, factors influencing their release, future prospects, and practical utility in dairy animals: an overview. Vet World. 11:567-577.

Andersen, S., I.R. Dohoo, R. Olde Riekerink, H. Stryhn, and Mastitis Research Workers' Conference. 2010. Diagnosing intramammary infections: evaluating expert opinions on the definition of intramammary infection using conjoint analysis. J Dairy Sci. 93:2966-2975.

Andrews, T., D.A. Neher, T.R. Weicht, and J.W. Barlow. 2019. Mammary microbiome of lactating organic dairy cows varies by time, tissue site, and infection status. PLoS One. 14:1-21.

Auldist, M.J. and I.B. Hubble. 1998. Effects of mastitis on raw milk and dairy products. Aust J Dairy Technol. 53:28-36.

Ballou, M.A. 2011. Growth and development symposium: inflammation: role in the etiology and pathophysiology of clinical mastitis in dairy cows. J Anim Sci. 90:1466-1478.

Barkema, H.W., Y.H. Schukken, T.J.G.M. Lam, D.T. Galligan, M.L. Beiboer, and A. Brand. 1997. Estimation of interdependence among quarters of the bovine udder with subclinical mastitis and implications for analysis. J Dairy Sci. 80:1591-1599.

Bhanderi, B.B., M.K. Jhala, V.B. Ahir, V.D. Bhatt, and C.G. Joshi. 2014. Cultural and metagenomic based identification of a microbiome from subclinical mastitis in cows. Vet Arh. 84:215-228.

Boehmer, J.L., J.L. Ward, R.R. Peters, K.J. Shefcheck, M.A. McFarland, and D.D. Bannerman. 2009. Proteomic analysis of the temporal expression of bovine milk proteins during coliform mastitis and label-free relative quantification. J Dairy Sci. 93:593-603.

Breen, J.E., M.J. Green, and A.J. Bradley. 2009. Quarter and cow risk factors associated with the occurrence of clinical mastitis in dairy cows in the United Kingdom. J Dairy Sci. 92:2551-2561.

Brink, L.R., A.W. Herren, S. McMillen, K. Fraser, M. Agnew, N. Roy, and B. Lönnerdal. 2019. Omics analysis reveals variations among commercial sources of bovine milk fat globule membrane. J Dairy Sci. 103:3002-3016.

Bronzo, V., V. Lopreiato, F. Riva, M. Amadori, G. Curone, M.F. Addis, P. Cremonesi, P. Moroni, E. Trevisi, and B. Castiglioni. 2020. The role of innate immune response and microbiome in resilience of dairy cattle to disease: the mastitis model. Animals. 10:1-20.

Burvenich, C., V. Van Merris, J. Mehrzad, A. Diez-Fraile, and L. Duchateau. 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. Vet Res. 34:521-564.

Cameron, M., S.L. McKenna, K.A. MacDonald, I.R. Dohoo, J.P. Roy, and G.P. Keefe. 2013. Evaluation of selective dry cow treatment following on-farm culture: risk of postcalving intramammary infection and clinical mastitis in the subsequent lactation. J Dairy Sci, 97:270-284.

Ceciliani, F., M. Audano, M.F. Addis, C. Lecchi, M.H. Ghaffari, M. Albertini, F. Tangorra, R. Piccinini, D. Caruso, N. Mitro, and V. Bronzo. 2021. The untargeted lipidomic profile of quarter milk from dairy cows with subclinical intramammary infection by non-*aureus* staphylococci. J Dairy Sci. 104:1-15.

Crispie, F., M. Alonso-Gómez, C. O'Loughlin, K. Klostermann, J. Flynn, S. Arkins, W. Meaney, R.P. Ross, and C. Hill. 2008. Intramammary infusion of a live culture for treatment of bovine mastitis: effect of live lactococci on the mammary immune response. J Dairy Res. 75:374-384.

Dahlberg, J., J.E. Williams, M.A. McGuire, H.K. Peterson, K. Östensson, S. Agenäs, J. Dicksved, and K. Persson Waller. 2020. Microbiota of bovine milk, teat skin, and teat canal: similarity and variation due to sampling technique and milk fraction. J Dairy Sci. 103:7322-7330.

Danielsen, M., M.C. Codrea, K.L. Ingvartsen, N.C. Friggens, E. Bendixen, and C.M. Røntved. 2010. Quantitative milk proteomics—host responses to lipopolysaccharide-mediated inflammation of bovine mammary gland. Proteomics. 10:2240-2249.

Derakhshani, H., K.B. Fehr, S. Sepehri, D. Francoz, J. De Buck, H.W. Barkema, J.C. Plaizier, and E. Khafipour. 2018. Invited review: microbiota of the bovine udder: contributing factors and potential implications for udder health and mastitis susceptibility. J Dairy Sci. 101:10605-10625.

Diez-Fraile, A., E. Meyer, and C. Burvenich. 2003. Sympathoadrenal and immune system activation during the periparturient period and their association with bovine coliform mastitis. A review. Vet Q. 25:31-44.

Djabri, B., N. Bareille, F. Beaudeau, and H. Seegers. 2002. Quarter milk somatic cell count in infection dairy cows: a meta-analysis. Vet Res. 33:335-357.

Dufour, S., I.R. Dohoo, H.W. Barkema, L. DesCôteaux, T.J. DeVries, K.K. Reyher, J.P. Roy, and D.T. Scholl. 2011. Manageable risk factors associated with the lactational incidence, elimination, and prevalence of *Staphylococcus aureus* intramammary infections in dairy cows. J Dairy Sci. 95:1283-1300.

Eberhart, R.J., H.C. Gilmore, L.J. Hutchinson, and S.B. Spencer. 1979. Somatic cell counts in DHI samples. National Mastitis Council (U.S.) Annual Meeting. 18:32-40.

Eberhart, R.J., L.J. Hutchinson, and S.B. Spencer. 1982. Relationships of bulk tank somatic cell counts to prevalence of intramammary infection and to indices of herd production. J. Food Prot. 45:1125-1128.

Fournier, C., P. Kuhnert, J. Frey, R. Miserez, M. Kirchhofer, T. Kaufmann, A. Steiner, and H.U. Graber. 2008. Bovine *Staphylococcus aureus*: association of virulence genes, genotypes, and clinical outcome. Res Vet Sci. 85:439-448.

Fox, L.K., S.T. Chester, J.W. Hallberg, S.C. Nickerson, J.W. Pankey, and L.D. Weaver. 1995. Survey of intramammary infections in dairy heifers at breeding age and first parturition. J Dairy Sci. 78:1619-1628.

Fox, L. 2009. Prevalence, incidence, and risk factors of heifer mastitis. Vet Microbiol. 134:82-88.

Gao, J., Y.C. Liu, Y. Wang, H. Li, X.M. Wang, Y. Wu, D.R. Zhang, S. Gao, and Z. Qi. 2020. Impact of yeast and lactic acid bacteria on mastitis and milk microbiota composition of dairy cows. AMB Express. 10:1-12.

Gröhn, Y.T., H.N. Erb, C.E. McCulloch, and H.S. Saloniemi. 1990. Epidemiology of mammary gland disorders in multiparous Finnish Ayrshire cows. Prev Vet Med. 8:241-252.

Gröhn, Y.T., S.W. Eicker, and J.A. Hertl. 1995. The association between previous 305-day milk yield and disease in New York state dairy cows. J Dairy Sci. 78:1693-1702.

Gulbe, G., M. Pilmane, V. Saulīte, S. Donina, J. Jermolajevs, L. Peskova, and A. Valdovska. 2020. Cells and cytokines in milk of subclinically infected bovine mammary glands after the use of immunomodulatory composition GLP 810. Mediators Inflamm. 1-12.

Hagnestam-Nielsen, C., U. Emanuelson, B. Berglund, and E. Strandberg. 2009. Relationship between somatic cell count and milk yield in different stages of lactation. J Dairy Sci. 92:3124-3133.

Harmon, R.J. 1994. Physiology of mastitis and factors affecting somatic cell counts. J Dairy Sci. 77:2103-2112.

Heikkilä, A.M., E. Liski, S. Pyörälä, and S. Taponen. 2018. Pathogen-specific production losses in bovine mastitis. J Dairy Sci. 101:9493-8504.

Hoque, M.N., A. Istiaq, R.A. Clement, M. Sultana, K.A. Crandall, A.Z. Siddiki, and M.A. Hossain. 2019. Metagenomic deep sequencing reveals association of microbiome signature with functional biases in bovine mastitis. Sci Rep. 9:1-14.

Hoque, M.N., A. Istiaq, M.S. Rahman, M.R. Islam, A. Anwar, A.M.A.M.Z. Siddiki, M. Sultana, K.A. Crandall, and M.A. Hossain. 2020. Microbiome dynamics of bovine mastitis progression and genomic determinants of bovine mastitis. Genomics. 112:5188-5203.

Horst, E.A., S.K. Kvidera, and L.H. Baumgard. 2021. Invited review: the influence of immune activation on transition cow health and performance—a critical evaluation of traditional dogmas. J Dairy Sci. 104:8380-8410.

Huang, Y., L. Shen, J. Jiang, Q. Xu, Z. Luo, Q. Luo, S. Yu, X. Yao, Z. Ren, Y. Hu, Y. Yang, and S. Cao. 2019. Metabolomic profiles of bovine mammary epithelial cells stimulated by lipopolysaccharide. Sci Rep. 9:19131.

Hugerth, L.W. and A.F. Andersson. 2017. Analyzing microbial community composition through amplicon sequencing: from sampling to hypothesis testing. Front Microbiol. 8:1-22.

Hughes, K. and C.J. Watson. 2018. The mammary microenvironment in mastitis in humans, dairy ruminants, rabbits and rodents: a one health focus. J Mammary Gland Biol Neoplasia. 23:27-41.

International Dairy Federation (IDF). Bulletin 215/1987 Machine milking and mastitis.

International Dairy Federation (IDF) Bulletin 466/2013 Guidelines for the use and interpretation of bovine milk somatic cell counts (SCC) in the dairy industry.

Jamali, H., H.W. Barkema, M. Jacques, E.M. Lavallée-Bourget, F. Malouin, V. Saini, H. Stryhn, and S. Dufour. 2018. Invited review: incidence, risk factors, and effects of clinical mastitis recurrence in dairy cows. J Dairy Sci. 101:4729-4746.

Johnzon, C.F., J. Dahlberg, A.M. Gustafson, I. Waern, A.A. Moazzami, K. Östensson, and G. Pejler. 2018. The effect of lipopolysaccharide-induced experimental bovine mastitis on clinical parameters, inflammatory markers, and the metabolome: a kinetic approach. Front Immunol. 9:1-14.

Kehrli Jr., M.E. and D.E. Shuster. 1994. Factors affecting milk somatic cells and their role in health of the bovine mammary gland. J Dairy Sci. 77:619-627.

Klostermann, K., F. Crispie, J. Flynn, R.P. Ross, C. Hill, and W. Meaney. 2008. Intramammary infusion of a live culture of *Lactococcus lactis* for treatment of bovine mastitis: comparison with antibiotic treatment in field trials. J Dairy Res. 75:365-373.

Koskinen, M.T., G.J. Wellenberg, O.C. Sampimon, J. Holopainen, A. Rothkamp, L. Salmikivi, W.A. van Haeringen, T.J.G.M. Lam, and S. Pyörälä. 2010. Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. J Dairy Sci. 93:5707-5715.

Kuehn, J.S., P.J. Gorden, D. Munro, R. Rong, Q. Dong, P.J. Plummer, C. Wang, and G.J. Phillips. 2013. Bacterial community profiling of milk samples as a means to understand culture-negative bovine clinical mastitis. PLoS One. 8:1-10.

Kümmel, J., B. Stessi, M. Gonano, G. Walcher, O. Bereuter, M. Fricker, T. Grunert, M. Wagner, and M. Ehling-Schulz. 2016. *Staphylococcus aureus* entrance into the dairy chain: tracking *S. aureus* from dairy cow to cheese. Front Microbiol. 7:1-11.

Kusebauch, U., L.E. Hernández-Castellano, S.L. Bislev, R.L. Moritz, C.M. Røntved, and E. Bendixen. 2018. Selected reaction monitoring mass spectrometry of mastitis milk reveals pathogen-specific regulation of bovine host response proteins. J Dairy Sci. 101:6532-6541.

Latorre, A.A., P.A. Pachá, G. González-Rocha, I.S. Martin, M. Quezada-Aguiluz, A. Aguayo-Reyes, H. Bello-Toledo, R. Oliva, A. Estay, J. Pugin, and M.A. Muñoz. 2019. On-farm surfaces in contact with milk: the role of *Staphylococcus aureus*-containing biofilms for udder health and milk quality. Foodborne Pathog Dis. 1-8.

Li, Q., Y. Zhao, D. Zhu, X. Pang, Y. Liu, R. Frew, and G. Chen. 2017. Lipidomics profiling of goat milk, soymilk, and bovine milk by UPLC-Q-Exactive Orbitrap Mass Spectrometry. Food Chem. 224:302-309.

Li, M., Q. Li, S. Kang, X. Cao, Y. Zheng, J. Wu, R. Wu, J. Shao, M. Yang, and X. Yue. 2020. Characterization and comparison of lipids in bovine colostrum and mature milk based on UHPLC-QTOF-MS lipidomics. Food Res Int. 136:1-8.

Lindmark-Månsson, H., C. Bränning, G. Aldén, and M. Paulsson. 2006. Relationship between somatic cell count, individual leukocyte populations and milk components in bovine udder quarter milk. Int Dairy J. 16:717-727.

Luangwilai, M., K. Duangmal, N. Chantaprasarn, and S. Settachaimongkon. 2020. Comparative metabolite profiling of faw milk from subclinical and clinical mastitis cows using ^{1}H-NMR combined with chemometric analysis. Int J Food Sci Technol. 1-11.

Ma, T., L. Shen, Q. Wen, R. Lv, Q. Hou, L.Y. Kwok, Z. Sun, and H. Zhang. 2021. PacBio sequencing revealed variation in the microbiota diversity, species richness and composition between milk collected from healthy and mastitis cows. Microbiology. 167:1-10.

Malik, T.A., M. Mohini, S.H. Mir, B.A. Ganaie, D. Singh, T.K. Varun, S. Howal, and S. Thakur. 2018. Somatic cells in relation to udder health and milk quality—a review. J Anim Health Prod. 6:18-26.

Massé, J., S. Dufour, and M. Archambault. 2019. Characterization of *Klebsiella* isolates obtained from clinical mastitis cases in dairy cattle. J Dairy Sci. 103:3392-3400.

Mavangira, V., J.C. Gandy, C. Zhang, V.E. Ryman, A.D. Jones, and L.M. Sordillo. 2015. Polyunsaturated fatty acids influence differential biosynthesis of oxylipids and other lipid mediators during bovine coliform mastitis. J Dairy Sci. 98:6202-6215.

Mavangira, V., and L.M. Sordillo. 2018. Role of lipid mediators in the regulation of oxidative stress and inflammatory responses in dairy cattle. Res Vet Sci. 116:4-14.

Metzger, S.A., L.L. Hernandez, J.H. Skarlupka, T.M. Walker, G. Suen, and P.L. Ruegg. 2018. A cohort study of the milk microbiota of healthy and inflamed bovine mammary glands from dryoff through 150 days in milk. Front Vet Sci. 5:1-11.

Mitina, A., P. Mazin, A. Vanyushkina, N. Anikanov, W. Mair, S. Guo, P. Khaitovich. 2020. Lipidome analysis of milk composition in humans, monkeys, bovids, and pigs. BMC Evol Biol. 20:1-8.

Morrison, E.I., T.J. DeVries, and S.J. LeBlanc. 2018. Short communication: associations of udder edema with health, milk yield, and reproduction in dairy cows in early lactation. J Dairy Sci. 101:9521-9526.

Mudaliar, M., R. Tassi, F.C. Thomas, T.N. McNeilly, S.K. Weidt, M. MacLaughlin, D. Wilson, R. Burchmore, P. Herzyk, P.D. Eckersall, and R.N. Zadoks. 2016. Mastitomics, the integrated omics of

bovine milk in an experimental model of *Streptococcus uberis* mastitis: 2. label-free relative quantitative proteomics. Mol Biosyst. 12:2748-2761.

Murphy, S.C., N.H. Martin, D.M. Barbano, and M. Wiedmann. 2016. Influence of raw milk quality on processed dairy products: how do raw milk quality test results relate to product quality and yield?. J Dairy Sci. 99:10128-10149.

Oikonomou, G., V.S. Machado, C. Santisteban, Y.H. Schukken, and R.C. Bicalho. 2012. Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. PLoS One. 7:1-14.

Oikonomou, G., M.L. Bicalho, E. Meira, R.E. Rossi, C. Foditsch, V.S. Machado, A.G.V. Teixeira, C. Santisteban, Y.H. Schukken, and R.C. Bicahlo. 2014. Microbiota of cow's milk; distinguishing healthy, sub-clinically, and clinically diseased quarters. PLoS One. 9:1-11.

Oliveira, C.S.F., H. Hogeveen, A.M. Botelho, P.V. Maia, S.G. Coelho, and J.P.A. Haddad. 2015. Cow-specific risk factors for clinical mastitis in Brazilian dairy cattle. Prevent Vet Med. 121:297-305.

Oliver, S.P., S.E. Murinda, and B.M. Jayaro. 2011. Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: a comprehensive review. Foodborne Pathog Dis. 8:337-355.

Ott, S. 1999. Costs of herd-level production losses associated with subclinical mastitis in US dairy cows. Pages 152-156 in Proc. 38th Ann. Mtg. Natl. Mastitis Counc. Arlington, VA. Natl. Mastitis Counc. Madison, WI.

Paape, M., J. Mehrzad, X. Zhao, J. Detilleux, and C. Burvenich. 2002. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. J Mammary Gland Biol Neoplasia. 7:109-121.

Paape, M.J., D.D. Bannerman, X. Zhao, and J. Lee. 2003. The bovine neutrophil: structure and function in blood and milk. Vet Res. 34:597-627.

Pang, M., X. Xie, H. Bao, L. Sun, T. He, H. Zhao, Y. Zhou, L. Zhang, H. Zhang, R. Wei, K. Xie, and R. Wang. 2018. Insights into the bovine milk microbiota in dairy farms with different incidence rates of subclinical mastitis. Front Microbiol. 9:1-13.

Park, J.Y., L.K. Fox, K.S. Seo, M.A. McGuire, Y.H. Park, F.R. Rurangirwa, W.M. Sischo, and G.A. Bohach. 2011a. Comparison of phenotypic and genotypic methods for the species identification of

coagulase-negative staphylococcal isolates from bovine intramammary infection. Vet Microbiol. 147:142-148.

Park, J.Y., L.K. Fox, K.S. Seo, M.A. McGuire, Y.H. Park, F.R. Rurangirwa, W.H. Sischo, and G.A. Bohach. 2011b. Detection of classical and newly described staphylococcal superantigen genes in coagulase-negative staphylococcal isolated from bovine intramammary infection. Vet Microbiol. 147:149-154.

Patel, K., D.M. Godden, E. Royster, B.A. Crooker, J. Timmerman, and L. Fox. 2019. Relationships among bedding materials, bedding bacteria counts, udder hygiene, milk quality, and udder health in US dairy herds. J Dairy Sci. 102.

Pearson, J.K.L., D.O. Greer, and B.K. Spence. 1971. The relationship between bulk milk cell counts and cow and quarter mastitis incidence. Vet. Rec. 88:488-494.

Pearson, J.K.L. and D.O. Greer. 1974. Relationship between somatic cell counts and bacterial infections of the udder. Vet. Rec. 95:252-257.

Pérez-Báez, J., C.A. Risco, R.C. Chebel, G.C. Gomes, L.F. Greco, S. Tao, I.M. Thompson, B.C. do Amaral, M.G. Zenobi, N. Martinez, C.R. Staples, G.E. Dahl, J.A. Hernández, J.E.P. Santos, and K.N. Galvão. 2019. Association of dry matter intake and energy balance prepartum and postpartum with health disorders postpartum: Part II. Ketosis and clinical mastitis. J Dairy Sci. 102:9151-9164.

Piepers, S., A. Prenafeta, J. Verbeke, A. De Visscher, R. March, and S. De Vliegher. 2016. Immune response after an experimental intramammary challenge with killed *Staphylococcus aureus* in cows and heifers vaccinated and not vaccinated with Startvac, a polyvalent mastitis vaccine. J Dairy Sci. 100:769-782.

Pyörälä, S. 2008. Mastitis in post-partum dairy cows. Reprod Dom Anim. 43:252-259.

Rainard, P. and G. Foucras. 2018. A critical appraisal of probiotics for mastitis control. Front Vet Sci. 5:1-13.

Rainard, P., F.B. Gilbert, P. Germon, and G. Foucras. 2021. Invited review: a critical appraisal of mastitis vaccines for dairy cows. J Dairy Sci. 104:10427-10448.

Rajala-Schultz, P.J., Y.T. Gröhn, C.E. McCulloch, and C.L. Guard. 1999. Effects of clinical mastitis on milk yield in dairy cows. J Dairy Sci. 82:1213-1220.

Reece, W.O. 2004. Endocrinology, reproduction, and lactation. Pages 720-742 in Duke's Physiology of Domestic Animals. Twelfth ed. Cornell University Press, New York, US.

Ren, Q., G. Liao, Z. Wu, J. Lv, and W. Chen. 2020. Prevalence and characterization of *Staphylococcus aureus* isolates from subclinical bovine mastitis in southern Xinjiang, China. J Dairy Sci. 103:3368-3380.

Rollin, E., K.C. Dhuyvetter, and M.W. Overton. 2015. The cost of clinical mastitis in the first 30 days of lactation: an economic modeling tool. Prevent Vet Med. 122:257-264.

Rowe, S.M., S.M. Godden, E. Royster, J. Timmerman, B.A. Crooker, and M. Boyle. 2019. Cross-sectional study of the relationships among bedding materials, bedding bacteria counts, and intramammary infection in late-lactation dairy cows. J Dairy Sci. 102:11384-11400.

Schukken, Y.H., F.J. Grommers, D. Van de Geer, H.N. Erb, and A. Brand. 1990. Risk factors for clinical mastitis in herds with a low bulk milk somatic cell count. 1. Data and risk factors for all cases. J. Dairy Sci. 73:3463-3471.

Schukken, Y.H., D.J. Wilson, F. Welcome, L. Garrison-Tikofsky, and R.N. Gonzalez. 2003. Monitoring udder health and milk quality using somatic cell counts. Vet Res. 34:579-596.

Schukken, Y.H., G.J. Bennett, M.J. Zurakowski, H.L. Sharkey, B.J. Rauch, M.J. Thomas, B. Ceglowski, R.L. Saltman, N. Belomestnykh, and R.N. Zadoks. 2011. Randomized clinical trial to evaluate the efficacy of a 5-day ceftiofur hydrochloride intramammary treatment on nonsevere gram-negative clinical mastitis. J Dairy Sci. 94:6203-6215.

Seroussi, E., S.E. Blum, O. Krifucks, Y. Lavon, and G. Leitner. 2018. Application of pancreatic phospholipase A2 for treatment of bovine mastitis. PLoS One. 13:1-13.

Shaheen, M., H.A. Tantary, and S.U. Nabi. 2016. A treatise on bovine mastitis: disease and disease economics, etiological basis, risk factors, impact on human health, therapeutic management, prevention, and control strategy. J Adv Dairy Res. 4:1-10.

Shpigel, N.Y., M. Winkler, G. Ziv, and A. Saran. 1998. Clinical, bacteriological, and epidemiological aspects of clinical mastitis in Israeli dairy herds. Prevent Vet Med. 35:1-9.

Smith, K.L., J.E. Hillerton, and R.J. Harmon. 2001. Guidelines on normal and abnormal raw milk based on somatic cell counts and signs of clinical mastitis. National Mastitis Council, Madison WI.

Souza, F.N., M.G. Blagitz, C.F. Batista, P.V. Takano, R.G. Gargano, S.A. Diniz, M.X. Silva, J.A. Ferronatto, K.R. Santos, M.B. Heinemann, S. De Vliegher, and A.M.M.P. Della Libera. 2020. Immune response in nonspecific mastitis: what can it tell us?. J Dairy Sci. 103:5376-5386.

Sumon, S.M.M.R., M.S. Parvin, M.A. Ehsan, and M.T. Islam. 2020. Dynamics of somatic cell count and intramammary infection in lactating dairy cows. J Adv Vet Anim Res. 7:314-319.

Sundekilde, U.K., N.A. Poulsen, L.B. Larsen, and H.C. Bertram. 2012. Nuclear magnetic resonance metabonomics reveals strong association between milk metabolites and somatic cell count in bovine milk. J Dairy Sci. 96:290-299.

Syväjärvi, J., H. Saloniemi, and Y. Gröhn. 1986. An epidemiological and genetic study on registered diseases in Finnish Ayrshire cattle IV. clinical mastitis. Acta Vet Scand. 27:223-234.

Taponen, S., L. Salmikivi, H. Simojoki, M. T. Koskinen, and S. Pyörälä. 2009. Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. J Dairy Sci. 92:2610-2617.

Taponen, S., E. Liski, A.M. Heikkilä, and S. Pyörälä. 2016. Factors associated with intramammary infection in dairy cows caused by coagulase-negative staphylococci, *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Corynebacterium bovis*, or *Escherichia coli*. J Dairy Sci. 100:493-503.

Taponen, S., D. McGuinness, H. Hiitiö, H. Simojoki, R. Zadoks, and S. Pyörälä. 2019. Bovine milk microbiome: a more complex issue than expected. Vet Res. 50:1-15.

Thomas, F.C., W. Mullen, R. Tassi, A. Ramirez-Torres, M. Mudaliar, T.N. McNeilly, R.N. Zadoks, R. Burchmore, and P.D. Eckersall. 2016a. Mastitomics, the integrated omics of bovine milk in an experimental model of *Streptococcus uberis* mastitis: 1. high abundance proteins, acute phase proteins, and peptidomics. Mol Biosyst. 12:2735-2747.

Thomas, F.C., M. Mundaliar, R. Tassi, T.N. McNeilly, R. Burchmore, K. Burgess, P. Herzyk, R.N. Zadoks, and P.D. Eckersall. 2016b. Mastitomics, the integrated omics of bovine milk in an experimental model of *Streptococcus uberis* mastitis: 3. untargeted metabolomics. Mol Biosyst. 12:2762-2769.

Tizard, I.R. 2013. Innate immunity: the complement system. Pages 61-73 in Veterinary Immunology. Ninth ed. Elsevier Saunders, Missouri, US.

Tong, J., H. Zhang, Y. Zhang, B. Xiong, and L. Jiang. 2019. Microbiome and metabolome analyses of milk from dairy cows with subclinical *Streptococcus agalactiae* mastitis—potential biomarkers. Front Microbiol. 10:1-14.

Trevisi, E. and A. Minuti. 2018. Assessment of the innate immune response in the periparturient cow. Res Vet Sci. 116:47-54.

Tsiafoulis, C.G., C. Papaemmanouil, D. Alivertis, O. Tzamaloukas, D. Miltiadou, S. Balayssac, M. Malet-Martino, and I.P. Gerothanassis. 2019. NMR-based metabolomics of the lipid fraction of organic and conventional bovine milk. Molecules. 24:1-18.

Turk, R., N. Rošić, J. Kuleš, A. Horvatić, A. Gelemanovic, A. Galen, B.B. Ljubić, M. Benić, V. Stevanović, V. Mrljak, C.C. Chadwick, and P.D. Eckersall. 2021. Milk and serum proteomes in subclinical and clinical mastitis in Simmental cows. J Proteomics. 244:1-15.

Valckenier, D., S. Piepers, A. De Visscher, R.M. Bruckmaier, and S. De Vliegher. 2019. Effect of intramammary infection with non-*aureus* staphylococci in early lactation in dairy heifers on quarter somatic cell count and quarter milk yield during the first 4 months of lactation. J Dairy Sci. 102:6442-6453.

Wanasinghe, D.D. and A.J. Frost. 1979. The prevalence of udder infection and mastitis in herds producing bulk milk with either consistently high or low cell count. Aust. Vet. J. 55:374-380.

Wang, Y., M.X. Nan, G.Y. Zhao, H. Wang, L.M. Wang, F. Zhang, F. Xue, K.D. Hua, S.L. Jiang, J. Liu, M.K. Li, J. Yao, and B. Xiong. 2020a. Coupling 16s rDNA and untargeted mass spectrometry for milk microbial composition and metabolites from dairy cows with clinical and subclinical mastitis. J Agric Food Chem. 1-38.

Wang, L., X. Li, L. Liu, H. da Zhang, Y. Zhang, Y.H. Chang, and Q.P. Zhu. 2020b. Comparative lipidomics analysis of human, bovine, and caprine milk by UHPLC-Q-TOF-MS. Food Chem. 310:1-8.

Ward, G.E. and L.H. Schultz. 1972. Relationship of somatic cells in quarter milk to type of bacteria and production. J. Dairy Sci. 55:1428-1431.

Williams, H.J., P.J. Cripps, and D.H. Grove-White. 2012. The association between high milk somatic cell counts in the first lactation and somatic cell counts in the second lactation. Vet J. 191:183-187.

Xi, X., L.Y. Kwok, Y. Wang, C. Ma, Z. Mi, and H. Zhang. 2016. Ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry MS^E-based untargeted milk metabolomics in dairy cows with subclinical or clinical mastitis. J Dairy Sci. 100:4884-4896.

Yu, J., Y. Ren, X. Xi, W. Huang, and H. Zhang. 2017. A novel lactobacilli-based teat disinfectant for improving bacterial communities in the milks of cow teats with subclinical mastitis. Front Microbiol. 8:1-12.

Zadoks, R.N., H.G. Allore, H.W. Barkema, O.C. Sampimon, G.J. Wellenberg, Y.T. Gröhn, and Y.H. Schukken. 2001. Cow- and quarter-level risk factors for *Streptococcus uberis* and *Staphylococcus aureus* mastitis. J Dairy Sci. 84:2649-2663.

Zandkarimi, F., J. Vanegas, X. Fern, C.S. Maier, and G. Bobe. 2018. Metabotypes with elevated protein and lipid catabolism and inflammation precede clinical mastitis in prepartal transition dairy cows. J Dairy Sci. 101:5531-5548.

Table 1-1: Summary of top five bacterial taxa in colostrum or milk collected from healthy cows, from cows with subclinical mastitis (SCM), and from cows with clinical mastitis (CM) using 16S rRNA assessment of the microbiome from several studies illustrating similarities and differences among results.

Reference	Confidence Level[1]	Healthy	SCM	CM
Oikonomou et al. 2012	low	*Propionibacterium* *Staphylococcus* *Bacteroides* *Streptococcus* unclassified *Ruminococcaceae*	*Streptococcus* *Fusobacterium* *Bacteroides* *Staphylococcus* unclassified *Enterobacteriaceae*	*Fusobacterium* *Streptococcus* *Staphylococcus* *Escherichia/Shigella* unclassified *Prevotellaceae*
Kuehn et al. 2013	high	*Ralstonia* *Pseudomonas* *Stenotrophomonas* *Psychrobacter* *Bradyrhizobium*		*Sphingomonas* *Stenotrophomonas* *Ralstonia* *Pseudomonas* *Corynebacterium*
Bhanderi et al. 2014	low		*Staphylococcus* *Ralstonia* *Serratia* *Pseudomonas* *Bacillus*	
Oikonomou et al. 2014	high	*Propionibacterium* *Aeribacillus* *Faecalibacterium* unclassified *Lachnospiraceae* unclassified *Ruminococcaceae*	*Propionibacterium* *Aeribacillus* *Staphylococcus* unclassified *Lachnospiraceae* unclassified *Ruminococcaceae*	*Asticcacoulis* *Fusobacterium* *Streptococcus* *Aeribacillus* unclassified *Bacteroidales*
Lima et al. 2017	high	*Staphylococcus* *Pseudomonas* *Fusobacterium* *Mycoplasma* *Corynebacterium*		*Staphylococcus* *Fusobacterium* *Mycoplasma* *Pseudomonas* *Corynebacterium*
Metzger et al. 2018	high	*Enhydrobacter* *Janthinobacterium* *Corynebacterium* *Knoellia* *Cupriavidus*	*Staphylococcus* *Enhydrobacter* *Corynebacterium* *Knoellia* *Coxiella*	*Staphylococcus* *Fibrobacter* *Enhydrobacter* *Corynebacterium* *Aerococcus*
Pang et al. 2018	high	*Acinetobacter* *Listeria* *Halomonas* *Atopostipes* *Ruminococcus*	*Halomonas* *Klebsiella* *Escherichia-Shigella* *Streptococcus* *Corynebacterium*	
Hoque et al. 2019	low	*Acinetobacter* *Serratia* *Campylobacter* *Pseudomonas* *Eubacterium*		*Acinetobacter* *Serratia* *Campylobacter* *Pseudomonas* *Eubacterium*

Table 1-1. cont'd				
Tong et al. 2019	low	*Acinetobacter* *Stenotrophomonas* *Microbacterium* *Corynebacterium* *Romboutsia*	*Streptococcus* *Acinetobacter* *Romboutsia* *Turicibacter* *Enterococcus*	
Dahlberg et al. 2020	high	*Dyella* *Delftia* *Janthinobacterium* *Stenotrophomonas* *Pseudomonas*		
Hoque et al. 2020	low	*Enterobacter* *Acinetobacter* *Pseudomonas* *Streptococcus* *Salmonella*	*Escherichia* *Shigella* *Serratia* *Lactococcus* *Enterobacter*	*Actinobacter* *Pseudomonas* *Escherichia* *Shigella* *Aeromonas*
Guo et al. 2021	low	*Bacillus* *Lactococcus* *Pseudomonas* *Lactobacillus* *Acinetobacter*		
Ma et al. 2021	high	*Lactobacillus* *Lactococcus* *Bacteroides* *Acinetobacter* *Massilia*		*Bacillus* *Clostridium* *Streptococcus* *Helcococcus* *Lactobacillus*

[1]Refers to confidence in the results based on thoroughness of details in the methods (such as aseptic sample collection, definitions/determinations of CM, SCM, and healthy, and use of healthy control samples for comparisons) provided.

Table 1-2: Summary of detected metabolite changes in milk collected from healthy (H) cows, from cows with subclinical mastitis (SCM), and from cows with clinical mastitis (CM) using metabolomic analysis.

		Response		
Reference	**Metabolites**	**CM vs H**	**SCM vs H**	**SCM vs CM**
Sundekilde et al., 2012	butyrate, acetate, lactate, isoleucine		↑	
	β-hydroxybutyrate		↑	
	fumarate, hippurate		↓	
Thomas et al., 2016b	lactate, bile acids, leucine		↑	
	hippurate, lactose		↓	
Xi et al., 2016	hippurate, citrate, glucose	↓		
	lactose			↑
	4-hydroxyphenyllactate	↓		↑
	L-carnitine	↓	↓	
	D-glycerol-1-phosphate	↓	↓	
	sn-glycero-3-phosphocholine	↓		
	cis-aconitate		↓	↑
	benzoic acid		↓	
	Leu-Leu, arginine	↑	↑	
	oxoglutarate			↑
	Phe-Pro-Ile, Val-Phe-Val-Tyr			↓
	Leu-Ala, Asn-Arg-Ala-Ile			↓
Johnzon et al., 2018	lactose	↓		
Tong et al., 2019	phenylpyruvic acid, glycerol		↑	
	xanthine:guanine, uridine		↑	
	Homogentisic acid:4-HPPA		↑	
Luangwilai et al., 2020	acetate, lactate, benzoate, formate	↑		
	β-hydroxybutyrate	↑		
	isoleucine, hippurate, leucine	↑	↑	
	valerate, histidine	↑	↑	
	alanine, phenylalanine	↑		
	valine, threonine	↑		
	N-acetylglucosamine	↑	↑	
Wang, Y. et al., 2020	uridine		↓	
	5-methyl-tetrahydrofolate	↑		↓
	testosterone glucuronide	↑		↓
	urea, creatine, brassidic acid	↓		
	L-arginine phosphate, melibiose	↓	↓	
	flavin mononucleotide	↓		↑
	orotic acid	↓	↓	↑
	xanthine, thiamine	↓	↓	
	5-aminoimidazole ribonucleotide	↓	↓	↑

Table 1-3: Summary of detected protein changes in milk collected from healthy (H) cows, from cows with subclinical mastitis (SCM), and from cows with clinical mastitis (CM) using proteomic analysis.

