ABSTRACT

Title of Document: PROTEOMIC PROFILING AND LABEL-FREE QUANTIFICATION OF BOVINE MILK PROTEINS DURING EXPERIMENTALLY INDUCED *ESCHERICHIA COLI* MASTITIS

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Coliform mastitis has been a primary focus of dairy cattle disease research due to staggering affiliated losses, severe systemic complications arising from host inflammatory response to lipopolysaccharide, and the poor response of coliform pathogens to antimicrobials. Reliable biomarkers are needed to evaluate the efficacy of adjunctive therapies for the treatment of inflammation associated with coliform mastitis, and to aid in the approval of new veterinary drugs. The aims of the current analyses were to utilize proteomic methodologies to evaluate protein expression in whey from cows with experimentally induced coliform mastitis, and to employ label-free quantification strategies to estimate changes in relative abundance of proteins

identified in milk over the course of clinical infection. Two-dimensional gel electrophoresis followed by matrix-assisted laser desorption/ionization time-of-flight (MALDI- TOF) mass spectrometry (MS) resulted in the identification of complement factors, antimicrobial proteins, and acute phase proteins in mastitic milk. Analysis using liquid chromatography (LC) inline with electrospray ionization - quadrupole TOF tandem mass spectrometry (MS/MS) resulted primarily in the identification of abundant whey and casein proteins, and the transient detection of proteins related to host response. Nano-LC- nanospray-MS/MS using a linear ion trap, however, led to the robust discovery of over fifty inflammatory proteins in whey from mastitic milk, including the novel markers kininogen-2 and inter-alpha trypsin inhibitor heavy chain-4. Normalized spectral counts were compared to enzyme-linked immunosorbant assay (ELISA) for select proteins to assess the accuracy of the spectral count data. Similar expression patterns were detected using spectral counts and ELISA. Results indicate that proteomic methodologies can detect biomarkers of coliform mastitis in bovine milk during clinical infections, and that spectral counts are a viable means of evaluating relative changes in protein biomarkers of mastitis, including those for which no antibody currently exists.

PROTEOMIC PROFILING AND LABEL-FREE QUANTIFICATION OF BOVINE MILK PROTEIN EXPRESSION DURING EXPERIMENTALLY INDUCED *ESCHERICHIA COLI* MASTITIS

By

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctorate of Philosophy
2009

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Preface

The research detailed in the following dissertation is part of an on-going joint effort between two offices within the U.S Food and Drug Administration's Center for Veterinary Medicine (CVM), the Office of Research (OR), and the Office of New Animal Drug Evaluation (ONADE). The primary goal of this research collaborative is to identify reliable biomarkers of inflammation associated with disease in food producing animals for the purpose of establishing industry guidance for the evaluation of the efficacy of new animal drugs that have claims to treat inflammation. As a part of the continued efforts to identify biomarkers of food animal disease, the following dissertation describes preliminary investigations designed primarily for the purposes of method development and discovery, which were conducted in collaboration with the Department of Animal and Avian Sciences at the University of Maryland.

Dedication

I have been fortunate throughout the course of my academic and professional career to have worked with, and been under the guidance of, some truly accomplished and inspiring professors and scientists. To all of my colleagues and mentors who have contributed to my career development, especially those who have provided guidance over the past four years, I extend my sincere gratitude. I would not be where I am today, however, had it not been for one man in particular, Dr. Lee Majeskie, who first encouraged my love of dairy science. Though I had not a shred of experience or knowledge of cows when I enrolled in his Dairy Cattle Management class as an undergraduate over a decade ago, Dr. Majeskie orchestrated opportunities for me to gain additional knowledge and practical skills working with dairy cattle, occasions that later afforded me career options in both the dairy industry and academia. I am forever indebted, and would like to dedicate my dissertation to the memory of my very first academic mentor, Dr. Lee Majeskie, to whom I owe all of my experiences with dairy cattle, and with whom I shared many laughs along the way.

Acknowledgements

There are a number of entities and individuals I would like to acknowledge as having provided financial, intellectual, or technical support throughout the course of the research that comprises the following dissertation. First I must recognize the Food and Drug Administration Center for Veterinary Medicine for funding and supporting my research efforts over the past 4 years, and the Department of Animal and Avian Sciences for accommodating an unconventional graduate student arrangement, and for providing the use of the University dairy herd.

I would also like to thank my committee, Dr. Bob Peters, Dr. Doug Bannerman, Dr. Jeffrey Ward, Dr. Catherine Fenselau, and Dr. Rich Erdman for their support and intellectual input. I was very fortunate to have a diverse committee comprised of accomplished scientists with an array of expertise who provided meaningful guidance. In particular, I would like to extend my gratitude to my academic advisor, Dr. Bob Peters, who graciously assumed the role of chair of my committee, and whose organization and experience most certainly kept me on track! A special thanks as well to my co-advisor Dr. Doug Bannerman for all of his advice and scientific support throughout the duration of my PhD research. Dr. Bannerman gave an inordinate amount of his time and has been most instrumental in my research and career development. I would not have had the courage to undertake graduate studies, however, had it not been for the encouragement and support of my CVM mentor, Dr. Jeffrey Ward. The day-to-day technical advice and guidance provided by Dr. Ward often served as my “second wind”, and I most certainly would never have come so far in my life, my studies, or my career without his influence.

I am also extremely grateful to Dr. John Callahan at the FDA Center for Food Safety and Applied Nutrition not only for providing the necessary instrumentation, but also for offering me the support of his research group, who became my lifeline for all things chromatography and mass spectrometry. I want to thank Dr. Kevin Shefcheck for his unbelievable patience and sense of humor in the early days when I did not know the difference between an autosampler and an ionization source, and for dedicating so much of his time to helping and educating me. I also want to thank Dr. Melinda McFarland for providing the MassSieve software, for assisting me with all of the semiquantitative analyses, and for teaching me the art of informatics. As well, I want to thank Dr. Jeff DeGrasse, for the unbelievable amount of support and technical assistance he provided on the ion-trap data, and for always being flexible. Drs. Callahan, Shefcheck, McFarland, and DeGrasse have been my second committee, and without "Team Awesomeness", my dissertation would never have been completed.

Finally, and most importantly, I want to thank my family, especially my husband Gregg, for 4 years of love, support, and tolerance of all my "bad behavior" when the stress reached maximum levels. Gregg took care of all aspects of my life that fell by the wayside while my research and job responsibilities consumed me, which was just about everything from laundry to oil changes in my car. There really aren't any words to adequately express the true depth of my appreciation and gratitude, so I will simply say thank you, and enjoy the Phelps Insignia!

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Chapter 1: Introduction

Mastitis, or intramammary inflammation, is a disease of primary significance in agricultural research due to staggering direct and affiliated economic losses, the inability of the host to eradicate causative pathogens, and the absence of efficacious treatment options when certain bacterial strains are the causative agent (DeGraves and Fetrow, 1993; Seegers et al., 2003). Manifestations of mastitis include decreased milk production, compositional changes in the milk, abnormal milk appearance, swelling and pain in the udder, elevated rectal temperature, depression, and decreased feed intake (Harmon, 1994). The host immune response elicited by the invading bacteria is variable, however, and can fluctuate in severity depending on the bacterial strain (Bannerman et al., 2004; Petzl et al., 2008).

Mastitis that results from the invasion of gram-negative environmental bacteria is of particular concern to producers and practitioners, due primarily to the presence of lipopolysaccharide (LPS), a toxin that is shed from the outer wall of gram negative pathogens and that induces a rapid inflammatory response in the bovine mammary gland (Paape et al., 1996; Lee et al., 2003). The profound inflammation and affiliated deleterious secondary effects caused by LPS are difficult to alleviate, and to date no entirely efficacious treatments exist. Antibiotics are not effective in treating coliform mastitis (Hogan et al., 1989; Wilson et al., 1999; Erskine et al., 2000), and while vaccines against coliforms are commercially available, immunization with the vaccines based on *E. coli* J5 bacterins only appeared to augment bacterial clearance rates in infected glands (Hogan et al., 1992; Tomita et al., 2000; Wilson et al., 2007). The only treatment that has shown promise as an adjunctive therapy for coliform

mastitis is the use of non-steroidal anti-inflammatory drugs or NSAIDs (Anderson, 1989; Lohuis et al., 1991; Vangroenweghe et al., 2005), but due to the lack of valid criteria to evaluate efficacy, only the nonselective NSAID Banamine® has been approved for use in lactating dairy cattle.

Because few viable treatment options are available, a tremendous amount of research has been dedicated to elucidating the mechanisms and mediators involved in the host response of the bovine mammary gland to infection with gram- negative pathogens. Because of the prevalence of *Escherichia coli* in the environment of dairy cattle, and the frequency of coliform mastitis, *E. coli* has been used experimentally to induce mastitis (Hirvonen et al., 1999; Riollet et al., 2000; Bannerman et al., 2004). Several inflammatory mediators have been reported to increase significantly following experimental induction of coliform mastitis with *E. coli* including: the cytokines tumor necrosis factor-alpha (TNFα), interleukin-1 beta (IL-1β), interleukin-8 (IL-8), and interleukin-6 (IL-6; Bannerman et al., 2004); several acute phase proteins including serum amyloid A (SAA), haptoglobin (Hp), and alpha-1-acid-glycoprotein (A1AG; Hoeben et al., 2000; Ekersall et al., 2001; Ceciliani et al., 2005); and complement factor 5a (C5a), the pro-inflammatory byproduct of complement activation (Shuster et al., 1997; Rainard and Poutrel, 2000). Additionally, knowledge of the role of toll-like receptors (TLR) and affiliated signaling pathways activated during the bovine innate immune response following infection with gram-negative pathogens has expanded in recent years (De Schepper et al., 2008; Ibeagha-Awemu et al., 2008; Yang et al., 2008).