		Response		
Reference	**Protein**	**CM vs H**	**SCM vs H**	**SCM vs CM**
Boehmer et al., 2009	transferrin, fibrinogen	↑		
	cathelicidin-1, apolipoprotein I	↑		
	peptidoglycan recognition receptor protein	↑		
	bovine serum albumin, lactoferrin	↑		
Danielsen et al., 2010	Apolipoproteins I, II, and IV	↑		
	α-, β-, and κ-casein	↑		
	complement proteins C3 and C4	↑		
	calgranulin B, growth-related α protein	↑		
Mudaliar et al., 2016	peptidoglycan recognition receptor protein		↑	
	serum amyloid A, haptoglobin		↑	
	cathelicidin proteins, LPS-binding protein		↑	
	histidine-rich glycoprotein		↑	
	mucin-1, cystatin-B, dystroglycan		↓	
	α-lactalbumin, myozenin-1		↓	
Kusebauch et al., 2018	cathepsin C, α-1 antitrypsin, vanin-1	↑		
	α-2 macroglobulin, interleukin-8	↑		
	galectin 1 and 3, haptoglobin	↑		
	cluster of differentiation 14	↑		
	calgranulin B, serum amyloid A3	↑		
Turk et al., 2021	serpins B1 and B3, β-defensin	↑	↑	
	Ig heavy chain, cathelicidin-3	↑	↑	
	Pregnancy zone protein	↑	↑	
	ATP-binding transporter, butyrophilin	↓	↓	
	calponin, variant 19 of α-2 macroglobulin			↓
	αs2-casein, β-lactoglobulin, κ-casein			↑
	thrombospondin			↑
	apolipoproteins I and II, cathelicidin-1			↓
	Serum amyloid A, haptoglobin	↑	↑	
	mucin-1	↓	↓	
	α-2 macroglobulin	↑	↑	

Table 1-4: Summary of detected lipid changes in milk from healthy (H) cows, cows with subclinical mastitis (SCM), and cows with clinical mastitis (CM) using lipidomic analysis.

		Response	
Reference	**Lipid**	**CM vs H**	**SCM vs H**
Mavangira et al., 2015	linoleic acid, arachidonic acid, oleic acid	↑	
	eicosapentaenoic acid, docosahexaenoic acid	↑	
	6-KetoPGF$_{1\alpha}$, PGE$_2$, 13-HODE, 9-HODE[3]	↑	
	14,15-DiHETE, 20-HETE[3], 11-HETE[3], 9-HETE[3]	↑	
Ceciliani et al., 2021	TAG 40:0 and 40:1		↓
	Cer 58:2, HexCer d82:15		↑

Chapter 2: Use of somatic cell count and sodium and potassium concentrations in milk aids in detection of subclinical and clinical mastitis in the first 22 days postpartum

H.K. Peterson[1], R.M. Pace[2], J.E. Williams[1], M.K. McGuire[2], and M.A. McGuire[1]

[1] Department of Animal, Veterinary and Food Sciences, University of Idaho

[2] Margaret Ritchie School of Family and Consumer Sciences, University of Idaho

Abstract

Bovine milk somatic cell count (SCC), lactose, protein, sodium (Na) and potassium (K) concentrations, and the ratio of Na to K (Na/K) are distinctly altered during mastitis. Consequently, they can be used to aid in the detection of clinical (CM) and subclinical (SCM) mastitis in early lactation. However, to date there have been no studies comprehensively evaluating all these parameters simultaneously. To help fill this research gap, we collected daily post-milking, quarter-milk samples from 107 cows across the first 22 d postpartum. Samples were classified as colostrum (0-1 d postpartum) or milk (2-21 d postpartum). Quarters were classified as having CM if visible signs (such as flakes, blood, clots) were present in the secretion. Quarters were classified as having SCM if SCC was ≥200,000 cells/mL and no visible signs of CM were present. Quarters were classified as healthy if SCC was <200,000 cells/mL and no visible signs of CM were present in either colostrum or milk. Somatic cell counts and lactose and protein concentrations were measured using near-infrared analysis; Na and K concentrations were measured using a handheld ion-selective meter and Na/K calculated. Receiver operating characteristic (ROC) curves were used to test the diagnostic capability of components. Very few cases of CM were detected in colostrum (0.6% of samples) and milk (1.2% of samples). Subclinical mastitis cases accounted for 18.2% of the milk samples. Somatic cell count and K concentration best identified colostrum collected from quarters without CM from colostrum collected from quarters with CM. In milk, Na and K concentrations and their ratios best identified quarters with CM whereas Na/K and lactose and Na concentrations best identified quarters with SCM. Somatic cell count and K concentration in colostrum and Na/K and the concentrations of Na, K, and lactose in milk assist in the detection of mastitis in individual mammary gland quarters of cows in early lactation.

Introduction

Inflammation of the mammary gland (mastitis) negatively impacts the dairy industry worldwide and, despite adoption of control strategies, remains a significant challenge (Bradley, 2002; Shaheen et al., 2016; Potter et al., 2018). Intramammary bacterial infection, a common cause of mastitis, is associated with an increase in somatic cell count (SCC), a measure of immune cells in milk (Eberhart et al., 1979). Therefore, determination of a SCC threshold to distinguish between infected and uninfected animals garnered much interest in early work. Various SCC thresholds have been proposed, assessed, and reviewed (e.g., Reichmuth, 1975; Klastrup, 1975; Schultz, 1977; Kitchen 1981; Dohoo et al., 1981; Dohoo and Meek, 1982; Dohoo and Leslie, 1991; Harmon 1994; Schepers et al., 1997). A milk SCC cutoff of 200,000 cells/mL to distinguish between infected and uninfected cows has become widely accepted in mastitis management [Harmon, 2001; Smith et al., 2001; International Dairy Federation (IDF), 2013)]. In particular, the most recent guidelines suggest diagnosing clinical mastitis (CM) via clinical examination and subclinical mastitis (SCM) via a milk SCC threshold of 200,000 cells/mL at the quarter-level (Farre et al., 2022). However, SCC is just one tool available for the management of mastitis and overall mammary gland health.

Several milk components, in addition to immune cell number, are affected by mastitis. For example, lysis of bacteria, increased presence of polymorphonuclear neutrophils (PMN), and macrophage-derived enzymes such as lipases, oxidases, proteases, and glycosidases contribute to mammary gland injury (Li et al., 2014). The blood-milk barrier, primarily through mammary epithelial cells that control the exchange of components between blood and milk, can become disrupted (Wellnitz and Bruckmaier, 2021). Leaky junctions between mammary epithelial cells caused by migration of PMN to the site of infection as well as loss of mammary epithelial cells can lead to milk components leaking out of, and blood components flowing into, the alveolar lumen (Harmon, 1994; Auldist and Hubble, 1998; Wellnitz and Bruckmaier, 2021). In addition to reducing milk yield, this damage causes compositional changes in the milk such as increased concentrations of albumin, immunoglobulins, and sodium (Na), and decreased caseins, lactose, and potassium (K) (Harmon, 1994; Malik et al., 2018; Wellnitz and Bruckmaier, 2021). As milk Na increases, milk K decreases to preserve milk osmolality (Harmon, 1994; Kandeel et al., 2019). Many studies have reported increased Na and decreased K in milk produced by cows with mastitis compared to milk produced by healthy cows (Tallamy and Randolph, 1970; Wegner and Stull, 1978; El Zubeir et al., 2005; Haron et al., 2014). However, Na and K concentration and the ratio of Na to K (Na/K) in milk is much more commonly used to assess mastitis risk and/or severity in humans (Willumsen et al., 2003; Aryeetey et al., 2008; Pace et al., 2022). Thus, in the present study we sought to apply those parameters used in

human health along with the parameters commonly used in the dairy industry (measurements of SCC and, to a lesser extent, protein and lactose concentrations) to a large dataset to detect mastitis in individual mammary gland quarters of cows in early lactation.

The aim of this study was to test the diagnostic ability of SCC, concentrations of lactose, protein, Na and K, and Na/K in identifying CM and SCM in colostrum (CM only) and milk (CM and SCM) collected from individual mammary gland quarters. We hypothesized thresholds of SCC, Na and K concentrations and Na/K would be able to distinguish colostrum produced by quarters with CM from colostrum produced by quarters without CM. We further hypothesized that thresholds of SCC, lactose, protein, Na, and K concentrations, and Na/K would be able to distinguish milk produced by quarters with CM from milk produced by healthy quarters. Finally, we hypothesized that thresholds of lactose, protein, Na, and K concentrations and Na/K would be able to distinguish milk collected from quarters with SCM from milk collected from healthy quarters.

Materials and Methods

Animals and milk collection procedures All procedures involving animals were approved by the University of Idaho Animal Care and Use Committee (protocol 2018-66). Milk samples (n = 8,888) were collected from 107 mostly Holstein cows on four commercial dairies in southern Idaho. Milk was collected daily from each mammary gland quarter after milking in the first 22 d postpartum. Samples were aseptically collected following National Mastitis Council (2004) recommendations. Briefly, after removal of the milking machine and while wearing gloves, each teat was wiped carefully using ethanol-soaked wipes (Ecolab Inc., St. Paul, MN), paying particular attention to the teat end. Approximately 40-50 mL of milk were collected from each quarter into sterile 50 mL centrifuge tubes (Stockwell Scientific, Scottsdale, AZ). Samples were placed in a cooler filled with ice packs until transported to the laboratory. After sample collection, teats were dipped or sprayed with post-milking teat disinfectant as per each dairy's protocol. Gloves were sanitized between animals or, when feasible, changed. Animals were monitored closely by both study and dairy personnel for signs of CM (such as milk with blood or clots and swelling, redness, or hardness of the mammary gland) and any evidence documented by quarter.

SCC, protein, and lactose evaluation Approximately 30 mL of colostrum/milk were transferred to a tube containing preservative for determination of SCC, protein concentration, and lactose concentration via near-infrared analysis (High Desert Dairy Lab Inc., Nampa, ID). For low-volume samples (<20 mL), SCC was determined by study personnel using a DeLaval cell counter (model #92740080, Tumba, Sweden).

Classification of mammary health status and sample type Quarters with clinical signs in milk (flakes, clots, or blood) and/or in mammary gland (redness, hardness, or swelling; Farre et al., 2022) were categorized as having CM. Quarters producing milk with SCC ≥200,000 cells/mL but no clinical signs in milk or mammary gland were categorized as having SCM. Quarters producing milk with SCC <200,000 cells/mL and no clinical signs in milk or the mammary gland were categorized as healthy. Samples were further divided into colostrum (0-1 d postpartum, with 0 d postpartum indicating the first milking after calving) and milk (2-21 d postpartum).

Electrolyte evaluation Sodium (Na) and potassium (K) concentrations were measured, retrospectively, in a subset of the samples (n= 745). Sodium and K concentrations were determined using handheld ion-selective meters (LAQUAtwin Na-11 and LAQUAtwin K-11, HORIBA Advanced Techno Co., Ltd. Kyoto, Japan). The subset was determined to provide a representative set of each mammary gland health status group (quarters with CM, quarters with SCM, and healthy quarters) in both colostrum and milk. Before use, meters were calibrated using appropriate solutions. Samples were thawed on ice at room temperature and 200 µL pipetted onto the sensor of the Na meter; after a few seconds, the result was recorded. This process was repeated for the K meter. Concentrations of Na and K (reported in ppm) were converted to mmol/L.

Statistical analyses All statistical analyses were performed using R (v4.1.2; R Core Team) or Prism 10 (v10.0.3; GraphPad Software, San Diego, USA). We used lme from the R package nlme (v3.1-162; Pinheiro et al., 2015) and emmeans from the R package emmeans (v1.8.8; Searle et al., 1980) to determine least-squares means for each colostrum or milk component. The model included the mammary gland quarter health status group (with CM or without CM for colostrum samples and healthy, SCM, or CM for milk samples) as a fixed effect and quarter nested within cow as a random effect. Tukey's test was used to identify differences in least-squares means of each colostrum or milk component among mammary gland quarter health status group. Due to excessive coagulation, lactose or protein concentrations could not be determined in twelve CM samples. Receiver operating characteristic (ROC) curves (Fawcett, 2006) were used to test for sensitivity and specificity for detection of mastitis of SCC, Na and K concentrations, and Na/K in colostrum and of SCC, concentrations of protein, lactose, Na and K, and Na/K in milk. Youden's index (Youden, 1950) was calculated (*sensitivity + specificity – 100*) and used to determine the optimum threshold of sensitivity and specificity for detection of mastitis for each component. Positive predictive values and negative predictive values were also calculated. Significance was declared at $P<0.05$ with trends noted at $P<0.10$ and $P>0.05$.

Results

Day-to-day variation in mean SCC, lactose concentration, and protein concentration in colostrum and milk from each mammary gland quarter health group is shown in **Figure 2-1**. The use of the IDF definition of SCM (≥200,000 cells/mL and no clinical signs) was deemed inappropriate for colostrum (0-1 d postpartum) samples because the IDF definition was established for milk only (Farre et al., 2022). Furthermore, SCC is naturally elevated in colostrum (Dohoo and Meek, 1982). Therefore, SCM (as per the IDF definition) was not defined in colostrum. In milk produced by quarters with CM, SCC appeared to be greater than SCC in milk produced by healthy quarters. In milk produced by quarters with SCM, SCC was greater than SCC in milk produced by healthy quarters. Lactose concentration increased in the transition from colostrum to milk. Lactose concentration appeared to be lower in milk produced by quarters with CM compared to milk produced by quarters with SCM or healthy quarters. Protein concentration declined rapidly in the first 3 d postpartum. Milk collected from quarters with CM appeared to have greater protein concentration than in milk produced by quarters with SCM or healthy quarters after 9 d postpartum (Figure 2-1).

Colostrum analyses Clinical mastitis was associated with 0.6% of the 827 colostrum samples collected. Somatic cell count was greater ($P<0.0001$) and concentration of Na lower ($P=0.0022$) in colostrum produced by quarters with CM compared to colostrum produced by quarters without CM (**Table 2-1**). Trends for differences in K concentration ($P=0.0908$) and Na/K ($P=0.0626$) between quarters without CM and quarters with CM were observed. Thresholds for SCC (564,500 cells/mL; $P=0.0009$) and concentration of K (21.10 mmol/L; $P=0.0135$) distinguished colostrum collected from quarters with CM from colostrum collected from quarters without CM with high sensitivity and specificity (**Table 2-2**).

Milk analyses The percentage of mastitis status types in 8,061 quarter milk samples was 1.2% for CM, 18.2% for SCM, and 80.6% for healthy in 107 cows over the first 22 d of lactation. Somatic cell count was lowest in milk collected from healthy quarters, greater in milk collected from quarters with SCM, and greatest in milk collected from quarters with CM (**Table 2-3**). Lactose concentration was greater in milk collected from healthy quarters than in milk collected from quarters with SCM or CM. Lactose concentration was lower in milk collected from quarters with CM than in milk collected from quarters with SCM. Protein concentration was lower in milk collected from healthy quarters than in milk collected from quarters with SCM or CM. Sodium concentration and Na/K were lowest in milk collected from healthy quarters, greater in milk collected from quarters with SCM, and greatest in milk collected from quarters with CM (Table 2-3). Concentration of K in milk collected from quarters with CM was lower than in milk collected from quarters with SCM or healthy quarters. Potassium

concentration in milk collected from quarters with SCM was higher than in milk collected from healthy quarters (Table 2-3). Thresholds for SCC (291,500 cells/mL; $P<0.0001$); lactose (4.03%; $P<0.0001$), protein (3.38%; $P<0.0001$), Na (22.84 mmol/L; $P<0.0001$), and K (19.95 mmol/L; $P<0.0001$) concentrations; and Na/K (1.11; $P<0.0001$) distinguished milk collected from quarters with CM from milk collected from quarters without CM (**Table 2-4**). All thresholds ($P<0.0001$) were the same to identify milk collected from quarters with CM from milk collected from healthy quarters only, except for a lower threshold of SCC (195,500 cells/mL; Table 2-4). Milk collected from quarters with SCM was differentiated from milk collected from healthy quarters through thresholds for lactose (4.30%; $P<0.0001$), protein (3.18%; $P<0.0001$) and Na (12.83 mmol/L; $P<0.0001$) concentrations; and Na/K (0.51; $P<0.0001$; Table 2-4).

Discussion

In the present study, the large sample size allowed for the examination of components in colostrum and milk for their ability to identify mastitis in individual mammary gland quarters of cows in early lactation. Intramammary bacterial infection was not included in our approach, unlike that of many others (such as Schultz, 1977; Dohoo et al., 1981; Schepers et al., 1997; Barkema et al., 1999), to avoid the pitfalls of conventional culture. Culture-dependent methods can fail to grow bacteria in >30% of milk samples collected from quarters with either CM or SCM (Taponen et al., 2009). Furthermore, it would be inappropriate to classify infected/non-infected interchangeably with mastitis/healthy since mastitis may not be solely caused by bacterial infection. Moreover, the term "culture-negative" to describe healthy milk fails to acknowledge the existence of a large and diverse bacterial population, as determined by culture-independent methods (Oikonomou et al., 2012; Kuehn et al., 2013). Culture-independent methods provide a plethora of information (like identification, relative abundance, and function) beyond presence/absence of infection and growth/no-growth of bacteria. Therefore, because mastitis can be the result of various factors, we chose to define CM (clinical signs of milk such as clots, blood, and/or mammary gland such as redness, swelling) and SCM (SCC ≥200,000 cells/mL and no clinical signs) based on the guidelines recently published by the IDF (Farre et al., 2022) rather than conventional culture and infection status. Furthermore, because distinct compositional changes were observed in colostrum (0-1 d postpartum) and milk (2-21 d postpartum), data from colostrum and milk were analyzed separately.

Characterization of CM via colostrum components As one of few reports available that compares colostrum collected from quarters with and without mastitis, Barkema et al. (1999) observed SCC was greater in colostrum produced by quarters infected with either a minor pathogen (*Corynebacterium bovis* and coagulase-negative staphylococci) or a major pathogen (*Staphylococcus*

aureus, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Escherichia coli*) than in colostrum produced by culture-negative quarters. Likely due (at least in part) to the limited number of observations, we only detected trends for differences in K concentration and Na/K between colostrum collected from quarters without CM versus with CM. However, SCC and Na concentration were different (Table 2-1). For diagnostic purposes, thresholds of SCC and K concentration were the only components in colostrum that might be capable of distinguishing between quarters with CM and quarters without CM (Table 2-2). Both thresholds are highly sensitive but have low positive predictive values, indicating a low probability of either threshold correctly identifying quarters with CM. If the goal is to correctly identify quarters with CM, K concentration would perform slightly better than SCC. Both thresholds are highly specific and have negative predictive values of 100% indicating a high probability of either threshold correctly identifying quarters without CM. If the goal is to correctly identify quarters without CM, SCC and K concentration would perform similarly (Table 2-2). No other studies to our knowledge have attempted to identify diagnostic thresholds for components in colostrum although our limited number of CM cases only provides preliminary determination of diagnostic capacity.

Characterization of CM and SCM via milk components In the current study, shifts in milk components align well with previously reported changes (Wegner and Stull, 1978; Auldist and Hubble, 1998; Le Maréchal et al., 2011; Li et al., 2014). Milk collected from quarters with mastitis (either SCM or CM) had elevated SCC compared with milk collected from healthy quarters (Table 2-3). However, many samples (n = 36) collected from quarters with CM had low SCC (<200,000 cells/mL). An increase in SCC would be expected given the massive influx of immune cells to defend the mammary gland against bacterial infection (Paape et al., 2002) but that is not always the case. Low SCC has been found to be a risk factor for the development of CM (Schukken et al., 1990; Suriyasathaporn et al., 2000; Burvenich et al., 2003) or reflective of transient infection of minor mastitis pathogens (Ward and Schultz, 1972; Harmon, 1994; Sharma et al., 2011). In agreement with Alhussien and Dang (2018), total protein concentration in milk produced by quarters with CM or SCM was greater than in milk produced by healthy quarters. However, several reviews (Kitchen, 1981; Auldist and Hubble, 1998; Le Maréchal et al., 2011) conclude that even though changes in individual proteins (such as caseins, ß-lactoglobulin, whey proteins, and serum albumin) are evident, total protein concentration does not vary significantly between milk produced by quarters with mastitis (CM or SCM) and milk produced by healthy quarters. Auldist and Hubble (1998) noted that an increase in proteins originating in the blood is compensated by a decrease in milk proteins due to the disruption in mammary epithelial cell integrity and blood-milk barrier. Although individual proteins were not measured in the current study, the increase in protein concentration in milk

collected from quarters with mastitis may be reflective of greater concentrations of blood proteins relative to milk proteins or reduced concentrations of milk proteins relative to blood proteins. Future studies should examine changes in individual proteins in conjunction with total protein concentration in milk collected post-milking from quarters with mastitis (CM or SCM) compared to milk collected from healthy quarters of cows in early lactation. In accordance with Kitchen et al. (1981), Harmon (1994), and Le Maréchal et al. (2011), lactose concentration was greatest in milk produced by healthy quarters compared to milk produced by quarters with SCM or CM. The greater Na concentration and lower K concentration in milk produced by quarters with SCM or CM compared to milk produced by healthy quarters (Table 2-3) agrees with previous studies (Reichmuth, 1975; Kitchen, 1981; El Zubeir et al., 2005). In accordance with Wegner and Stull (1978) and El Zubeir et al. (2005), Na/K was greater in milk produced by quarters with SCM or CM compared to milk produced by healthy quarters.

Diagnostic SCC thresholds The threshold of SCC (195,500 cells/mL) that distinguished milk produced by quarters with CM from milk produced by healthy quarters had greater specificity, sensitivity, and positive predictive value compared with the threshold of SCC (291,500 cells/mL) for milk produced by quarters with CM versus those without CM (Table 2-4). The latter threshold of SCC had reasonably high sensitivity but a low positive predictive value reducing the probability of correctly identifying milk produced by quarters with CM from milk produced by quarters without CM (Table 2-4). This is most likely attributable to the fact that both milk collected from quarters with CM and milk collected from quarters with SCM had greater SCC relative to milk collected from healthy quarters (Table 2-3). McDermott et al. (1982) tested several SCC thresholds to distinguish milk produced by infected cows from milk produced by uninfected cows using composite samples. Although not directly comparable (CM/healthy versus infected/not infected as well as quarter versus cow), their thresholds of 200,000 and 300,000 cells/mL had greater sensitivities and positive predictive values, but lower specificities and negative predictive values compared with the SCC thresholds in the present study (Table 2-4). Dohoo and Leslie (1991) determined a SCC threshold of 200,000 cells/mL to distinguish milk produced by infected cows (culture-positive) from milk produced by non-infected (culture-negative) cows. Schepers et al. (1997) and Petzer et al. (2017) also determined the 200,000 cells/mL threshold was capable of distinguishing milk produced by infected quarters from milk produced by non-infected quarters. Despite the differences (such as CM/healthy vs infected/non-infected and quarter vs cow), the diagnostic capabilities of the SCC thresholds in the present study performed similarly to the SCC thresholds in these other studies.

Diagnostic lactose concentration thresholds The threshold for concentration of lactose (4.03%, wt/vol) distinguished milk produced by quarters with CM from milk produced by quarters without CM and from milk produced by healthy quarters alone. Although the sensitivity and negative predictive values are the same, the slightly greater specificity and positive predictive value indicate better diagnostic performance when the quarters with SCM were removed from the test (Table 2-4). A lactose concentration threshold of 4.30% performed reasonably well when used to identify milk produced by quarters with SCM from milk produced by healthy quarters. No other studies to our knowledge have attempted to identify diagnostic thresholds for lactose in milk.

Diagnostic protein concentration thresholds The protein concentration threshold of 3.38% distinguished milk produced by quarters with CM from milk produced by quarters without CM and from milk produced by healthy quarters alone. The sensitivity and negative predictive values were the same, but the specificity and positive predictive values were a little higher (Table 2-4). Removing the quarters with SCM resulted in a slight improvement in the diagnostic performance of this protein concentration threshold. The protein concentration threshold of 3.18% has the lowest sensitivity and negative predictive values and a low specificity, indicating it has the lowest diagnostic performance when used to identify milk produced by quarters with SCM from milk produced by healthy quarters. No other studies to our knowledge have attempted to identify diagnostic thresholds for protein in milk.

Diagnostic Na concentration thresholds The Na concentration threshold of 22.84 mmol/L distinguished milk produced by quarters with CM from milk produced by quarters without CM and from milk produced by healthy quarters only. The sensitivity and negative predictive values were the highest and were the same for both comparison groups (Table 2-4). The high specificity and positive predictive values indicate the threshold performed the best when it was used to distinguish quarters with CM from healthy quarters. The threshold of 12.83 mmol/L that distinguished quarters with SCM from healthy quarters had the lowest diagnostic performance of the Na concentration thresholds (Table 2-4). Kandeel et al. (2019) reported a higher Na threshold (24 mmol/L) with greater sensitivity, but lower specificity compared to the Na threshold (12.83 mmol/L) determined in the present study to identify milk produced by quarters with SCM from milk produced by healthy quarters.

Diagnostic K concentration thresholds The K concentration threshold of 19.95 mmol/L distinguished milk produced by quarters with CM from milk produced by quarters without CM and from milk produced by healthy quarters alone. The sensitivity and negative predictive values were the highest and were the same for both comparison groups (Table 2-4). The specificity and positive predictive

values were slightly higher, thus the diagnostic performance was marginally better, when the quarters with SCM were removed from the test. Unlike Kandeel et al. (2019), it was not possible to determine a reliable threshold to distinguish milk produced by quarters with SCM from milk produced by healthy quarters based on K concentration in the present study.

Diagnostic Na/K thresholds The Na/K threshold of 1.10 had the highest sensitivity, specificity, positive predictive value, and negative predictive value of the Na/K thresholds determined (Table 2-4). This indicates it has a high probability of correctly identifying quarters with CM as well as identifying healthy quarters. A nearly identical Na/K threshold of 1.11 was determined to distinguish milk produced by quarters with CM from milk produced by quarters without CM (Table 2-4). The positive predictive value is lower, so the performance is slightly lower than the 1.10 Na/K threshold. Although it had the lowest diagnostic performance of all the Na/K thresholds, the threshold of 0.51 performed reasonably well in correctly identifying quarters with SCM from healthy quarters.

Summary of diagnostic performance of milk components The ratio of Na/K had the best diagnostic performance as determined by the area under the curve (AUC) in identifying quarters with SCM or CM. Sodium and K concentrations and Na/K appeared to be better indicators than SCC in identifying quarters with CM (Table 2-4). Implementing Na and K sensors into automatic milking systems, as is already done to measure SCC with varying levels of success (van der Voort et al., 2021), would strengthen detection of CM in instances when milk is not visually inspected. Protein concentration performed similarly to SCC in identifying quarters with CM whereas lactose concentration performed worse. Quarters with SCM were also well identified through lactose and Na concentrations but protein and K concentrations had a lower diagnostic performance (Table 2-4). In summary, Na/K and individual Na and K concentrations have better diagnostic capabilities than the traditional measure of SCC in identifying CM in individual mammary gland quarters.

The dairies utilized in this study had low incidence of naturally occurring CM. This resulted in limited applicability and interpretation of the CM findings, particularly in colostrum samples. Another limitation was our use of the IDF definition of SCM (Farre et al., 2022). Although an SCC cutoff of 200,000 cells/mL at quarter-level is recommended, it is not a precise characterization of non-visible inflammation in the mammary gland. Several studies (Reichmuth, 1975; Kitchen, 1981; Harmon, 1994; Schukken et al., 2003; Malik et al., 2018) report compositional changes in milk at a range of SCC 100,000-1,000,000 cells/mL. Therefore, the SCM results of this study are only applicable to the SCM definition of SCC ≥200,000 cells/mL. The results of this study may also not apply to post-milking samples after the first 22 d postpartum. While the diagnostic factors may be reflective of mastitis in the dairy industry of southern Idaho, our results may not be applicable to the

dairy industry in other regions of the United States. Future studies should determine diagnostic thresholds of SCC, protein, lactose, Na, and K concentrations, and Na/K for different geographical regions, different breeds, and across all stages of lactation.

Conclusion

In the current study, SCC, Na and K concentrations, and Na/K were evaluated to characterize CM in colostrum (0-1 d postpartum) from individual mammary gland quarters. Additionally, SCC, lactose, protein, Na, and K concentrations, and Na/K were appraised to more closely define SCM and CM in milk (2-21 d postpartum). Thresholds of SCC and K concentration in colostrum are the first to identify specific mammary gland quarters with CM. Our calculated SCC thresholds of 291,500 and 195,500 cells/mL distinguished quarters with CM from quarters without CM and from healthy quarters only. These cutoffs are consistent with earlier research and support current advice to dairy farmers to adopt an SCC cutoff of 200,000 cells/mL to identify SCM. The use of Na and K concentrations and their ratio to identify mastitis is novel in the dairy industry. The ratio of Na/K had the best diagnostic performance of milk components, followed by Na and K concentration, in identifying quarters with CM. Milk Na/K and lactose and Na concentrations best identified quarters with SCM. Implementing use of Na and K concentrations and their ratio on-farm, such as with sensors to measure Na and K concentrations in automatic milking systems, may provide reliable diagnostic tools for the detection of CM and SCM in early lactation.

References

Alhussien, M.N., and A.K. Dang. 2018. Milk somatic cells, factors influencing their release, future prospects, and practical utility in dairy animals: an overview. Vet World. 11:562-577.

Aryeetey, R.N.O., G.S. Marquis, L. Timms, A. Lartey, and L. Brakohiapa. 2008. Subclinical mastitis is common among Ghanaian women lactating 3 to 4 months postpartum. J Hum Lact. 24:263-267.

Auldist, M. J. and I. B. Hubble. 1998. Effects of mastitis on raw milk and dairy products. Aust J Dairy Technol. 53:28-36.

Barkema, H.W., H.A. Deluyker, Y.H. Schukken, and T.J.G.M. Lam. 1999. Quarter-milk somatic cell count at calving and at the first six milkings after calving. Prev Vet Med. 38:1-9.

Bradley, A. J. 2002. Bovine mastitis: an evolving disease. Vet J. 164:116-128.

Burvenich, C., V. Van Merris, J. Mehrzad, A. Diez-Fraile, and L. Duchateau. 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. Vet Res. 34:521-564.

Dohoo, I. R., A. H. Meek, S. W. Martin, and D. A. Barnum. 1981. Use of total and differential somatic cell counts from composite milk samples to detect mastitis in individual cows. Can Comp Med. 45:8-14.

Dohoo, I. R. and A. H. Meek. 1982. Somatic cell counts in bovine milk. Can Vet J. 23:119-125.

Dohoo, I. R. and K. E. Leslie. 1991. Evaluation of changes in somatic cell counts as indicators of new intramammary infections. Prev Vet Med. 10:225-237.

Eberhart, R.J., H.C. Gilmore, L.J. Hutchinson, and S.B. Spencer. 1979. Somatic cell counts in DHI samples. National Mastitis Council Annual Meeting 1979. 32-40.

El Zubeir, I. E. M., O. A. O. El Owni, and G. E. Mohamed. 2005. Effect of mastitis on macro-minerals of bovine milk and blood serum in Sudan. J S Afr Vet Assoc. 76:22-25.

Farre, M., A. Zecconi, and D. Kelton. 2022. Guidelines for defining quarter and udder health status and cured clinical and subclinical mastitis cases. International Dairy Federation (IDF) Bulletin 515/2022. 1-28.

Fawcett, T. 2006. An introduction to ROC analysis. Pattern Recognit Lett. 27:861-874.

Harmon, R. J. 1994. Physiology of mastitis and factors affecting somatic cell counts. J Dairy Sci. 77:2103-2112.

Harmon, R. J. 2001. Somatic cell counts: a primer. National Mastitis Council Annual Meeting Proceedings. 3-9.

Haron, A. W., F. F. J. Abdullah, A. Tijjani, Y. Abba, L. Adamu, K. Mohammed, A. M. M. Amir, M. A. Sadiq, and M. A. M. Lila. 2014. The use of Na+ and K+ ion concentrations as potential diagnostic indicators of subclinical mastitis in dairy cows. Vet World. 7:966-969.

International Dairy Federation (IDF) Bulletin 466/2013 Guidelines for the use and interpretation of bovine milk somatic cell counts (SCC) in the dairy industry.

Kandeel, S. A., A. A. Megahed, and P. D. Constable. 2019. Evaluation of hand-held sodium, potassium, calcium, and electrical conductivity meters for diagnosing subclinical mastitis and intramammary infection in dairy cattle. J Vet Intern Med. 33:2343-2353.

Kitchen, B.J. 1981. Review of the progress of dairy science: bovine mastitis: milk compositional changes and related diagnostic tests. J Dairy Res. 48:167-188.

Klastrup, O. 1975. Discussion opening in diagnosis of mastitis and intramammary infection. Proceedings of the IDF seminar on mastitis control 1975, reading university, April 7-11. 41-52.

Kuehn, J.S., P.J. Gorden, D. Munro, R. Rong, Q. Dong, P.J. Plummer, C. Wang, and G.J. Phillips. 2013. Bacterial community profiling of milk samples as a means to understand culture-negative bovine clinical mastitis. PLoS One. 8:1-10.

Le Maréchal, C., R. Thiéry, E. Vautor, and Y. Le Loir. 2011. Mastitis impact on technological properties of milk and quality of milk products—a review. Dairy Sci Technol. 91:247-282.

Li, N., R. Richoux, M. Boutinaud, P. Martin, and V. Gagnaire. 2014. Role of somatic cells on dairy processes and products: a review. Dairy Sci & Technol. 94:517-538.

Malik, T. A., M. Mohini, S. H. Mir, B. A. Ganaie, D. Singh, T. K. Varun, S. Howal, and S. Thakur. 2018. Somatic cells in relation to udder health and milk quality—a review. J Anim Health Prod. 6:18-26.

McDermott, M.P., H.N. Erb, and R.P. Natzke. 1982. Predictability by somatic cell counts related to prevalence of intramammary infection within herds. J Dairy Sci. 65:1535-1539.

National Mastitis Council. 2004. Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality. NMC Publication.

Oikonomou, G., V.S. Machado, C. Santisteban, Y.H. Schukken, and R.C. Bicalho. 2012. Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. PLoS One. 7:1-14.

Paape, M., J. Mehrzad, X. Zhao, J. Detilleux, and C. Burvenich. 2002. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. J Mammary Gland Biol Neoplasia. 7:109-121.

Pace, R.M., C.D.W. Pace, B.D. Fehrenkamp, W.J. Price, M. Lewis, J.E. Williams, M.A. McGuire, and M.K. McGuire. 2022. Sodium and potassium concentrations and somatic cell count of human milk produced in the first six weeks postpartum and their suitability as biomarkers of clinical and subclinical mastitis. Nutrients. 14:4708. Doi: 10.3390/nul4224708.

Petzer, I-M., J. Karzis, E. F. Donkin, E. C. Webb, and E. M. C. Etter. 2017. Somatic cell count thresholds in composite and quarter milk samples as indicator of bovine intramammary infection status. Onderstepoort J Vet Res. 84:1-10.

Pinheiro, J., D. Bates, and S. DebRoy. 2015. Nlme: linear and nonlinear mixed effects models. R package nlme version.

Potter, T.L., C. Arndt, and A.N. Hristov. 2018. Short communication: increased somatic cell count is associated with milk loss and reduced feed efficiency in lactating dairy cows. J Dairy Sci. 101:9510-9515.

R Core Team. 2021. R: a language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria.

Reichmuth, J. 1975. Somatic cell counting—interpretation of results. Proceedings of the IDF seminar on mastitis control 1975, reading university, April 7-11. 93-109.

Schepers, A. J., T. J. G. M. Lam, Y. H. Schukken, J. B. M. Wilmink, and W. J. A. Hanekamp. 1997. Estimation of variance components for somatic cell counts to determine thresholds for uninfected quarters. J Dairy Sci. 80:1833-1840.

Schukken, Y.H., F.J. Grommers, D. Van de Geer, H.N. Erb, and A. Brand. 1990. Risk factors for clinical mastitis in herds with a low bulk milk somatic cell count. 1. Data and risk factors for all cases. J Dairy Sci. 73:3463-3471.

Schukken, Y.H., D.J. Wilson, F. Welcome, L. Garrison-Tikofsky, and R.N. Gonzalez. 2003. Monitoring udder health and milk quality using somatic cell counts. Vet Res. 34:579-596.

Schultz, L. H. 1977. Somatic cell counting of milk in production testing programs as a mastitis control technique. J Am Vet Med Assoc. 170:1244-1246.

Searle, S.R., F.M. Speed, and G.A. Milliken. 1980. Population marginal means in the linear model: an alternative to least squares means. Am Stat. 34:216-221.

Shaheen, M., H.A. Tantary, and S.U. Nabi. 2016. A treatise on bovine mastitis: disease and disease economics, etiological basis, risk factors, impact on human health, therapeutic management, prevention and control strategy. J Adv Dairy Res. 4:1-10.

Sharma, N., N.K. Singh, and M.S. Bhadwal. 2011. Relationship of somatic cell count and mastitis: an overview. Asian-Aust J Anim Sci. 24:429-438.

Smith, K. L., J. E. Hillerton, and R. J. Harmon. 2001. Guidelines on normal and abnormal raw milk based on somatic cell counts and signs of clinical mastitis. National Mastitis Council, Madison WI.

Suriyasathaporn, W., Y.H. Schukken, M. Nielen, and A. Brand. 2000. Low somatic cell count: a risk factor for subsequent clinical mastitis in a dairy herd. J Dairy Sci. 83:1248-1255.

Tallamy, P. T. and H. E. Randolph. 1970. Influence of mastitis on properties of milk. V. total and free concentrations of major minerals in skimmilk. J Dairy Sci. 53:1386-1388.

Taponen, S., L. Salmikivi, H. Simojoki, M.T. Koskinen, and S. Pyörälä. 2009. Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. J Dairy Sci. 92:2610-2617.

van der Voort, M., D. Jensen, C. Kamphuis, I.N. Athanasiadis, A. De Vries, and H. Hogeveen. 2021. Invited review: toward a common language in data-driven mastitis detection research. J Dairy Sci. 104:10449-10461.

Ward, G.E. and L.H. Schultz. 1972. Relationship of somatic cells in quarter milk to type of bacteria and production. J Dairy Sci. 55:1428-1431.

Wegner, T. N. and J. W. Stull. 1978. Relation between mastitis test score, mineral composition of milk, and blood electrolyte profiles in Holstein cows. J Dairy Sci. 61:1755-1759.

Wellnitz, O. and R. M. Bruckmaier. 2021. Invited review: the role of the blood-milk barrier and its manipulation for the efficacy of the mammary immune response and milk production. J Dairy Sci. 104:6376-6388.

Willumsen, J.F., S.M. Filteau, A. Coutsoudis, M.L. Newell, N.C. Rollins, H.M. Coovadia, and A.M. Tomkins. 2003. Breastmilk RNA viral load in HIV-infected South African women: effects of subclinical mastitis and infant feeding. AIDS. 17:407-414.

Youden, W. J. 1950. Index for rating diagnostic tests. Cancer. 3:32-35.

Table 2-1**:** Summary measurements of components in colostrum from quarters with or without clinical mastitis (CM)[1].

Component	**Without CM**	**With CM**	***P*-value**
SCC (cells/mL)	386,180 ± 29,902	1,818,091 ± 232,120	<0.0001
Na (mmol/L)	17.0 ± 1.3	29.3 ± 3.9	0.0022
K (mmol/L)	31.0 ± 1.7	24.1 ± 4.2	0.0908
Na/K	0.7 ± 0.2	1.2 ± 0.3	0.0626

[1]Values represent least-squares means ± standard errors of the mean. The numbers of observations for SCC were 822 in quarters without CM and 5 in quarters with CM, respectively. The numbers of observations for Na, K, and Na/K measured in quarters without CM and in quarters with CM were 100 and 3, respectively. *P*-value obtained via Tukey's test.

Table 2-2: Receiver operating characteristic curves and area under the curve for the efficacy of components in colostrum from mammary gland quarters as predictors of clinical mastitis (CM).

Component	**Threshold**	**Sensitivity (%)**	**Specificity (%)**	**Positive Predictive Value (%)**	**Negative Predictive Value (%)**	**Area Under the Curve**	**95% CI**	***P*-value**
SCC (cells/mL)	564,500	100	80	3	100	0.93	0.87 to 1.00	0.0009
Na (mmol/L)	13.27	67	67	4	98	0.52	0.09 to 0.95	0.9141
K (mmol/L)	21.10	100	87	19	100	0.92	0.84 to 1.00	0.0135
Na/K	0.62	67	79	13	98	0.73	0.44 to 1.00	0.1791

Table 2-3: Summary measurements of milk components by healthy quarters, quarters with subclinical mastitis (SCM), and quarters with clinical mastitis (CM)[1].

Component	**Healthy**	**SCM**	**CM**
SCC (cells/mL)	93,325 ± 16,955[a]	687,356 ± 20,461[b]	1,876,232 ± 58,322[c]
Lactose (%, wt/vol)	4.43 ± 0.03[a]	4.13 ± 0.03[b]	3.46 ± 0.05[c]
Protein (%, wt/vol)	2.91 ± 0.03[a]	3.25 ± 0.04[b]	3.40 ± 0.07[b]
Na (mmol/L)	13.70 ± 0.89[a]	19.00 ± 1.02[b]	36.40 ± 2.16[c]
K (mmol/L)	27.96 ± 1.32[a]	30.07 ± 1.44[b]	6.71 ± 2.72[c]
Na/K	0.51 ± 0.05[a]	0.74 ± 0.08[b]	6.18 ± 0.23[c]

[1]Values represent least-squares means ± standard errors of the mean. Different superscripts indicate differences in means within component across quarter health status ($P<0.05$). The number of observations for SCC, lactose, and protein measured in healthy quarters was 6414, 6309, and 6347, respectively; in SCM quarters was 1548, 1471, and 1520, respectively; and in CM quarters was 99, 91, and 91, respectively. The number of observations for Na, K, and Na/K measured in healthy, SCM, and CM quarters was 531, 105, and 6, respectively.

Table 2-4: Assessment of milk components collected by mammary gland quarter to predict clinical (CM) and subclinical (SCM) mastitis through receiver operating characteristic analysis.

Milk Component	Threshold	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Area Under the Curve	95% CI	P-value
SCC (cells/mL)								
CM vs Not CM	291,500	64	86	8	100	0.79	0.74 to 0.84	<0.0001
CM vs Healthy	195,500	68	100	80	100	0.86	0.81 to 0.91	<0.0001
Lactose (%, wt/vol)								
CM vs Not CM	4.03	54	88	9	99	0.72	0.66 to 0.79	<0.0001
CM vs Healthy	4.03	54	93	16	99	0.75	0.69 to 0.81	<0.0001
SCM vs Healthy	4.30	61	73	47	92	0.73	0.72 to 0.75	<0.0001
Protein (%, wt/vol)								
CM vs Not CM	3.38	67	84	7	100	0.78	0.72 to 0.84	<0.0001
CM vs Healthy	3.38	67	87	10	100	0.80	0.74 to 0.85	<0.0001
SCM vs Healthy	3.18	42	77	52	88	0.63	0.61 to 0.64	<0.0001
Na (mmol/L)								
CM vs Not CM	22.84	100	94	14	100	0.98	0.96 to 1.00	<0.0001
CM vs Healthy	22.84	100	97	26	100	0.99	0.98 to 1.00	<0.0001
SCM vs Healthy	12.83	83	49	28	97	0.71	0.66 to 0.77	<0.0001
K (mmol/L)								
CM vs Not CM	19.95	100	89	7	100	0.98	0.95 to 1.00	<0.0001
CM vs Healthy	19.95	100	91	10	100	0.98	0.96 to 1.00	<0.0001
SCM vs Healthy	33.25	76	38	22	96	0.53	0.47 to 0.59	0.3953
Na/K								
CM vs Not CM	1.11	100	97	27	100	0.99	0.99 to 1.00	<0.0001
CM vs Healthy	1.10	100	99	67	100	1.00	1.00 to 1.00	<0.0001
SCM vs Healthy	0.51	70	72	40	94	0.75	0.70 to 0.80	<0.0001

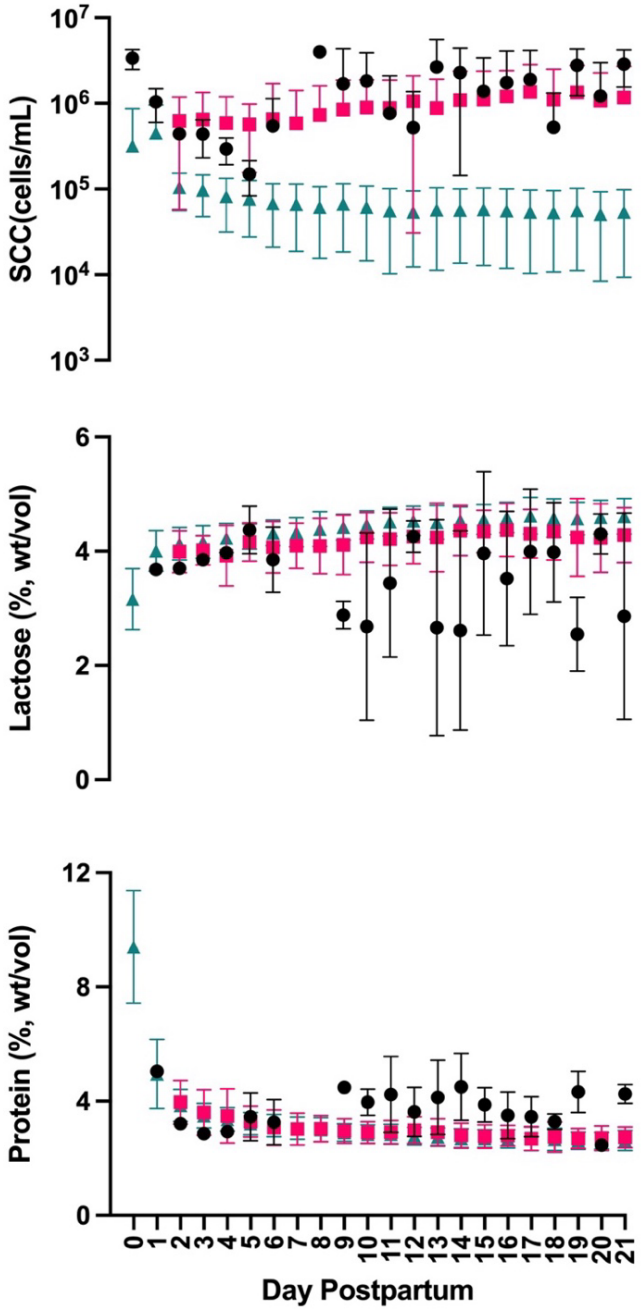


Figure 2-1: Mean (± SD) somatic cell count (SCC), lactose concentration, and protein concentration in milk produced by healthy quarters (n = 7236, 6993, and 7141, respectively; ▲) and those with clinical mastitis (CM; n = 104, 92, and 92, respectively; ●) or subclinical mastitis (SCM; n = 1548, 1471, and 1520, respectively; ■) across the first three weeks postpartum. No distinction between SCM and healthy quarters occurred on days 0 and 1 (colostrum) where use of the IDF definition of SCM was inappropriate. The day of calving is denoted by day 0.

Supplementary Material

Supplementary Table 2-1: Components of colostrum (0-1 d postpartum) produced by quarters without and with clinical mastitis (CM).

	Without CM[1]	**With CM[2]**
SCC		
Number of quarters	822	5
Mean (cells/mL)	384,376	1,973,800
Range (cells/mL)	9,000-3,936,000	565,000-4,000,000
Lactose		
Number of quarters	684	1
Mean (%, wt/vol)	3.60	3.68
Range (%, wt/vol)	0.61-4.82	NA
Protein		
Number of quarters	794	1
Mean (%, wt/vol)	7.16	5.04
Range (%, wt/vol)	2.63-13.37	NA
Na		
Number of quarters	100	3
Mean (mmol/L)	17.38	24.94
Range (mmol/L)	6.53-47.85	9.57-52.20
K		
Number of quarters	100	3
Mean (mmol/L)	31.94	15.77
Range (mmol/L)	18.42-61.38	5.37-20.97
Na/K		
Number of quarters	100	3
Mean	0.57	3.60
Range	0.26-2.08	0.46-9.72

[1]Quarters without CM did not have clinical signs (such as blood, flakes in colostrum and/or redness, swelling of the mammary gland)
[2]Quarters with CM did have clinical signs in colostrum and/or mammary gland
NA= not applicable with only one value

Supplementary Table 2-2: Components of milk (2-21 d postpartum) produced by quarters classified as healthy or as having subclinical (SCM) or clinical mastitis (CM).

	Healthy	**SCM[1]**	**CM[2]**
SCC			
Number of quarters	6,414	1,548	99
Mean (cells/mL)	62,864	831,016	1,649,273
Range (cells/mL)	1,000-199,000	200,000-7,832,000	4,000-8,998,000
Lactose			
Number of quarters	6,309	1,471	91
Mean (%, wt/vol)	4.46	4.14	3.42
Range (%, wt/vol)	1.74-5.37	0.66-5.48	0.05-5.05
Protein			
Number of quarters	6,347	1,520	91
Mean (%, wt/vol)	2.89	3.19	3.79
Range (%, wt/vol)	1.19-6.06	0.51-9.52	2.35-6.71
Na			
Number of quarters	531	105	6
Mean (mmol/L)	13.56	19.71	41.47
Range (mmol/L)	5.22-36.54	7.40-65.25	23.05-69.60
K			
Number of quarters	531	105	6
Mean (mmol/L)	29.36	27.99	9.34
Range (mmol/L)	13.04-58.83	11.00-46.04	4.35-19.95
Na/K			
Number of quarters	531	105	6
Mean	0.47	0.83	6.42
Range	0.23-1.53	0.34-5.14	1.16-16.01

[1]SCM defined as milk with an SCC ≥200,000 cells/mL and no clinical signs (such as blood, flakes in milk and/or redness, swelling of the mammary gland)
[2]CM defined as the presence of clinical signs in milk and/or mammary gland

Chapter 3: Milk microbiomes of healthy cows, cows with subclinical mastitis, and cows with clinical mastitis

H.K. Peterson[1], R.M. Pace[2], J.E. Williams[1], M.K. McGuire[2], and M.A. McGuire[1]

[1] Department of Animal, Veterinary and Food Sciences, University of Idaho

[2] Margaret Ritchie School of Family and Consumer Sciences, University of Idaho

Abstract

Bovine milk production can be severely hampered by inflammation of the mammary gland, or mastitis. Many factors, including age, stage of lactation, housing, and milking procedures can affect incidence of mastitis on dairy farms. Mastitis mostly occurs in the first few weeks postpartum and is generally caused by a bacterial infection. Mastitis can be clinical (CM; presence of clinical signs such as flakes or clots in the milk and/or redness or swelling of the mammary gland) or subclinical [SCM; milk somatic cell count (SCC) ≥200,000 cells/mL and no clinical signs]. It may be possible for producers to more effectively treat mastitis in their herds if they are aware of the variations between the bacterial communities in the milk of cows with CM or SCM. The aim of this study was to apply full-length sequencing of the 16S rRNA gene, present in all bacteria, to a subset (n = 748) of daily quarter-milk samples (n = 8,888) collected post-milking from healthy cows (milk SCC <200,000 cells/mL and no clinical signs), from cows with SCM (milk SCC ≥200,000 cells/mL and no clinical signs), and from cows with CM (presence of clinical signs) on four dairies in southern Idaho in the first 22 d postpartum. Samples collected from quarters with CM were matched, as closely as possible based on dairy, parity, and d postpartum, to samples collected from healthy quarters and quarters with SCM for comparisons both before the matched event (1-3 d prior to first observance of clinical signs) and during the matched event (first observance of clinical signs). A higher relative abundance of *Staphylococcus*, a taxon commonly associated with mastitis, was identified in milk collected from quarters with SCM compared to milk collected from healthy quarters and quarters with CM. Interestingly, *Bifidobacterium* was identified in high relative abundance in all milk samples, regardless of mammary gland quarter health status group. Understanding the impact of mastitis incidence and environmental influences on milk microbiota would provide important information to expand our knowledge of these complex and intertwined entities.

Introduction

Mastitis, or inflammation of the mammary gland, is a disease that significantly affects lactating dairy cows (Pyörälä, 2008; Angelopoulou et al., 2019). Mastitis presents an economic challenge (~$2 billion/y) to the US dairy industry due to discarded milk, decreased milk production, and compromised animal health (Rollin et al., 2015; Shaheen et al., 2016). Mastitis tends to occur in the few first weeks postpartum and usually causes elevated somatic cell count (SCC), an inflammatory marker measurable in milk. Consisting of mostly white blood cells such as macrophages, lymphocytes, and neutrophils, SCC is a good indicator of inflammation of the mammary gland (Ballou, 2011). Mastitis can be clinical (CM; presence of clinical signs such as flakes, clots, blood in the milk and/or redness, swelling, hardness of the mammary gland) or subclinical (SCM; milk SCC ≥200,000 cells/mL without clinical signs) (National Mastitis Council, 1999; Farre et al., 2022). Mastitis is generally caused by a bacterial infection commonly with *Escherichia coli*, *Streptococcus uberis*, *Streptococcus agalactia*, *Staphylococcus aureus*, and non-aureus *staphylococci* (NAS) (Bradley, 2002; Shaheen et al., 2016).

Bacterial culturing is customarily used to identify the offending mastitis pathogen(s) so the appropriate treatment(s) (such as antibiotics) could be administered. However, these culture-dependent methods fail to grow bacteria in 20-30% of CM cases in cows (Taponen et al., 2009). Advanced methods, such as culture-independent techniques based on DNA, specifically the 16S rRNA gene present in all bacteria, improve the identification of bacterial pathogens in mastitis cases (Koskinen et al., 2010; Oikonomou et al., 2020; Ruegg, 2022). Targeted regions (conserved regions and hypervariable regions) of the 16S rRNA gene can be amplified via polymerase chain reaction (PCR). These amplicons, or amplified products, are then subject to DNA sequencing. The resulting sequences can be checked against databases of known sequences for identification of specific taxa (Hugerth and Andersson, 2017) allowing for the identification of bacterial species or groups in a specific sample. Some studies (Kuehn et al., 2013; Oikonomou et al., 2014; Pang et al., 2018) target certain hypervariable regions of the 16S rRNA gene while others (Abellan-Schneyder et al., 2021; Guo et al., 2021; Ma et al., 2021) target the entire gene using full-length gene sequencing. Several reports (Derakhshani et al., 2018; Oikonomou et al., 2020; Parente et al., 2020) show a myriad of bacteria present even in milk produced by healthy quarters (milk SCC <200,000 cells/mL and no clinical signs). Interestingly, milk was once thought to be sterile, and that any bacterial presence must be due to cases of infection, like mastitis. However, microbiome research indicates milk produced by all cows, regardless of mammary health status, contains distinct bacterial communities (Kuehn et al., 2013; Oikonomou et al., 2014; Metzger et al., 2018).

Observing shifts in bacterial communities shortly before the actual mastitis event could benefit and enhance our approach to cure or prevent mastitis. Therefore, the objective of the current study was to identify differences in milk microbiomes among samples collected from quarters with CM that were matched, based on dairy, parity, and d postpartum, to samples collected from quarters with SCM and healthy quarters both before the matched event (1-3 d prior to first observance of clinical signs) and during the matched event (first observance of clinical signs) within early-lactation cows. Another objective was to identify differences in milk microbiomes between the two timepoints (before and during the matched event) within quarters with CM, quarters with SCM, and healthy quarters. We hypothesized that bacterial communities in milk collected from quarters with mastitis (either CM or SCM) would be different from bacterial communities in milk collected from healthy quarters at both timepoints. We further hypothesized that a distinct shift would occur in bacterial communities in milk collected from quarters with CM before the matched event to during the matched event. We also hypothesized that bacterial communities in milk collected from quarters with SCM would be different before the matched event compared to during the matched event. Finally, we hypothesized milk microbiomes of healthy quarters would remain unchanged in the shift between the timepoint before the matched event to during the matched event.

Materials and Methods

Animals and milk collection procedures All procedures involving animals were approved by the University of Idaho Animal Care and Use Committee (protocol 2018-66). Daily quarter-milk samples (n = 8,888) were collected from 107 cows on four commercial dairies in southern Idaho across the first 22 d postpartum. Samples were aseptically collected post-milking following the National Mastitis Council recommendations for collection (National Mastitis Council, 2004). Briefly, while wearing gloves, each teat was wiped carefully using Sani-wipes (Ecolab Inc., St. Paul, MN) paying particular attention to the teat end. Approximately 40-50 mL of milk was collected from each mammary gland quarter into sterile 50 mL centrifuge tubes (Stockwell Scientific, Scottsdale, AZ), taking care not to touch the teat end to the tube or anything else. After sample collection, teats were dipped or sprayed with post-milking teat disinfectant as per the dairy's protocol. Gloves were sanitized with Sani-wipes between animals or changed completely when feasible. Animals were monitored closely by both study and dairy personnel for any signs of CM (such as milk with flakes or blood and/or swelling or redness of the mammary gland). Study personnel documented any clinical signs and noted which quarter(s) were affected. Animals exhibiting clinical signs were moved into hospital pens for treatment where study personnel continued to collect samples until either the animal responded to treatment and was returned to her original milking pen, the 22-day sample period was

reached, or the animal was removed from the herd (n= 1). Milk samples were placed in a cooler filled with ice packs immediately after collection before transportation to a lab.

Sample preparation and SCC evaluation Samples were aliquoted into five 1.7 mL microcentrifuge tubes (VWR, Radnor, PA) in 1 mL amounts and frozen at -20 °C until further analysis. Approximately 30-40 mL of milk was transferred to a tube containing preservative for measurement of SCC (High Desert Dairy Lab Inc., Nampa, ID). The SCC for low-volume milk samples (<20 mL) was determined by study personnel using a DeLaval cell counter (Sweden). Some milk samples collected from cows exhibiting CM signs were too clotted to obtain a successful reading from the cell counter (n= 32).

Classification of mammary health status A subset of the samples (n = 748) was used to be representative of healthy quarters (milk SCC <200,000 cells/mL and no clinical signs), quarters with SCM (milk SCC ≥200,000 cells/mL and no clinical signs), and quarters with CM (presence of clinical signs) among the four dairies.

DNA extraction DNA was extracted using the ZymoBIOMICS 96 MagBead DNA Kit (Zymo Research Corp.) following the automation protocol on a KingFisher Flex system (ThermoFisher Scientific). Briefly, 1 mL aliquots of samples (n= 748) were thawed on ice at room temperature and then centrifuged at 13,000 x g for 10 min at 4 °C. The supernatant was carefully removed to leave the cell pellet and fat layer for extraction. ZymoBIOMICS Lysis Solution (750 µL) was added to the sample tube to resuspend the cell pellet and fat layer which was then transferred to a ZR Bashing Bead Lysis Tube. Samples were attached to tube adaptors on Vortex Genies (VWR) and processed for 40 min at max speed. Samples were centrifuged at 13,000 x g for 1 min to pellet the beads and 200 µL of supernatant were transferred to a 96-deep-well plate. The sample plate, reagents, and plasticware were assembled on the KingFisher Flex according to the setup guide and then the Zymo protocol was executed. Samples were eluted in 50 µL ZymoBIOMICS DNase/RNase Free Water and stored at -20 °C until further analysis.

Amplification of full-length 16S rRNA genes For SMRT barcode sequencing, full-length 16S rRNA gene sequences were amplified by PCR from the extracted DNA. Universal primer pair 27F (5'-AGRGTTYGATYMTGGCTCAG-3') and 1492R (5'-RGYTACCTTGTTACGACTT-3') were used with 16-base barcodes (Integrated DNA Technologies, Coralville, IA) to distinguish between samples. All PCR were performed in 25 µL reactions containing 12.5 µL of 2x KAPA HiFi HotStart ReadyMix (Roche Molecular System Inc., Pleasanton, CA), 1.5 µL of nuclease-free water (ThermoFisher Scientific, Waltham, MA), 3 µL of barcoded forward primers (2.5 µM), 3 µL of

barcoded reverse primers (2.5 μM), and 5 μL of template DNA. The thermocycling program was 1 cycle of 95 °C for 3 min, followed by 27 cycles of 95 °C for 30 sec, 57 °C for 30 sec, and 72 °C for 60 sec. PCR amplification results were checked via QIAxcel (Qiagen, Hilden, Germany).

Sequencing by SMRT The 16S rRNA gene amplicons were used to construct DNA libraries with the Pacific Biosciences (PacBio) SMRT Bell Template Prep Kit 3.0, according to the manufacturer's instructions. Sequencing was achieved by adding the constructed library, DNA polymerase, and sequencing primers to the SMRT cells and running on a PacBio Sequel II instrument.

Bioinformatics analysis Raw data were processed via SMRT link v11 with Detect And Split Heteroduplex Reads (DASHR) turned on. Specific filtering criteria were as follows: (1) minimum full passes of 3; (2) minimum predicted accuracy of 90; (3) minimum insert read length of 1,000; and maximum insert read length of 1,700. All reads were sorted into different samples based on their barcodes and then barcodes and primer sequences were removed. High-quality sequences were processed into amplicon sequence variants (ASV) with chimeric sequences removed via DADA2 (v1.22.0) in R (v4.1.2). The Silva database (v138) was used to assign the taxonomy of each ASV representative sequence with an 80% confidence threshold. Contaminant sequences were removed via the decontam R package (v1.14.0; Davis et al., 2017). Due to incomplete taxonomic assignment, we decided to drop taxonomic rank to fill in missing taxonomic information.

Statistical analysis Statistical analyses were performed using R (v.4.1.2). Chao1 index, Shannon diversity index, and Simpson diversity index were used to estimate richness and diversity of bacterial taxa of milk samples. Principal coordinate analysis (PCoA) was used to observe differences in milk microbiota among samples collected from quarters with CM, from quarters with SCM, and from healthy quarters. Milk samples collected from quarters with CM were matched, as closely as possible based on dairy, parity, and day postpartum, to milk samples collected from healthy quarters and from quarters with SCM. Samples were further separated into two timepoints: before the matched event (1-3 d prior to first observance of clinical signs) and during the matched event (first observance of clinical signs). Two cows were diagnosed with CM on 0 d postpartum (day of calving) and therefore did not have before matched event samples. We used adonis2 from the R package vegan (v2.6-4; Oksanen et al., 2022) to perform permutational multivariate analysis of variance (PERMANOVA) to identify the effect of different mammary gland quarter health status groups on bacterial microbiota composition. The pairwise.wilcox.test from the R package stats (v4.1.2; R Core Team, 2021) was used to compare mammary gland quarter health status group levels with Benjamini-Hochberg correction for multiple testing. We used lmer from the R package lme4 (v1.1.32; Bates et al., 2015) to perform analysis of variance (ANOVA) to test for differences among relative abundances of the top

20 taxa identified in milk collected from quarters with CM, from quarters with SCM, and from healthy quarters at both timepoints. The model included the mammary gland quarter health status group (healthy, SCM, or CM), timepoint (before the matched event and during the matched event), and the interaction of mammary gland quarter health status group and timepoint as fixed effects and quarter nested within cow as a random effect. Tukey's test was used to identify which mammary gland quarter health status group(s) and/or timepoint(s) were different. Significance was declared at $P<0.05$ with trends declared at $P>0.05$ but <0.10.

Results

A total of 8,033,417 high-quality 16S rRNA reads were produced from the 748 milk samples in the current study (mean = 10,740 ± 26,395 reads per sample). These reads were processed into 62,569 ASVs. After removal of primers and chimeric sequences, 2,790 ASVs remained in 735 milk samples for downstream analysis. Eight quarter-milk samples were dropped after filtering out contaminants.

Figure 3-1 depicts the top 20 bacterial taxa by overall relative abundance, prevalence, and individual sample relative abundance in milk samples collected from healthy quarters, from quarters with SCM, and from quarters with CM across all samples with successful sequencing. In milk produced by healthy quarters, *Bifidobacterium* was the most abundant taxon, but *Romboutsia* was the most prevalent taxon. In milk produced by quarters with SCM, *Staphylococcus* was the most abundant taxon whereas *Corynebacterium* was the most prevalent taxon. In milk produced by quarters with CM, *Escherichia-Shigella* was the most abundant taxon whereas *Bifidobacterium, Corynebacterium*, *Paeniclostridium*, *Clostridium sensu stricto 1*, *Atopostipes*, and *Planococcus* were the most prevalent taxa (Figure 3-1). Distinct separation was not observed among mammary gland quarter health status groups (**Figure 3-2**). The Chao1 alpha diversity index was higher (P=0.02) in quarters with SCM compared to healthy quarters (**Figure 3-3**). The Shannon alpha diversity index tended to be higher (P=0.06) in quarters with SCM compared to healthy quarters (**Figure 3-4**). The Simpson alpha diversity index tended to be higher (P=0.06) in quarters with SCM compared to quarters with CM (**Figure 3-5**).

Differences in relative abundance of the top 20 taxa were observed (**Figure 3-6**) among mammary gland quarter health status group (healthy, SCM, and CM) and timepoint (before matched event and during matched event). The relative abundance of *Atopostipes* was different (P=0.047) in milk collected from healthy quarters compared to milk collected from quarters with CM (**Table 3-1**). The relative abundance of *Jeotgalicoccus* was higher (P=0.001) in milk samples collected before the matched event compared to milk samples collected during the matched event for all mammary gland quarter health status groups. The relative abundance of *Staphylococcus* was higher (P=0.005) in milk

produced by SCM compared to milk produced by healthy quarters and quarters with CM both before and during the matched event. The relative abundance of *Tissierella* tended to be higher (P=0.089) in milk samples collected from healthy quarters during the matched event compared to milk samples collected from healthy quarters before the matched event (Table 3-1).

Discussion

In the present study, milk samples were collected from cows on four dairies in southern Idaho and evaluated for differences in bacterial communities among samples collected from quarters with CM, from quarters with SCM, and from healthy quarters both before and during the matched event via full-length 16S rRNA gene sequencing. The most interesting aspect of the current study is that it appears to be the first to report *Bifidobacterium* as a highly abundant taxon identified in milk samples collected from healthy quarters, from quarters with SCM, and from quarters with CM (Figure 3-1). Although the relative abundance of *Bifidobacterium* appeared to numerically decrease in milk produced by quarters with mastitis (both SCM and CM) from before the matched event to during the matched event (Figure 3-6; Table 3-1), it is likely that insufficient experimental power was likely to detect a significant difference. Gastrointestinal tracts of mammals are inhabited by *Bifidobacterium,* but it is not normally found in high relative abundance in bovine milk (Oikonomou et al., 2012; Kuehn et al., 2013; Oikonomou et al., 2014; Lima et al., 2016; Metzger et al., 2018; Pang et al., 2018; Andrews et al., 2019; Taponen et al., 2019; Dahlberg et al., 2020; Guo et al., 2021; Ma et al., 2021; Sokolov et al., 2021). In contrast, *Bifidobacterium* is commonly detected in human milk (Martín et al., 2008; Soto et al., 2014; Kordy et al., 2020). Various *Bifidobacterium* species, such as *B. infantis*, *B. breve*, and *B. bifidum*, colonize the infant gastrointestinal tract and promote a healthy immune system (Meng et al., 2020; Laursen et al., 2021). An enteromammary pathway has been suggested as the route through which *Bifidobacterium* from the gastrointestinal tract ends up in human milk (Rodríguez, 2014; Jost et al., 2015). An enteromammary pathway has been theorized for bovine milk as well (Derakhshani et al., 2018; Oikonomou et al., 2020; Ruegg, 2022). Much controversy surrounds the concept of enteromammary pathways so future studies are warranted to establish the source of *Bifidobacterium* in bovine milk.

In the present study, Chao1 and Shannon diversity indices were higher in quarters with SCM than in healthy quarters but not different in quarters with CM. Milk collected from healthy quarters has previously been found to be more diverse than milk collected from quarters with mastitis (Lima et al., 2016; Metzger et al., 2018; Andrews et al., 2019). In contrast to Pang et al. (2018), Shannon diversity index was higher in quarters with SCM compared to healthy quarters (Figure 3-4). Ma et al. (2021) also reported higher Shannon diversity in samples collected from healthy cows compared to samples

collected from cows with mastitis. Like Sokolov et al. (2021) however, Chao1 and Shannon diversity indices were higher in samples collected from cows with mastitis (both CM and SCM) compared to samples collected from healthy cows (Figures 3-3 and 3-4).

The relative abundance of *Atopostipes* was different between healthy quarters and quarters with CM, particularly during the matched event (Table 3-1). A few other studies (Pang et al., 2018; Sokolov et al., 2021) have also identified *Atopostipes* in bovine milk. *Atopostipes* was first isolated from pig manure (Cotta et al., 2004) but has also been identified as a member of the fecal microbiome of the black rhino (Antwis et al., 2019). Antwis et al. (2019) noted an association of *Atopostipes* with improved reproduction parameters in the black rhino. Like *Bifidobacterium*, it appears *Atopostipes* may be another gastrointestinal microbe identifiable in bovine milk and perhaps supportive of an enteromammary pathway (Ruegg, 2022). The relative abundance of *Jeotgalicoccus,* typically associated with the environment (Schwalger et al., 2010; Cao et al., 2012), was higher before the matched event than during the matched event, regardless of mammary gland quarter health status group. Milk produced by healthy quarters tended to have a lower relative abundance of *Tissierella* before compared to during the matched event (Table 3-1). *Tissierella* is generally found in the environment, but it has been observed in human gastrointestinal tracts (Caméléna et al., 2016; Yang et al., 2022). It appears shifts in milk microbiota occur even in healthy quarters between timepoints, which could be helpful in detecting mastitis in other mammary gland quarters.

Bacterial taxa (*Escherichia-Shigella* and *Staphylococcus*) associated with common mastitis pathogens such as *E. coli* and *S. aureus* (Akers, 2002) were identified in milk produced by healthy quarters (*Staphylococcus* only), by quarters with SCM, and by quarters with CM (*Escherichia-Shigella* only; Figure 3-1). This is in accordance with several reports (Kuehn et al., 2013; Lima et al., 2016; Metzger et al., 2018; Dahlberg et al., 2020). Species of *Escherichia-Shigella*, specifically *E. coli*, are important pathogens of CM (Burvenich et al., 2003; Blum et al., 2018; Sharifi et al., 2019; Orsi et al., 2022). The relative abundance of *Staphylococcus* was higher in milk produced by quarters with SCM than in milk produced by healthy quarters and quarters with CM (Figure 3-6; Table 3-1). A numerical increase in relative abundance of *Staphylococcus* was further observed in milk produced by quarters with SCM from the timepoint before the matched event to the timepoint during the matched event (Table 3-1). Incidence of subclinical mastitis is commonly associated with *Staphylococcus*, in particular *S. aureus* (Castilho et al., 2017; Bonsaglia et al., 2018; Nedic et al., 2019; Ren et al., 2019), so this suggests a high relative abundance of *Staphylococcus* could be used to detect SCM even before an increase in SCC is observed. This knowledge could assist in early detection of CM.

Conclusion

The microbiome of bovine milk is incredibly complex, highly varied, and greatly influenced by mastitis (either CM or SCM) and environmental input. We utilized full-length 16S rRNA gene sequencing to provide more information on our understanding of bovine mastitis and its relationship with the milk microbiome. Of immense interest was the detection of *Bifidobacterium* in high relative abundance among all milk samples, regardless of mammary gland quarter health status group (CM, SCM, and healthy). Few differences in relative abundance of milk microbiota (*Atopostipes* and *Staphylococcus*) were observed among samples collected from quarters with CM, from quarters with SCM, and from healthy quarters, regardless of timepoint. The relative abundances of only a couple of taxa, *Jeotgalicoccus* and *Tissierella*, were different between the two timepoints. A key aspect of the present study was matching quarters with SCM and healthy quarters to quarters with CM based on dairy, parity, and d postpartum. As mastitis was naturally occurring, a main limitation of the present study was the low number of mastitis cases. Another limitation was the issue of incomplete taxonomic assignment. Future studies should take a closer look at the presence of gastrointestinal microbiota such as *Bifidobacterium* and *Atopostipes* in bovine milk and how they may (or may not) influence bovine mastitis.