The desire to elucidate targets for novel drug therapies for coliform mastitis, coupled with the need to identify biomarkers to evaluate the efficacy of new and existing drug therapies, has provided a stimulus for investigations into the bovine milk proteome. Despite hindrances related to biological complexity, numerous post-translational modifications (PTMs), and the dynamic range of proteins in milk (O'Donnell et al., 2004), attempts have been made to study the dynamics of differential protein expression during bovine mastitis (Hogarth et al., 2004; Smolenski et al., 2007). Although significant limitations prevent the full characterization of the bovine milk proteome (O'Donnell et al., 2004; Garbis et al., 2005; Lippolis and Reinhardt, 2008), advances have been made in identifying low abundance proteins in normal bovine whey (Fong et al., 2008), the milk fat globular membrane (MFGM; Reinhardt and Lippolis, 2006; 2008; Fong et al., 2007), and in various other bovine milk fractions (Yamada et al., 2002; Smolenski et al., 2007). The proteomic analyses of mastitic bovine milk performed thus far, however, have been on milk samples collected from cows with mastitis that was established based on clinical parameters (Hogarth et al., 2004), or the analysis was conducted on samples from only one cow with a naturally occurring clinical infection (Smolenski et al., 2007). Additionally, neither of the previous analyses of differential protein expression in bovine milk during mastitis involved samples collected over the course of a clinical infection (Hogarth et al., 2004; Smolenski et al., 2007). Nonetheless, the use of proteomic methodologies to evaluate protein changes in milk during the inflammatory response associated with coliform mastitis, coupled with existing knowledge of soluble mediators of inflammation, could lead to the identification of a biomarker, or pattern of

biomarkers, indicative of the disease. In order to be considered robust, biomarkers of coliform mastitis must exhibit consistent expression patterns across a group of cows at more than one time point following infection. Likewise, a reliable means of quantifying the relative abundance of differentially expressed bovine milk proteins over the course of clinical mastitis must be established, especially if no bovine-specific antibodies are available for the protein(s) of interest. If the difficulties inherent to the characterization of the bovine milk proteome could be overcome, and the criteria for accuracy, sensitivity, and specificity met, biomarkers of mastitis could prove useful in evaluating the efficacy of existing or new drugs to treat secondary inflammation caused by gram-negative pathogens, or for the discovery of potential new drug targets

Chapter 2: Literature Review

Bovine Mastitis

Mastitis, or inflammation of the mammary gland as a result of invasion of bacterial pathogens or injury to the gland, has been both a primary concern for dairy producers, as well as a main focus of dairy cattle disease research for over a century. Of the causes of mastitis, bacteria are by far the most common. Bacteria gain access to the mammary gland via the teat canal, and cause infections that may decrease milk production, change the composition and appearance of milk, cause swelling and pain in the udder, elevate rectal temperature, and decrease feed intake (Harmon, 1994). Mastitis can vary in severity, and is classified as subclinical, clinical, or chronic (Eberhart et al., 1987; Harmon, 1994). Subclinical infections are usually only manifest by increases in bulk tank somatic cell counts (SCC) and decreased production (Eberhart et al., 1987), whereas clinical infections are marked by changes in the milk, such as clots or flakes, that can be detected during fore-stripping prior to milking, or by the appearance of the udder, which can become swollen and hot to the touch (Harmon, 1994). The economic losses attributed to mastitis are staggering, and result from decreased milk yields, discarded milk due to treatment with antibiotics, loss of quality premiums, reduced longevity of animals, lowered salvage value of culled cows, elevated labor costs, higher veterinary and treatment bills, and increased cost of replacing animals in the lactating herd (Eberhart et al., 1987; DeGraves and Fetrow, 1993; Seegers et al., 2003).

Bacteria that cause mastitis are classified as either contagious or environmental pathogens. The most common contagious mastitis pathogens are *Streptococcus agalactiae* and *Staphylococcus aureus*, gram positive bacteria that usually cause decreased milk production, clinical mastitis, and elevated somatic cell counts in some cases (SCC; Eberhart et al., 1987; Schroeder, 1997). Contagious organisms are well adapted to grow and survive in the mammary gland, and frequently cause infections lasting weeks, months or years. The major reservoir for contagious pathogens is the infected udder. Contagious pathogens can spread easily from cow to cow during milking time as the result of improper sanitation of milking units, by the recycling of dairy towels used on infected cows, or the contaminated hands of milking personnel (Harmon, 1994). Most species of gram positive bacteria, excepting *S. aureus,* are effectively eliminated by treatment with antibiotics. *S. aureus* can be more problematic to clear, however, and is often associated with subclinical chronic inflammation characterized by periodic flare-ups of clinical symptoms, and often recurs in subsequent lactations (Schroeder, 1997).

Environmental pathogens, on the other hand, are most prevalent in the cow's environment including bedding, soil, and manure, and are often isolated from acute cases of clinical mastitis (Zehner et al., 1986; Schroeder, 1997; Hogan et al., 1999). Environmental mastitis pathogens are typically gram-negative bacteria, and include the coliforms *Escherichia coli, Klebsiella pneumoniae, Enterobacter* spp.*,* and *Serratia marcescens* (Schroeder, 1997). Gram-negative bacteria are characterized by the presence of lipopolysaccharide (LPS) in their outer membrane, a compound known to stimulate a rapid inflammatory response in the bovine mammary gland

(Paape et al., 1996; Lee et al., 2003). LPS causes the secondary clinical signs associated with coliform mastitis including high fever, depressed appetite, rapid weight loss, abnormal milk, and decreased milk production (Baumann and Gauldie, 1994; Hirvonen et al., 1999; Hoeben et al., 2000). Severe cases of coliform mastitis can also result in bacteremia and septicemia, as the blood-milk barrier is destroyed and LPS leaks into circulation (Wenz et al., 2001). The ensuing acute systemic symptoms of coliform mastitis may include respiratory distress, metabolic imbalances, tissue injury, and death (Lohuis et al., 1990; Wenz et al., 2001).

Mastitis infections caused by gram-negative pathogens are problematic to treat, mainly because the deleterious effects of the endotoxin released by gram negative bacteria are not ameliorated by antimicrobials. In fact, several studies have demonstrated that antibiotics have little or no efficacy in treating clinical or subclinical cases of coliform mastitis (Hogan et al., 1989; Erskine et al., 2000). Herd management practices that are effective in controlling and reducing incidence of mastitis caused by gram- positive bacteria, such as post-milking teat dips and dry cow antibiotic therapies, have likewise proven to be ineffective in the control of coliform mastitis due mainly to the prevalence of coliforms in the environment of the cow (Smith et al., 1985).

The core and lipid A regions of LPS in the cell wall of coliforms are conserved across many of the pathogens and have, as a result, been targeted as viable antigens for vaccine development (Tyler et al., 1991; 1992). Challenge studies have revealed, however, that immunization with the commercially available vaccines based on *E. coli* J5 bacterins increased antibody titers to *E. coli* J5 and augmented bacterial

clearance rates in infected quarters, but did not minimize the severity of coliform mastitis or prevent development of the disease (Hogan et al., 1992; Tomita et al., 2000; Wilson et al., 2007).

To date, the only treatment that has shown promise as an adjunctive therapy for coliform mastitis is the use of non-steroidal anti-inflammatory drugs or NSAIDs (Anderson, 1989; Lohuis et al., 1991; Vangroenweghe et al., 2005). Nonsteroidal anti-inflammatory drugs function by inhibiting cyclooygenase (COX), the enzyme responsible for the oxidation and subsequent conversion of arachidonic acid to prostaglandins, a class of compounds that are well known mediators of pain and inflammation (Soberman and Christmas, 2003). Two forms of COX have been identified, the constitutively expressed COX-1 that is associated with the production of cytoprotective prostacyclin in the gastric mucosa (Whittle et al., 1980), and COX-2, the inducible form that is involved in inflammation (Xie et al., 1992). COX-2 is only expressed following induction by inflammatory stimuli such as cytokines and intracellular messengers; thus, COX-2 has been the focus of several studies regarding the anti-inflammatory action of NSAIDs (Vane and Botting, 1998). Currently the efficacy of NSAIDS in treating inflammation associated with coliform mastitis is evaluated by the ability to cause a 2 degree drop in rectal temperature, and a decrease in serum concentration of prostaglandin E2 (PGE_2; ONADE, personal communication). Reliance on measuring a reduction in PGE_2 concentration as an indicator of NSAID efficacy is problematic, however, due to the fact that PGE_2 can vary significantly between animals, and the compound is known to metabolize rapidly *in vivo*. Thus, due to lack of established bovine protein biomarkers to measure

efficacy, only the nonselective NSAID Banamine® has been approved for use in lactating dairy cattle.

The economic impact of mastitis, coupled with the need to develop novel adjunctive therapies, has inspired decades of research on the subject of coliform mastitis with topics as varied as: the effects of nutrition on immune function (Smith et al., 1984; Chew, 1987); the relationship between quantitative measures of productive life, udder health, and incidence of mastitis (Coffey et al., 1986; Shook and Schultz, 1994; Cranford and Pearson, 2001); induction of cytokines and soluble inflammatory mediators during coliform infections, (reviewed in Bannerman, 2009); and the role of the Toll-like receptors (TLR) in the bovine innate immune response (De Schepper et al., 2008; Ibeagha-Awemu et al., 2008; Yang et al., 2008). Following advances in techniques for protein and peptide analysis, studies regarding host response during coliform mastitis have likewise gravitated towards the identification of differentially expressed proteins in bovine milk during mastitis infections, and annotation of the bovine milk proteome (Hogarth et al., 2004; Smolenski et al., 2007; Fong et al., 2007; 2008; Reinhardt and Lippolis, 2006; 2008).

Studies of the bovine innate immune response during coliform mastitis have established that LPS, via association with LPS-binding protein (LBP), cluster of differentiation-14 (CD-14), and various other scaffold proteins such as MyD88, binds to Toll-like receptor-4 (TLR4), and induces an inflammatory response through the activation of nuclear factor kappa-B (NF-kB), a cytoplasmic factor that controls transcription of many genes, including proinflammatory cytokines (Pahl, 1999; Hatada et al., 2000). Recently, NF-kB activity during *E. coli*-induced mastitis was

investigated (Notebaert et al., 2008). Intramammary concentrations of NF-kB were shown to increase *in vivo* in murine mammary glands following inoculation with *E. coli,* with corresponding activation in hepatic tissue, and increases in systemic circulation levels of both cytokines and acute phase proteins (Notebaert et al., 2008).

Cytokines known to be upregulated following infection with gram negative bacteria include: tumor necrosis factor-alpha (TNFα), interleukin-1 (IL-1), interferon-gamma (IFN-γ), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12 (IL-12), transforming growth factor-alpha (TGF-α), and transforming growth factor-beta (TGF-β; reviewed in Bannerman, 2009). In particular, studies have demonstrated that the acute symptoms of coliform mastitis are closely related to milk and serum levels of the cytokines TNFα, IL-1β, and IL-8 (Sordillio and Peel, 1992; Riollet et al., 2000). IL-1β is a potent inducer of fever (Dinarello, 1998), as well as the acute phase response, and is produced by a variety of cell types (reviewed in Bannerman, 2009). IL-1β and TNFα act redundantly in the activation of leukocytes and endothelial cells, and both can elicit very harmful secondary effects including altered vascular permeability, shock, and tissue injury (Bannerman, 2009). TNFα and IL-1β also upregulate the expression of vascular adhesion molecules such as E-selectin, which promotes the adhesion of leukocytes to vascular walls, and ultimately results in passage of leukocytes through blood vessel walls (Beutler, 2004). During intra-mammary infections, both TNFα and IL-1β have been reported to exhibit increased concentrations in milk by 18 h following experimental induction of mastitis (Shuster et al., 1997; Lee et al., 2003 Bannerman et al., 2004).

IL-8 is a chemokine that stimulates the migration of neutrophils into the mammary gland from circulation during intramammary infection (Beutler, 2004; Bannerman, 2009). In addition to attracting neutrophils to the site of inflammation, IL-8 has also been shown to directly augment neutrophil phagocytosis, priming, degranulation, and oxygen radical production (Watson et al., 1995; McClenahan et al., 2000). Increases in IL-8 have been detected in milk within 16 h following experimental challenge with *E. coli* (Lee et al., 2003 Bannerman et al., 2004). Similarly, recent studies have indicated that platelet activating factor (PAF) is produced by mammary endothelial cells in response to challenge with LPS, and that in addition to IL-8 and IL-1β, PAF likewise serves as a chemotactic factor to recruit leukocytes to the gland during mastitis (Corl et al., 2008).

The actions of IL-6, another prominent cytokine involved in the bovine innate immune response, include fever induction, B-cell differentiation, T-cell activation, and the inhibition of TNFα and IL-1β (reviewed in Bannerman, 2009). A more prominent function of IL-6 during inflammation, however, is the stimulation of the hepatic production of acute phase proteins (APP) including serum amyloid A (SAA), haptoglobin (Hp), and α-1-acid glycoprotein (A1AG; reviewed in Heinrich et al., 1990). Similar to IL-8, milk concentrations of IL-6 have been observed within 16 h following experimental induction of coliform mastitis with *E. coli* (Shuster et al., 1997).

In contrast to data available on the expression and actions of cytokines during coliform mastitis, less is currently known regarding the roles of APP during inflammation. Studies directed at the innate immune response in the bovine

mammary gland following challenge with either LPS or *E. coli* have reported increases in the APP SAA, Hp, A1AG, and LBP in milk during clinical mastitis (Hirvonen et al., 1999; Eckersall et al., 2001; Bannerman et al., 2003; Hiss et al., 2004; Ceciliani et al., 2005). While the primary source of APP is the liver, local production of SAA, Hp, and A1AG in the bovine mammary gland have been demonstrated (Hiss et al., 2004; Jacobsen et al., 2005; Ceciliani et al., 2005; Larson et al., 2006). In addition to local and hepatic production, the granules of bovine neutrophils are likewise a reported source of A1AG (Rahman et al., 2008). A recent analysis of the temporal expression of SAA, Hp, and LBP during experimentally induced *E. coli* mastitis indicated that increases in all three major APP began as early as 12 h following infection, but unlike peak expression of the major cytokines involved in inflammation during coliform mastitis, maximum levels of the APP were not detected in milk until 36-60 h after challenge (Suojala et al., 2008).

Until the past decade, the discovery of biomarkers specific to the host response during gram-negative intramammary infection has been limited to the study of the expression of cytokines and other soluble mediators of inflammation during clinical mastitis, and information has been derived primarily from enzyme-linked immunosorbent assays (ELISA). The chief limitation of utilizing methodologies such as ELISAs in biomarker discovery analyses, however, is that ELISAs are designed to measure only one protein at a time, and are limited by antibody development and availability. Recent advances in both genomic and proteomic strategies have given rise to novel assessments of the bovine innate immune response following *E. coli* challenge that are not restricted to single marker analyses. Examples include the use

of a DNA-microarray to profile changes in the transcriptome of mammary epithelial cells (MEC) after gram-negative bacterial infection (Gunther et al., 2009), and the proteomic analysis of bovine milk fractions using liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) to identify proteins related to host response in milk during clinical mastitis (Smolenski et al., 2007). DNA microarray of MEC following challenge with *E. coli* revealed upregulation of genes encoding chemokines, interleukins, beta-defensins, SAA, and Hp, results which are all in accord with previous analyses (Eckersall et al., 2001; Bannerman et al., 2004; Gunther et al., 2009). Proteomic analyses of mastitic bovine milk, on the other hand, led to the identification primarily of antimicrobial and antigen recognition proteins (Smolenski et al., 2007). While no strategy is without limitations, the advances in mastitis research made in the past decade in regards to elucidation of cytokine expression, signaling pathways, and differential gene and protein expression during coliform mastitis will prove useful in the discovery and establishment of biomarkers of mastitis for use in disease detection and diagnosis, and in the evaluation of novel therapeutics.

Biomarker Discovery

By definition, a biomarker is "a characteristic that can be measured and evaluated as an indicator of normal biological processes, pathological processes or pharmacologic responses to therapeutic interventions" (NIH Biomarkers Definitions Working Group, 1998). Several types of biomarkers have been described, including: diagnostic biomarkers used to identify the presence or absence of a disease (Taba et al., 2005; Kijanka and Murphy, 2009); predictive biomarkers used to evaluate the

efficacy of drug therapies to treat disease (Boenisch and Chandraker, 2008; Sakurada and Tsao, 2009); and surrogate biomarkers that are regarded as valid substitutes for measuring clinical outcomes (Bose et al., 2004). To be considered a "good" biomarker, an indicator of disease must exhibit accuracy, sensitivity, and specificity; thus, more simply stated, the expression of a biomarker must be specific to a disease, unaffected by unrelated disorders, and reliable and reproducible quantification of the biomarker must be possible (LaBaer, 2005; Issaq and Blonder, 2009).

Proteomics has been defined as a scientific approach used to elucidate all proteins within a cell or tissue (Colantonio and Chan, 2005). Over the past decade, proteomic methodologies including two dimensional electrophoresis (2D-GE) and mass spectrometry (MS), have become the most prominent approaches used to identify novel biomarkers (Mann et al., 2001; Issaq and Blonder, 2009). Genomic methodologies, including gene arrays and the evaluation of mRNA expression using polymerase chain reaction (PCR), have also been used for biomarker discovery, but proteomics boasts a significant advantage over genomic analyses because of the demonstrated weak correlation that often exists between mRNA levels and actual protein concentration. Thus, quantitative mRNA data is often an inadequate indicator of protein expression (Gygi et al., 1999; Ideker et al., 2001; Griffin et al., 2002; Tian et al., 2004).

Chromatography and electrophoresis, when coupled with mass spectrometry, are the combined methodologies of choice when considering the identification of proteins in complex biological matrices (Mann et al., 2001; Roe and Griffin, 2006; Issaq and Blonder, 2009). Advances in ionization techniques in mass spectrometry,

including electro-spray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), have broadened the applications of mass spectrometry to include intact protein and peptide analysis, and have paved the way for ground-breaking studies into the dynamics of differential protein expression (reviewed in Mann et al., 2001). The basic distinguishing features between MALDI and ESI are the reliance of MALDI ionization on a crystallizing energy absorbing matrix for ion formation, as opposed to a liquid solvent matrix utilized by ESI, and the fact that MALDI ionization routinely results in the production of singly-charged ions, whereas ESI produces multiply-charged ions (Karas et al., 1987; Tanaka et al., 1988; Fenn et al., 1989). Another distinction between the two forms of soft ionization is that the generation of ions in MALDI is initiated by short pulse irradiation with a laser (Karas and Krüger, 2003), typically nitrogen or neodymium-doped yttrium aluminium garnet (Nd:YAG), whereas ESI generates ions via the passage of a liquid mixture of solvent and the target analyte through a metal capillary tube to which a charge is applied (Fenn et al., 1989). Nanospray is another very popular ionization method employed in mass spectrometry-based proteomic analyses that is very similar in principle to ESI. The primary distinctions between ESI and nanospray are the significantly lower flow rates and smaller needle diameters used for nano- spray (Kinter and Sherman, 2000).

In MALDI-MS analyses, the analytes, which can include peptides, proteins, synthetic polymers, oligonucleotides, oligosaccharides, or drugs and metabolite systems (Hillenkamp et al., 1991; Nordhoff et al., 1992), are mixed with an energy absorbing matrix that facilitates the production of intact gas-phase ions by excitation

of matrix molecules and subsequent proton transfer to the thermally labile analyte molecules (Hillenkamp et al., 1991; Karas and Krüger, 2003). Common matrices used in MALDI analyses are 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid; SPA), α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB; Beavis and Chait, 1989; Kosaka and Kinoshita, 1996).

Conversely, the formation of ions in ESI mass spectrometry begins with the mixing of the sample to be analyzed with a solution containing a volatile solvent as well as volatile acids, bases, or buffers (Fenn et al., 1989; 1990). The mixture is then passed through a highly charged metal capillary at a very low flow rate (Fenn et al., 1989; 1990). Due to charge repulsion, the solvent:analyte mixture propels itself through the capillary and emerges as an aerosol mist consisting of small charged droplets about 10 μm across (Fenn et al., 1989; 1990; Nguyen and Fenn, 2007). Solvent evaporation leads to the decomposition of the initial droplets into smaller and more highly charged offspring droplets, a process that eventually leads to observable ions (Kebarle, 2000; Nguyen and Fenn, 2007).

Mass spectrometers measure the mass-to-charge ratio (*m/z)* of analytes, including proteins and peptides, by mass separation (Mann et al., 2001). The three basic means of achieving mass separation include: 1) separation based on time-of-flight (TOF MS); 2) separation by quadrupole electric fields generated by metal rods and the application of direct current (dc) and radio frequency (rf) (quadrupole MS); and 3) separation by selective ejection of ions from a three-dimensional trapping field (ion trap/ Fourier transform ion cyclotron MS; Mann et al., 2001). Peptide sequencing is achieved by performing two steps of mass spectrometry in tandem

(MS/MS), which can be done by using the same separation principle twice, or utilizing two different MS separation principles (Mann et al., 2001).

MALDI is a discontinuous ionization technique and is therefore typically coupled to a TOF mass analyzer (Mann et al., 2001). Time-of- flight instruments are well suited to MALDI ion sources because MALDI produces brief pulses of ions that are followed by relatively long periods of measurement during which no additional ions can be allowed into the instrument's analyzer (Fenyö and Beavis, 2008). Structural information can be obtained when peptide ions formed by MALDI decay as the result of energy departing during the desorption process, an event referred to as post source decay (PSD; Mann et al., 2001). MALDI-TOF instruments are also typically used to generate peptide mass fingerprints (PMF) of proteins, or a set of peptide masses resulting from enzymatic digestion with a protease such as trypsin (Mann et al., 2001).