References

Abellan-Schneyder, I., A. Siebert, K. Hofmann, M. Wenning, and K. Neuhaus. 2021. Full-length SSU rRNA gene sequencing allows species-level detection of bacteria, archaea, and yeasts present in mik. Microorganisms. 9:1-15.

Akers, R.M., 2002. Pages 125-128 in Lactation and the mammary gland. First ed. Blackwell Publishing Professional, Iowa, US.

Andrews, T., D.A. Neher, T.R. Weicht, and J.W. Barlow. 2019. Mammary microbiome of lactating organic dairy cows varies by time, tissue site, and infection status. PLoS One. 14:1-21.

Angelopoulou, A., R. Holohan, M.C. Rea, A.K. Warda, C. Hill, and R.P. Ross. 2019. Bovine mastitis is a polymicrobial disease requiring a polydiagnostic approach. Int Dairy J. 99:1-9.

Antwis, R.E., K.L. Edwards, B. Unwin, S.L. Walker, and S. Shultz. 2019. Rare gut microbiota associated with breeding success, hormone metabolites and ovarian cycle phase in the critically endangered eastern black rhino. Microbiome. 1-12.

Ballou, M.A. 2011. Growth and development symposium: inflammation: role in the etiology and pathophysiology of clinical mastitis in dairy cows. J Anim Sci. 90:1466-1478.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. J Stat Softw. 67:1-48.

Blum, S.E., R.J. Goldstone, J.P.R. Connolly, M. Répérant-Ferter, P. Germon, N.F. Inglis, O. Krifucks, S. Mathur, E. Manson, K. Mclean, P. Rainard, A.J. Roe, G. Leitner, and D.G.E. Smith. 2018. Postgenomics characterization of an essential genetic determinant of mammary pathogenic *Escherichia coli*. mBio. 9:1-11.

Bonsaglia, E.C.R., N.C.C. Silva, B.F. Rossi, C.H. Camargo, S.T.A. Dantas, H. Langoni, F.F. Guimarães, F.S. Lima, J.R. Fitzgerald, A. Fernandes Júnior, and V.L.M. Rall. 2018. Molecular epidemiology of methicillin-susceptible *Staphylococcus aureus* (MSSA) isolated from milk of cows with subclinical mastitis. Microb Pathog. 124:130-135.

Bradley, A.J. 2002. Bovine mastitis: an evolving disease. Vet J. 164:116-128.

Burvenich, C., V. van Merris, J. Mehrzad, A. Diez-Fraile, and L. Duchateau. 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. Vet Res. 34:521-564.

Caméléna, F., B. Pilmis, B. Mollo, A. Hadj, A. Le Monnier, and A. Mizrahi. 2016. Infections caused by *Tissierella praeacuta*: a report of two cases and literature review. Anaerobe. 40:15-17.

Cao, Y.R., Y. Jiang, R.X. Jin, L. Han, W.X. He, Y.L. Li, X.S. Huang, and Q.H. Xue. 2012. *Enteractinococcus coprophilus* gen. nov., sp. nov., of the family *Micrococcaceae*, isolated from *Panthera tigris amoyensis faeces*, and transfer of *Yaniella fodinae* to the genus *Enteractinococcus* as *Enteractinococcus fodinae* comb. nov. Int J Syst Evol Microbiol. 62:2710-2716.

Castilho, I.G., S.T.A. Dantas, H. Langoni, J.P. Araújo Jr., A. Fernandes Jr., F.C.L. Alvarenga, L. Maia, D.Q. Cagnini, and V.L.M. Rall. 2017. Host-pathogen interactions in bovine mammary epithelial cells and HeLa cells by *Staphylococcus aureus* isolated from subclinical bovine mastitis. J Dairy Sci. 100:6414-6421.

Cotta, M.A., T.R. Whitehead, M.D. Collins, and P.A. Lawson. 2004. Atopostipes suicloacale gen. nov., sp. nov., isolated from an underground swine manure storage pit. Anaerobe. 191-195.

Dahlberg, J., J. E. Williams, M. A. McGuire, H. K. Peterson, K. Östensson, S. Agenäs, J. Dicksved, and K. Persson Waller. Microbiota of bovine milk, teat skin, and teat canal: similarity and variation due to sampling technique and milk fraction. 2020. J Dairy Sci. 103:7322-7330.

Davis, N.M., D. Proctor, S.P. Holmes, D.A. Relman, and B.J. Callahan. 2017. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. bioRxiv. 1-39.

Derakhshani, H., K.B. Fehr, S. Sepehri, D. Francoz, J. De Buck, H.W. Barkema, J.C. Plaizier, and E. Khafipour. 2018. Invited review: microbiota of the bovine udder: contributing factors and potential implications for udder health and mastitis susceptibility. J Dairy Sci. 101:10605-10625.

Farre, M., A. Zecconi, and D. Kelton. 2022. Guidelines for defining quarter and udder health status and cured clinical and subclinical mastitis cases. International Dairy Federation (IDF) Bulletin 515/2022. 1-28.

Guo, X., Z. Yu, F. Zhao, Z. Sun, L.Y. Kwok, and S. Li. 2021. Both sampling seasonality and geographic origin contribute significantly to variations in raw milk microbiota, but sampling seasonality is the more determining factor. J Dairy Sci. 104:10609-10627.

Hugerth, L.W. and A.F. Andersson. 2017. Analysing microbial community composition through amplicon sequencing: from sampling to hypothesis testing. Front Microbiol. 8:1-22.

Jost, T., C. Lacroix, C. Braegger, and C. Chassard. 2015. Impact of human milk bacteria and oligosaccharides on neonatal gut microbiota establishment and gut health. Nutr Rev. 73:426-437.

Kordy, K., T. Gaufin, M. Mwangi, F. Li, C. Cerini, D.J. Lee, H. Adisetiyo, C. Woodward, P.S. Pannaraj, N.H. Tobin, and G.M. Aldrovandi. 2020. Contributions to human breast milk microbiome and enteromammary transfer of *Bifidobacterium breve*. PLoS One. 15:1-10.

Koskinen, M.T., G.J. Wellenberg, O.C. Sampimon, J. Holopainen, A. Rothkamp, L. Salmikivi, W.A. van Haeringen, T.J.G.M. Lam, and S. Pyörälä. 2010. Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. J Dairy Sci. 93:5707-5715.

Kuehn, J. S., P. J. Gorden, D. Munro, R. Rong, Q. Dong, P. J. Plummer, C. Wang, and G. J. Phillips. Bacterial community profiling of milk samples as a means to understand culture-negative bovine clinical mastitis. 2013. PLoS One. 8:1-10.

Laursen, M.F., M. Sakanaka, N. von Burg, U. Mörbe, D. Andersen, J.M. Moll, C.T. Pekmez, A. Rivollier, K.F. Michaelsen, C. Mølgaard, M.V. Lind, L.O. Dragsted, T. Katayama, H.L. Frandsen, A.M. Vinggaard, M.I. Bahl, S. Brix, W. Agace, T.R. Licht, and H.M. Roager. 2021. *Bifidobacterium* species associated with breastfeeding produce aromatic lactic acids in the infant gut. Nat Microbiol. 6:1367-1382.

Lima, S.F., A.G. Teixeira, F.S. Lima, E.K. Ganda, C.H. Higgins, G. Oikonomou, and R.C. Bicalho. 2016. The bovine colostrum microbiome and its association with clinical mastitis. J Dairy Sci. 100:3031-3042.

Ma, T., L. Shen, Q. Wen, R. Lv, Q. Hou, L.Y. Kwok, Z. Sun, and H. Zhang. 2021. PacBio sequencing revealed variation in the microbiota diversity, species richness and composition between milk collected from healthy and mastitis cows. Microbiology. 167:1-10.

Martín, R., E. Jiménez, H. Heilig, L. Fernández, M.L. Marín, E.G. Zoetendal, and J.M. Rodríguez. 2008. Isolation of Bifidobacteria from breast milk and assessment of the Bifidobacterial population by PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR. Appl Environ Microbiol. 75:965-969.

Meng, D., E. Sommella, E. Salvlati, P. Campiglia, K. Gangull, K. Djebali, W. Zhu, and W.A. Walker. 2020. Indole-3-lactic acid, a metabolite of tryptophan, secreted by *Bifidobacterium longum* subspecies *infantis* is anti-inflammatory in the immature intestine. Pediatr Res. 88:209-217.

Metzger, S.A., L.L. Hernandez, J.H. Skarlupka, T.M. Walker, G. Suen, and P.L. Ruegg. 2018. A cohort study of the milk microbiota of healthy and inflamed bovine mammary glands from dryoff through 150 days in milk. Front Vet Sci.

National Mastitis Council. 1999. Laboratory Handbook on Bovine Mastitis, 2nd rev. ed. Natl. Mastitis Counc. Inc.. New Prague. MN.

National Mastitis Council. 2004. Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality. NMC Publication.

Nedic, S., S. Vakanjac, M. Samardzija, and S. Borozan. 2019. Paraoxonase 1 in bovine milk and blood as marker of subclinical mastitis caused by *Staphylococcus aureus*. Res Vet Sci. 125:323-332.

Oikonomou, G., V. S. Machado, C. Santisteban, Y. H. Schukken, and R. C. Bicalho. Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. 2012. PLoS One. 7:1-14.

Oikonomou, G., M. L. Bicalho, E. Meira, R. E. Rossi, C. Foditsch, V. S. Machado, A. G. V. Teixeira, C. Santisteban, Y. H. Schukken, and R. C. Bicahlo. Microbiota of cow's milk; distinguishing healthy, sub-clinically, and clinically diseased quarters. 2014. PLoS One. 9:1-11.

Oikonomou, G., M.F. Addis, C. Chassard, M.E.F. Nader-Macias, I. Grant, C. Delbès, C.I. Bogni, Y. Le Loir, and S. Even. 2020. Milk microbiota: what are we exactly talking about?. Front Microbiol. 11:1-15.

Orsi, H., F.F. Guimarães, D.S. Leite, S.T. Guerra, S.F. Joaquim, J.C.F. Pantoja, R.T. Hernandes, S.B. Lucheis, M.G. Ribeiro, H. Langoni, and V.L.M. Rall. 2022. Characterization of mammary pathogenic *Escherichia coli* reveals the diversity of *Escherichia coli* isolates associated with bovine clinical mastitis in Brazil. J Dairy Sci. 106:1403-1413.

Oksanen, J., G.L. Simpson, F.G. Blanchet, R. Kindt, P. Legendre, P.R. Minchin, R.B. O'Hara, P. Solymos, M.H.H. Stevens, E. Szoecs, H. Wagner, M. Barbour, M. Bedward, B. Bolker, D. Borcard, G. Carvalho, M. Chirico, M. De Caceres, S. Durand, H.B.A. Evangelista, R. FitzJohn, M. Friendly, B. Furneaux, G. Hannigan, M.O. Hill, L. Lahti, D. McGlinn, M-H. Ouellette, E.R. Cunha, T. Smith, A. Stier, C.J.F. Ter Braak, and J. Weedon. 2022. Vegan: community ecology package. R package version 2.6-4.

Pang, M., X. Xie, H. Bao, L. Sun, T. He, H. Zhao, Y. Zhou, L. Zhang, H. Zhang, R. Wei, K. Xie, and R. Wang. Insights into the bovine milk microbiota in dairy farms with different incidence rates of subclinical mastitis. 2018. Front Microbiol. 9:1-13.

Parente, E., A. Ricciardi, and T. Zotta. 2020. The microbiota of dairy milk: a review. Int Dairy J. 107:1-16.

Pyörälä, S. Mastitis in post-partum dairy cows. 2008. Reprod Dom Anim. 43:252-259.

R Core Team. 2021. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ren, Q., G. Liao, Z. Wu, J. Lv, and W. Chen. 2019. Prevalence and characterization of *Staphylococcus aureus* isolates from subclinical bovine mastitis in southern Xinjiang, China. J Dairy Sci. 103:3368-3380.

Rodrigues, M.X., S.F. Lima, S.G. Canniatti-Brazaca, and R.C. Bicalho. 2017. The microbiome of bulk tank milk: characterization and associations with somatic cell count and bacterial count. J Dairy Sci. 100:2536-2552.

Rollin, E., K. C. Dhuyvetter, and M. W. Overton. The cost of clinical mastitis in the first 30 days of lactation: an economic modeling tool. 2015. Prevent Vet Med. 122:257-264.

Ruegg, P.L. 2022. The bovine milk microbiome—an evolving science. Domest Anim Endocrinol. 79:1-6.

Schwalger, K., C. Hölzel, M. Mayer, and J. Bauer. 2010. Notes on the almost unknown genus *Jeotgalicoccus*. Lett Appl Microbiol. https://doi.org/10.1111/j.1472-765X.2010.02811.x.

Shaheen, M., H.A. Tantary, and S.U. Nabi. 2016. A treatise on bovine mastitis: disease and disease economics, etiological basis, risk factors, impact on human health, therapeutic management, prevention, and control strategy. J Adv Dairy Res. 4:1-10.

Sharifi, S., A. Pakdel, E. Ebrahimie, Y. Aryan, M.G. Zefrehee, and J.M. Reecy. 2019. Prediction of key regulators and downstream targets of *E. coli* induced mastitis. J Appl Genet. 60:367-373.

Sokolov, S., K. Fursova, I. Shulcheva, D. Nikanova, O. Artyemieva, E. Kolodina, A. Sorokin, T. Dzhelyadin, M. Shchannikova, A. Shepelyakovskaya, N. Zinovieva, and F. Brovko. 2021. Comparative analysis of milk microbiomes and their association with bovine mastitis in two farms in central Russia. Animals. 11:1-12.

Soto, A., V. Martín, E. Jiménez, I. Mader, J.M. Rodríguez, and L. Fernández. 2014. Lactobacilli and Bifidobacteria in human breast milk: influence of antibiotherapy and other host and clinical factors. JPGN Rep. 59:78-88.

Taponen, S., L. Salmikivi, H. Simojoki, M. T. Koskinen, and S. Pyörälä. Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. 2009. J Dairy Sci. 92:2610-2617.

Taponen, S., D. McGuinness, H. Hiitiö, H. Simojoki, R. Zadoks, and S, Pyörälä. 2019. Bovine milk microbiome: a more complex issue than expected. Vet Res. 50:1-15.

Yang, J., D. Gilbert, L. Meece, and A. Afroze. 2022. Tissierella praeacuta infection in the setting of chronic sacral wounds. Cureus. 14:1-3.

Table 3-1: Differences in the top 20 bacterial taxa identified in milk samples collected from healthy quarters, quarters with subclinical mastitis (SCM), and quarters with clinical mastitis (CM) between two timepoints (before the matched event and during the matched event[1]).

	Healthy		SCM		CM			
Taxa	**Before**	**During**	**Before**	**During**	**Before**	**During**	**SEM[2]**	***P*-value[3]**
Acetitomaculum	1.0	1.3	0.9	0.2	1.7	1.4	0.2	0.506
Atopostipes	8.9^{a}	8.8^{a}	7.9^{ab}	8.8^{ab}	9.1^{b}	2.4^{b}	1.1	0.047
Bifidobacterium	12.4	11.0	11.5	4.1	14.5	7.1	1.6	0.309
Clostridium sensu stricto 1	5.2	4.2	3.7	7.3	4.4	2.7	0.6	0.285
Corynebacterium	8.0	6.6	7.8	6.6	9.0	5.2	0.5	0.631
Dietzia	1.1	1.5	0.7	0.9	1.1	0.5	0.1	0.879
Enteractinococcus	1.8	1.6	1.9	2.0	1.6	0.8	0.2	0.612
Facklamia	3.6	5.0	2.8	3.0	3.2	0.5	0.6	0.345
Family_ Carnobacteriaceae	1.7	2.5	1.4	2.0	1.8	0.5	0.3	0.237
Jeotgalicoccus	6.8^{a}	5.2^{b}	6.0^{a}	5.3^{b}	8.1^{a}	2.1^{b}	0.8	0.001
Paeniclostridium	4.6	4.5	3.3	2.4	3.7	3.0	0.4	0.941
Planococcus	2.7	2.8	3.3	2.9	2.4	1.8	0.2	0.423
Pseudomonas	3.4	1.6	1.2	0.2	0.4	1.7	0.5	0.590
Psychrobacter	2.7	3.7	2.3	4.1	3.1	0.4	0.5	0.100
Romboutsia	7.7	6.3	5.6	4.1	6.6	4.8	0.5	0.999
Salinicoccus	1.2	1.1	1.0	1.5	1.0	0.3	0.2	0.296
Staphylococcus	2.4^{a}	1.5^{a}	13.1^{b}	20.1^{b}	2.2^{a}	0.3^{a}	3.3	0.005
Tissierella	1.2^{a}	1.9^{b}	1.6^{ab}	1.7^{ab}	1.5^{ab}	0.5^{ab}	0.2	0.089
Turicibacter	4.2	3.8	4.1	3.5	3.9	2.4	0.3	0.898
Other	19.5^{a}	25.0^{b}	19.9^{ab}	19.0^{ab}	20.7^{abc}	61.6^{d}	6.9	<0.05

[1]Quarters with CM were matched, as closely as possible, to quarters with SCM and healthy quarters based on dairy, parity, and d postpartum both before the matched event (1-3 d prior to first observance of clinical signs such as blood or flakes in milk and/or redness or swelling of the mammary gland) and during the matched event (first observance of clinical signs).

[2] Standard error of the mean

[3]Values represent mean relative abundance (%). Means within taxa that do not share superscripts among mammary gland quarter health groups and timepoints differ at $P<0.05$.

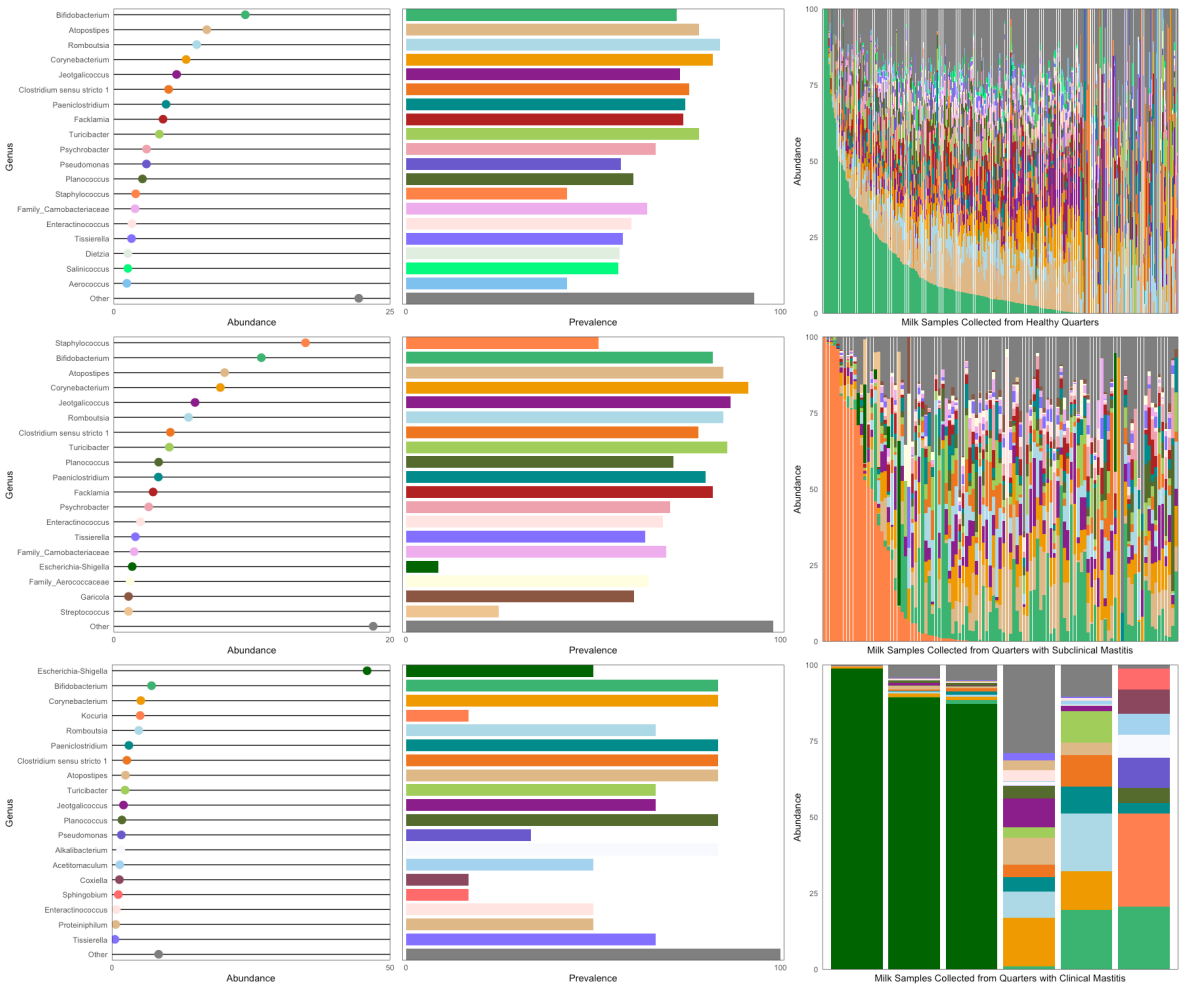


Figure 3-1: The top 20 bacterial taxa by overall relative abundance, prevalence, and individual sample relative abundance across milk samples collected from healthy quarters [n = 592; milk somatic cell count (SCC) <200,000 cells/mL and no clinical signs such as blood or flakes in milk and/or redness or swelling of the mammary gland], across milk samples collected from quarters with subclinical mastitis (SCM; n = 137; milk SCC ≥200,000 cells/mL and no clinical signs), and across milk samples collected from quarters with clinical mastitis (CM; n = 6; presence of clinical signs).

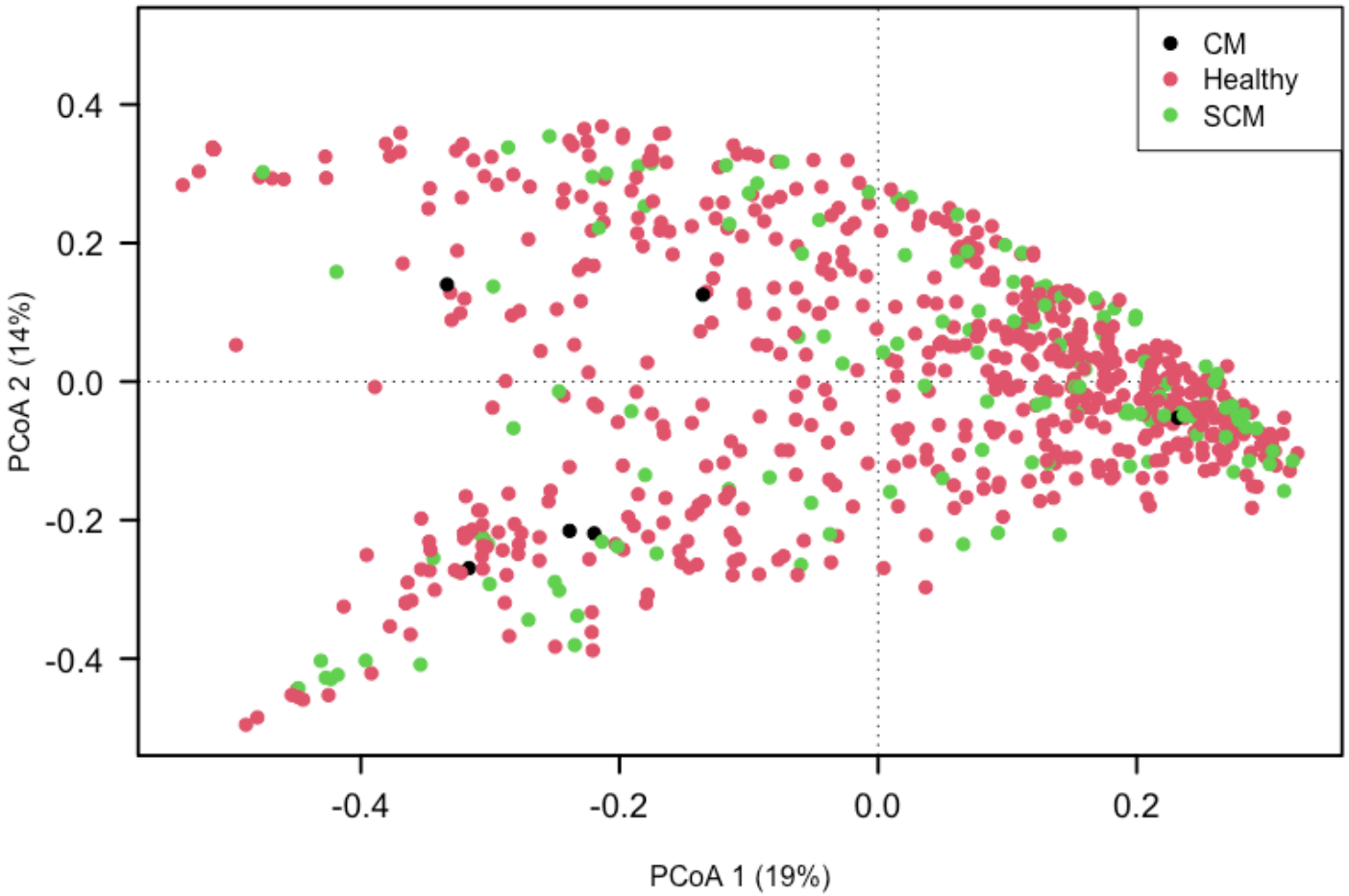


Figure 3-2: Principal coordinate analysis (PCoA; Bray-Curtis distance) of bacterial taxa in all milk samples grouped by mammary gland quarter mastitis status [clinical mastitis (CM) defined as presence of clinical signs such as clots or blood in milk and/or swelling or redness of the mammary gland; subclinical mastitis (SCM) defined as somatic cell count (SCC) ≥200,000 cells/mL and no clinical signs; and healthy defined as SCC <200,000 cells/mL and no clinical signs]. Results of permutational multivariate analysis of variance (PERMANOVA) are shown in the PCoA plot.

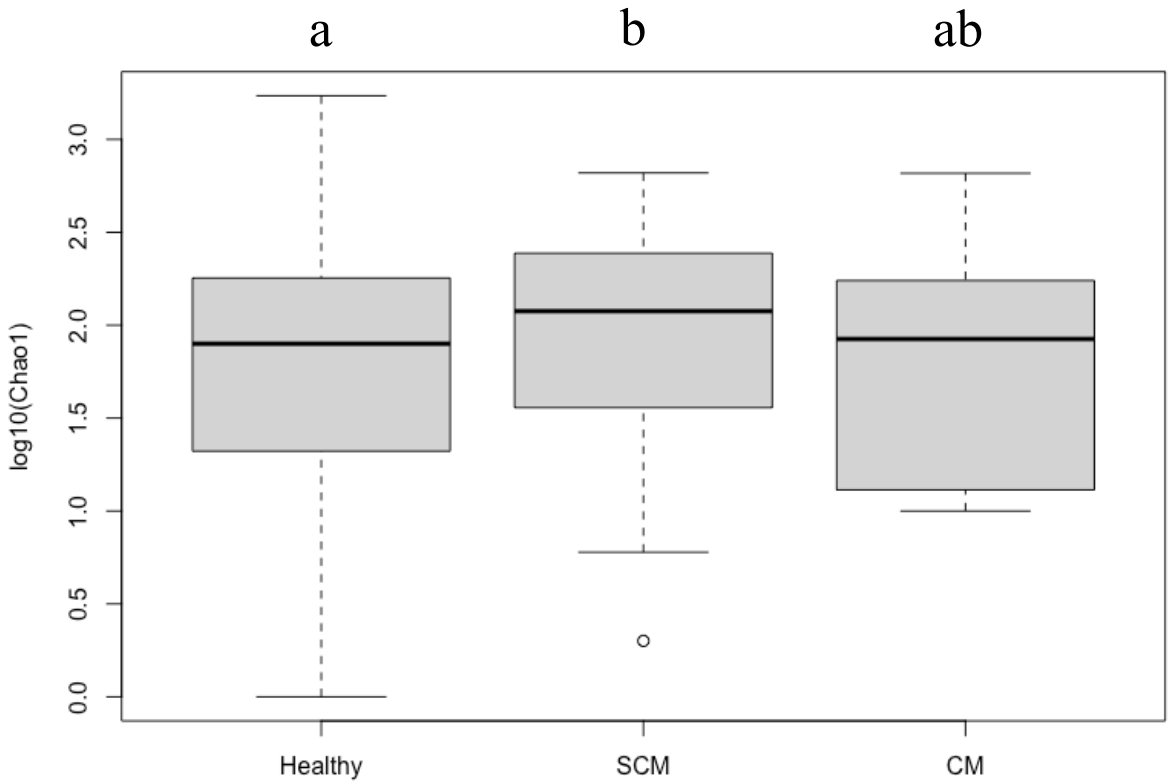


Figure 3-3: Chao1 index (log10-transformed) of milk microbiota of healthy quarters, quarters with subclinical mastitis (SCM), and quarters with clinical mastitis (CM). Columns that do not share superscripts differ (P=0.02) in Chao1 alpha diversity among mammary gland quarter health groups.

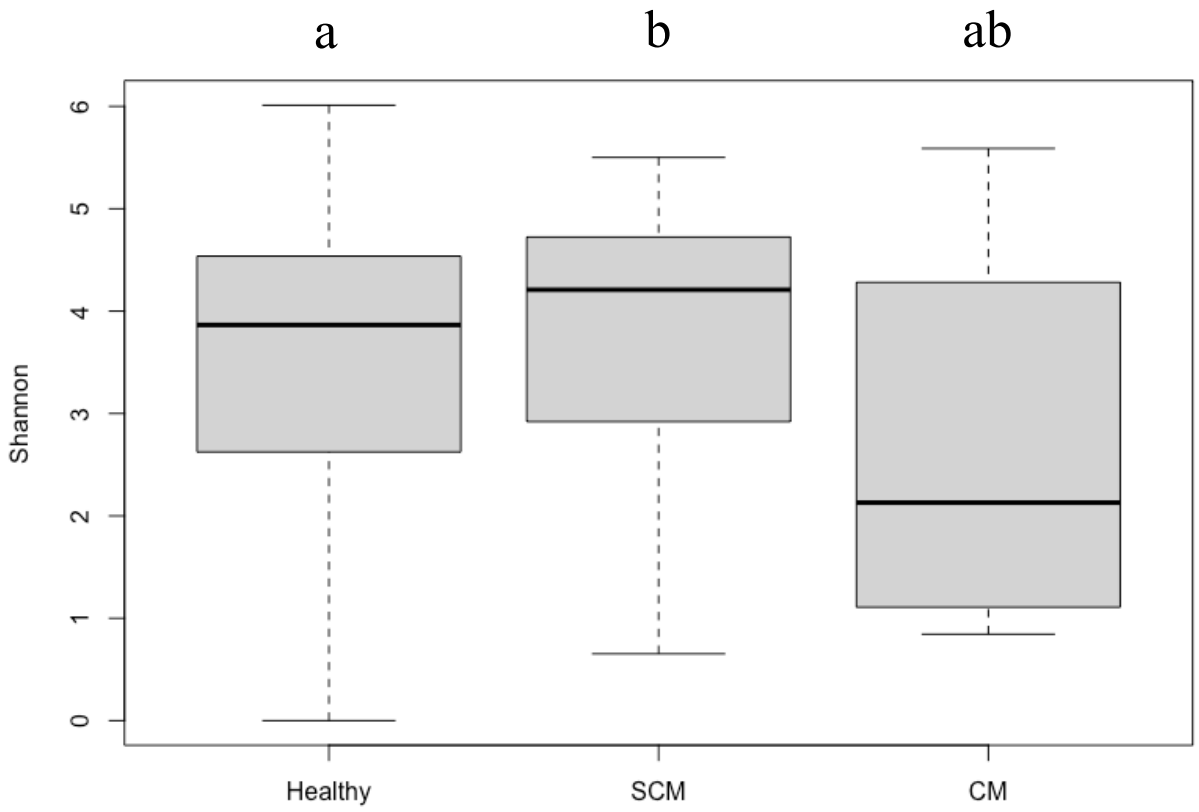


Figure 3-4: Shannon diversity index of milk microbiota of healthy quarters, quarters with subclinical mastitis (SCM), and quarters with clinical mastitis (CM). Columns that do not share superscripts indicate a trend (P=0.06) for differences in Shannon diversity among mammary gland quarter health groups.

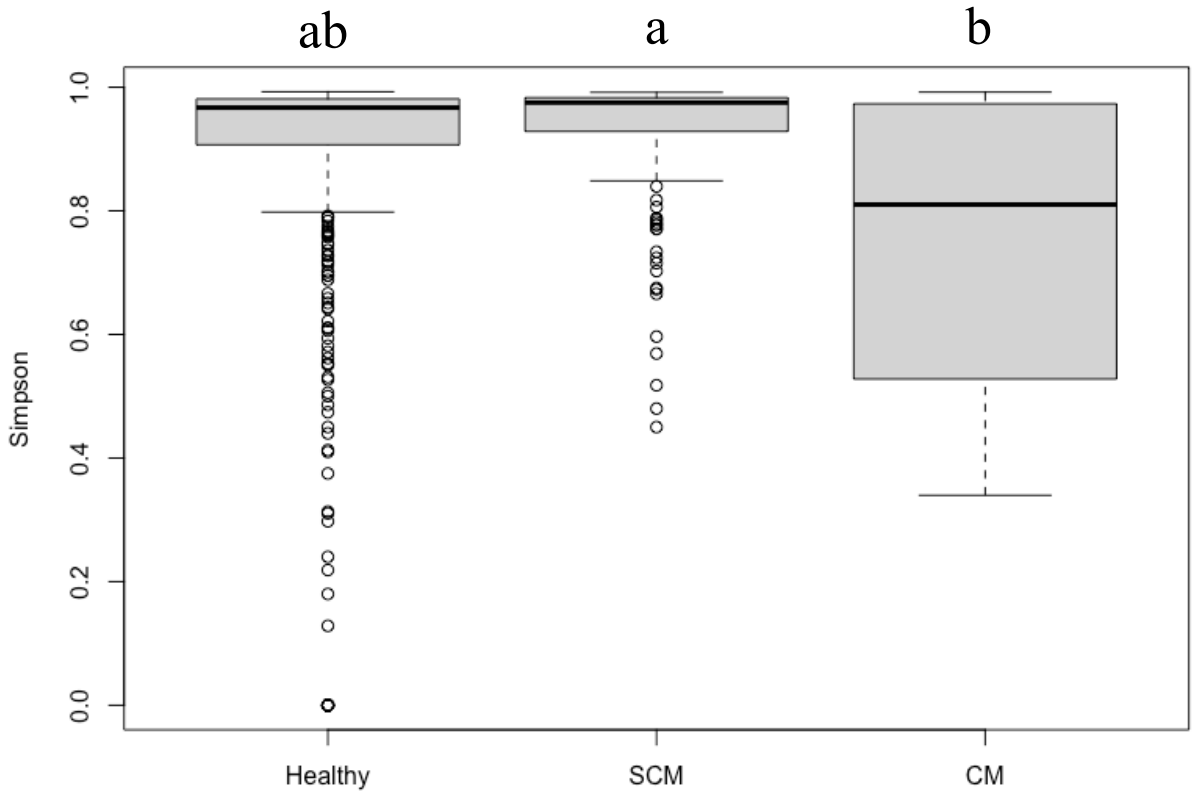


Figure 3-5: Simpson diversity index of milk microbiota of healthy quarters, quarters with subclinical mastitis (SCM), and quarters with clinical mastitis (CM). Columns that do not share superscripts indicate a trend (P=0.06) for differences in Simpson diversity among mammary gland quarter health groups.