ESI is most often combined with quadrupole mass separation wherein the quadrupole serves as a mass filter that, following the application of an oscillating electric field, only lets ions of a certain mass to pass through (Mann et al., 2001). Triple quadrupole mass spectrometers have also been merged with the TOF instruments wherein the third quadrupole section is replaced by a TOF analyzer (Q-TOF; Morris et al., 1996). ESI is likewise used often with ion-trapping instruments that utilize three-dimensional electric fields to trap ions, and additional electric fields to eject ions one species at a time from the trap (reviewed in Mann et al., 2001). While the ion-trap instruments offer high performance, the resolution and mass

accuracy of the trap instruments do not approach those of the Q-TOF instruments (Mann et al., 2001).

Advances in mass spectrometry have provided a means for the accurate and sensitive identification of differentially expressed proteins in complex biological samples, but another important criterion of quality biomarkers is reliable quantification. Relative and absolute quantification of changes in abundance of biomarkers in biological matrices using proteomic strategies is a topic that has garnered significant attention in recent years (Roe and Griffin, 2006; Fenselau, 2007; Mueller et al., 2008; Simpson et al., 2009). Quantification methods can be assigned to one of two broad categories: a labeled approach that requires the incorporation of labels into proteins or peptides prior to MS analysis, or the alternative, which is to use a semiquantitative label-free method (Simpson et al., 2009).

The basis of most labeled quantification methods is the stable isotope dilution principle, or the theory that a labeled peptide will behave chemically in the same fashion as its unlabeled counterpart, and therefore the two peptides will have identical chromatographic and MS properties (Simpson et al., 2009). The addition of a label, however, does impart a mass difference between the two peptides, and thus relative abundance can be inferred by comparing the respective signal intensities of the labeled and unlabeled peptides in the same MS run (Simpson et al., 2009). Labels can be incorporated in several ways, with the most popular being either metabolically, chemically, or enzymatically (Fenselau, 2007; Simpson et al., 2009). Examples of labeling strategies include: metabolic labeling commonly known as stable isotope labeling by amino acids in cell culture or SILAC (Ong et al., 2002); proteolytic

labeling with ^{18}O (Yao et al., 2003); or isotope incorporation by several means including chemical derivatization also known as isotope coded affinity tags or ICAT (Gygi et al., 1999), isobaric tags for relative and absolute quantitation (iTRAQ; Ross et al., 2004), or global internal standard technology (GIST; Chakraborty and Regnier, 2002).

Some labeling strategies can be cost-limiting, however, and thus there has been interest in quantification without the incorporation of labels. Label-free strategies are based on the correlation between the abundance of a protein or peptide in a sample, and the MS signal (Simpson et al., 2009). One of the most popular methods of label-free quantification is extracted ion chromatograms (XIC), in which the number and intensity of selected precursor ions at a particular *m/z* are summed and the peaks areas used as a measure of relative abundance (Old et al., 2005). An alternative approach that is gaining popularity is spectral counting, or the number of MS/MS fragment spectra that contribute to the identification of a given protein (Liu et al., 2004; Zybailov et al., 2005). Similarly, the number of unique peptides identified for each protein in a sample has likewise been used as a measure of relative protein abundance (Liu et al., 2004). An inherent drawback of label-free methods, though, is the assumption that the linearity of response will be the same for each protein, which often does not hold true because the chromatographic behavior of peptides tends to vary (Simpson et al., 2009). Thus, many spectra must be acquired and spectral counts or XIC normalized when using label- free methods (Old et al., 2005). Nevertheless, label-free quantification does not require any extra sample processing and can be performed retrospectively; two attributes which make non-labeled quantification

methods the continued focus of development in biomarker discovery research (Simpson et al., 2009).

Milk Proteomics

Milk proteins have been the subject of intense research for over fifty years. Despite numerous in-depth analyses however, many questions still remain regarding expression of bovine milk proteins, interactions between milk proteins, and the structure, function, and modification of milk proteins (O'Donnell et al., 2004). A major limitation in studying the bovine milk proteome has been the biological complexity of milk, a problem made even more complicated by the numerous post-translational modifications (PTMs) that occur in milk proteins including glycosylation, phosphorylation, disulphide bond formation, and proteolysis (O'Donnell et al., 2004). Additionally, analyses of the bovine milk proteome are hampered by the dynamic range of proteins in milk, which are known to vary in concentration by as much as a factor of 10^6 (O'Donnell et al., 2004; Gagnaire et al., 2009). Despite the somewhat overwhelming abundance of just a few select proteins, however, the bovine milk proteome is extremely complex due to the existence of numerous low abundance proteins that comprise approximately 5% of the total protein concentration of bovine milk (Gagnaire et al., 2009). Since the beginning of the 1990s, proteomic methodologies have been applied to the study of milk proteins and several advances have been made including the identification of minor or low abundance proteins in bovine milk and affiliated subcellular compartments, elucidation of variations in milk proteins due to species and stage of lactation, and the

characterization of several PTMs that occur frequently in milk proteins (Gagnaire et al., 2009).

Proteomic analyses typically employ the two-step strategy of protein or peptide separation followed by identification of the protein (reviewed in Garbis et al., 2005). The most popular approaches to the two-fold process of protein discovery are 2D-GE for the separation of proteins followed by identification using MALDI–TOF-MS, and one- or two-dimensional liquid chromatography (LC) for protein or peptide separation followed by protein identification using ESI and tandem mass spectrometry (MS/MS; O'Donnell et al., 2004; Garbis et al., 2005). Protein discovery also entails digestion of isolated intact proteins or mixtures of proteins with an enzyme such as trypsin either prior to or following separation by either 2D-GE or LC (O'Donnell et al., 2004; Garbis et al., 2005; Gagnaire et al., 2009).

Proteomic approaches are broadly classified as either a top-down approach, which entails separation of intact proteins prior to digestion and identification of the protein by either peptide mass mapping or peptide sequencing, or a bottoms-up analysis that involves digestion of complex protein mixtures followed by the separation of peptides using one- or two-dimensional LC. Typically, pre-fractionation steps are employed in most proteomic analyses to reduce sample complexity and increase proteome coverage (Righetti et al., 2005; Gagnaire et al., 2009). Common pre-fractionation techniques used in proteomic analyses include: separation of proteins or peptides using centrifugation, electrophoresis or chromatography; fractionating based on isoeletric point (IEF) or size; and selective depletion of high abundance proteins (Righetti et al., 2005). Attempts to reduce the complexity of bovine milk samples

have included the removal of caseins by acid precipitation (Baeker et al., 2002; Hogarth et al., 2004; Fong et al., 2008), immunoabsorption (Yamada et al., 2004), or by ultra-centrifugation (Smolenski et al., 2007); removal of the milk fat by centrifugation at reduced temperatures (Galvani et al., 2001; Baeker et al., 2002; Yamada et al., 2002; Hogarth et al., 2004; Smolenski et al., 2007; Fong et al., 2008); and the fractionation of whey following removal of the fat and caseins using fast protein liquid chromatography (FPLC; Fong et al., 2008). While attempts to reduce the complexity of bovine milk and affiliated subcellular fractions have resulted in more thorough comprehension of the complete bovine milk proteome, a tremendous gap in knowledge still exists regarding bovine milk protein modulation during disease (O'Donnell et al., 2004).

Proteomic strategies were first applied to the study of the bovine milk proteome in the late 1980s, and were predominantly focused on analysis and characterization of the most abundant milk proteins (O'Donnell et al., 2004; Gagnaire et al., 2009). The early forays utilized either 2D-GE or LC alone, and were performed only on bovine whole milk (Shimazaki et al., 1983; Holt and Zeece, 1988; Bobe et al., 1998). The preliminary attempts to analyze bovine milk proteins were successful only in separating the major whey proteins serum albumin, β-lactoglobulin, and α-lactalbumin, and the caseins α_S-casein and β-casein (Shimazaki et al., 1983; Holt and Zeece, 1988). Additionally, protein identification was based solely on a previous report of the isolelectric points and molecular weights of bovine milk proteins (Whitney et al., 1977). Sample preparation methods in the early milk proteome studies focused mainly on the removal of caseins from the milk samples prior to

analysis, and were comprised of either acid precipitation followed by centrifugation (Shimazaki et al., 1983), or centrifugation alone (Holt and Zeece, 1988).

The earliest attempt to identify bovine milk proteins via LC coupled with MS (LC-MS; Léonil et al., 1995) were limited to the detection of only the major milk proteins β-lactoglobulin, α-lactalbumin, α-casein, β-casein, and κ-casein, and lacked peptide sequencing data to support the authors' identification of milk proteins on the basis of molecular mass. Pre-fractionation methods utilized by Léonil and colleagues involved separating the casein and whey proteins by acid precipitation of the caseins, followed by dialysis of both protein fractions (Léonil et al., 1995).

One of the first studies to employ MALDI to analyze bovine milk proteins generated mass spectra on the purified milk proteins β-lactoglobulin, α-lactalbumin, α-caseins, β-casein, and κ-casein, and then compared these spectra to a MALDI mass spectrum generated from the protein extract of a bulk milk sample (Catinella et al., 1996). In the bulk milk sample, the intact proteins β-lactoglobulin, α-lactalbumin, proteose peptones, β-casein, and γ-casein were identified. Sample preparation was limited and did not involve attempts to remove high abundance proteins prior to analysis (Catinella et al., 1996). In the same study, MALDI mass spectra of milk from several different breeds of dairy cattle including Holstein, Brown Swiss, Jersey, and Reggiana cows were likewise examined for differences in protein content and abundance (Catinella et al., 1996). Results indicated that the protein content of milk collected from Jersey cattle was markedly different from other dairy breeds. Likewise, Catinella et al., were able to demonstrate via the comparison of MALDI mass spectra generated from milk samples of each dairy breed collected throughout

the course of a lactation cycle that compositional differences existed at different stages of lactation.

The first analyses of bovine milk to combine the use of 2D-GE and MALDI-TOF-MS were analyses conducted on both powdered milk and commercially available milk that primarily focused on the identification of the casein and whey proteins, and variants of the major milk proteins (Galvani et al 2000; 2001). Using MALDI-TOF-MS to identify intact proteins in commercial whole milk, Galvani and colleagues were able to identify the whey proteins β-lactoglobulin, and α-lactalbumin, and the caseins γ3-casein A_2 and γ_2-casein A_2, both fragments of β-casein A_2, as well as α_{S1}-casein, α_{S2}-casein, β-casein, and κ-casein. 2D-GE profiles were also generated using the whole milk samples, but no additional milk proteins were identified (Galvani et al, 2001).

One of the first attempts to identify bovine milk proteins, other than the abundant whey and casein proteins, compared bovine colostrum and mature milk collected from healthy Holstein cows using 2D-GE, *N*-terminal sequencing, MALDI-TOF-MS, and ESI-MS/MS (Yamada et al., 2002). In order to identify lower abundance milk proteins, the major proteins β-casein and immunoglobulin G (IgG) were removed from the milk and colostrum samples using immunoabsorption. *N*-terminal sequencing of protein spots separated on a 2-DE gel identified α_{S1}-casein, β-casein fragments, and β-lactoglobulin and α-lactalbumin (Yamada et al., 2002). MALDI peptide mass fingerprinting (PMF) following in-gel trypsin digestion of proteins isolated using 2D-GE allowed for the identification of lactoferrin, serotransferrin, β-casein fragments, secretory component, apolipoprotein H, and transthyretin, while

tandem mass spectrometry of tryptic peptides added fibrinogen β-chain, chitinase 3-like 1, IgM heavy chain, α_1-antritrypsin, complement C3 α-chain, and gelsolin to the list of identified proteins (Yamada et al., 2002).

The first demonstrated success of utilizing proteomic techniques to identify novel markers for bovine mastitis was the work of Baeker and colleagues, who were the first to attempt to characterize differentially expressed proteins in normal and mastitic milk using 2D-GE followed by MALDI-TOF-MS. The presence of lipocalin-type prostaglandin D synthase was observed only in the 2D gels generated from samples taken from cows with clinical mastitis, and did not appear on gels generated from control samples (Baeker et al., 2002). While Baeker and colleagues were only successful in identifying a single differentially expressed protein in mastitic bovine milk, the results nonetheless marked the first instance of published literature on a marker of inflammation in bovine milk identified using proteomic strategies.

Following the work of Baeker et al., more focused attempts to identify differentially expressed proteins in whey from healthy cows versus cows with clinical mastitis were initiated by Hogarth et al., in 2004. Changes in protein composition of milk during clinical mastitis were examined using 2D-GE followed by enzymatic digestion of isolated proteins and PMF using MALDI-TOF-MS. The samples were collected from cows residing on a commercial dairy, and mastitis was defined strictly by identification of clinical signs and not by bacterial culturing or experimental induction (Hogarth et al., 2004). Milk sample preparation prior to analysis included fat removal and precipitation of the casein proteins by the addition of salt followed by dialysis to remove traces of ammonium sulphate (Hogarth et al., 2004). Despite

attempts to remove the casein fraction from the milk prior to proteomic analysis, α_{S1}-casein, β-casein, and κ-casein were still found in rather high abundance in normal milk. Also identified were the proteins serum albumin, transferrin, microsomal triglyceride protein, β-lactoglobulin and α-lactalbumin (Hogarth et al., 2004). Marked increases in serum albumin and transferrin, concurrent with apparent decreases in the casein proteins as well as in β-lactoglobulin and α-lactalbumin, were apparent in the 2D-GE profiles generated from whey samples from cows with mastitis, and supported the well established theory that serum protein concentration increased in milk during mastitis as a result of the breakdown of the blood milk barrier during mastitis infections (Hogarth et al., 2004). While no additional information was gained, Hogarth et al., were the first to demonstrate temporal changes in bovine milk protein expression during mastitis, though the analysis was limited to only normal and mastitic samples, and confirmation of mastitis was based only on clinical observations and not experimental induction of the disease.

Knowledge of more extensive protein changes in bovine milk during a naturally-occurring mastitis infection was reported by a research group in New Zealand three years after the work of Hogarth and colleagues. The endeavors of Smolenski et al., in 2007 resulted in the first report of extensive examinations of protein expression in separate subcellular fractions of bovine milk during different physiological states (Smolenski et al., 2007). The proteomic techniques employed included direct LC-MS/MS, as well as 2D-GE followed by MALDI-TOF-MS or MS/MS (Smolenski et al., 2007). The milk fractions analyzed included milk from peak lactation, colostrum, and milk from a cow with clinical mastitis. Though milk was collected from only one

cow that was recently fresh, one cow that was in mid-lactation, and one cow that was suspected to have mastitis based on clinical indications of the disease, the results of the analyses conducted by Smolenski et al., were the most comprehensive to date in terms of number of low abundance proteins indentified and the number of host response proteins that were isolated from mastitic milk (Smolenski et al., 2007). An additional novel aspect to the proteomic analyses conducted by Smolenski and colleagues was the fact that no sample clean-up or attempts to fractionate the milk were carried out on the samples prior to analysis. Smolenski et al., were the first to identify proteins such as apolipoprotein A-1, cathelicidin-1, fatty acid binding protein (FABP), glycosylation-dependent call adhesion molecule 1 (Glycam-1), H3 histone, heat shock protein 70, nucleobindin, peptidoglycan recognition protein (PGRP), and SAA in milk fractions collected from a cow with clinical mastitis (Smolenski et al., 2007).

Not all endeavors to characterize low abundance proteins in bovine milk have focused on proteins differentially expressed during disease states. Significant efforts have also been made to fractionate whey derived from the milk of healthy cows in attempt to identify low abundance proteins. Fong and colleagues sought to characterize proteins other than the abundant caseins and whey proteins in normal whey by first removing the milk fat and caseins by centrifugation at 4ºC and acid precipitation, respectively. The remaining intact whey proteins were then separated using fast protein liquid chromatography (FPLC; Fong et al., 2008). The major whey proteins β-lactoglobulin and α-lactalbumin were isolated using semi-coupled anion and cation exchange, and the remaining minor or low abundance proteins in the basic

and acidic whey fractions resulting from the FPLC were analyzed using 2D-GE (Fong et al., 2008). Proteins visualized on the 2D gels generated by Fong and colleagues were excised, digested with trypsin, and identified using nano-reversed phase- LC followed by ESI-MS/MS. Despite the removal of high abundance casein proteins and the elaborate fractionation strategies employed to sequester high abundance whey proteins, all of the casein proteins as well as β-lactoglobulin and α-lactalbumin were still identified in spots cut from the series of 2D gels (Fong et al., 2008). Additionally, the complete fractionation of the abundant milk proteins was not entirely achieved as caseins and the whey proteins β-lactoglobulin and α-lactalbumin appeared in gels generated from different fractions obtained using FPLC. A significant number of previously unidentified whey proteins were detected in the 2D gels generated on the whey fractions however, including butyrophilin, alpha-1B-glycoprotein, alpha-2-HS-glycoprotein, kininogen-1, vitamin D binding protein, antithrombin III, several immunoglobulins, complement components C3, C4, C7, and C9, polymeric immunoglobulin receptor (PIGR), hemopexin, and lactoperoxidase (Fong et al., 2008).

In addition to the characterization of the whey fraction of bovine milk, there have been several proteomic analyses of the bovine milk fat globular membrane (MFGM; Reinhardt and Lippolis, 2006; 2008; Fong et al., 2007). In initial proteomic studies of the MFGM, Reinhardt and Lippolis utilized 1-dimensional gel electrophoresis (1D-GE) to fractionate proteins followed by in-gel digestion of protein slices and identification of tryptic peptides via MS/MS. A total of 120 proteins that comprise the bovine MFGM were identified, including proteins associated with membrane

trafficking, cell signaling, fat transport, and immune function (Reinhardt and Lippolis, 2006). The identification of CD9, CD14, immunoglobulin M (IgM), TLR2, and TLR4 in the bovine MFGM were novel findings, and confirmed the already suspected role of the bovine mammary gland in surveillance and disease detection (Reinhardt and Lippolis, 2006). Follow-up proteomic analyses of the bovine MFGM identified fewer proteins, but determined that lipid composition of the MFGM consisted primarily of triglycerides and phospholipids (Fong et al., 2007).

Significant strides have been made in the characterization of proteins that comprise the bovine milk proteome since the initial studies that focused primarily on describing the abundant milk proteins and their affiliated genetic variants (Shimazaki et al., 1983; Holt and Zeece, 1988). Following advances in mass spectrometry, including the development and application of soft ionization techniques to the study of proteins and peptides, proteomic studies of the bovine milk proteome have led to the characterization of low abundance proteins in normal and mastitic whey, as well as in colostrum and the MFGM (Reinhardt and Lippolis, 2006; Smolenski et al., 2007; Fong et al., 2008). Successful application of proteomic methodologies to the identification of differentially expressed proteins in milk collected from cows with clinical mastitis has likewise been demonstrated (Hogarth et al., 2004; Smolenski et al., 2007). Nonetheless, challenges arising from the biological complexity of milk and dynamic range of proteins present in bovine milk remain a hurdle in the characterization of the complete bovine milk proteome (O'Donnell et al., 2004; Gagnaire et al., 2009). Additionally, while proteomic techniques have contributed significantly to the systematic identification of both high and low abundance bovine

milk proteins, a gap still exists between the identification of proteins and the quantification of differentially expressed bovine milk proteins manifest during disease and at varying stages in lactation (Gagnaire et al., 2009). Results thus far indicate the utility of proteomics in prospective biomarker discovery, and in the identification of potential novel drug targets. However, subsequent research endeavors must strive to address apparent inefficiencies in reducing the biological complexity of milk in order to accurately target low abundance markers in bovine milk, and to bridge the current distance between qualitative and quantitative proteomic methodologies applied to the characterization of the bovine milk proteome.

Chapter 3: Objectives

The Objectives of the following series of analyses were:

Objective 1

To generate proteomic profiles of normal bovine whey and whey following experimental induction of *Escherichia coli* mastitis using 2D gel electrophoresis, and to identify differentially expressed proteins by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry with post source decay (PSD).

Objective 2

To develop more high-throughput methods of detecting differentially expressed bovine milk proteins in whey from normal versus mastitic milk using in-solution digestion methods, followed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using both an LC-ESI-QTOF instrument system and a nano-LC- nano-spray linear ion trap instrument system.

Objective 3

To compare mass spectrometry-based label-free semiquantitiative methods including spectral counts and number of unique peptides identified, with temporal expression patterns determined using enzyme-linked immunosorbant assay (ELISA), and to evaluate changes in relative abundance of select

differentially expressed protein biomarkers in bovine milk over a time course following experimental induction of mastitis with *Escherichia coli*.

Chapter 4: Proteomic Analysis of Differentially Expressed Proteins in Bovine Milk during Experimentally Induced *Escherichia coli* Mastitis.