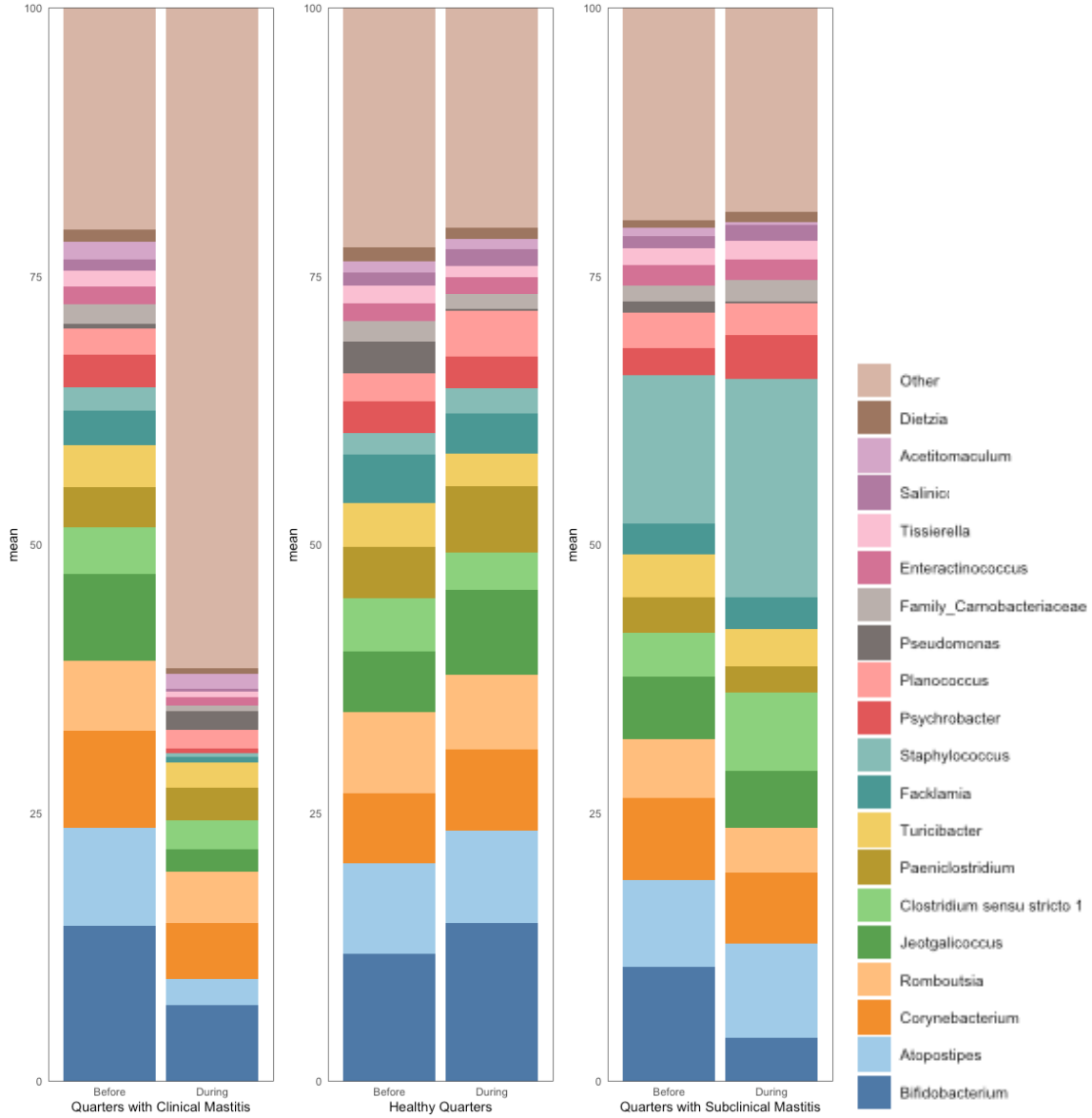


Figure 3-6: The top 20 bacterial taxa across all milk samples by mean (relative abundance) grouped by mammary gland quarter health status [clinical mastitis (CM) defined as presence of clinical signs such as blood or flakes in milk and/or redness or swelling of the mammary gland; healthy defined as milk somatic cell count (SCC) <200,000 cells/mL and no clinical signs; and subclinical mastitis (SCM) defined as milk SCC ≥200,000 cells/mL and no clinical signs] before the matched event (1-3 d prior to first observance of clinical signs) and during the matched event (first observance of clinical signs). Quarters with CM were matched, as closely as possible, to quarters with SCM and healthy quarters based on dairy, parity, and d postpartum.

Chapter 4: Exploring the proteome, metabolome, and lipidome of milk produced by healthy cows, cows with subclinical mastitis, and cows with clinical mastitis

H.K. Peterson[1], J.E. Williams[1], S.P. Couvillion[2], C.D. Nicora[2], B.M. Webb-Robertson[2], E.S. Nakayasu[2], T.O. Metz[2], R.M. Pace[3], M.K. McGuire[3], and M.A. McGuire[1]

[1]Department of Animal, Veterinary, and Food Sciences, University of Idaho

[2]Pacific Northwest National Laboratory

[3]Margaret Ritchie School of Family and Consumer Sciences, University of Idaho

Abstract

Bovine mastitis is an inflammation of the mammary gland that negatively impacts milk production, animal welfare, and is a significant economic burden to the dairy industry. Mastitis can cause severe disease with obvious local/systemic manifestations, or it can be virtually undetectable. Milk somatic cell count (SCC) is a common tool used in identifying mastitis, but the addition of other biological indicators of inflammation, or biomarkers, is of interest. The aim of this study was to apply proteomic, lipidomic, and metabolomic analyses to identify components in milk collected from individual mammary gland quarters altered by mastitis. Quarter-milk samples (n = 8,888) were collected from cows with clinical mastitis (CM; presence of clinical signs of milk such as blood, clots, and/or mammary gland such as swelling, hardness), from cows with subclinical mastitis (SCM; SCC ≥200,000 cells/mL and no clinical signs), and from healthy cows (SCC <200,000 cells/mL and no clinical signs) in the first 22 d postpartum. A subset of the samples (n = 112) was used to compare healthy quarters to quarters with mastitis (CM or SCM) both within and among cows. Milk samples collected from healthy quarters and from quarters with SCM were matched to milk samples collected from quarters with CM based on dairy, parity, and d postpartum. Milk samples produced by quarters with CM or SCM were compared to milk samples produced by healthy quarters within cows with CM or SCM. Furthermore, milk samples collected from two healthy quarters were compared to each other within healthy cows. Milk samples collected from healthy quarters were compared among healthy cows, cows with SCM, and cows with CM. Milk samples produced by quarters with CM of cows with CM were compared to milk samples produced by healthy quarters of healthy cows. Milk samples produced by quarters with SCM of cows with SCM were compared to milk samples produced by healthy quarters of healthy cows. Finally, milk samples collected from quarters with SCM of cows with SCM were compared to milk samples collected from quarters with CM of cows with CM. We performed untargeted proteomics using liquid-chromatography coupled with tandem mass spectrometry, untargeted lipidomics using liquid-chromatography mass spectrometry, and untargeted metabolomics using gas-chromatography mass spectrometry. Machine learning was performed to determine whether any features could predict CM in individual mammary gland quarters before clinical signs were first observed. Milk composition of quarters with mastitis (CM or SCM) was different from the milk composition of healthy quarters within cows with either CM or SCM. Several proteins and metabolites related to immune function were at higher levels in milk produced by quarters with mastitis (CM or SCM) relative to milk produced by healthy quarters. Variation in milk composition of healthy quarters among healthy cows, cows with SCM, and cows with CM was observed. Lipids abundant in cell membranes were at higher levels and prominent milk lipids were at lower levels in milk produced by quarters with CM relative to milk produced by healthy quarters.

Lipid level differences were not so apparent in milk produced by quarters with SCM compared to milk produced by healthy quarters. Machine learning identified features in milk, such as transitional endoplasmic reticulum ATPase, complement C8 gamma chain, alpha-L-iduronidase, fructose-bisphosphatase, L-leucine, 3-indolelactic acid, trans-3-hydroxyl-L-proline, citric acid, N-acetyl-alpha-glucosaminidase, L-lysine, L-isoleucine, osteoclast stimulating factor 1, Ras-related protein Rab-18, a triglyceride species, and two phosphatidylcholine species that may be able to, with reasonable accuracy, predict CM in individual mammary gland quarters before clinical signs are observed.

Introduction

Mastitis, or inflammation of the mammary gland, affects the dairy industry in many aspects, from milk production and animal welfare to management decisions and economics (Potter et al., 2018; Gussmann et al., 2018; Stevens et al., 2018; Puerto et al., 2021). A bacterial infection of the mammary gland is usually the cause of mastitis but injury through other means (like mechanical, chemical, or physical) can also lead to mastitis. Common mastitis pathogens include *Streptococcus agalactiae*, *Staphylococcus aureus*, non-*aureus* staphylococci and *Escherichia coli* (Erskine et al., 1988). Somatic cell count (SCC), a marker of inflammation measurable in milk, has traditionally been used to monitor mastitis in dairy cows (Kirkeby et al., 2019). McDermott et al. (1982) observed an increased risk of mastitis was associated with increased milk SCC across several herds. Mastitis can be clinical (CM; presence of clinical signs such as blood or clots in the milk, and/or redness or swelling of the mammary gland) or subclinical (SCM; milk SCC ≥200,000 cells/mL but no clinical signs) with difficulty of detection and subsequent treatment. Hand et al. (2012) noted a linear relationship of SCC and milk loss as average SCC increased across lactation; projected milk loss (up to 919 kg/cow/lactation) also increased. Identifying mastitis quickly is critical for producers to be able to make appropriate management decisions. Although SCC is a useful tool for monitoring milk quality and animal health, finding and utilizing additional biomarkers of mastitis would be of great benefit to the dairy industry.

The use of metabolomics, proteomics, and lipidomics in dairy cattle research is still in its infancy but shows promise in elucidating the complex milk environment in the mammary gland. Harnessing technological advancements to measure milk components such as metabolites, proteins, and lipids more thoroughly and accurately could be a better approach to identify potential biological indicators of mastitis. A variety of metabolites, including sugars, free amino acids, and free fatty acids, can be detected and measured in milk (Sundekilde et al., 2012; Luangwilai et al., 2020; Bobbo et al., 2021). Furthermore, milk contains many proteins related to the immune system (such as cathelicidins, lysozymes, and cytokines) that could be excellent biomarker candidates (Boehmer et al., 2009; Danielsen et al., 2010; Kusebauch et al., 2018). Milk lipids, such as phospholipids (PL) and sphingomyelin (SM), can signal disruption in the mammary gland. The primary component of milk fat globule membranes (MFGM) is phosphatidylcholine (PC), followed by phosphatidylethanolamine (PE), SM, phosphatidylinositol (PI), and phosphatidylserine (PS) (Jensen, 2002). The PL and SM function as emulsifiers and stabilizers of the MFGM. They are also sources of long-chain polyunsaturated fatty acids (Jensen et al., 1991). Moreover, bacterial cell membranes are primarily composed of PE (Sohlenkamp and Geiger, 2016). Differences in milk metabolites, including

oligopeptides and bile acids, between cows with mastitis (CM or SCM) and healthy cows have been shown (Xi et al., 2016; Johnzon et al., 2018; Tong et al., 2019; Wang et al., 2020). Major milk proteins, such as caseins and whey proteins, as well as other proteins like histones and peptidoglycan recognition protein 1, are altered in milk collected from cows with mastitis (CM or SCM) compared with milk collected from healthy cows (Boehmer et al., 2009; Thomas et al., 2016a; Turk et al., 2021). Lipids, including triacylglycerols, oxylipids, and ceramides (Cer), have been shown to be different in milk collected from cows with mastitis (CM or SCM) compared to milk collected from healthy cows (Mavangira et al., 2015; Ceciliani et al., 2021). Identifying and monitoring alterations of milk metabolites, proteins, and lipids may allow for the application of other biological markers to indicate cases of mastitis (CM or SCM) as well as to provide more insight into the disease itself.

The objective of the current study was to perform untargeted proteomics, lipidomics, and metabolomics on quarter-milk samples collected from cows with CM, from cows with SCM, and from healthy cows. Further objectives were to compare the metabolomes, proteomes, and lipidomes of milk collected from quarters with CM, from quarters with SCM, and from healthy quarters at two different timepoints: before the matched event (1-3 d prior to the first observance of clinical signs in quarters with CM) and during the matched event (when clinical signs are first observed in quarters with CM) both within and among cows. A final objective was to use a machine learning approach to identify predictors of CM in individual mammary gland quarters before the matched event. We hypothesized that milk proteins, metabolites, and lipids would not be differentially abundant before the matched event in samples collected from quarters with mastitis (CM or SCM) compared to samples collected from healthy quarters within cow, in samples collected from healthy quarters of cows with mastitis (CM or SCM) compared to samples collected from healthy quarters of healthy cows, and in samples collected from quarters with mastitis (CM or SCM) of cows with mastitis (CM or SCM) compared to samples collected from healthy quarters of healthy cows. We further hypothesized that milk proteins, metabolites, and lipids would be differentially abundant during the matched event in samples collected from quarters with mastitis (CM or SCM) compared to samples collected from healthy quarters within cow, in samples collected from healthy quarters of cows with mastitis (CM or SCM) compared to samples collected from healthy quarters of healthy cows, and in samples collected from quarters with mastitis (CM or SCM) of cows with mastitis (CM or SCM) compared to samples collected from healthy quarters of healthy cows. Finally, we hypothesized a few specific proteins, metabolites, and lipids would be identified as early predictors of CM in individual mammary gland quarters.

Materials and Methods

Sample collection Post-milking quarter-milk samples (n= 8,888) were collected in the first 22 d postpartum from cows with CM (clinical signs of milk such as flakes, blood, and/or mammary gland such as swelling, redness), from cows with SCM (milk SCC ≥200,000 cells/mL and no clinical signs), and from healthy cows (milk SCC <200,000 cells/mL and no clinical signs) on four southern Idaho dairies. A subset of the samples (n = 112) was used to compare milk collected from quarters with mastitis (CM or SCM) to milk collected from healthy quarters. Samples were separated into two timepoints based on detection of CM: before the matched event (1-3 d prior to observation of clinical signs) and during the matched event (first observance of clinical signs). Milk samples collected from cows with SCM and from healthy cows were matched, as closely as possible, to milk samples collected from cows with CM based on dairy, parity, and d postpartum. This allowed for quarter-level comparisons among cows as well as within cows both before and during the matched event.

Global proteomics, lipidomics, and metabolomics analyses were performed at the Pacific Northwest National Laboratory (Richland, WA). Briefly, samples were extracted with the MPLEx (Metabolite, Protein and Lipid Extraction) approach by adding 4 volumes of chloroform/methanol (2:1, v/v). Samples were then vortexed and incubated for 10 min on ice before centrifuging for 10 min at 16,000 x g to separate into a hydrophilic layer with polar metabolites, a hydrophobic layer with lipids, and precipitated proteins (Nakayasu et al., 2016). The hydrophilic and hydrophobic layers were separately collected into glass autosampler vials and dried *in vacuo*. Methanol was used to rinse the precipitated proteins and then dried. All fractions were stored at -80 °C until further processing.

Proteomic analysis Precipitated milk proteins were solubilized with 8 M urea in 50 mM NH_4HCO_3, pH 8.5. Protein disulfide bonds were reduced by adding 500 mM dithiothreitol to a final concentration of 5 mM and incubating for 30 min at 60 ºC. Cysteine residues were then alkylated by adding 400 mM iodoacetamide to a final concentration of 20 mM and incubated in the dark at 24 °C for 1 h. The digestion was performed with 1/50 sequencing-grade trypsin/protein ratio for 3 h at 37 ºC. Peptides were desalted with C18 solid phase extraction cartridges and labeled with 16-plex tandem mass tags (TMT, Thermo Fisher Scientific) following manufacturer recommendations. Labeled peptides were multiplexed and fractionated by basic pH reversed phase chromatography (Wang et al., 2011) prior to the liquid chromatography-tandem mass spectrometry analysis. A Thermo Dionex Ultimate 3000 UPLC system (Thermo Scientific, San Jose, CA) was configured to load a 5µL injection directly onto the column at a flow rate of 200 nL/min, allowing 40 min to load the sample onto the column before the elution gradient was started. The analytical column was made using an integrated emitter capillary (75 µm i.d. x 25 cm long), packed in-house using Waters BEH

C18 media (Milford, MA) in 1.7µm particle size. Columns were heated to 45 ºC using the MonoSLEEVE controller and a 15 cm heater (Analytical Sales and Services Inc., Flanders, NJ). Mobile phases consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile with the following gradient profile (min, %B): 0,1; 40, 1; 50, 8; 145, 25; 155, 35; 160, 75; 165, 5; 170, 95; 175, 1. Mass spectrometry analysis was performed using an Q Exactive HF-X mass spectrometer (Thermo Scientific, San Jose, CA) outfitted with a Nanospray Flex™ Ion Source (Thermo Scientific, San Jose, CA) ionization interface. The ion transfer tube temperature and spray voltage were 300 ºC and 2.2 kV, respectively. Data were collected for 120 min following a 60 min delay from sample injection. Fourier transform-mass spectrometry spectra were acquired from 300-1800 m/z at a resolution of 60k (AGC target 3e6) and while the top 12 FT-HCD-MS/MS spectra were acquired in data dependent mode with an isolation window of 0.7 m/z and at a resolution of 45K (AGC target 1e5) using a normalized collision energy of 30 and a 45 sec exclusion time. An internal standard of a pooled reference sample was used to enable comparison of different sets of LC-MS/MS analyses. To determine possible bacteria present in the samples, peptides were submitted to de novo sequencing and sequenced peptides were searched against the whole non-redundant Uniprot Knowledgebase using an in-house developed tool named Kaiko (Lee et al., 2022). The species with the highest coverage for the top 100 genera, along with *Bos taurus*, had their protein database sequences downloaded from Uniprot to build the database for peptide identification. Peptides were identified by searching against this database using the MS-GF+ tool (Kim and Pevzner, 2014). A target-decoy approach was used to control false discovery. Only tryptic peptide identifications of no more than 2 missed cleavage sites were kept for further evaluation. The peptide-spectrum-matches (PSM) were then filtered with mass error < 4.66 ppm and MS-GF+ spectra probability score < 7.20e-11 to ensure peptide-level false discovery rate (FDR) of 1%. Tandem mass tag reporter ion intensities were extracted from each PSM using the MASIC software (Monroe et al., 2008). Within each TMT set, for each peptide, the PSM with the highest sum of reporter ion intensities was used for the subsequent calculation of peptide abundances. Peptide abundances were measured based on the TMT reporter ion intensities.

Lipidomic analysis Lipid LC-MS/MS was carried out essentially as described previously (Farley et al., 2022). Briefly, extracted lipids were dissolved in 1:9 chloroform:methanol (v/v) solution and loaded into a Waters CSH reverse phase column (3.0 mm × 150 mm × 1.7 µm particle size) and eluted with a 34-min gradient (mobile phase A: acetonitrile/water (40:60) containing 10 mM ammonium acetate; mobile phase B: acetonitrile/isopropanol (10:90) containing 10 mM ammonium acetate) with a flow rate of 250 µL/min with a Waters Aquity UPLC H-class system. Mass spectra were collected with a Velos-ETD Orbitrap mass spectrometer (Thermo Fisher Scientific) in both positive and

negative ionization modes. Lipids were fragmented with both higher-energy collision dissociation and collision-induced dissociation. Mass spectrometry raw files were converted to ABF format using Reifycs Abf (Analysis Base File) Converter and analyzed with MS-DIAL (v4.92) for lipid identification and extraction of peak areas. A parent mass tolerance of 0.05 Da and a fragment mass tolerance of 0.5 Da were used for identification. A 0.05 Da mass tolerance and a 0.2 min retention time tolerance were used for peak alignment. Identifications were manually inspected based on their fragmentation profile and retention time to eliminate lipids formed by in-source fragmentation, such as fatty acids and lysophospholipids.

Metabolomic analysis The metabolite extracts were reconstituted with pyridine-containing methoxyamine for derivatizing carbonyl groups by methoxyamination. N-methl-N-trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane was used to subsequently derivatize the hydroxl and amine groups (Kim et al., 2013). Analysis of the derivatized metabolites occurred via gas chromatography-mass spectrometry (GC-MS) on an Agilent GC 8890 coupled with a single quadrupole MSD 5977B (Agilent Technologies, Inc). An HP-5MS column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies) was used for untargeted metabolomic analyses. The sample injection mode was splitless and 1 μl of each sample was injected. The injection port temperature was held at 250 °C throughout the analysis. The GC oven was held at 60 °C for 1 min after injection and the temperature was then increased to 325 °C by 10 °C min^{-1}, followed by a 5 min hold at 325 °C (Couvillion et al., 2023). The helium gas flow rates for each experiment were determined by the Agilent Retention Time Locking function based on analysis of deuterated myristic acid and were in the range of 0.45–0.5 ml min^{-1}. Data were collected over the mass range 50–550 *m/z*. A mixture of fatty acid methyl esters (FAMEs) (C8–C28) was analyzed once per day together with the samples for retention index alignment purposes during subsequent data analysis. The GC-MS data were deconvoluted and chromatographically aligned using MetaboliteDetector (Hiller et al., 2009) and metabolites were identified by matching against the Fiehn Metabolomics Library method (Kind et al., 2009) based on retention time and mass spectral profiles. All identifications were manually validated to reduce deconvolution errors and to eliminate false identifications. The NIST 14 GC-MS library was also used to cross-validate the spectral matching scores obtained using the Agilent library and to provide identifications of unmatched metabolites.

Statistical analysis All data were first subjected to quality control processing using standard principal component analysis (PCA) for visual inspection for potential outliers. The proteomics data were additionally evaluated via a robust Mahalanobis distance with Peptide Abundance Vectors (rMd-PAV) to attain a quantitative evaluation of outlier behavior (Matzke et al., 2011). The metabolomics

dataset had 1.7% of the measured values that were not detected and the lipidomics data had 0.2% and 0.7% missing across the datasets for positive and negative modes, respectively. These two datasets were imputed with probabilistic PCA. The proteomics data had 56.1% missing data. An initial filter was applied to remove proteins that were identified by a single peptide as well as peptides that did not have enough data present for statistical analysis, which was defined as peptides not observed in at least 3 of the individual batches (10 batches based on TMT-16). This reduced the total missing data to 7.9%, a total of 9,490 unique peptides mapping to 5,461 proteins. There were multiple proteins defined by identical peptides with 2,933 unique protein families noted. Proteomics data were not imputed but were normalized to the reference pool within each of the 10 batches. All datasets were normalized to total abundance via median centering.

To identify statistical associations, data were analyzed using Analysis of Variance (ANOVA) with an effect for mammary gland quarter health status group (CM, SCM, and healthy) adjusted for dairy. Models were run comparing the healthy quarter before and during the matched event, comparing the quarter with mastitis (CM or SCM) before and during the matched event, and the ratio of the quarter with mastitis (CM or SCM) to the healthy quarter before and during the matched event (9 total models). Comparisons are illustrated in **Figure 4-1**. Changes were considered significant at $P<0.05$.

Pathway enrichment analysis was performed on differential proteins and metabolites identified in each mammary gland quarter health status group. For the proteins, UniProt accession numbers were converted into genes by the DAVID conversion tool (https://david.ncifcrf.gov/tools.jsp) to be plugged into the KEGG pathway database (https://www.genome.jp/kegg/pathway.html). For the metabolites, MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) combined with the KEGG Pathway database was used.

Machine learning was performed in MatLab®, using logistic regression with 3- fold cross-validation. Feature selection was performed using Repeated Optimization for Feature Interpretation (ROFI) (Frohnert et al., 2020; Webb-Robertson et al., 2021; Webb-Robertson et al., 2022) using 100 repetitions, 10 iterations, δ of 1E-5, and γ of 1E-3. Features selected by ROFI with greater than 20% frequency were considered as important based on a ROFI analysis with random outcome assignment.

Results

A total of 2,933 proteins, 193 metabolites, and 291 lipids were detected in 112 bovine quarter-milk samples via proteomic, metabolomic, and lipidomic analyses (**Table 4-1**).

Two healthy quarters within healthy cows (Figure 1 line 1) A total of 48 features were different (22 features before the matched event and 26 features during the matched event) between milk samples

collected from two separate healthy quarters within healthy cows (Table 4-1). The detected variation represented 0.6% of proteins, 1.0% of metabolites, and 1.0% of lipids before the matched event and 0.8% of proteins, 0.5% of metabolites, and 0.7% of lipids during the matched event. Before the matched event, proteins and metabolites that mapped to pathways of cell motility and the immune system were at higher levels in milk collected from one set of healthy quarters relative to milk collected from the other set of healthy quarters within healthy cows (**Table 4-2**). One PE species and one triglyceride (TG) species were at higher levels, whereas another PE species was at a lower level, in milk collected from one set of healthy quarters relative to milk collected from the other set of healthy quarters within healthy cows. During the matched event, one PC species was at a higher level, and one PE species was at a lower level in milk collected from one set of healthy quarters relative to milk collected from the other set of healthy quarters within healthy cows (**Table 4-3**).

Quarters with SCM vs healthy quarters within cows with SCM (Figure 1 line 2) A total of 317 features were different (4 features before the matched event and 313 features during the matched event) in milk produced by quarters with SCM compared to milk produced by healthy quarters within cows with SCM (Table 4-1). The detected variation represented 0.1% of proteins, 0% of metabolites, and 0% of lipids before the matched event and 10.5% of proteins, 0.5% of metabolites, and 1.0% of lipids during the matched event. During the matched event, proteins and metabolites that mapped to pathways of immune, circulatory, nervous, and endocrine systems; transport and catabolism; cell motility; cell growth and death; carbohydrate and amino acid metabolism; transcription; and signal transduction were at higher levels in milk produced by quarters with SCM relative to milk produced by healthy quarters within cows with SCM (Table 4-2). Proteins and metabolites that mapped to pathways of the nervous system; signal transduction; glycan biosynthesis and metabolism; and membrane transport were at lower levels in milk produced by quarters with SCM relative to milk produced by healthy quarters within cows with SCM (Table 4-2). One PC species was at a higher level; one diglyceride (DG) species and one PE species were at lower levels in milk produced by quarters with SCM relative to milk produced by healthy quarters within cows with SCM (Table 4-3).

Quarters with CM vs healthy quarters within cows with CM (Figure 1 line 3) A total of 774 features were different (7 features before the matched event and 767 features during the matched event) in milk collected from quarters with CM compared to milk collected from healthy quarters within cows with CM (Table 4-1). The detected variation represented 0.2% of proteins, 0.5% of metabolites, and 0.3% of lipids before the matched event and 21.5% of proteins, 15.0% of metabolites, and 37.1% of lipids during the matched event. Before the matched event, one PC species was at a lower level in milk collected from quarters with CM relative to milk collected from healthy quarters within cows

with CM (Table 4-3). During the matched event, proteins and metabolites that mapped to pathways of immune, circulatory, nervous, and endocrine systems; cell motility; cell growth and death; carbohydrate and amino acid metabolism; transcription; signal transduction; energy metabolism; development and regeneration; folding, sorting and degradation; replication and repair; translation; and metabolism of cofactors and vitamins were at higher levels in milk collected from quarters with CM relative to milk collected from healthy quarters within cows with CM (Table 4-2). Proteins and metabolites that mapped to pathways of immune and endocrine systems; transport and catabolism; cell growth and death; carbohydrate metabolism; signal transduction; glycan biosynthesis and metabolism; development and regeneration; folding, sorting and degradation; and signaling molecules and interaction were at lower levels in milk collected from quarters with CM relative to milk collected from healthy quarters within cows with CM (Table 4-2). Two Cer species, 36 PC species, four PE species, one PI species, one PS species, and eight SM species were at higher levels and one DG species, seven PE species, and 48 TG species were at lower levels in milk collected from quarters with CM relative to milk collected from healthy quarters within cows with CM (Table 4-3).

Healthy quarters of cows with SCM vs healthy quarters of healthy cows (Figure 1 line 4) A total of 31 features were different (22 features before the matched event and 9 features during the matched event) in milk produced by healthy quarters of cows with SCM compared to milk produced by healthy quarters of healthy cows (Table 4-1). The detected variation represented 0.6% of proteins, 0.5% of metabolites, and 1.0% of lipids before the matched event and 0.3% of proteins, 0% of metabolites, and 0.3% of lipids during the matched event. Before the matched event, proteins and metabolites that mapped to pathways of the immune system were at higher levels and others were at lower levels in milk produced by healthy quarters of cows with SCM relative to milk produced by healthy quarters of healthy cows (**Table 4-4**). Three TG species were at lower levels in milk produced by healthy quarters of cows with SCM relative to milk produced by healthy quarters of healthy cows (Table 4-3**)**. During the matched event, proteins and metabolites that mapped to pathways of the immune system were at higher levels and transport and catabolism were at lower levels in milk produced by healthy quarters of cows with SCM relative to milk produced by healthy quarters of healthy cows (Table 4-4). One PC species was at a higher level in milk produced by healthy quarters of cows with SCM relative to milk produced by healthy quarters of healthy cows (Table 4-3).

Healthy quarters of cows with CM vs healthy quarters of healthy cows (Figure 1 line 5) A total of 250 features were different (41 features before the matched event and 209 features during the matched event) in milk collected from healthy quarters of cows with CM compared to milk collected from healthy quarters of healthy cows (Table 4-1). The detected variation represented 1.3% of

proteins, 1.0% of metabolites, and 0.3% of lipids before the matched event and 6.0% of proteins, 8.8% of metabolites, and 5.5% of lipids during the matched event. Before the matched event, proteins and metabolites that mapped to pathways of transcription were at higher levels in milk collected from healthy quarters of cows with CM relative to milk collected from healthy quarters of healthy cows (Table 4-4). One Cer species was at a lower level in milk collected from healthy quarters of cows with CM relative to milk collected from healthy quarters of healthy cows (Table 4-3). During the matched event, proteins and metabolites that mapped to pathways of transport and catabolism; signal transduction; cell motility; the nervous system; metabolism of cofactors and vitamins; carbohydrate and amino acid metabolism; and translation were at lower levels in milk collected from healthy quarters of cows with CM relative to milk collected from healthy quarters of healthy cows. Proteins and metabolites that mapped to pathways of carbohydrate metabolism were at higher levels in milk collected from healthy quarters of cows with CM relative to milk collected from healthy quarters of healthy cows (Table 4-4). Four PC species and one SM species were at higher levels, and one PC species, six PE species, and two PI species were at lower levels in milk collected from healthy quarters of cows with CM relative to milk collected from healthy quarters of healthy cows (Table 4-3).

Healthy quarters of cows with SCM vs healthy quarters of cows with CM (Figure 1 line 6) A total of 157 features were different (32 features before the matched event and 125 features during the matched event) in milk produced by healthy quarters of cows with SCM compared to milk produced by healthy quarters of cows with CM (Table 4-1). The detected variation represented 1.0% of proteins, 0% of metabolites, and 0.7% of lipids before the matched event and 3.5% of proteins, 7.3% of metabolites, and 2.7% of lipids during the matched event. Before the matched event, proteins and metabolites that mapped to pathways of carbohydrate and amino acid metabolism and translation were at lower levels in milk produced by healthy quarters of cows with SCM relative to milk produced by healthy quarters of cows with CM (Table 4-4). Two Cer species were at lower levels in milk produced by healthy quarters of cows with SCM relative to milk produced by healthy quarters of cows with CM (Table 4-3). During the matched event, proteins and metabolites that mapped to pathways of the immune system and carbohydrate metabolism were at higher levels in milk produced by healthy quarters of cows with SCM relative to milk produced by healthy quarters of cows with CM. Proteins and metabolites that mapped to pathways of metabolism of cofactors and vitamins; translation; and amino acid metabolism were at lower levels in milk produced by healthy quarters of cows with SCM relative to milk produced by healthy quarters of cows with CM (Table 4-4). Five PE species, one PC species, and two PI species were at lower levels in milk produced by healthy quarters of cows with SCM relative to milk produced by healthy quarters of cows with CM (Table 4-3).

Quarters with SCM of cows with SCM vs healthy quarters of healthy cows (Figure 1 line 7) A total of 185 features were different (28 features before the matched event and 157 features during the matched event) in milk collected from quarters with SCM of cows with SCM compared to milk collected from healthy quarters of healthy cows (Table 4-1). The detected variation represented 1.0% of proteins, 0% of metabolites, and 0% of lipids before the matched event and 5.3% of proteins, 0.5% of metabolites, and 0% of lipids during the matched event. Before the matched event, proteins and metabolites that mapped to pathways of the immune system were at higher levels whereas transport and catabolism were at lower levels in milk collected from quarters with SCM of cows with SCM relative to milk collected from healthy quarters of healthy cows (**Table 4-5**). During the matched event, proteins and metabolites that mapped to pathways of cell motility; immune and circulatory systems; signal transduction; and carbohydrate metabolism were at higher levels in milk collected from quarters with SCM of cows with SCM relative to milk collected from healthy quarters of healthy cows. Proteins and metabolites that mapped to pathways of the immune system; cell growth and death; and signaling molecules and interaction were at lower levels in milk collected from quarters with SCM of cows with SCM relative to milk collected from healthy quarters of healthy cows (Table 4-5).

Quarters with CM of cows with CM vs healthy quarters of healthy cows (Figure 1 line 8) A total of 1,489 features were different (13 features before the matched event and 1,476 features during the matched event) in milk produced by quarters with CM of cows with CM compared to milk produced by healthy quarters of healthy cows (Table 4-1). The detected variation represented 0.4% of proteins, 0.5% of metabolites, and 0.3% of lipids before the matched event and 45.2% of proteins, 23.8% of metabolites, and 36.1% of lipids during the matched event. Before the matched event, proteins and metabolites that mapped to pathways of nucleotide metabolism were at lower levels in milk produced by quarters with CM of cows with CM relative to milk produced by healthy quarters of healthy cows (Table 4-5). One PE species was at a lower level in milk produced by quarters with CM of cows with CM relative to milk produced by healthy quarters of healthy cows (Table 4-3). During the matched event, proteins and metabolites that mapped to pathways of cell motility; immune, circulatory, and digestive systems; signal transduction; carbohydrate and amino acid metabolism; cell growth and death; energy metabolism; folding, sorting and degradation; transcription; metabolism of cofactors and vitamins; and translation were at higher levels in milk produced by quarters with CM of cows with CM relative to milk produced by healthy quarters of healthy cows (Table 4-5). Proteins and metabolites that mapped to pathways of transport and catabolism; immune, endocrine, nervous, and digestive systems; signal transduction; carbohydrate and amino acid metabolism; cell growth and death; signaling molecules and interaction; folding, sorting and degradation; glycan biosynthesis and

metabolism; lipid metabolism; metabolism of cofactors and vitamins; and development and regeneration were at lower levels in milk produced by quarters with CM of cows with CM relative to milk produced by healthy quarters of healthy cows (Table 4-5). Two Cer species, 38 PC species, ten PE species, two PI species, nine SM species, and one PS species were at higher levels and 11 PE species, two PI species, and 30 TG species were at lower levels in milk produced by quarters with CM of cows with CM relative to milk produced by healthy quarters of healthy cows (Table 4-3).

Quarters with SCM of cows with SCM vs quarters with CM of cows with CM (Figure 1 line 9) A total of 982 features were different (28 features before the matched event and 954 features during the matched event) in milk collected from quarters with SCM of cows with SCM compared to milk collected from quarters with CM of cows with CM (Table 4-1). The detected variation represented 1.0% of proteins, 0% of metabolites, and 0% of lipids before the matched event and 27.6% of proteins, 25.9% of metabolites, and 32.6% of lipids during the matched event. Before the matched event, proteins and metabolites that mapped to pathways of transport and catabolism were at higher levels and nucleotide metabolism were at lower levels in milk collected from quarters with SCM of cows with SCM relative to milk collected from quarters with CM of cows with CM (Table 4-5). During the matched event, proteins and metabolites that mapped to pathways of transport and catabolism; the immune system, signal transduction, carbohydrate and amino acid metabolism; cell growth and death; folding, sorting and degradation; transcription; metabolism of cofactors and vitamins; replication and repair; and translation were at higher levels in milk collected from quarters with SCM of cows with SCM relative to milk collected from quarters with CM of cows with CM (Table 4-5). Proteins and metabolites that mapped to pathways of transport and catabolism; signal transduction; amino acid metabolism; the endocrine and nervous systems; folding, sorting and degradation; glycan biosynthesis and metabolism; lipid metabolism; and development and regeneration were at lower levels in milk collected from quarters with SCM of cows with SCM relative to milk collected from quarters with CM of cows with CM (Table 4-5). Seven PE species, 39 PC species, two PI species, one PS species, and eight SM species were at higher levels and eight PE species, one PI species, and 29 TG species were at lower levels in milk collected from quarters with SCM of cows with SCM relative to milk collected from quarters with CM of cows with CM (Table 4-3).

Machine learning Fourteen features (six proteins, five metabolites, and three lipids) identified in milk collected from healthy quarters of cows with CM predicted, with reasonable accuracy, the development of CM 1-3 days before clinical signs appear in early lactation. The proteins VPS37B subunit of ESCRT-I and alpha-L-iduronidase, the metabolites L-leucine and L-tyrosine, and the lipids

SM(d18:0/18:2) and PC(0:0/18:0) were at lower levels in milk collected from healthy quarters of cows with CM relative to milk collected from healthy quarters of cows with SCM and healthy cows before the matched event. The proteins transitional endoplasmic reticulum ATPase, fructose-bisphosphatase, complement C8 gamma chain, and cartilage acidic protein 1, the metabolites citric acid, palatinose, and 3-indolelactic acid, and the coeluted lipids PC(17:0/17:0);PC(16:0/18:0) were at higher levels in milk collected from healthy quarters of cows with CM relative to milk collected from healthy quarters of cows with SCM and healthy cows before the matched event (**Table 4-6**). Thirteen features (six proteins, five metabolites, and two lipids) identified in milk from quarters with CM of cows with CM predicted, with high accuracy, the development of CM 1-3 days before clinical signs appear. The proteins VPS37B subunit of ESCRT-I and two isoforms of osteoclast stimulating factor 1, the metabolites L-tyrosine, L-lysine, and palatinose, and the lipid TG(47:2) were at lower levels in milk collected from quarters with CM of cows with CM relative to milk collected from quarters with SCM of cows with SCM and healthy quarters of healthy cows before the matched event. The proteins N-acetyl-alpha-glucosaminidase, cartilage acidic protein 1, and ras-related protein Rab-18, the metabolites L-isoleucine and trans-3-hydroxyl-L-proline, and the lipid SM(d18:0/18:2) were at higher levels in milk collected from quarters with CM of cows with CM relative to milk collected from quarters with SCM of cows with SCM and healthy quarters of healthy cows before the matched event (Table 4-6).

Discussion

In the current study, proteomic, metabolomic, and lipidomic analyses were performed on post-milking quarter-milk samples (n = 112) collected prospectively from cows before determination of quarter health status. Samples collected from quarters with SCM and from healthy quarters were matched, as closely as possible, to samples collected from quarters with CM based on dairy, parity, and d postpartum. This approach was used to best match cows with naturally occurring mastitis. Previous studies compared milk through proteomic analysis (Boehmer et al., 2009; Danielsen et al., 2010; Mudaliar et al., 2016; Thomas et al., 2016a; Kusebauch et al., 2018) and metabolomic analysis (Thomas et al., 2016b; Johnzon et al., 2018) collected from cows with experimentally induced mastitis, which allowed for a more specific evaluation of changes due to specific mastitis pathogens.

Comparisons before mastitis In the present study, few features and pathways were different before mastitis was determined. Quarters that would become SCM or CM had no differential pathways identified noting the relative acuteness of mastitis. Within cow comparisons between healthy quarters before the matched event (Table 4-2) detected 3 sub pathways in 3 super pathways. The differential pathways included regulation of actin cytoskeleton, a critical component in maintaining the shape and

structure of cells and involved in cell migration and trafficking (Balta et al., 2021); adherens junction, or the junctions that mechanically hold cells together (Alberts et al., 1983); and leukocyte transendothelial migration, or the transient movement of leukocytes through the endothelium of blood vessels as part of the inflammatory response (Schimmel et al., 2017). These pathways all relate to cell structure and movement and could reflect the variation in mammary tissue remodeling as lactation becomes established.

Comparisons during mastitis As expected, a large number of immune system pathways were altered in quarters from cows during CM and SCM compared to their healthy quarters (Table 4-2). Many of the pathways in both SCM and CM reflect the immune response to a pathogen and enhanced inflammatory signaling (Tizard, 2013; Parham, 2015). In particular, neutrophils, macrophages, and eosinophils phagocytose invading bacteria as part of the innate immune response. Neutrophils can also release nuclear contents to trap and destroy bacteria (Tizard, 2013). Various signaling pathways, such as interleukin-17, chemokine, and Fc epsilon RI pathways, activate and recruit leukocytes to the site of infection (Parham, 2015). B cell and T cell receptor signaling pathways were at higher levels in quarters with CM. These signaling pathways are responsible for activating B cells (antibody-mediated response) and T cells (cell-mediated response) as part of the adaptive immune response (Parham, 2015). This indicates quarters with CM may elicit a more advanced immune response compared to quarters with SCM. Higher levels of complement and coagulation cascades imply the innate immune response is still active in healthy quarters of cows with SCM relative to healthy quarters of cows with CM. These cascades include opsonization of bacteria, elimination of apoptotic cells, production of proinflammatory cytokines, augmentation of blood coagulation, and instigation of chemotaxis (Tizard, 2013; Parham, 2015). In quarters with CM, a reduction in glutathione metabolism, a key player in antioxidant defense and production of cytokines, possibly reflects oxidative stress (Wu et al., 2004; Tizard, 2013). These immune system pathways are intricately woven together to work in harmony as part of the complexity of CM and SCM.

Lower levels of galactose, arginine, histidine, beta-alanine, and vitamin B6 metabolism as well as pantothenate, CoA, and aminoacyl-tRNA biosynthesis were observed in milk produced by healthy quarters of cows with CM (Table 4-4). This could indicate reduced synthesis of milk in the healthy quarter (Stryer, 1981; Reece, 2004). Thus, CM appears to have an effect on the healthy quarter. Synthesis of milk components was further disrupted in healthy quarters of cows with SCM compared to healthy quarters of cows with CM (Table 4-4). Impacts of CM and SCM beyond the inflamed quarter are detectable.

Lower levels of a few Cer species were observed in healthy quarters of cows with SCM or CM (Table 4-3). Ceramides are a necessary unit of sphingolipids such as sphingomyelin (an important component of mammalian cell membranes), cerebroside (abundant in nerve myelin sheaths), and ganglioside (present on surface of neuronal cells) (Kolter, 2012; Slotte, 2013; Blanco and Blanco, 2017).

Increases in circulatory and nervous system pathways were observed (Table 4-2) in quarters with mastitis (SCM or CM). The pathway for vascular smooth muscle contraction was at higher levels in quarters with SCM or CM relative to healthy quarters within cows with SCM or CM (Table 4-2). Furthermore, this pathway was at higher levels in quarters with SCM or CM of cows with SCM or CM compared to healthy quarters of healthy cows (Table 4-5). The sub pathway of vascular smooth muscle contraction refers to the contraction of blood vessels, such as those located in the mammary gland (Reece, 2004; Brozovich et al., 2016). An increase in vascular smooth muscle contraction results in vasoconstriction of blood vessels to decrease blood flow, reduce nutrient delivery, and allow for leukocyte transendothelial migration (Tizard, 2013; Schimmel et al., 2017).

Within cows with mastitis (SCM or CM), quarters with mastitis (SCM or CM) exhibited higher levels of long-term potentiation and neurotrophin signaling pathway compared to healthy quarters. The neurotrophin signaling pathway regulates long-term potentiation (Mitre et al., 2017). Long-term potentiation refers to synaptic plasticity or an escalation in synaptic strength that results in an enduring signal transmission between neurons (Verslegers et al., 2013; Fu and Jhamandas, 2020). A lower level of a nervous system pathway, long-term potentiation, was observed in healthy quarters of cows with CM relative to healthy quarters of healthy cows suggesting a decrease in signal transmission. Clinical mastitis appears to affect neuronal function even in noninflamed quarters.

The dopaminergic synapse pathway is at lower levels in quarters with SCM relative to healthy quarters within cows with SCM (Table 4-2). Dopamine is a neurotransmitter that regulates gene expression and release of prolactin, a hormone associated with mammary gland growth and the initiation and maintenance of lactation (Ben-Jonathan and Hnasko, 2001; Reece, 2004). Prolactin is also associated with the immune system as it regulates humoral and cellular inflammatory response (Freeman et al., 2000; Akers, 2002; Boutet et al., 2007). Lower levels of long-term depression and cholinergic synapse pathways were observed in quarters with SCM or CM (Table 4-5). In contrast to long-term potentiation, long-term depression refers to decreased capability of synapses to transmit neuronal signals between parallel fibers, axons of small neurons located in the brain, and Purkinje cells, large neurons with several branching dendrites also located in the brain (Ferrari and Goda, 2009). Purkinje cells release gamma-aminobutyric acid, an inhibitory neurotransmitter that decreases

nerve cell stimulation (Jewett and Sharma, 2023). Gamma-aminobutyric acid may play an anti-inflammatory role in mastitis by minimizing consequences of cell apoptosis and hindering expression of proinflammatory cytokines (Wang et al., 2018). Cholinergic synapse refers to the use of acetylcholine as a neurotransmitter with excitatory or inhibitory properties in the central nervous system (Stryer, 1981; Alberts et al., 1983; Thany and Tricoire-Leignel, 2011). In quarters with CM, serotonergic synapse was at lower levels (Table 4-5). Serotonin is a neurotransmitter produced in the mammary gland that has been shown to regulate lactation through feedback inhibition (Hernandez et al., 2008; Collier et al., 2012). In summary, quarters with mastitis exhibited differences in milk protein and metabolite pathways related to the circulatory and nervous systems.

Machine learning Milk composition appears to be very consistent in healthy mammary glands and any differences may illustrate the natural variation among healthy quarters within healthy cows whereas healthy quarters that remain among healthy cows, cows with SCM, and cows with CM still exhibit differences in milk composition. Even though milk composition was similar in quarters before mastitis, we employed machine learning in an attempt to tease apart predictive features. A combination of differentially abundant features that may predict CM were ascertained from the data (Table 4-6). Most of the proteins and metabolites identified were involved in carbohydrate metabolism. Higher levels of fructose-bisphosphatase, citric acid, L-isoleucine, and trans-3-hydroxyl-L-proline indicate an increase in glycolysis whereas lower levels of L-leucine, L-tyrosine, and L-lysine imply lower glycolytic activity (Alberts et al., 1983; Reece, 2004). A higher level of palatinose (trade name of isomaltulose), a sugar alternative consisting of one glucose molecule and one fructose molecule, was identified from the data as being predictive of CM (Table 4-6). Isomaltulose is naturally found in sugarcane, honey, and beet extracts and has been identified as an animal metabolite (Livesey, 2014). Two proteins at higher levels, transitional endoplasmic reticulum ATPase and ras-related protein Rab-18, and one protein at a lower level, vacuolar protein sorting-associated protein 37B (VPS37B) subunit of endosomal sorting complex required for transport I (ESCRT-I), involved in vesicle trafficking and endocytosis, or the intracellular transport of proteins and other molecules (Stryer, 1981; Alberts et al., 1983) were also noted. Other features found through machine learning as predictive of CM include two proteins involved in glycosaminoglycan breakdown, N-acetyl-alpha-glucosaminidase (at a higher level in quarters with CM) and alpha-L-iduronidase (at a lower level in healthy quarters of cows with CM) (Table 4-6). Glycosaminoglycans include heparan sulfate, a cell surface receptor that elicits effects on cell tissue structure, and heparin, an anticoagulant found in mast cells (Reece, 2004; Sarrazin et al., 2011). Connective tissue of the bovine mammary gland is composed of proteoglycans, which include heparan sulfate, and contains mast cells, which incorporate heparin (Stryer, 1981; Akers, 2002). Key players in mammary cell apoptosis, lysosomes,

contain N-acetyl-alpha-glucosaminidase and alpha-L-iduronidase (Tizard, 2013). The higher level of N-acetyl-alpha-glucosaminidase in quarters with CM and the lower level of alpha-L-iduronidase could indicate connective tissue remodeling in the mammary gland as part of the immune system response. Furthermore, a protein involved in the immune response was at a higher level in healthy quarters of cows with CM. Complement C8 gamma chain is a component of the membrane attack complex of the complement system (Parham, 2015). In summary, disruption in mammary gland metabolism appears to be evident before clinical signs of mastitis are observed.

Interestingly, 3-indolelactic acid was identified from the data as predictive of CM (Table 4-6). It is a gut-associated metabolite of tryptophan shown to have anti-inflammatory properties, including neutralizing free radicals and reducing oxidative stress (National Center for Biotechnology Information, 2023). According to Meng et al. (2020), 3-indolelactic acid is produced by *Bifidobacterium longum* subsp. *infantis*, a commensal organism associated with human milk. Other species of *Bifidobacterium*, including *B. breve* and *B. bifidum* have been shown to produce 3-indolelactic acid as well (Laursen et al., 2021). Ehrlich et al. (2020) reported higher levels of 3-indolelactic acid in infant feces that contained a higher level of *B*. *infantis*. This metabolite appears to have the most anti-inflammatory effect in immature infant intestinal enterocytes as opposed to mature enterocytes (Ehrlich et al., 2020; Meng et al., 2020; Laursen et al., 2021). The present study identified 3-indolelactic acid in bovine milk whereas others have detected it in human milk. Future studies are needed to elucidate factors that impact the concentration of 3-indolelactic acid in bovine milk and its relationship with risk of mastitis.

Conclusion

In the present study, proteomic, metabolomic, and lipidomic analyses were performed on milk samples collected from quarters with CM, quarters with SCM, and healthy quarters both within and among cows in early lactation. Matching quarters with SCM and healthy quarters to quarters with CM based on dairy, parity, and d postpartum was a main strength of the current study. As mastitis was naturally occurring, a main limitation of the present study was the low number of mastitis cases. Very few changes in milk proteins, metabolites, and lipids were observed 1-3 days before mastitis occurred. However, several pathways related to the immune system were differentially abundant during mastitis incidence. Pathways of the innate immune response included neutrophil extracellular trap formation, Fc gamma R-mediated phagocytosis, and natural killer cell mediated cytotoxicity. Pathways of the adaptive immune response included antigen processing and presentation as well as B cell and T cell signaling pathways. This indicates a logical immune response was noted in quarters with CM or SCM. Several components, such as palatinose, alpha-L-iduronidase, and 3-indolelactic

acid, were identified from the data as being predictive of CM. Further testing of these identified components will help advance bovine mastitis detection and prevention.

References

Akers, R.M., 2002. Pages 3-240 in Lactation and the mammary gland. First ed. Blackwell Publishing Professional, Iowa, US.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J.D. Watson. 1983. Pages 682-683 in Molecular biology of the cell. First ed. Garland Publishing, Inc., New York, US.

Balta, E., J. Kramer, and Y. Samstag. 2021. Redox regulation of the actin cytoskeleton in cell migration and adhesion: on the way to a spatiotemporal view. Front Cell Dev Biol. 8:1-11.

Ben-Johnson, N., and R. Hnasko. 2001. Dopamine as a prolactin (PRL) inhibitor. Endocr Rev. 22:724-763.

Blanco, A., and G. Blanco. 2017. Chapter 5—Lipids. Pages 99-119 in Medical Biochemistry. Academic Press, Elsevier Inc.

Bobbo, T., G. Meoni, G. Niero, L. Tenori, C. Luchinat, M. Cassandro, and M. Penasa. 2021. Nuclear magnetic resonance spectroscopy to investigate the association between milk metabolites and udder quarter health status in dairy cows. J Dairy Sci. 105:535-548.

Boehmer, J.L., J.L. Ward, R.R. Peters, K.J. Shefcheck, M.A. McFarland, and D.D. Bannerman. 2009. Proteomic analysis of the temporal expression of bovine milk proteins during coliform mastitis and label-free relative quantification. J Dairy Sci. 93:593-603.

Boutet, P., J. Sulon, R. Closset, J. Detilleux, J.F. Beckers, F. Bureau, and P. Lekeux. 2007. Prolactin-induced activation of nuclear factor kB in bovine mammary epithelial cells: role in chronic mastitis. J Dairy Sci. 90:155-164.

Brozovich, F.V., C.J. Nicholson, C.V. Degen, Y.Z. Gao, M. Aggarwal, and K.G. Morgan. 2016. Mechanisms of vascular smooth muscle contraction and the basis for pharmacologic treatment of smooth muscle disorders. Pharmacol Rev. 68:476-532.

Ceciliani, F., M. Audano, M.F. Addis, C. Lecchi, M.H. Ghaffari, M. Albertini, F. Tangorra, R. Piccinini, D. Caruso, N. Mitro, and V. Bronzo. 2021. The untargeted lipidomic profile of quarter milk from dairy cows with subclinical intramammary infection by non-*aureus* staphylococci. J Dairy Sci. 104.

Collier, R.J., L.L. Hernandez, and N.D. Horseman. 2012. Serotonin as a homeostatic regulator of lactation. Domest Anim Endocrinol. 43:161-170.

Couvillion, S.P., R.E. Danczak, D. Naylor, M.L. Smith, K.G. Stratton, V.L. Paurus, K.J. Bloodsworth, Y. Farris, D.J. Schmidt, R.E. Richardson, L.M. Bramer, S.J. Fansler, E.S. Nakayasu, J.E. McDermott, T.O. Metz, M.S. Lipton, J.K. Jansson, and K.S. Hofmockel. 2023. Rapid remodeling of the soil lipidome in response to a drying-rewetting event. Microbiome. 11:1-20.

Danielsen, M., M.C. Codrea, K.L. Ingvartsen, N.C. Friggens, E. Bendixen, and C.M. Røntved. 2010. Quantitative milk proteomics—host responses to lipopolysaccharide-mediated inflammation of bovine mammary gland. Proteomics. 10:2240-2249.

Ehrlich, A.M., A.R. Pacheco, B.M. Henrick, D. Taft, G. Xu, M.N. Huda, D. Mishchuk, M.L. Goodson, C. Slupsky, D. Barile, C.B. Lebrilla, C.B. Stephensen, D.A. Mills, and H.E. Raybould. 2020. Indole-3-lactic acid associated with *Bifidobacterium*-dominated microbiota significantly decreases inflammation in intestinal epithelial cells. BMC Microbiol. 20:1-13.

Erskine, R.J., R.J. Eberhart, L.J. Hutchinson, S.B. Spence, and M.A. Campbell. 1988. Incidence and types of clinical mastitis in dairy herds with high and low somatic cell counts. J Am Vet Med Assoc. 192:761-765.

Farley, S.E., J.E. Kyle, H.C. Leier, L.M. Bramer, J.B. Weinstein, T.A. Bates, J.Y. Lee, T.O. Metz, C. Schultz, and F.G. Tafesse. 2022. A global lipid map reveals host dependency factors conserved across SARS-CoV-2 variants. Nat Commun. 13:1-14.

Ferrari, A., and Y. Goda. 2009. Cytoskeleton in plasticity. Pages 311-316 in Encyclopedia of Neuroscience. Academic Press, Elsevier Ltd.

Freeman, M.E., B. Kanyicska, A. Lerant, and G. Nagy. 2000. Prolactin: structure, function, and regulation of secretion. Physiol Rev. 80:1523-1585.

Frohnert, B.I., B.J. Webb-Robertson, L.M. Bramer, S.M. Reehl, K. Waugh, A.K. Steck, J.M. Norris, and M. Rewers. 2020. Predictive modeling of type I diabetes stages using disparate data sources. Diabetes. 69:238-248.

Fu, W., and J.H. Jhamandas. 2020. Chapter 20—Amylin and amylin receptors in Alzheimer's disease. Pages 309-324 in Genetics, Neurobiology, Behavior, and Diet in Dementia: The Neuroscience of Demetia, Volume 2. Academic Press, Elsevier Inc.

Gussmann, M., W. Steeneveld, C. Kirkeby, H. Hogeveen, M. Nielen, M. Farre, and T. Halasa. 2018. Economic and epidemiological impact of different intervention strategies for clinical contagious mastitis. J Dairy Sci. 102:1483-1493.

Hand, K.J., A. Godkin, and D.F. Kelton. 2012. Milk production and somatic cell counts: a cow-level analysis. J Dairy Sci. 95:1358-1362.

Hernandez, L.L., C.M. Stiening, J.B. Wheelock, L.H. Baumgard, A.M. Parkhurst, and R.J. Collier. 2008. Evaluation of serotonin as a feedback inhibitor of lactation in the bovine. J Dairy Sci. 91:1834-1844.

Hiller, K., J. Hangebrauk, C. Jäger, J. Spura, K. Schreiber, and D. Schomburg. 2009. MetaboliteDetector: comprehensive analysis tool for targeted and nontargeted GC/MS based metabolome analysis. Anal Chem. 81:3429-3439.

Jensen, R.G., A.M. Ferris, and C.J. Lammi-Keefe. 1991. The composition of milk fat. J Dairy Sci. 74:3228-3243.

Jensen, R.G. 2002. The composition of bovine milk lipids: January 1995 to December 2000. J Dairy Sci. 85:295-350.

Jewett, B.E., and S. Sharma. 2023. Physiology, GABA. StatPearls Publishing, Florida, US.

Johnzon, C.F., J. Dahlberg, A.M. Gustafson, I. Waern, A.A. Moazzami, K. Östensson, and G. Pejler. 2018. The effect of lipopolysaccharide-induced experimental bovine mastitis on clinical parameters, inflammatory markers, and the metabolome: a kinetic approach. Front Immunol. 9:1-14. Nat Commun. 5:1-10.

Kim, S. and P.A. Pevzner. 2014. MS-GF+ makes progress towards a universal database search tool for proteomics.

Kim, Y.M., B.J. Schmidt, A.S. Kidwai, M.B. Jones, B.L.D. Kaiser, H.M. Brewer, H.D. Mitchell, B.O. Palsson, J.E. McDermott, E. Jason, F. Heffron, R.D. Smith, S.N. Peterson, C. Ansong, D.R. Hyduke, T.O. Metz, and J.N. Adkins. 2013. Salmonella modulates metabolism during growth under conditions that induce expression of virulence genes. Mol Biosyst. 9:1522-1534.

Kolter, T. 2012. Ganglioside biochemistry. ISRN Biochem. 1-36.

Kind, T. G. Wohigemuth, D.Y. Lee, Y. Lu, M. Palazoglu, S. Shahbaz, and O. Fiehn. 2009. FiehnLib: mass spectral and retention index libraries for metabolomics based on quadrupole and time-of-flight gas chromatography/mass spectrometry. Anal Chem. 81:10038-10048.

Kirkeby, C. N. Toft, D. Schwarz, M. Farre, S.S. Nielsen, L. Zervens, S. Hechinger, and T. Halasa. 2019. Differential somatic cell count as an additional indicator for intramammary infection in dairy cows. J Dairy Sci. 103:1759-1775.

Kusebauch, U., L.E. Hernández-Castellano, S.L. Bislev, R.L. Moritz, C.M. Røntved, and E. Bendixen. 2018. Selected reaction monitoring mass spectrometry of mastitis milk reveals pathogen-specific regulation of bovine host response proteins. J Dairy Sci. 101:6532-6541.

Laursen, M.F., M. Sakanaka, N. von Burg, U. Mörbe, D. Andersen, J.M. Moll, C.T. Pekmez, A. Rivollier, K.F. Michaelsen, C. Mølgaard, M.V. Lind, L.O. Dragsted, T. Katayama, H.L. Frandsen, A.M. Vinggaard, M.I. Bahl, S. Brix, W. Agace, T.R. Licht, and H.M. Roager. 2021. *Bifidobacterium* species associated with breastfeeding produce aromatic lactic acids in the infant gut. Nat Microbiol. 6:1367-1382.

Lee, J.Y., H.D. Mitchell, M.C. Burnet, R. Wu, S.C. Jenson, E.D. Merkley, E.S. Nakayasu, C.D. Nicora, J.K. Jansson, K.E. Burnum-Johnson, and S.H. Payne. 2022. Uncovering hidden members and functions of the soil microbiome using de novo metaproteomics. J Proteome Res. 21:2023-2035.

Livesey, G. 2014. Carbohydrate digestion, absorption, and fiber. Third ed. Elsevier reference module in biomedical sciences.

Luangwilai, M., K. Duangmal, N. Chantaprasarn, and S. Settachaimongkon. 2020. Comparative metabolite profiling of raw milk from subclinical and clinical mastitis cows using ^{1}H-NMR combined with chemometric analysis. Int J Food Sci Technol. 1-11.

Matzke, M.M., K.M. Waters, T.O. Metz, J.M. Jacobs, A.C. Sims, R.S. Baric, J.G. Pounds, and B.J.M. Webb-Robertson. 2011. Improved quality control processing of peptide-centric LC-MS proteomics data. Bioinformatics. 27:2866-2872.

Mavangira, V., J.C. Gandy, C. Zhang, V.E. Ryman, A.D. Jones, and L.M. Sordillo. 2015. Polyunsaturated fatty acids influence differential biosynthesis of oxylipids and other lipid mediators during bovine coliform mastitis. J Dairy Sci. 98:6202-6215.

McDermott, M.P., H.N. Erb, and R.P. Natzke. 1982. Predictability by somatic cell counts related to prevalence of intramammary infection within herds. J Dairy Sci. 65:1535-1539.

Meng, D., E. Sommella, E. Salvlati, P. Campiglia, K. Gangull, K. Djebali, W. Zhu, and W.A. Walker. 2020. Indole-3-lactic acid, a metabolite of tryptophan, secreted by *Bifidobacterium longum* subspecies *infantis* is anti-inflammatory in the immature intestine. Pediatr Res. 88:209-217.

Metz, and J.N. Adkins. 2013. Salmonella modulates metabolism during growth under conditions that induce expression of virulence genes. Mol Biosyst. 9:1522-1534.

Mitre, M., A. Mariga, and M.V. Chao. 2017. Neurotrophin signaling: novel insights into mechanisms and pathophysiology. Clin Sci. 131:13-23.

Monroe, M.E., J.L. Shaw, D.S. Daly, J.N. Adkins, and R.D. Smith. 2008. MASIC: a software program for fast quantitation and flexible visualization of chromatographic profiles from detected LC-MS(/MS) features. Comput Biol Chem. 32:215-217.

Mudaliar, M., R. Tassi, F.C. Thomas, T.N. McNeilly, S.K. Weidt, M. McLaughlin, D. Wilson, R. Burchmore, P. Herzyk, P.D. Eckersall, and R.N. Zadoks. 2016. Mastitomics, the integrated omics of bovine milk in an experimental model of *Streptococcus uberis* mastitis: 2. Label-free relative quantitative proteomics. Mol Biosyst. 12:2748-2761.

Nakayasu, E.S., C.D. Nicora, A.C. Sims, K.E. Burnum-Johnson, Y.M. Kim, J.E. Kyle, M.M. Matzke, A.K. Shukla, R.K. Chu, A.A. Schepmoes, J.M. Jacobs, R.S. Baric, B.J. Webb-Robertson, R.D. Smith, and T.O. Metz. 2016. MPLEx: a robust and universal protocol for single-sample integrative proteomic, metabolomic, and lipidomic analyses. mSystems. 1:1-14.

Parham, P. 2015. Innate immunity: the immediate response to infection and Innate Immunity: the induced response to infection. Pages 31-39 and 57-62 in The Immune System. Fourth ed. Garland Science, Taylor and Francis Group, LLC., New York, US.

Potter, T.L., C. Arndt, and A.N. Hristov. 2018. Short communication: increased somatic cell count is associated with milk loss and reduced feed efficiency in lactating dairy cows. J Dairy Sci. 101:9510-9515.

Puerto, M.A., E. Shepley, R.I. Cue, D. Warner, J. Dubuc, and E. Vasseur. 2021. The hidden cost of disease: I. impact of the first incidence of mastitis on production and economic indicators of primiparous dairy cows. J Dairy Sci. 104:7932-7943.

Reece, W.O. 2004. Digestion, absorption, and metabolism and endocrinology, reproduction, and lactation. Pages 516-553 and 720-742 in Duke's Physiology of Domestic Animals. Twelfth ed. Cornell University Press, New York, US.

Sarrazin, S., W.C. Lamanna, and J.D. Esko. 2011. Heparan sulfate proteoglycans. Cold Spring Harb Perspect Biol. 3:1-33.

Schimmel, L., N. Heemskerk, and J.D. van Buul. 2017. Leukocyte transendothelial migration: a local affair. Small GTPases. 8:1-15.

Slotte, J.P. 2013. Biological functions of sphingomyelins. Prog Lipid Res. 52:424-437.

Sohlenkamp, C. and O. Geiger. 2016. Bacterial membrane lipids: diversity in structures and pathways. FEMS Micobiol Revs. 40:133-159.

Stevens, M., S. Piepers, and S. De Vliegher. 2018. The effect of mastitis management input and implementation of mastitis management on udder health, milk quality, and antimicrobial consumption in dairy herds. J Dairy Sci. 102:2401-2415.

Stryer, L. 1981. Pentose phosphate pathway and gluconeogenesis. Pages 343-345, 887 in Biochemistry. Second ed. W.H. Freeman and Company, California, US.

Sundekilde, U.K., N.A. Poulsen, L.B. Larsen, and H.C. Bertram. 2012. Nuclear magnetic resonance metabonomics reveals strong association between milk metabolites and somatic cell count in bovine milk. J Dairy Sci. 96:290-299.

Thany, S.H., and H. Tricoire-Leignel. 2011. Emerging pharmacological properties of cholinergic synaptic transmission: comparison between mammalian and insect synaptic and extra-synaptic nicotinic receptors. Current Neuropharmacol. 9:706-714.

Thomas, F.C., W. Mullen, R. Tassi, A. Ramirez-Torres, M. Mudaliar, T.N. McNeilly, R.N. Zadoks, R. Burchmore, and P.D. Eckersall. 2016a. Mastitomics, the integrated omics of bovine milk in an experimental model of *Streptococcus uberis* mastitis: 1. High abundance proteins, acute phase proteins, and peptidomics. Mol Biosyst. 12:2735-2747.

Thomas, F.C., M. Mudaliar, R. Tassi, T.N. McNeilly, R. Burchmore, K. Burgess, P. Herzyk, R.N. Zadoks, and P.D. Eckersall. 2016b. Mastitomics, the integrated omics of bovine milk in an experimental model of *Streptococcus uberis* mastitis: 3. Untargeted metabolomics. Mol Biosyst. 12:2762-2769.

Tizard, I.R. 2013. Innate immunity: the complement system. Pages 61-73 in Veterinary Immunology. Ninth ed. Elsevier Saunders, Missouri, US.

Tong, J., H. Zhang, Y. Zhang, B. Xiong, and L. Jiang. 2019. Microbiome and metabolome analyses of milk from dairy cows with subclinical *Streptococcus agalactiae* mastitis—potential biomarkers. Front Immunol. 10:1-14.

Turk, R., N. Rošic, J. Kuleš, A. Horvatic, A. Gelemanovic, A. Galen, B.B. Ljubic, M. Benic, V. Stevanovic, V. Mrljak, C.C. Chadwick, and P.D. Eckersall. 2021. Milk and serum proteomes in subclinical and clinical mastitis in Simmental cows. J Proteomics. 244:1-15.

Verslegers, M., K. Lemmens, I. Van Hove, and L. Moons. 2013. Matrix metalloproteinase-2 and -9 as promising benefactors in development, plasticity, and repair of the nervous system. Prog Neurobiol. 105:60-78.

Wang, Y., F. Yang, M.A. Gritsenko, Y. Wang, T. Clauss, L. Tao, Y.Shen, M.E. Monroe, D. Lopez-Ferrer, T. Reno, R.J. Moore, R.L. Klemke, D.G. Camp, and R.D. Smith. 2011. Reverse-phase chromatography with multiple fraction concentration strategy for proteome profiling of human MCF10A cells. Proteomics. 11:2019-2026.

Wang, Y.Y., S.P. Sun, H.S. Zhu, X.Q. Jiao, K. Zhong, Y.J. Guo, G.M. Zho, L.Q. Han, G.Y. Yang, and H.P. Li. 2018. GABA regulates the proliferation and apoptosis of MAC-T cells through the LPS-induced TLR4 signaling pathway. Res Vet Sci. 118:395-402.

Wang, Y., X. Nan, Y. Zhao, H. Wang, M. Wang, L. Jiang, F. Zhang, F. Xue, D. Hua, K. Li, J. Liu, J. Yao, and B. Xiong. 2020. Coupling 16S rDNA sequencing and untargeted mass spectrometry for milk microbial composition and metabolites from dairy cows with clinical and subclinical mastitis. J Agric Food Chem. 68:8496-8508.

Webb-Robertson, B.J., E.S. Nakayasu, B.I. Frohnert, L.M. Bramer, S.M. Akers, J.M. Norris, K. Vehik, A. Ziegler, T.O. Metz, S.S. Rich, and M.J. Rewers. 2022. Integration of infant metabolite, genetic, and islet autoimmunity signatures to predict type I diabetes by age 6 years. J Clin Endocrinol Metab. 107:2329-2338.

Webb-Robertson, B.J., L.M. Bramer, B.A. Stanfill, S.M. Reehl, E.S. Nakayasu, T.O. Metz, B.I. Frohnert, J.M. Norris, R.K. Johnson, S.S. Rich, and M.J. Rewers. 2021. Prediction of the development of islet autoantibodies through integration of environmental, genetic, and metabolic markers. J Diabetes. 13:143-153.

Wu, G., Y.Z. Fang, S. Yang, J.R. Lupton, and N.D. Turner. 2004. Glutathione metabolism and its implications for health. J Nutr. 134:489-492.

Xi, X., L.Y. Kwok, Y. Wang, C. Ma, Z. Mi, and H. Zhang. 2016. Ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry MS^E-based untargeted milk metabolomics in dairy cows with subclinical or clinical mastitis. J Dairy Sci. 100:4884-4896

Table 4-1: Number of differential features in each comparison group before and during the matched event[1].

		Before the Matched Event			During the Matched Event		
Line[1]	**Comparison Group**	**Proteins**	**Metabolites**	**Lipids**	**Proteins**	**Metabolites**	**Lipids**
1	Healthy vs healthy quarters within healthy cows	17	2	3	23	1	2
2	Quarters with SCM vs healthy quarters within cows with SCM	4	0	0	309	1	3
3	Quarters with CM vs healthy quarters within cows with CM	5	1	1	630	29	108
4	Healthy quarters from cows with SCM vs healthy quarters from healthy cows	18	1	3	8	0	1
5	Healthy quarters from cows with CM vs healthy quarters from healthy cows	38	2	1	176	17	16
6	Healthy quarters from cows with SCM vs healthy quarters from cows with CM	30	0	2	103	14	8
7	Quarters with SCM from cows with SCM vs healthy quarters from healthy cows	28	0	0	156	1	0
8	Quarters with CM from cows with CM vs healthy quarters from healthy cows	11	1	1	1325	46	105
9	Quarters with SCM from cows with SCM vs quarters with CM from cows with CM	28	0	0	809	50	95

Table 4-1. cont'd						

[1]Quarters with subclinical mastitis (SCM) and healthy quarters were matched, as closely as possible, to quarters with clinical mastitis (CM) based on dairy, parity, and d postpartum both before (1-3 d prior to first observance of clinical signs, i.e., blood, flakes in milk and/or redness, swelling of the mammary gland) and during (first observance of clinical signs).
[2]Corresponds to the line number in **Figure 1** depicting the various comparisons that were performed.

Table 4-2: Response of pathways comparing milk produced by quarters with clinical mastitis (CM) and by quarters with subclinical mastitis (SCM) to milk produced by healthy (H) quarters before and during the matched event using differential features from proteomic and metabolomic analyses within cows[1].