Abstract

The objectives of the current study were to profile changes in protein composition using 2-dimensional gel electrophoresis (2D-GE) on whey samples from a group of 8 cows prior to and 18 hours after infection with *Escherichia coli*, and to identify differentially expressed milk proteins by peptide sequencing using matrix-assisted laser desorption/ ionization time-of-flight (MALDI-TOF/TOF) mass spectrometry post source decay (PSD). Only proteins present in whey fractions of all 8 cows were sequenced to avoid reporting a protein response unique to only a subset of infected cows. Despite the overwhelming presence of casein and β-lactoglobulin, the low abundance proteins transthyretin, lactadherin, β-2-microglobulin precursor, α-1 acid glycoprotein and complement C3 precursor could be identified in whey samples from healthy cows. Whey samples at 18 hours post infection were characterized by an abundance of serum albumin, in spots of varying mass and isoelectric point, as well as increased transthyretin and complement C3 precursor levels. Also detected at 18 hours post inoculation were the antimicrobial peptides cathelicidin, indolicidin, and bactenicin 5 and 7, and the proteins β-fibrinogen, α-2-HS-glycoprotein, S100-A12, and α-1-antiproteinase. Most notable was the detection of the acute phase protein α-1

acid glycoprotein (A1AG) in mastitic whey samples, a result not previously reported. In contrast to methods used in previous proteomic analyses of bovine milk, the methods used in the current study enabled the rapid identification of milk proteins with minimal sample preparation. Use of a larger sample size than previous analyses also allowed for more robust protein identification. Results indicate that examination of the protein profile of whey samples from cows following inoculation with *E. coli* could provide a rapid survey of milk protein modulation during coliform mastitis and aid in the identification of biomarkers of this disease.

Keywords: proteomic analysis, coliform mastitis, milk protein

Introduction

For decades, mastitis has been the most costly disease to impact the dairy industry, mainly due to economic losses that stem from decreased milk production, reduced milk quality, discarded milk, cow mortality, and increased cull rates (Schepers and Dijkhuizen, 1991). Cases of mastitis arising from infection by gram-negative bacteria are of particular concern to dairy producers for several reasons: 1) coliform pathogens do not respond well to traditional mastitis treatments including antimicrobial drugs (Shim et al., 2004); 2) a vaccine developed against coliforms has exhibited only limited efficacy (Hogan et al., 1992); and 3) nearly 25% of the cows suffering from gram-negative mastitis will either die or be culled as a result of systemic complications arising from the host inflammatory response (Eberhart et al., 1987). Because there are a limited number of treatment options available, coliform mastitis and the innate immune response in the bovine mammary gland have been the focus of a considerable amount of research (Hirvonen et al, 1999; Bannerman et al., 2003; 2004). *Escherichia coli* remains the most prevalent gram-negative bacteria species to cause bovine mastitis and thus *E. coli* strains have often been used in research to experimentally induce mastitis (Hirvonen et al, 1999; Bannerman et al., 2004; Lee at al., 2006).

Dramatic changes in milk protein profiles during coliform mastitis occur in response to the release of a toxin from gram-negative bacteria cell walls during bacterial replication or death, known as endotoxin or lipopolysaccharide (LPS). LPS is a proinflammatory molecule that stimulates a rapid inflammatory response in the

mammary gland (Shuster et al., 1993; Lee et al., 2003). Epithelial cells in the mammary gland respond to LPS by producing cytokines including IL-8, TNFα, and lactoferrin (Pareek et al., 2005; McClenehan et al., 2005), and it has been established that IL-1β, IL-6, IL-8, and TNFα are all upregulated by LPS (Shuster et al., 1993; Bannerman et al., 2003). The cytokines IL-1β, IL-8, and TNFα have also been demonstrated to increase in response to *E. coli* infection in vivo (Bannerman et al., 2004). IL-1β and TNFα and are established inducers of fever, the hepatic acute phase response, and the expression of vascular endothelial adhesion molecules, while the chemokine IL-8 is known to play a major role in the recruitment of neutrophils from peripheral sites to the mammary gland during mastitis. Cytokine expression following exposure to LPS or *E. coli* is also thought to cause increased permeability of mammary vasculature, which subsequently results in a dramatic increase in levels of vascular derived proteins in milk, most notably bovine serum albumin (BSA).

Cytokine expression and the effects of the innate immune response on milk composition during coliform mastitis have been previously characterized; however, changes in actual expression profiles of milk proteins during coliform mastitis are not entirely known. Data reported on cytokine expression following *E. coli* or LPS infection have been based on enzyme-linked immunosorbent assays (ELISA), which are limited by antibody availability and reagent development (e.g., generation of recombinant protein to be used as a standard). Proteomic analysis does not rely on antibodies however, and therefore is not limited to the detection of only a certain number of proteins. Previous attempts to characterize bovine milk proteins using a proteomic approach have been performed on only a small number of cows with

mastitis confirmed only by clinical parameters, or have involved the removal of highly abundant proteins prior to analysis. The desire to better understand underlying changes that occur in the mammary gland in response to invading pathogens, and the need to identify potential targets for new mastitis therapies coupled with advances in proteomic technology have led to investigations into the bovine milk proteome. Comparative analyses of bovine whey have been conducted using 2-dimensional gel electrophoresis (2D-GE) followed by matrix assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry (Yamada et al., 2002; Hogarth et al., 2004; Smolenski et al., 2007). In the previous analyses, high-speed ultracentrifugation was used to produce the whey fraction analyzed (Yamada et al., 2002; Smolenski et al., 2007) and the caseins were removed by either acid precipitation followed by dialysis (Hogarth et al., 2004) or by immunoabsorption (Yamada et al., 2002). All previous analyses were successful in identifying the abundant whey proteins serum albumin, α-casein, β-casein, κ-casein, β-lactoglobulin, and α-lactalbumin. Lower abundance proteins, including transthyretin and complement C3 α-chain were also identified in whey from three healthy mid-lactation cows (Yamada et al., 2002), but these analyses did not compare normal and mastitic whey samples and were limited in the number of low-abundance proteins identified despite prior sample clean-up. In comparative proteomic analyses of proteins in normal and mastitic whey, mastitis was established based on clinical parameters (heat in the gland, milk clots, swelling of udder) (Hogarth et al., 2004), or the analysis was conducted on samples from only one cow with a naturally occurring clinical infection (Smolenski et al., 2007). The more inclusive of the proteomic studies (Smolenski et

al., 2007), that incorporated both a top-down proteomic approach using 2D-GE followed by mass spectrometry (MS), and a bottoms-up approach using liquid chromatography followed by MS (LC-MS) analysis of trypsin digested skim milk, whey, and milk fat globular membrane fractions, succeeded in identifying host defense related proteins. However, a very small sample size was used (n=1), and either few peptides were sequenced or the proteins were only identified in one of the types of milk fraction analyzed (Smolenski et al., 2007).

The goal of the current study was to utilize samples collected from a larger number of cows (n=8) with experimentally induced coliform mastitis, and to eliminate time consuming sample clean-up steps such as removal of high-abundance proteins and high-speed ultracentrifugation. Caseins have been the principle target of high-abundance protein removal in sample preparation of milk from healthy cows. Since the removal of caseins requires acid precipitation followed by dialysis to remove acid traces, it is reasonable to theorize that both precipitation and dialysis could result in the loss of other proteins. Following pathogen invasion and stimulation of the innate immune response, it has been well established that altered vascular permeability, as a result of local cytokine production, leads to leakage of the serum proteins into milk and the subsequent increase in the milk concentration of BSA. Thus, the primary target of high-abundance protein removal in mastitic whey samples would be BSA. A number of the commercially available serum albumin removal columns contain the non-specific dye ligand Cibacron Blue F3G-A, which efficiently binds serum albumin, but could also result in the loss of other proteins due to its non-specificity. It has also recently been demonstrated that several small

molecules and proteins bind to serum albumin and thus, they could be lost as a result of serum albumin removal from samples (Gundry et al., 2007). In addition, other whey proteins including β-lactoglobulin have been shown to participate in the non-specific binding of other ligands (Konuma et al., 2007) and thus it is reasonable to speculate that some milk proteins could become bound to a cellular component of the milk. Should a binding event occur, chances of a decrease in abundance or loss of proteins are increased with high- speed ultra-centrifugation. The goal of maintaining the large dynamic range of the milk proteins prior to proteomic analysis by minimizing potential protein losses during sample clean-up steps was to enhance the probability of identifying a greater number of differentially expressed proteins in mastitic versus normal whey samples. The time points chosen for 2D-GE analysis in the study, prior to (time 0) and 18 h after *E. coli* infection, were based on previous reports indicating peak cytokine expression at or around 18 h following infection with *E. coli* (Bannerman et al., 2004), and preliminary 2D-GE followed by MALDI-TOF-MS analyses of a full time course (12, 18, 24, and 36 hours following infection) that revealed, on average, the most protein identifications and the highest level of differential protein expression to be at 18 h following infection with *E. coli* (Supplemental Fig.1). Further knowledge of protein modulation during coliform mastitis could ultimately aid in the identification of biomarkers that could prove useful in evaluating the efficacy of adjunctive therapies in reducing inflammation associated with clinical mastitis.

Experimental Procedures

Milk Samples

Whey samples that were analyzed in the current study were obtained from an *E. coli* challenge study conducted at the Bovine Functional Genomics Laboratory at the USDA-Agricultural Research Service in Beltsville, MD. Briefly, 8 clinically healthy Holstein cows in middle to late lactation were infected in one quarter with *E. coli* strain P4. Preparation of the inoculum and experimental infection were performed as previously described (Bannerman et al., 2004). Plating of aliquots of the final inoculum on blood agar plates confirmed that cows were infused with ~200 CFU's of *E. coli*. For the preparation of whey, milk samples were centrifuged at 44,000 x *g* at 4ºC for 30 min and the fat layer removed with a spatula. The skimmed milk was decanted into a clean tube, centrifuged again for 30 min, and the translucent supernatant collected and stored at −70ºC. The use and care of all animals in this study were approved by the USDA-ARS Beltsville Agricultural Research Center's Animal Care and Use Committee.