			Response		
			H	SCM	CM
Timepoint	**Super Pathway**	**Sub Pathway**	**H vs H**	**SCM vs H**	**CM vs H**
Before Matched Event	Cell Motility	Regulation of actin cytoskeleton	↑	--	--
	Cellular Community--Eukaryotes	Adherens junction	↑	--	--
	Immune System	Leukocyte transendothelial migration	↑	--	--
During Matched Event	Immune System	Neutrophil extracellular trap formation	--	↑	↑
		Fc gamma R-mediated phagocytosis	--	↑	↑
		Leukocyte transendothelial migration	--	↑	↑
		Platelet activation	--	↑	↑
		IL-17 signaling pathway	--	↑	--
		Chemokine signaling pathway	--	--	↑
		B cell receptor signaling pathway	--	--	↑
		Fc epsilon RI signaling pathway	--	--	↑
		Natural killer cell mediated cytotoxicity	--	--	↑
		T cell receptor signaling pathway	--	--	↑
		Antigen processing and presentation	--	--	↓
	Circulatory System	Vascular smooth muscle contraction	--	↑	↑
	Nervous System	Long-term potentiation	--	↑	↑
		Neurotrophin signaling pathway	--	↑	↑
		Dopaminergic synapse	--	↓	--
	Endocrine System	Glucagon signaling pathway	--	↑	--
		Thyroid hormone signaling pathway	--	--	↑
		Growth hormone synthesis, secretion and action	--	--	↑
		Oxytocin signaling pathway	--	--	↑
		GnRH signaling pathway	--	--	↑

Table 4-2. cont'd

		Estrogen signaling pathway	--	--	↓
	Cellular Community--Eukaryotes	Tight junction	--	↑	--
		Gap junction	--	↑	--
		Focal adhesion	--	↑	↑
	Transport and Catabolism	phagosome	--	↑	--
		lysosome	--	--	↓
	Cell Motility	Regulation of actin cytoskeleton	--	↑	↑
	Cell Growth and Death	apoptosis	--	↑	--
		Cellular senescence	--	--	↓
	Carbohydrate Metabolism	Glycolysis	--	↑	--
		Pentose phosphate pathway	--	↑	↑
		Pyruvate metabolism	--	↑	↑
		Starch and sucrose metabolism	--	↑	↑
		Galactose metabolism	--	--	↑
		Fructose and mannose metabolism	--	--	↓
	Amino Acid Metabolism	Cysteine and methionine metabolism	--	↑	↑
		Selenocompound metabolism	--	--	↑
		Valine, leucine and isoleucine biosynthesis	--	--	↑
		Valine, leucine and isoleucine degradation	--	--	↑
		Phenylalanine, tyrosine and tryptophan biosynthesis	--	--	↑
		Arginine and proline metabolism	--	--	↑
	Transcription	spliceosome	--	↑	↑
	Signal Transduction	HIF-1 signaling pathway	--	↑	↑
		cAMP signaling pathway	--	↓	↑
		Rap1 signaling pathway	--	--	↑
		cGMP-PKG signaling pathway	--	--	↑
		VEGF signaling pathway	--	--	↑
		ErbB signaling pathway	--	--	↑
		Sphingolipid signaling pathway	--	--	↑
		Ras signaling pathway	--	--	↑
		MAPK signaling pathway	--	--	↑
		AMPK signaling pathway	--	--	↓
	Glycan Biosynthesis and Metabolism	Glycosaminoglycan biosynthesis—heparan sulfate/heparin	--	↓	--

Table 4-2. cont'd					
		Various types of N-glycan biosynthesis	--	--	↓
	Membrane Transport	ABC transporters	--	↓	--
	Energy Metabolism	Sulfur metabolism	--	--	↑
	Development and Regeneration	Axon guidance	--	--	↓
	Folding, Sorting and Degradation	proteasome	--	--	↑
		Protein processing in endoplasmic reticulum	--	--	↓
	Replication and Repair	DNA replication	--	--	↑
	Signaling Molecules and Interaction	Cell adhesion molecules	--	--	↓
	Translation	Aminoacyl-tRNA biosynthesis	--	--	↑
	Metabolism of Cofactors and Vitamins	Thiamine metabolism	--	--	↑

[1]Quarters with SCM and healthy quarters were matched, as closely as possible, to quarters with CM based on dairy, parity, and d postpartum both before (1-3 d prior to first observance of clinical signs such as blood, flakes in milk and/or redness, swelling of the mammary gland) and during (first observance of clinical signs) the matched event.

Table 4-3: Differential lipids in milk collected from healthy quarters, from quarters with subclinical mastitis (SCM), and from quarters with clinical mastitis (CM) both within and among cows before and during the matched event[1].

Quarters with CM or SCM vs healthy quarters within cows	**Timepoint**	**Lipid[2,3,4]**	**Response**
Healthy quarters vs healthy quarters	Before Matched Event	1 PE species; 1 TG species	↑
		1 PE species	↓
	During Matched Event	1 PC species	↑
		1 PE species	↓
Quarters with SCM vs healthy quarters	During Matched Event	1 PC species	↑
		1 DG species; 1 PE species	↓
Quarters with CM vs healthy quarters	Before Matched Event	1 PC species	↓
	During Matched Event	2 Cer species; 36 PC species; 4 PE species	↑
		1 PI species; 1 PS species; 8 SM species	↑
		1 DG species; 7 PE species; 48 TG species	↓
Healthy quarters among cows			
Healthy quarters of cows with SCM vs healthy quarters of healthy cows	Before Matched Event	3 TG species	↓
	During Matched Event	1 PC species	↑
Healthy quarters of cows with CM vs healthy quarters of healthy cows	Before Matched Event	1 Cer species	↓
	During Matched Event	4 PC species; 1 SM species	↑
		1 PC species; 6 PE species; 2 PI species	↓
Healthy quarters of cows with SCM vs healthy quarters of cows with CM	Before Matched Event	2 Cer species	↓
	During Matched Event	5 PE species; 1 PC species; 2 PI species	↓
Quarters with CM or SCM vs healthy quarters among cows			
Quarters with CM of cows with CM vs healthy quarters of healthy cows	Before Matched Event	1 PE species	↓
	During Matched Event	38 PC species; 2 Cer species	↑
		10 PE species; 2 PI species	↑
		1 PS species; 9 SM species	↑
		11 PE species; 2 PI species; 30 TG species	↓

Table 4-3. cont'd			
Quarters with SCM of cows with SCM vs quarters with CM of cows with CM	During Matched Event	39 PC species; 7 PE species	↑
		2 PI species; 1 PS species; 8 SM species	↑
		8 PE species; 1 PI species; 29 TG species	↓

[1]Quarters with SCM and healthy quarters were matched, as closely as possible, to quarters with CM based on dairy, parity, and d postpartum both before (1-3 d prior to first observance of clinical signs such as blood, flakes in milk and/or redness, swelling of the mammary gland) and during (first observance of clinical signs) the matched event.
[2]Phospholipids: PC = phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, PS= phosphatidylserine
[3]Sphingolipid: SM= sphingomyelin,
[4]Other lipids: Cer = ceramide, DG = diglyceride, TG = triglyceride

Table 4-4: Response of pathways comparing milk produced by healthy (H) quarters of healthy cows, of cows with subclinical mastitis (SCM), and of cows with clinical mastitis (CM) both before and during the matched event using differential features from proteomic and metabolomic analyses among cows[1].

			Response		
Timepoint	**Super Pathway**	**Sub Pathway**	**SCM vs H**	**CM vs H**	**SCM vs CM**
Before Matched Event	Immune System	Fc gamma R-mediated phagocytosis	↑	--	--
		Antigen processing and presentation	↓	--	--
	Transcription	spliceosome	--	↑	--
	Amino Acid Metabolism	Tyrosine metabolism	--	--	↓
	Carbohydrate Metabolism	Glycolysis	--	--	↓
	Translation	ribosome	--	--	↓
During Matched Event	Immune System	NOD-like receptor signaling pathway	↑	--	--
		Complement and coagulation cascades	--	--	↑
	Transport and Catabolism	endocytosis	↓	--	--
		Autophagy and mitophagy	--	↓	--
	Signaling Molecules and Interaction	ECM-receptor interaction	--	↑	--
	Signal Transduction	AMPK signaling pathway	--	↓	--
		Ras signaling pathway	--	↓	--
	Cell Motility	Regulation of actin cytoskeleton	--	↓	--
	Nervous System	Long-term potentiation	--	↓	--
	Metabolism of Cofactors and Vitamins	Vitamin B6 metabolism	--	↓	↓
		Pantothenate and CoA biosynthesis	--	↓	↓
	Carbohydrate Metabolism	Galactose metabolism	--	↓	--
		Pentose phosphate pathway	--	↑	↑
	Translation	ribosome	--	--	↓
		Aminoacyl-tRNA biosynthesis	--	↓	↓
	Amino Acid Metabolism	Glutathione metabolism	--	↓	↓
		Arginine biosynthesis	--	↓	↓
		Histidine metabolism	--	↓	↓

Table 4-4. cont'd	Beta-Alanine metabolism	--	↓	--

[1]Quarters with SCM and healthy quarters were matched, as closely as possible, to quarters with CM based on dairy, parity, and d postpartum both before (1-3 d prior to first observance of clinical signs such as blood, flakes in milk and/or redness, swelling of the mammary gland) and during (first observance of clinical signs) the matched event.

Table 4-5: Response of pathways comparing milk produced by healthy (H) quarters, by quarters with subclinical mastitis (SCM), and by quarters with clinical mastitis (CM) both before and during the matched event using differential features from proteomic and metabolomic analyses among cows[1].

			Response		
Timepoint	**Super Pathway**	**Sub Pathway**	**SCM vs H**	**CM vs H**	**SCM vs CM**
Before Matched Event	Immune System	NOD-like receptor signaling pathway	↑	--	--
	Transport and Catabolism	endocytosis	↓	--	↑
		phagosome	--	--	↑
	Nucleotide Metabolism	Pyrimidine metabolism	--	↓	↓
		Purine metabolism	--	--	↓
During Matched Event	Cellular Community--Eukaryotes	Tight junction	↑	--	↓
		Gap junction	↑	--	↑
		Focal adhesion	--	↑	↑
	Cell Motility	Regulation of actin cytoskeleton	↑	↑	--
	Transport and Catabolism	phagosome	--	--	↑
		endocytosis	--	--	↑
		lysosome	--	↓	↓
		autophagy	--	↓	↓
		peroxisome	--	--	↑
	Immune System	Neutrophil extracellular trap formation	↑	↑	--
		Fc gamma R-mediated phagocytosis	↑	↑	↑
		Leukocyte transendothelial migration	↑	↑	↑
		Antigen processing and presentation	↓	↓	--
		Platelet activation	--	↑	↑
		Complement and coagulation cascades	--	↑	↑
		NOD-like receptor signaling pathway	--	↑	--
		B cell receptor signaling pathway	--	↑	--
		C-type lectin receptor signaling pathway	--	↑	--
	Circulatory System	Vascular smooth muscle contraction	↑	↑	--
	Signal Transduction	HIF-1 signaling pathway	↑	↑	↑
		ErbB signaling pathway	--	↑	--
		VEGF signaling pathway	--	↑	↑
		AMPK signaling pathway	--	↓	↓
		Phospholipase D signaling pathway	--	↓	↓
		Apelin signaling pathway	--	↓	↓
		Sphingolipid signaling pathway	--	↓	↓

Table 4-5. cont'd

		Ras signaling pathway	--	--	↓
	Carbohydrate Metabolism	Glycolysis/gluconeogenesis	↑	--	--
		Pentose phosphate pathway	↑	↑	↑
		Pyruvate metabolism	↑	--	--
		Citric acid cycle	--	↑	↑
		Starch and sucrose metabolism	--	↑	↑
		Amino sugar and nucleotide sugar metabolism	--	↓	↓
		Galactose metabolism	--	↓	↓
		Fructose and mannose metabolism	--	↓	--
		Butanoate metabolism	--	--	↑
	Cell Growth and Death	Cellular senescence	↓	↓	--
		necroptosis	--	↑	--
		apoptosis	--	↑	--
		Cell cycle	--	--	↑
	Signaling Molecules and Interaction	Cell adhesion molecules	↓	↓	--
	Amino Acid Metabolism	Cysteine and methionine metabolism	--	↑	↑
		Alanine, aspartate and glutamate metabolism	--	↑	--
		Tyrosine metabolism	--	↓	--
		Glutathione metabolism	--	↓	↑
		Valine, leucine and isoleucine biosynthesis	--	↑	↑
		Valine, leucine and isoleucine degradation	--	↑	↑
		Phenylalanine, tyrosine and tryptophan biosynthesis	--	↑	↑
		Arginine and proline metabolism	--	↑	↑
		Histidine metabolism	--	--	↑
	Energy Metabolism	Sulfur metabolism	--	↑	--
	Endocrine System	Insulin signaling pathway	--	--	↓
		PPAR signaling pathway	--	↓	--
		Adipocytokine signaling pathway	--	↓	↓
		GnRH signaling pathway	--	--	↓
	Digestive System	Cholesterol metabolism	--	↓	--
	Folding, Sorting and Degradation	proteasome	--	↑	↑
		RNA degradation	--	↑	--
		Protein processing in endoplasmic reticulum	--	↓	--
		SNARE interactions in vesicular transport	--	↓	↓
	Transcription	spliceosome	--	↑	↑
	Glycan Biosynthesis and Metabolism	N-glycan biosynthesis	--	↓	↓
		Other glycan degradation	--	↓	--

Table 4-5. cont'd

	Lipid Metabolism	Fatty acid biosynthesis	--	↓	↓
		Glycerolipid metabolism	--	↓	↓
	Metabolism of Cofactors and Vitamins	Vitamin B6 metabolism	--	↓	--
		Pantothenate and CoA biosynthesis	--	↑	↑
	Nervous System	Long-term depression	--	↓	↓
		Cholinergic synapse	--	↓	↓
		Serotonergic synapse	--	↓	--
	Development and Regeneration	Axon guidance	--	↓	↓
	Replication and Repair	DNA replication	--	--	↑
	Translation	Aminoacyl-tRNA biosynthesis	--	↑	↑

[1]Quarters with SCM and healthy quarters were matched, as closely as possible, to quarters with CM based on dairy, parity, and d postpartum both before (1-3 d prior to first observance of clinical signs such as blood, flakes in milk and/or redness, swelling of the mammary gland) and during (first observance of clinical signs) the matched event.

Table 4-6: Features from machine learning which predict the development of clinical mastitis (CM) by comparing healthy quarters of cows with CM to healthy quarters of cows without CM and by comparing quarters with CM of cows with CM to quarters without CM of cows without CM before the matched event[1].

	Healthy Quarters[2]	**Response**	**Quarters with CM[3]**	**Response**
Proteins	VPS37B subunit of ESCRT-I	↓	VPS37B subunit of ESCRT-I	↓
	Transitional endoplasmic reticulum ATPase	↑	Osteoclast stimulating factor 1 (A)	↓
	Fructose-bisphosphatase	↑	Osteoclast stimulating factor 1 (B)	↓
	Complement C8 gamma chain	↑	N-acetyl-alpha-glucosaminidase	↑
	Cartilage acidic protein 1	↑	Cartilage acidic protein 1	↑
	Alpha-L-iduronidase	↓	Ras-related protein Rab-18	↑
Metabolites	Citric acid	↑	L-isoleucine	↑
	L-leucine	↓	L-tyrosine	↓
	L-tyrosine	↓	L-lysine	↓
	Palatinose	↑	Palatinose	↓
	3-indolelactic acid	↑	Trans-3-hydroxyl-L-proline	↑
Lipids	SM(d18:0/18:2)	↓	SM(d18:0/18:2)	↑
	PC(0:0/18:0)	↓	TG(47:2)	↓
	PC(17:0/17:0);PC(16:0/18:0)	↑		

[1]Quarters with SCM and healthy quarters were matched, as closely as possible, to quarters with CM based on dairy, parity, and d postpartum before the matched event (1-3 d prior to first observance of clinical signs such as blood, flakes in milk and/or redness, swelling of the mammary gland).
[2]Area under the curve (AUC) = 0.763; 95% confidence interval (95% CI) = 0.617 to 0.900
[3]AUC = 0.869; 95% CI = 0.728 to 1.000

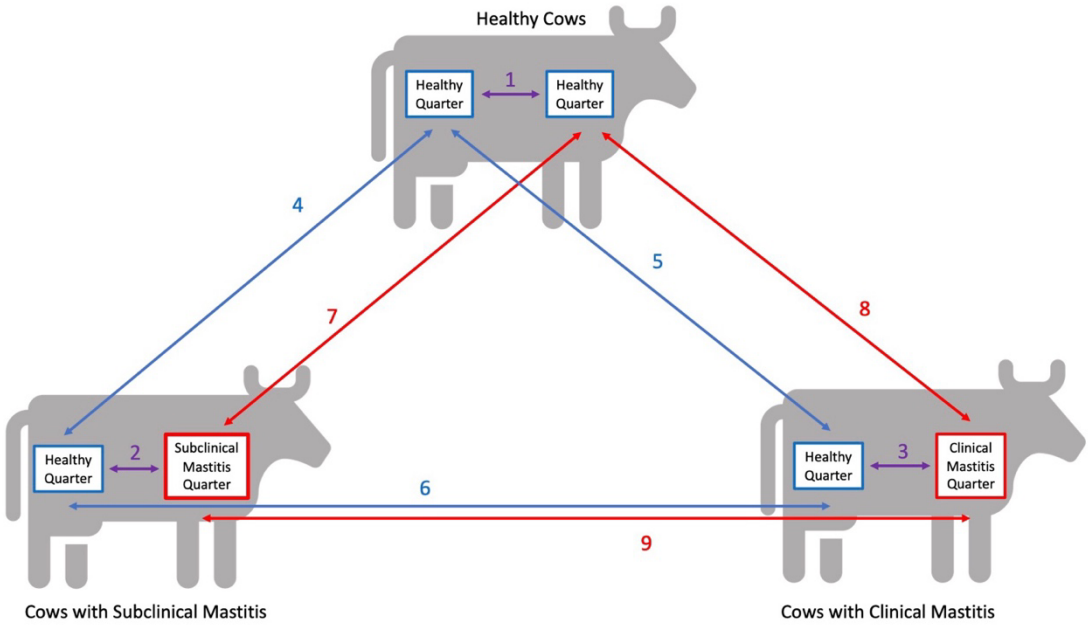


Figure 4-1: Comparisons before the matched event (1-3 d prior to observation of clinical signs such as blood or flakes in milk and/or redness or swelling of the mammary gland) and during the matched event (first observance of clinical signs) were made as shown by the arrows. Blue arrows (→) show comparisons between healthy quarters of healthy cows (n = 20), of cows with subclinical mastitis (SCM; n = 20), and of cows with clinical mastitis (CM; n = 16). Red arrows (→) show comparisons between healthy quarters of healthy cows (n = 20), quarters with SCM of cows with SCM (n = 20), and quarters with CM of cows with CM (n = 16). Purple arrows (→) show quarter comparisons within cows.

Chapter 5: Conclusion

Lactating dairy cows are critical in the production of nutritious milk to feed an ever-growing world population. However, despite decades of research, bovine mastitis presents an enormous challenge in terms of treatment and prevention. The first chapter in this dissertation provides a brief overview of mastitis research and its relationship with milk components from somatic cell count (SCC) to milk bacterial communities to specific milk proteins, metabolites, and lipids. Mastitis can be clinical (CM; presence of clinical signs such as flakes, clots, or blood in milk and/or redness, swelling, or hardness of the mammary gland) or subclinical (SCM; milk SCC ≥200,000 cells/mL and no clinical signs). Milk produced by a healthy mammary gland quarter is generally defined as having an SCC <200,000 cells/mL and no clinical signs. Milk SCC contains mostly white blood cells such as macrophages, lymphocytes, and neutrophils. Milk protein, lactose, sodium (Na), and potassium (K) concentrations and the ratio of Na to K (Na/K) are altered during mastitis. Historically, milk was thought to be sterile, and any bacterial presence was attributed to contamination during collection or during cases of infection (like mastitis). Culture-dependent methods established presence of bacteria and were used for isolation and characterization of known bacteria particularly related to mastitis. However, sometimes these methods fail to grow bacteria in milk collected from cows with mastitis. Culture-independent methods based on the 16S rRNA gene, which is present in all bacteria, aid in identifying bacterial pathogens for every case of mastitis. Bovine milk microbiome research indicates bacterial communities differ in milk collected from healthy cows compared to milk collected from cows with CM or SCM. Multiomic methods, including proteomics, metabolomics, and lipidomics, allow for a more in-depth analysis and comparison of specific milk proteins, lipids, and metabolites in milk produced by healthy quarters, by quarters with SCM, and by quarters with CM. Milk casein and whey proteins; free fatty acids and amino acids; sugars, and different phospholipid classes are typically altered during mastitis.

The second chapter of this dissertation examines the differences in colostrum/milk SCC; protein, lactose, Na, and K concentrations; and Na/K among quarters with mastitis (CM and SCM) and healthy quarters. An SCC threshold of 564,500 cells/mL and a K concentration threshold of 21.10 mmol/L accurately distinguished colostrum collected from quarters with CM from colostrum collected from quarters without CM. An Na/K threshold of 1.10 distinguished, with the highest accuracy, milk collected from quarters with CM from milk collected from healthy quarters. An Na/K threshold of 0.51 distinguished, with the highest accuracy, milk collected from quarters with SCM from milk collected from healthy quarters. In the age of automatic milking systems and shifting management styles, this study provides the dairy industry with information to aid in the detection of

CM and SCM in individual mammary gland quarters of early lactation cows. The third chapter of this dissertation explores differences in milk bacterial communities among samples collected from healthy quarters, from quarters with SCM, and from quarters with CM at two timepoints (before the matched event and during the matched event). Understanding how bacteria in milk impact CM and SCM incidence, or vice versa, is of immense interest. The relative abundance of *Atopostipes* was different between milk collected from healthy quarters and milk collected from quarters with CM. The relative abundance of *Jeotgalicoccus* was higher in milk samples collected before the matched event compared to milk samples collected during the matched event among all mammary gland quarter health status groups. *Staphylococcus* was in higher relative abundance in milk collected from quarters with SCM compared to milk collected from healthy quarters and from quarters with CM. Curiously, *Bifidobacterium* was identified in high relative abundance in milk samples, regardless of mammary gland quarter health status (CM, SCM, or healthy). This study aids mastitis research and the dairy industry in understanding how mastitis incidence, management differences, and environmental influences impact milk microbiota. The fourth chapter of this dissertation provides a closer look at changes in specific proteins, metabolites, and lipids in milk produced by quarters with mastitis (CM and SCM) as well as identified predictive features of CM. Many proteins and metabolites related to immune function were at higher levels in milk produced by quarters with either CM or SCM. In milk produced by quarters with CM, phospholipids abundant in cell membranes were at higher levels and triacylglycerols were at lower levels relative to milk produced by healthy quarters. Lipid differences were less noticeable in milk produced by quarters with SCM. Machine learning identified certain features such as L-leucine, three phosphatidylcholine species, L-lysine, and a phosphatidylinositol species, that may predict CM in individual mammary gland quarters with reasonable accuracy. This knowledge allows mastitis research and the dairy industry to better understand the cow's biological response which can be used to aid in detection of CM and SCM and prediction of CM.

Despite such a large dataset, some aspects were not able to be fully explored due to relatively low numbers of samples collected from quarters with SCM and even lower numbers of samples collected from quarters with CM. Overwhelmingly, the cows sampled on each dairy were healthy with rates of CM ranging 4.5-9.3%, which is a good thing from an industry perspective but provides limitations in research. Bacterial culturing was performed on the milk samples collected from quarters with CM to identify common mastitis pathogens, but most results were inconclusive. Cultured samples with predominantly *Escherichia coli* tended to match the most abundant taxa, *Escherichia-Shigella* identified in those same CM samples via full-length 16S rRNA gene sequencing. Metagenomic analysis was also performed on some of the same milk samples and, although 16S rRNA sequencing and metagenomic sequencing are different methods, a few of the taxa were identified as highly

abundant in both methods. Due to insufficient sequencing depth, functional annotation was not achievable in the metagenomic analysis. Future studies with greater cow numbers and/or a longer sampling period to boost mastitis sample numbers are warranted to answer some of these questions.

The studies in this dissertation evaluate naturally occurring mastitis and its relationship with general milk components, milk microbiome, and specific proteins, metabolites, and lipids in milk collected daily from individual mammary gland quarters of cows in early lactation on four commercial dairies in southern Idaho. A unique aspect of the studies in this dissertation is the large sample number that allowed for performing the various analyses described (full-length 16S rRNA gene sequencing, proteomics, metabolomics, and lipidomics) on the same set of milk samples. Furthermore, this work provides a snapshot of the similarities and differences among commercial dairy farms located in the vibrant Magic Valley dairy community of southern Idaho. This framework could be applied to other regions to not only benefit the dairy industry but also provide valuable information on specific communities worldwide. This research contributes to the continued advancement of bovine mastitis management using newer technologies to provide a deeper understanding of the complexities of the milk environment that will be imperative to ensuring the health of cows, the sustainability of dairy farm family livelihoods, and the reliability of a nutritious food source.

Appendix A: Protocol Approval Letter

University of Idaho
Office of Research Assurances
Institutional Animal Care and Use Committee
875 Perimeter Drive, MS 3010
Moscow, ID 83844-3010
Phone: 208-885-6162
Fax: 208-885-6014
Email: iacuc@uidaho.edu

Date: February 21, 2019
To: Mark A. McGuire
From: University of Idaho Institutional Animal Care and Use Committee
Re: Protocol IACUC-2018-66 *Mammary and milk microbiomes and metabolomes - Understanding early variation and impacts on risk for mammary inflammation and mastitis*

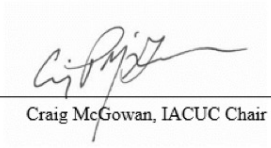
Your requested amendment of the animal care and use protocol listed above was reviewed and approved by the Institutional Animal Care and Use Committee on 02/21/2019.

This amendment request, 004704, was submitted for review on: 02/21/2019 10:18:31 AM PST
The original approval date for this protocol was: 11/16/2018
This approval will remain in effect until: 02/20/2020
The protocol may be continued by annual updates until: 11/15/2021

Currently approved internal personnel on this protocol are: McGuire, Mark A.; McGuire, Michelle; Peterson, Haley; Williams, Janet

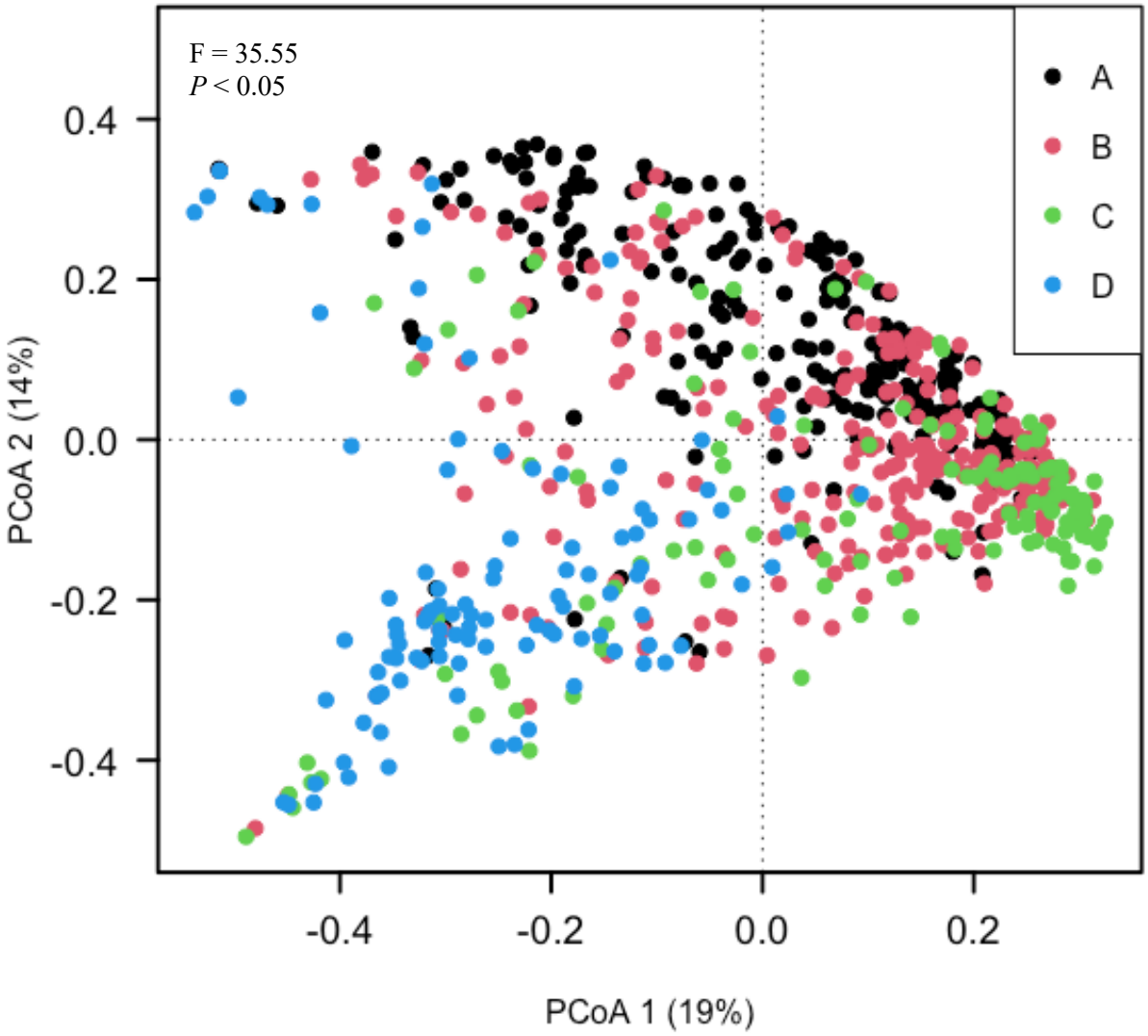
Currently approved external personnel on this protocol are: Matt Luth

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

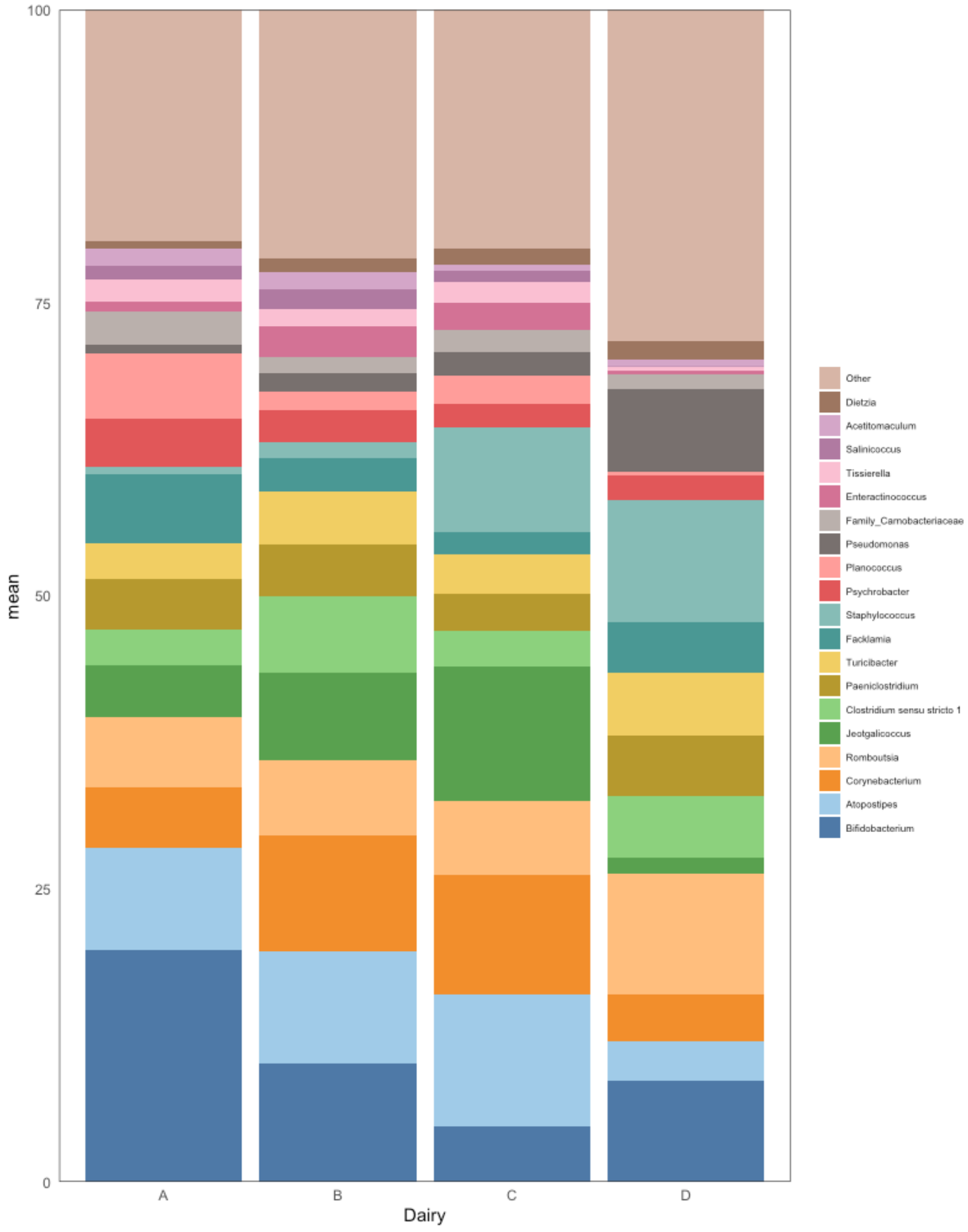


Craig McGowan, IACUC Chair

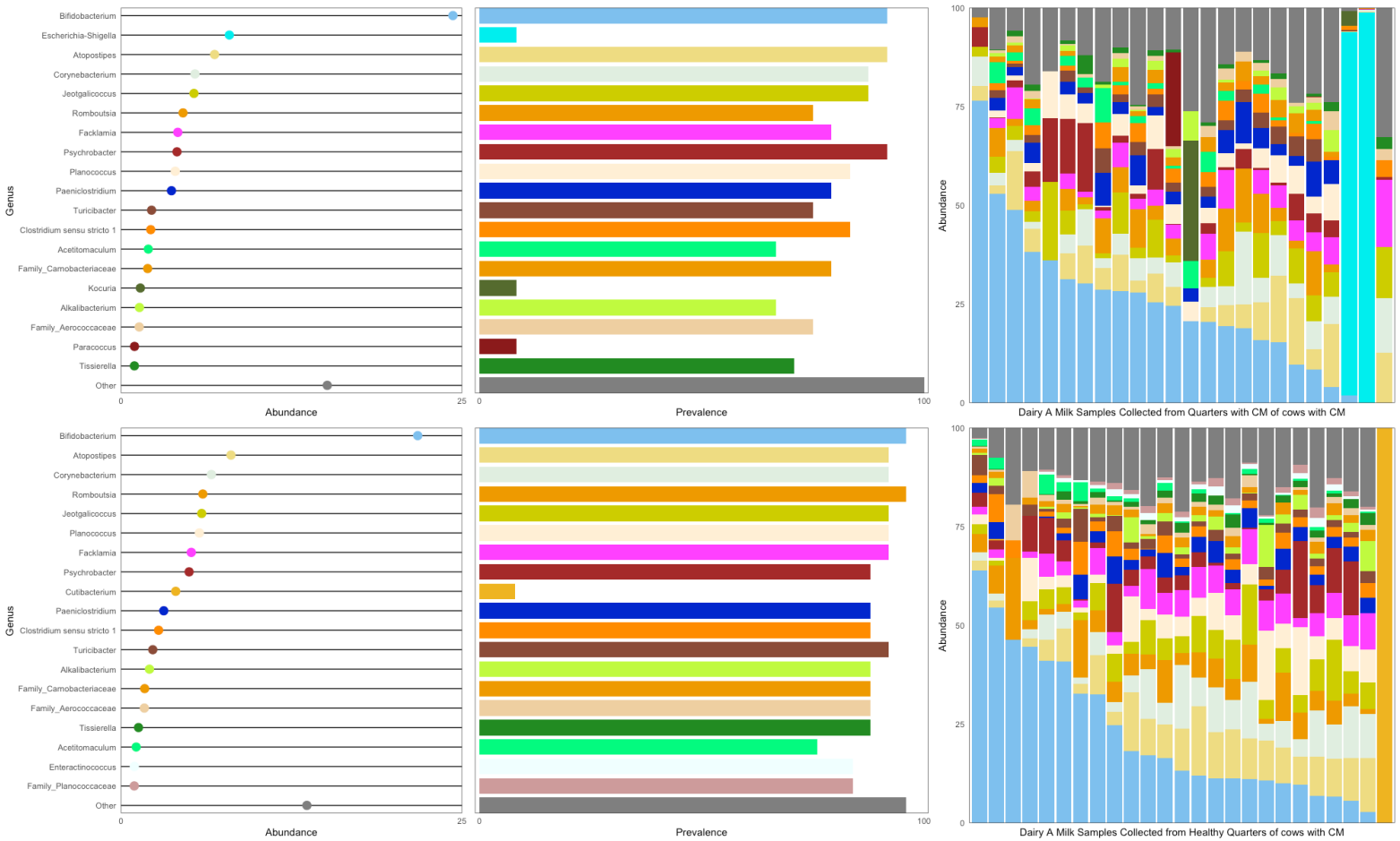
Appendix B: Milk Microbiome Differences Among Dairies