2D- Gel Electrophoresis

The protein content of the 0 hour (pre-infection) and 18 hour (post- infection) whey fractions from each cow (n=8) was determined with a Coomassie binding assay (Coomassie Plus Bradford Assay, Pierce Biotechnology, Rockford, IL) using BSA as a standard. Approximately 200 µg of each whey protein sample was diluted in rehydration buffer (8 M urea, 2% CHAPS, 50 mM dithiothreitol (DTT), 0.2% BioLyte™ 3/10 ampholyte, 0.001% Bromophenol Blue; Bio-Rad Laboratories,

Hercules, CA) to a final sample volume of 200 µl. Following dilution in rehydration buffer, samples were applied to 11 cm pH 3-10 nonlinear immobilized pH gradient (IPG) strips (Bio-Rad Laboratories, Hercules, CA) and focused in a Bio-Rad Protean IEF Cell for 20 h using the following voltage intervals: 500 V for 1 h, 1000 V for 1 h, 2000 V for 2 h, 4000 V for 4 h, and 8000 V for 12 h. After focusing, IPG strips were equilibrated in buffer I, which consisted of 6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, 20% glycerol, and 2% w/v DTT (Bio-Rad Laboratories, Hercules, CA), at room temperature for 30 min, then alkylated in buffer II which consisted of 6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, 20% glycerol, and 2.5% w/v iodoacetamide (Bio-Rad Laboratories, Hercules CA), at room temperature for 30 min. The IPG strips were run in the second dimension on 10-20% Tris-HCl polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) in 1X Tris/Glycine/SDS buffer at 200 V for 1 h. Gels were fixed in 40% methanol (MeOH) /10% acetic acid for 1 h and then stained overnight in SYPRO® Ruby stain (Lonza, Walkersville, MD). Gels were destained twice in 10% MeOH/7% acetic acid for 1 h at room temperature and then imaged on a Typhoon™ 8600 multimode image scanner (GE Healthcare Bio-Sciences, Piscataway, NJ). All samples were prepared in duplicate for a total of 2 gels per cow for both normal whey and mastitic whey.

Tryptic Digestion

Protein spots of interest were excised from the gel with a sterile 1000 µl pipet tip or a sterile stainless scalpel blade. Only spots present in all gels from all cows for each time point were excised for identification. Identical spots from replicate gels were pooled and destained at room temperature for 1 h in 25 mM ammonium bicarbonate/

50% acetonitrile. Gel spots were dehydrated in 100% acetonitrile for 5 min at room temperature and then dried to completeness in a vacuum centrifuge for 20 min at 22°C. Spots were rehydrated in 15 μg/mL tryspin (Sigma-Aldrich, St. Louis, MO) in 25 mM ammonium bicarbonate and allowed to digest for 16 h at 37°C. Following digestion, peptides were extracted with 50% acetonitrile/5% trifluoroacetic acid (TFA) at room temperature for 1 h. Peptides were concentrated in a vacuum centrifuge for 20 min at 22°C.

MALDI-TOF MS and MALDI TOF/TOF PSD

MALDI-TOF mass spectrometry was used to analyze tryptic peptides of digested proteins and MALDI-TOF/TOF post source decay (PSD) was used for peptide sequencing. Prior to analysis, 2 μl of digested sample was mixed with 2 μl of a 5 mg/mL α-cyano-4-hydroxycinnamic (CHCA; Sigma-Aldrich, St. Louis, MO) solution prepared in 70% acetonitrile/0.1% TFA. One microliter of the 1:1 sample: matrix mixture was spotted in triplicate onto a stainless steel MALDI plate, and allowed to air dry. Mass spectra were acquired on a 4800 Plus MALDI TOF/TOF™ Analyzer (Applied Biosystems, Foster City, CA) in positive reflectron mode. A mass range of 900 to 4000 Daltons was used for MS analysis and a total of 100 shots were collected per spectrum at a laser intensity of 4100. The 6 most intense ions per spectrum exhibiting a minimum signal to noise ratio of 10:1 were automatically selected for MS/MS PSD analyses. A laser intensity of 4450 was used for PSD analyses and 100 shots were collected per spectrum.

Protein Identification

MALDI-TOF/TOF PSD spectra were searched against the "other mammalian" taxonomy of the Swiss Prot database using Mascot Daemon (v.2.1.0; Matrix Sciences, London, UK). The enzyme used was set to trypsin, maximum number of missed cleavages allowed was 1, and the peptide mass tolerance was set at 0.5 Daltons. The fixed and variable modifications were carbamidomethyl and oxidation (methionine), respectively. Protein identities reported by Mascot were individually inspected and matched to protein spots on the gels by comparing the precursor mass list for each spot recorded for each run on the instrument software with the charge-to-mass ratio (*m/z*) of sequenced peptides on the Mascot search results report. Unless noted, only proteins with a minimum of two unique peptides sequenced with Mascot identity scores greater than 30 were reported.

Results

Infection with E. coli Results in Differential Expression of Several Milk Proteins

Whey from normal bovine milk samples (collected prior to infection with *E. coli*) and the whey fraction from mastitic milk samples (collected 18 h following infection with *E. coli*) were separated by 2D-GE and identified by peptide sequencing using MALDI-TOF/TOF PSD. Variation in the protein profiles between cows was minimal, and thus representative gels from both normal and mastitic whey were chosen to illustrate the protein profiles generated in the current study. Only protein spots clearly detected in all gels run on samples collected prior to or after infection from all 8 cows were selected for follow-up identification. Proteins were identified in

whey samples from healthy cows (Table 1) and in mastitic whey samples (Table 2) by the sequencing of at least two peptides, with the exception of two proteins that had only one peptide sequenced.

Normal bovine whey 2D-GE profiles (Fig. 1) exhibited an abundance of casein proteins, including α-S1-casein, β-casein, and κ-casein variants, and the whey proteins serum albumin, β-lactoglobulin, and α-lactalbumin. Also identified in whey from healthy cows were polymeric-immunoglobin receptor (PIGR) and several low abundance proteins including α-1-acid glycoprotein (A1AG), transthyretin, complement C3, epididymal secretory protein E1, lactadherin, fatty acid-binding protein (heart), and β-2 microglobulin.

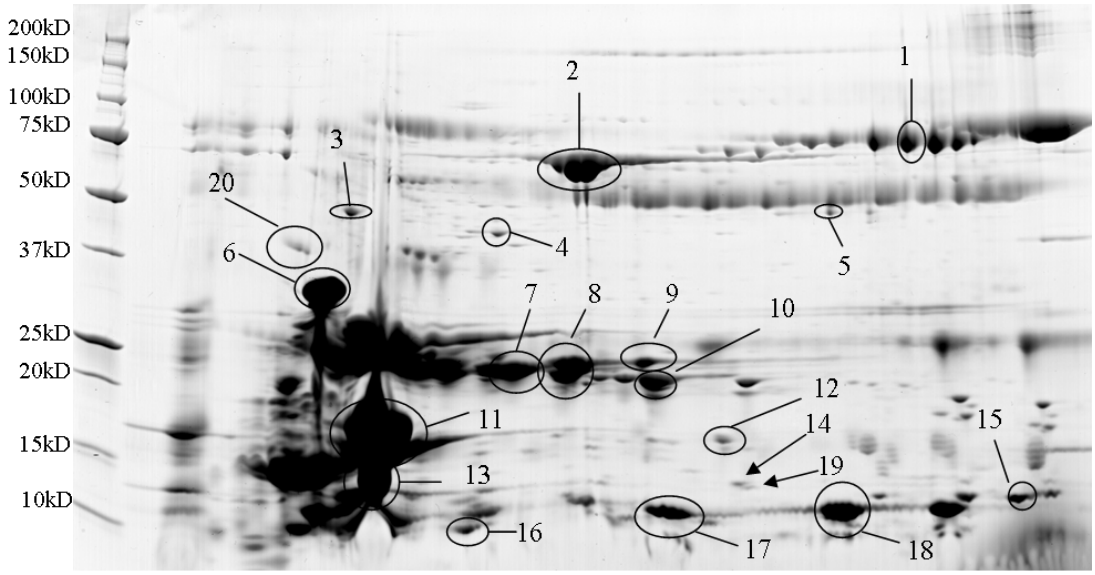


Figure 1 2D-GE SYPRO Ruby stained gel of normal bovine whey separated on an 11cm pH3-10 nonlinear IPG strip and 10-20% Tris-HCl SDS PAGE. Spot numbers correspond to Spot ID in Table 1.

Table 1 Proteins identified in normal bovine whey using MALDI-TOF PSD.

Spot ID[1]	Protein Name	M_r [2]	Peptides Identified	Peptide Score	MH^+ [3]
1	Polymeric-immunoglobin receptor	83695	YGETAAVYVAVESR	89	1514.91
			ADKINIDLQVLEPEPELVYGDLR	118	2639.63
			GLNFDVSLEVSQDPAQASHAHVYTVDLGR	93	3125.85
2	Serum Albumin	71244	LGEYGFQNALIVR	88	1479.98
			VPQVSTPTLVEVSR	85	1511.95
			ECCHGDLLECADDR	88	1749.78
3,6	α-S1-casein precursor	24570	HQGLPQEVLNENLLR	86	1760.19
			EPMIGVNQELAYFYPELFR	94	2316.48
			HPIKHGGLPQEVLNENLLR	135	2235.14
4	Complement C3 precursor	188675	SEETKENER	40	1121.64
			VYSYYNLDETCIR	111	1695.99
			VHQYFNVGLIQPGAVK	119	1770.16
5	Lactadherin	48520	QFQFIQVAGR	68	1193.83
			DFGHIQYVAAYR	116	1439.96
			INLFDTPLETQYVR	95	1709.19
7,8,9	κ-casein	21370	YIPIQYVLSR	51	1251.89
			HPHPHLSFMAIPPK. + Oxidation (M)	31	1625.04
			SPAQILQWQVLSNTVPAK	113	1980.39
10,16, 17,18	ß-casein	25148	DMPIQAFLLYQEPVLGPVR	101	2186.60
			FQSEEQQQTEDE LQDL	113	1982.17
11	ß-lactoglobulin precursor	20269	LSFNPTQLEEQCHI	110	1715.69
			YLLFCMENSAEPEQSLACQCLVR	137	2818.23
12	Epididymal secretory protein E1 precursor	16972	VVVEWELTDDKNQR	96	1731.08
13	α-lactalbumin	16692	VGINYWLAHQ	65	1200.51
			ILDKVGINY WLAHK	107	1669.76
			IWCKDDQNPHSSNICNISCDK	35	2590.78
14	Transthyretin	15831	TSESGELHGLTTEDKFVEGLYK	69	2440.37
			SLGISPFHEFAEVVFTANDSGPR	107	2477.34
15	ß-2-microglobulin precursor	13782	HVTLEQPR	59	979.69
			DWSFYLLSHAEFTPNSK	78	2042.32
			HPPEDGKPNYLNCYVYGFHPPQIEIDLLK	43	3454.36
19	Fatty acid-binding protein, heart	14827	SLGVGFATR	49	907.55
			LGVEFDETTADDR	86	1467.72
20	α-1-acid glycoprotein	23453	WFYIGSAFR	27	1147.22
			AIQAAFFYLER	82	1426.01

[1] Corresponds to the number assignment in Figure 1.[2]Average mass of protein. [3] Mass of charged peptide.