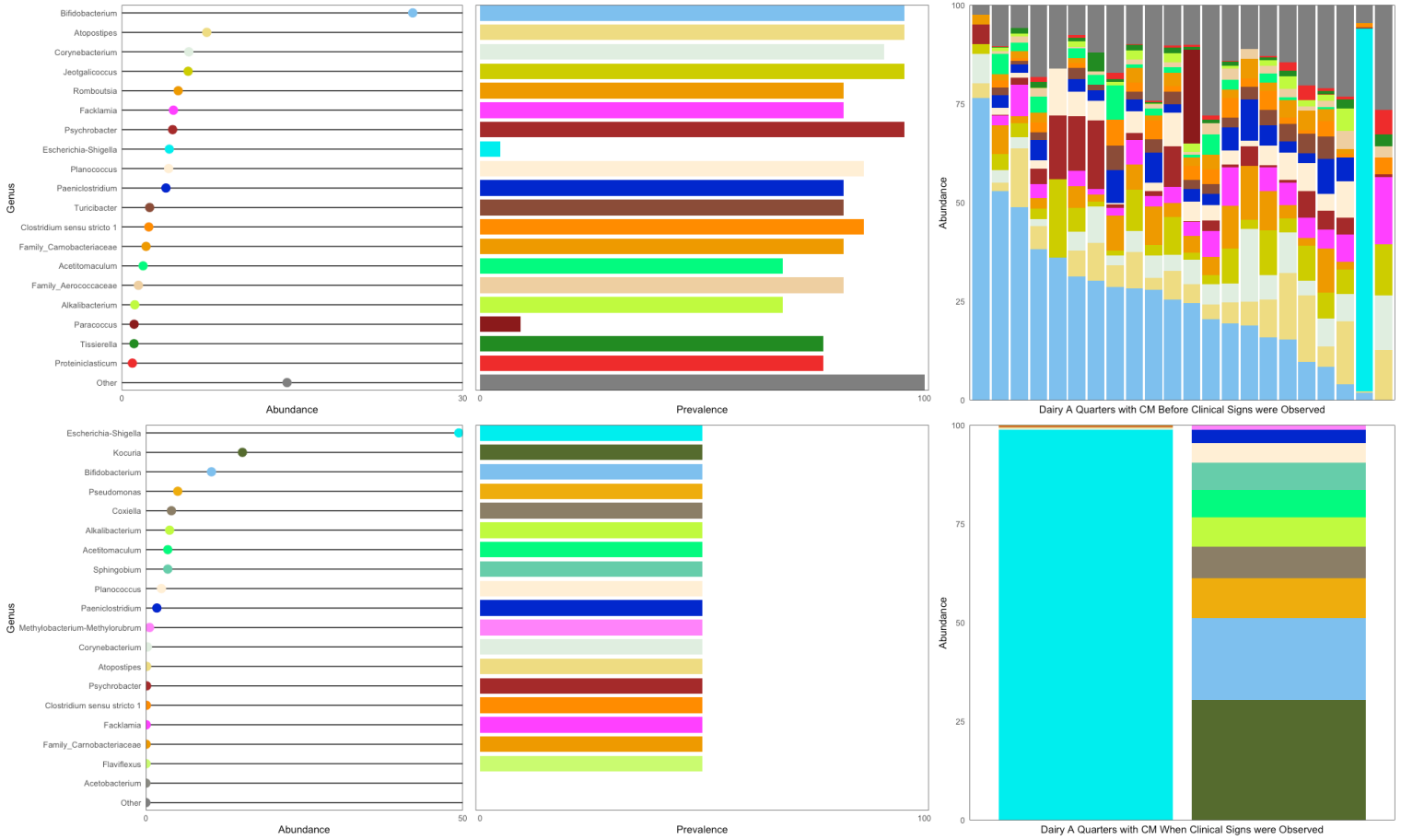
Appendix B Figure 1: Principal coordinate analysis (PCoA) of the top 20 bacterial taxa of milk samples collected grouped by dairy. The four dairies are represented by 'A', 'B', 'C', and 'D'. Results of permutational multivariate analysis of variance (PERMANOVA) are shown in the PCoA plot. Dairies A and B are different (P=0.003). Dairies A and C are different (P<0.05). Dairies A and D are different (P<0.05). Dairies B and C tended to be different (P=0.086). Dairies B and D are different (P<0.05). Dairies C and D are different (P<0.05).



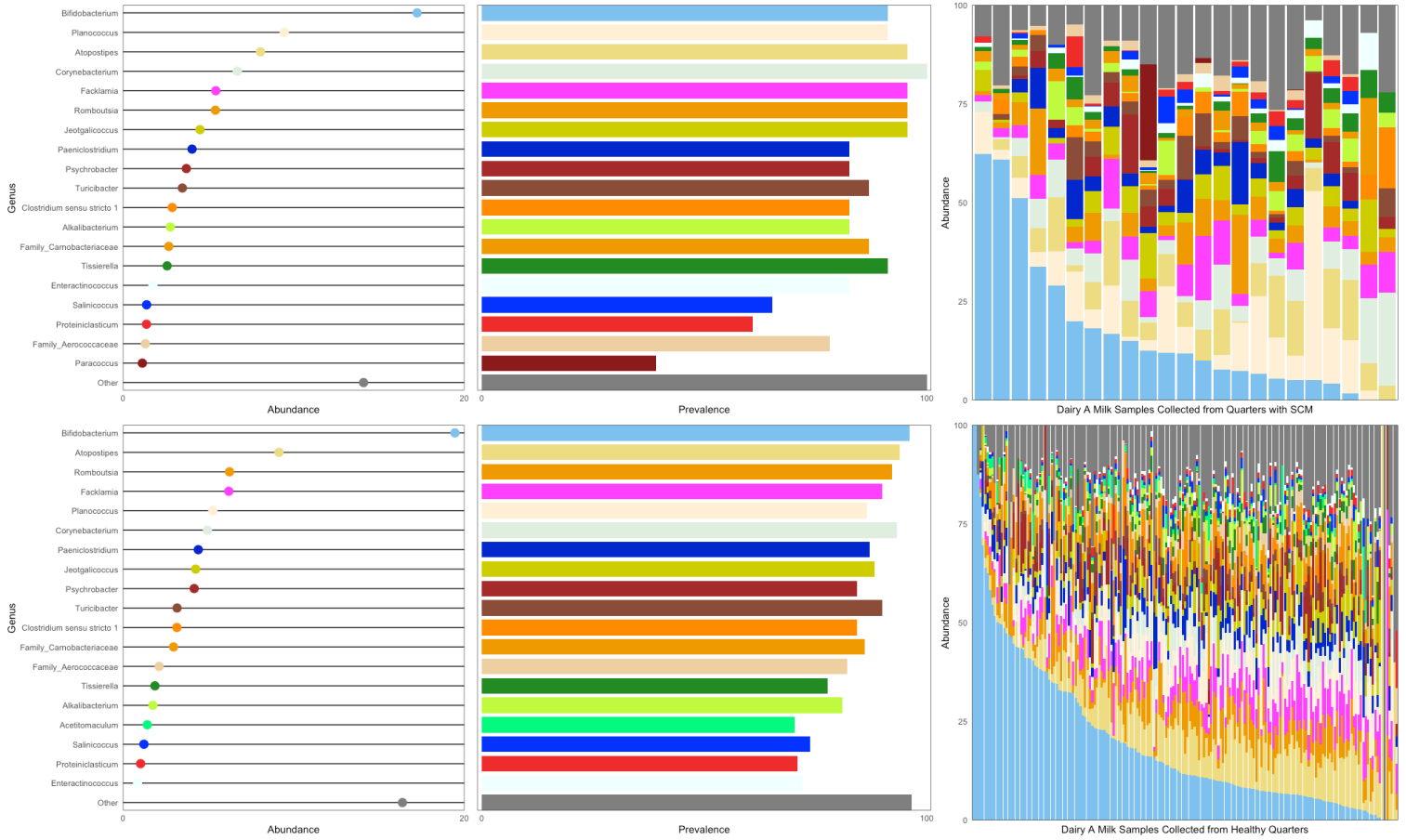
Appendix B Figure 2: Mean (relative abundance) of the top 20 bacterial taxa from milk samples collected grouped by dairy.



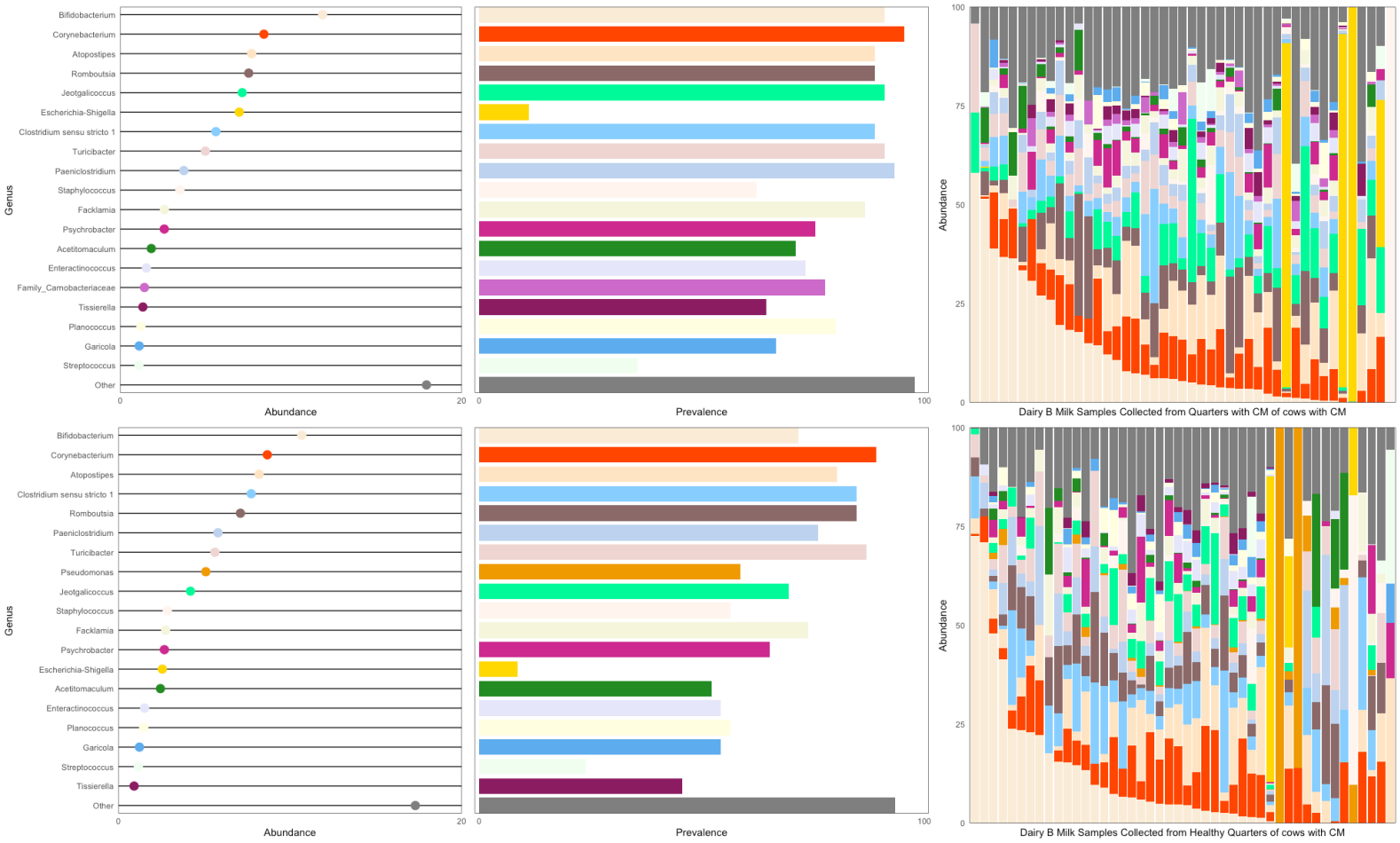
Appendix B Figure 3: The top 20 bacterial taxa by overall relative abundance, prevalence, and individual sample relative abundance across milk samples collected from quarters with clinical mastitis (CM; presence of clinical signs such as blood or flakes in milk and/or redness or swelling of the mammary gland) and from healthy quarters [somatic cell count (SCC) <200,000 cells/mL and no clinical signs] within cows with CM from Dairy A. The top five taxa (relative abundance) identified in milk samples collected from quarters with CM were: *Bifidobacterium* (24.3%), *Escherichia-Shigella* (7.9%), *Atopostipes* (6.9%), *Corynebacterium* (5.4%) and *Jeotgalicoccus* (5.4%). The top five taxa identified in milk samples collected from healthy quarters within the same cow were: *Bifidobacterium* (21.8%), *Atopostipes* (8.1%), *Corynebacterium* (6.6%), *Romboutsia* (6.0%), and *Jeotgalicoccus* (5.9%).



Appendix B Figure 4: The top 20 bacterial taxa by overall relative abundance, prevalence, and individual sample relative abundance across milk samples collected from quarters with CM before clinical signs were observed and from quarters with CM when clinical signs were observed within cows with CM from Dairy A. The top five taxa identified in milk samples collected quarters with CM before clinical signs were observed were: *Bifidobacterium* (25.6%), *Atopostipes* (7.5%), *Corynebacterium* (5.9%), *Jeotgalicoccus* (5.8%), and *Romboutsia* (5.0%). The top five taxa identified in milk samples collected from quarters with CM when clinical signs were first observed were: *Escherichia-Shigella* (49.4%), *Kocuria* (15.2%), *Bifidobacterium* (10.3%), *Pseudomonas* (5.0%), and *Coxiella* (4.0%).

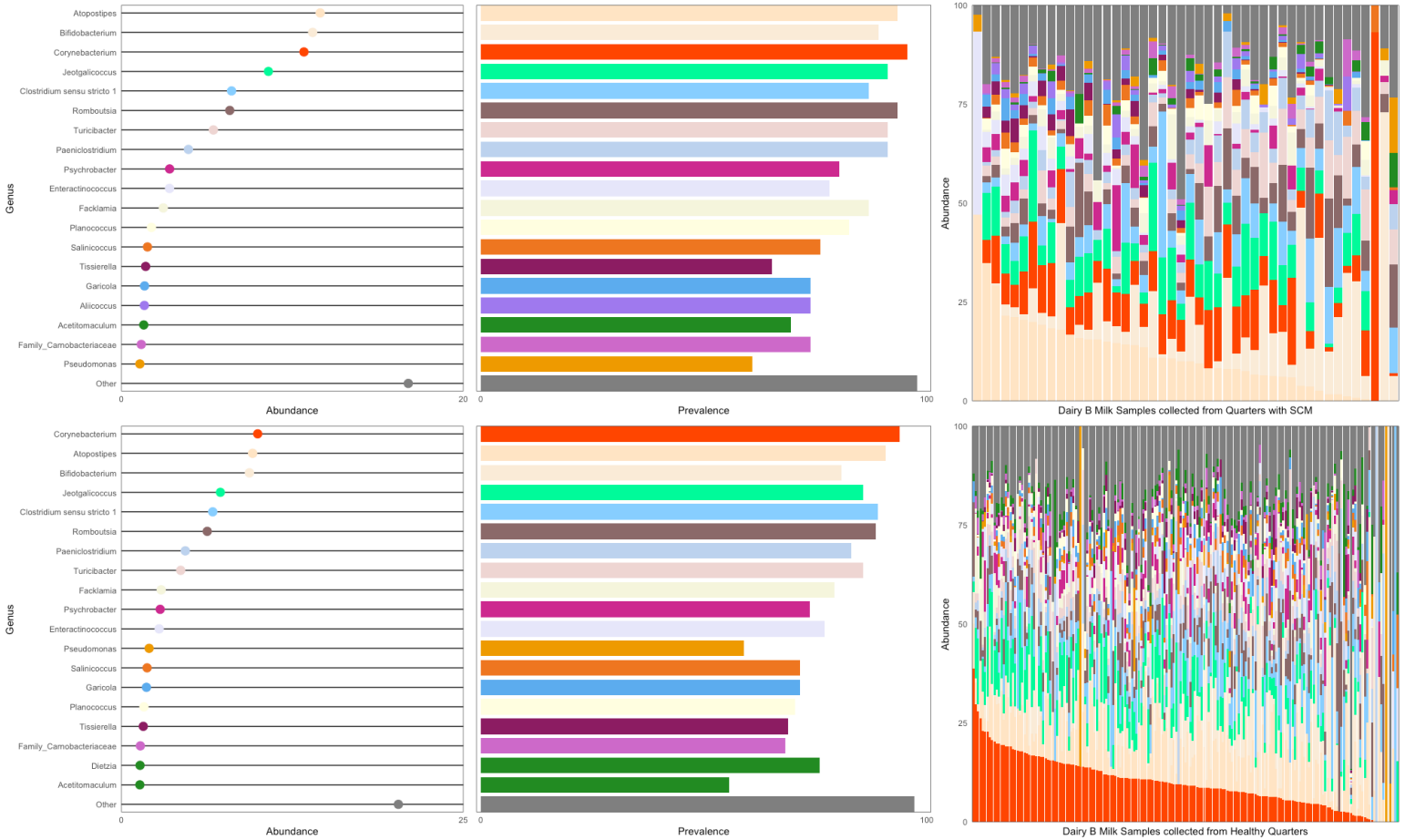


Appendix B Figure 5: The top 20 bacterial taxa by overall relative abundance, prevalence, and individual sample relative abundance across milk samples collected from quarters with subclinical mastitis (SCM; SCC ≥200,000 cells/mL and no clinical signs) and from healthy quarters (SCC <200,000 cells/mL and no clinical signs) from Dairy A. The top five taxa identified in milk samples collected from quarters with SCM were: *Bifidobacterium* (17.2%), *Planococcus* (9.5%), *Atopostipes* (8.1%), *Corynebacterium* (6.7%), and *Facklamia* (5.4%). The top five taxa identified in milk samples collected from healthy quarters were: *Bifidobacterium* (19.4%), *Atopostipes* (9.1%), *Romboutsia* (6.2%), *Facklamia* (6.2%), and *Planococcus* (5.3%).

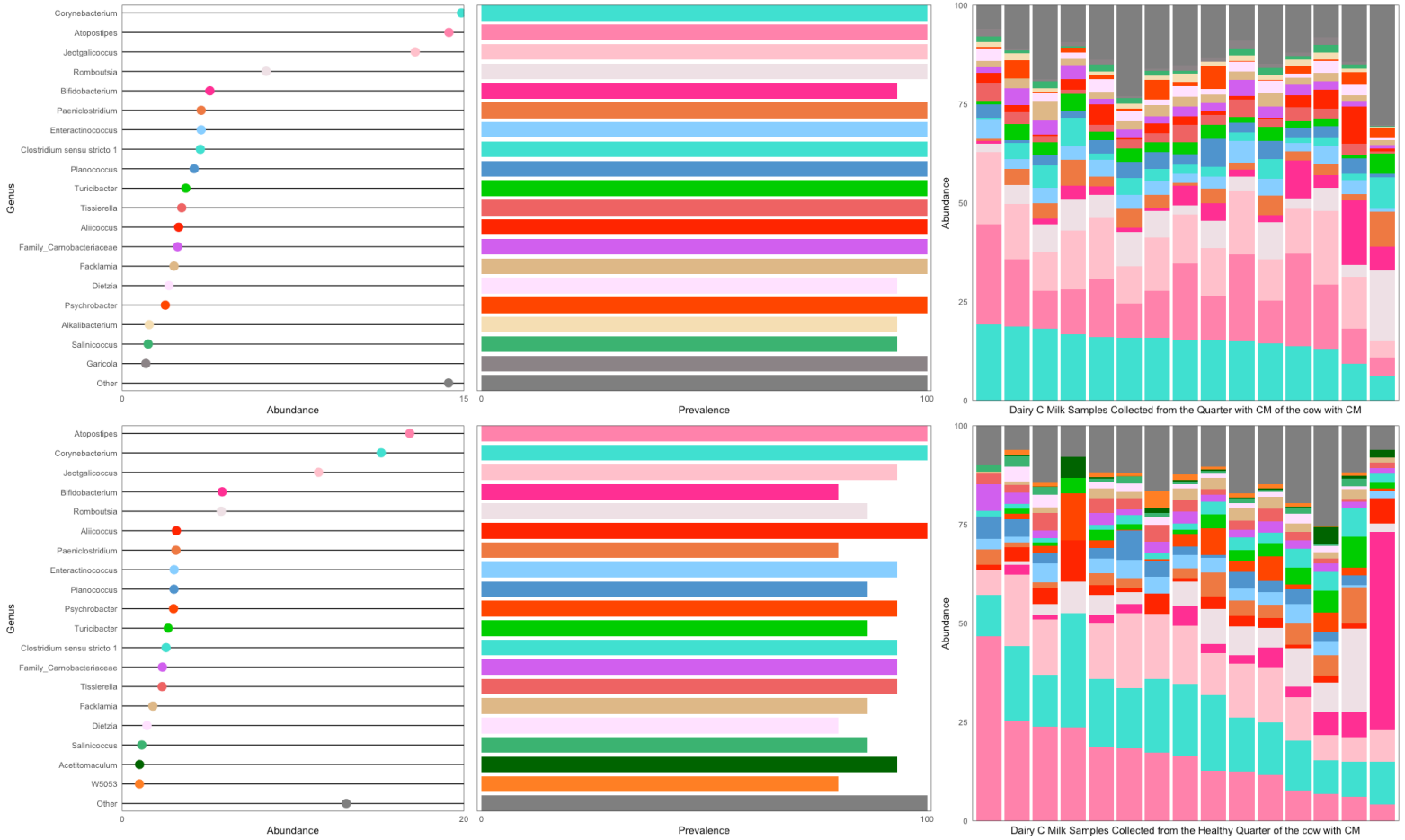


Appendix B Figure 6: The top 20 bacterial taxa by overall relative abundance, prevalence, and individual sample relative abundance across milk samples collected from quarters with CM and from healthy quarters within cows with CM from Dairy B. The top five taxa identified in milk samples collected from quarters with CM were: *Bifidobacterium* (11.9%), *Corynebacterium* (8.4%), *Atopostipes* (7.7%), *Romboutsia* (7.5%), and *Jeotgalicoccus* (7.1%). The top five taxa identified in milk samples collected from healthy quarters were: *Bifidobacterium* (10.7%), *Corynebacterium* (8.7%), *Atopostipes* (8.2%), *Clostridium sensu stricto 1* (7.7%), and *Romboutsia* (7.1%).

Appendix B Figure 7: The top 20 bacterial taxa by overall relative abundance, prevalence, and individual sample relative abundance across milk samples collected from quarters with CM before clinical signs were observed and from quarters with CM when clinical signs were observed within cows with CM from Dairy B. The top five taxa identified in milk samples collected from quarters with CM before clinical signs were observed were: *Bifidobacterium* (12.2%), *Corynebacterium* (8.7%), *Atopostipes* (8.1%), and *Jeotgalicoccus* (7.6%), and *Romboutsia* (7.6%). The top five taxa identified in milk samples collected from quarters with CM when clinical signs were first observed were: *Escherichia-Shigella* (58.8%), *Bifidobacterium* (7.0%), *Romboutsia* (6.8%), *Corynebacterium* (4.9%), and *Clostridium sensu stricto 1* (3.9%).

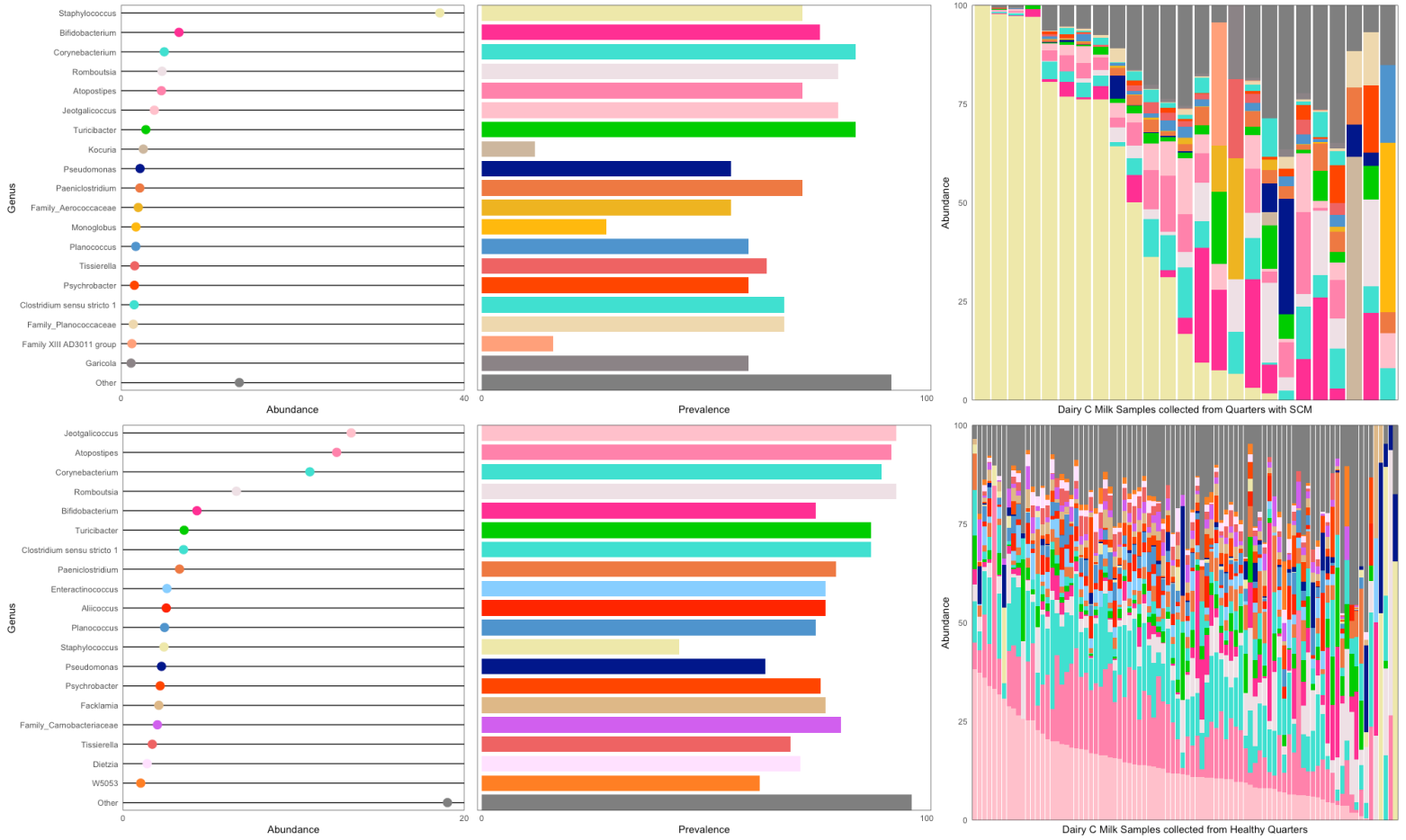


Appendix B Figure 8: The top 20 bacterial taxa overall relative abundance, prevalence, and individual sample relative abundance across milk samples collected from quarters with SCM and from healthy quarters from Dairy B. The top five taxa identified in milk samples collected from quarters with SCM were: *Atopostipes* (11.6%), *Bifidobacterium* (11.2%), *Corynebacterium* (10.7%), *Jeotgalicoccus* (8.6%), and *Clostridium sensu stricto 1* (6.4%). The top five taxa identified in milk samples collected from healthy quarters were: *Corynebacterium* (10.0%), *Atopostipes* (9.6%), *Bifidobacterium* (9.4%), *Jeotgalicoccus* (7.2%), and *Clostridium sensu stricto 1* (6.7%).



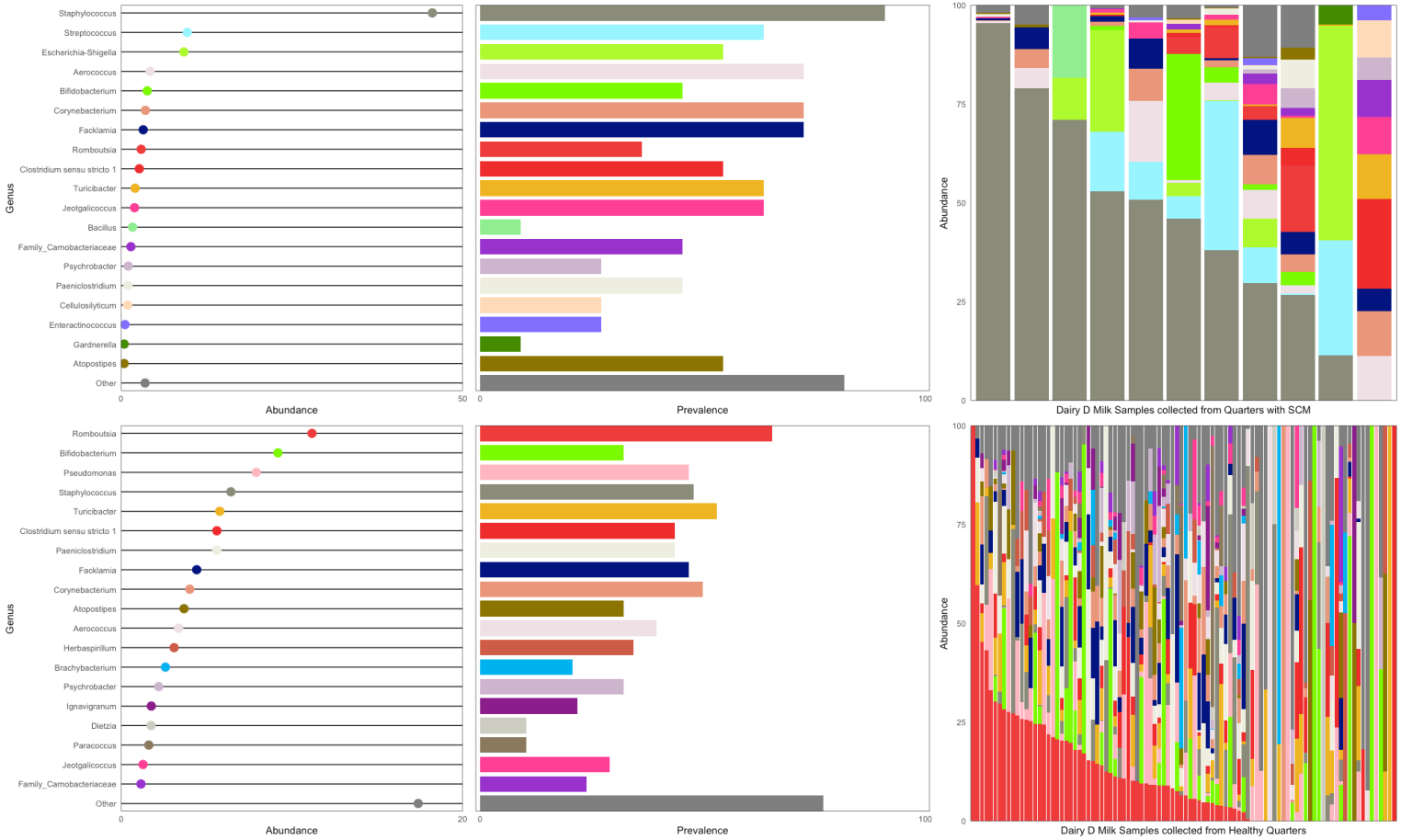
Appendix B Figure 9: The top 20 bacterial taxa by overall relative abundance, prevalence, and individual sample relative abundance across milk samples collected from the quarter with CM and from the healthy quarter within the cow with CM from Dairy C. The top five taxa identified in milk samples collected from the quarter with CM were: *Corynebacterium* (14.9%), *Atopostipes* (14.3%), *Jeotgalicoccus* (12.9%), *Romboutsia* (6.3%), and *Bifidobacterium* (3.9%). The top five taxa identified in milk samples collected from the healthy quarter were: *Atopostipes* (16.8%), *Corynebacterium* (15.2%), *Jeotgalicoccus* (11.5%), *Bifidobacterium* (5.9%), and *Romboutsia* (5.8%).

Appendix B Figure 10: The top 20 bacterial taxa by overall relative abundance, prevalence, and individual sample relative abundance across milk samples collected from the quarter with CM before clinical signs were observed and from the quarter with CM when clinical signs were observed within the cow with CM from Dairy C. The top five taxa identified in milk samples collected from the quarter with CM before clinical signs were observed were: *Corynebacterium* (14.8%), *Atopostipes* (14.7%), *Jeotgalicoccus* (13.1%), *Romboutsia* (6.2%), and *Bifidobacterium* (4.0%). The top five taxa identified in the milk sample collected from the quarter with CM when clinical signs were first observed were: *Corynebacterium* (15.9%), *Jeotgalicoccus* (9.5%), *Atopostipes* (8.7%), *Romboutsia* (8.6%), and *Paeniclostridium* (4.7%).



Appendix B Figure 11: The top 20 bacterial taxa by overall relative abundance, prevalence, and individual sample relative abundance across milk samples collected from quarters with SCM and from healthy quarters from Dairy C. The top five taxa identified in milk samples collected from quarters with SCM were: *Staphylococcus* (37.1%), *Bifidobacterium* (6.8%), *Corynebacterium* (5.0%), *Romboutsia* (4.8%), and *Atopostipes* (4.7%). The top five taxa identified in milk samples collected from healthy quarters were: *Jeotgalicoccus* (13.4%), *Atopostipes* (12.5%), *Corynebacterium* (11.0%), *Romboutsia* (6.7%), and *Bifidobacterium* (4.3%).

Appendix B Figure 12: The top 20 bacterial taxa by overall relative abundance, prevalence, and individual sample relative abundance in the milk sample collected the healthy quarter within the cow with CM from Dairy D. The cow with CM was diagnosed on 0 d postpartum so it was not possible to compare samples before clinical signs were observed to samples collected when clinical signs were observed. The top five taxa identified in the milk sample collected from the healthy quarter were: *Facklamia* (21.6%), *Aerococcus* (14.6%), *Corynebacterium* (10.7%), *Bifidobacterium* (8.9%), and *Staphylococcus* (8.4%).



Appendix B Figure 13: The top 20 bacterial taxa by overall relative abundance, prevalence, and individual sample relative abundance across milk samples collected from quarters with SCM and from healthy quarters from Dairy D. The top five taxa identified in milk samples collected from quarters with SCM were: *Staphylococcus* (45.6%), *Streptococcus* (9.7%), *Escherichia-Shigella* (9.2%), *Aerococcus* (4.3%), and *Bifidobacterium* (3.8%). The top five taxa identified in milk collected from healthy quarters were: *Romboutsia* (11.2%), *Bifidobacterium* (9.2%), *Pseudomonas* (7.9%), *Staphylococcus* (6.4%), and *Turicibacter* (5.8%).