In contrast, 2D-GE performed on mastitic bovine whey samples (Fig. 2A) revealed reduced abundance of α-S1-casein and β-casein, and the whey proteins β-lactoglobulin and α-lactalbumin (Fig. 3) but a profound increase in bovine serum albumin.. κ-casein variants were still evident in the mastitic whey, but in reduced amounts. Interestingly, spot 10 on the 2D-gels of mastitic whey appeared to be similar to spot 8 on the 2D-gels of normal whey, but the peptides sequenced from spot 10 were from apolipoprotein A-I. Other highly abundant proteins found in mastitic whey included serotransferrin, fetuin (α-2-HS-glycoprotein), fibrinogen, and A1AG. Low abundance proteins identified in mastitic whey included α-1-antiproteinase, complement C3 and C4, transthyretin, protein S100- A12, and several antimicrobial peptides in the cathelicidin family including cyclic dodecapeptide (cathelicidin-1), indolicidin (cathelicidin-4), bactenecin-5 (cathelicidin-2), and bactenecin-7(cathelicidin-3), as well as apolipoproteins A-I, A-II, and C-III (Fig. 2B). Levels of A1AG, transthyretin, and complement C3 levels appeared to increase in comparison to levels in normal whey, but no absolute quantification was performed.

Table 2 Proteins identified in mastitic milk samples 18 hours post inoculation

Spot ID[1]	Protein Name	M_r [2]	Peptides Identified	Score	MH^+ [3]
1	Serotransferrin precursor	79870	WCAIGHQER	71	1157.07
			TYDSYLGDDYVR	103	1467.34
			FDEFFSAGCAPGSPR	100	1645.51
2,6,14, 15,17,18, 18,20	Serum Albumin precursor	71244	LGEYGFQNALIVR	99	1479.71
			DAFLGSFLYEYSR	90	1567.69
			KVPQVSTPTLVEVSR	90	1639.86
3	α-1-antiproteinase precursor (α-1-antitrypsin)	46417	NLYHSEAFSINFR	78	1598.39
			LQQLEDKLNNELLAK	68	1769.67
4	α-2-HS-glycoprotein Precursor	39193	QDGQFSVLFTK	52	1270.14
			TPIVGQPSIPGGPVR	91	1475.42
5	α-1-acid glycoprotein	23453	WFYIGSAFR	75	1146.98
			AIQAAFFYLEPR	91	1426.28
7,8	Fibrinogen β-chain	53933	EDGGGWWYNR	58	1239.42
			YCGVPGEYWLGNDR	61	1685.62
			KYCGVPGEYWLGNDR	66	1813.71
9	Complement C4 precursor	103018	CSVFYGAPR	50	1055.87
			GQQDLEGYR	41	1065.94
10	Apolipoprotein A-I precursor	30258	VAPLGEEFR	48	1017.87
			LSPLAQELR	26	1026.93
			QQLAPYSDDLR	71	1306.07
11,13	Cyclic dodecapeptide precursor	17931	QPWAPPQAAR	30	1121.48
			AVDQLNEQSSEPNIYR	131	1862.80
			LLELDQPPQDDEDPDSPKR	101	2206.92
12	Transthyretin precursor	15831	FVEGLYKVELDTK	66	1540.74
			TSESGELHGLTTEDKFVEGLYK	130	2440.06
			SLGISPFHEFAEVVFTANDSGPR	95	2477.07
16	Complement C3 precursor	188675	VYSYYNLDETCIR	90	1695.99
			VHQYFNVGLIQPGAVK	118	1770.21
21	Indolicidin precursor	16697	AVDQLNELSSEANLYR	138	1822.16
			LLELDPPPKDNEDLGTR	62	1922.27
	Bactenecin-7 precursor	21782	SSEANLYR	63	939.85
			LLELDPPPKDVEDR	60	1636.56
22	Bactenecin-5 precursor	20303	SSEANLYR	57	939.50
			AVDQFNER	53	978.50
			LLELDPTPNDDLDPGTR	87	1881.08
23	α-S1-casein precursor	24570	EPMIGVNQELAYFYPELFR + Oxidation	66	2332.93
24	Apolipoprotein A-II precursor	11252	TQEELTPFFK	75	1239.99
			AGTDLLNFLSSFIDPK	83	1738.44
25	Apolipoprotein C-III precursor	10685	DWMTESFSSLK + Oxidation (M)	40	1347.04
			DALSSVQESQVAQQAR	134	1717.41
26	Indolicidin precursor	16697	AVDQLNELSSEANLYR	143	1822.67
			LLELDPPPKDNEDLGTR	68	1922.76
27	Protein S100-A12	10679	VGHFDTLNKR	78	1186.69
			DGAVSFEEFVVLVSR	79	1653.95

[1] Corresponds to the number assignment in Figure 2. [2]Average mass of protein. [3]Mass of charged peptide.

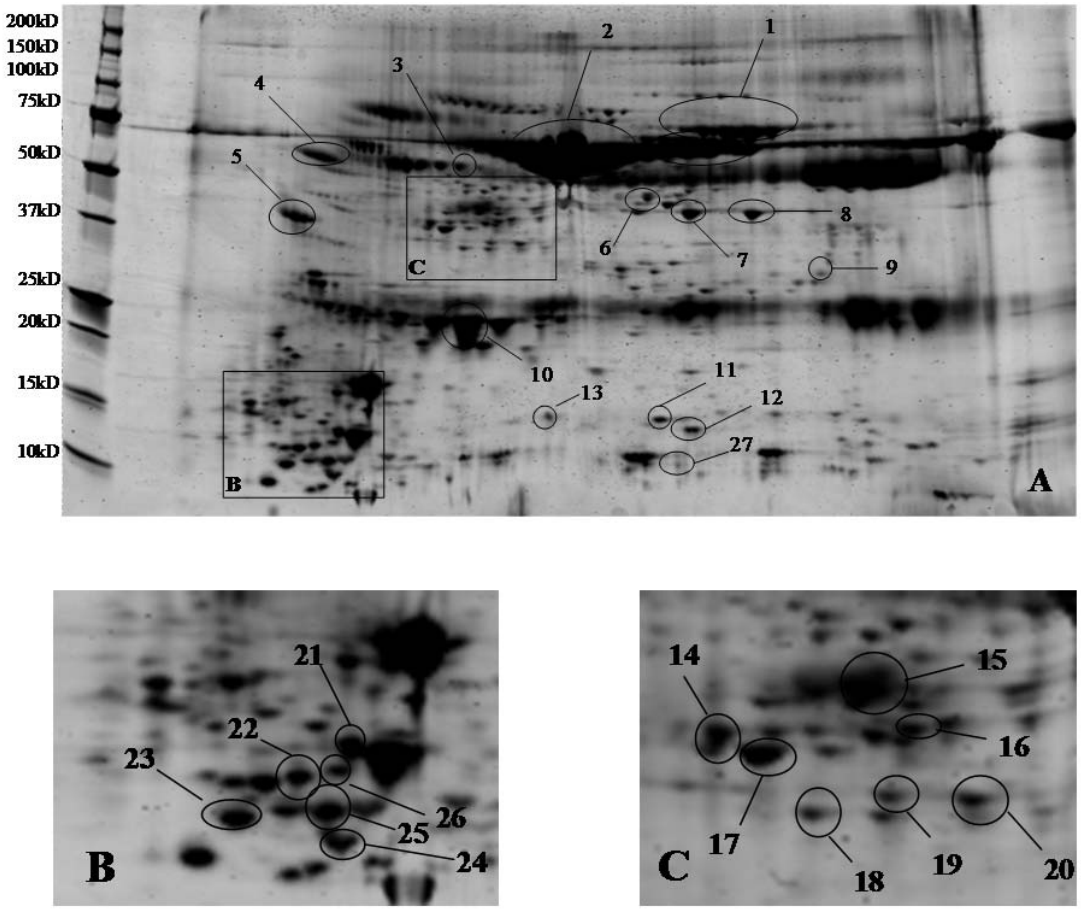


Figure 2 A) 2D-GE SYPRO Ruby stained gel of mastitic bovine whey separated on an 11cm pH3-10 nonlinear IPG strip and 10-20% Tris-HCl SDS PAGE. B) 2D-GE of antimicrobial peptides and apolipoproteins. C) 2D-GE of variant forms of bovine serum albumin. Spot numbers correspond to Spot ID in Table 2.

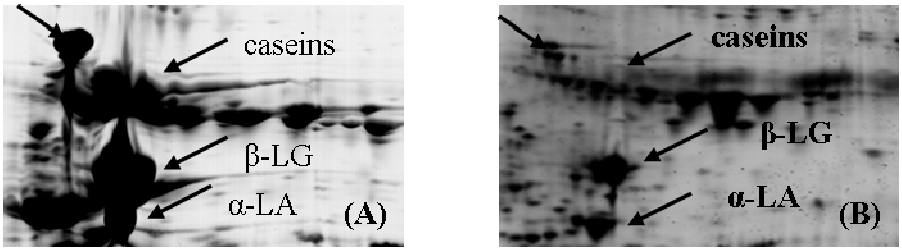


Figure 3 Comparison of abundance levels of caseins, β-lactoglobulin and α-lactalbumin in normal (A) and mastitic (B) milk. Arrows indicate caseins which include α-S1, β, and κ, and β-lactoglobulin (β – LG) and α-lactalbumin (α-LA).

Other proteins including calbindin, acetylcholine receptor subunits beta and delta, matrix metalloproteinase-20, apolipoprotein A-IV, insulin-like peptide, lactotransferrin, and toll-like receptor 2 proteins were identified (data not shown) in normal and mastitic whey, but too few peptides were sequenced, and the Mascot identity scores were low.

Serum Albumin is Present in Multiple Spots in Mastitic Whey Profiles

2D-GE performed on whey samples collected 18 h following infection with *E. coli* revealed a marked increase in abundance of serum albumin. In addition to intact serum albumin however, several smaller forms of BSA with varying mass and isoelectric points were present in mastitic whey samples (Fig. 2C). MALDI-TOF mass spectra of the abundant form of BSA found in normal whey and in mastitic whey ($M_r \approx 70$kD) differed slightly in terms of intensity of select ions and mass of tryptic peptides (Fig. 4). The most notable difference in the peptide mass maps of bovine serum albumin isolated from normal and mastitic whey samples was the presence a peptide with *m/z* 1749, which was unique to the spectrum of normal whey, and the presence a peptide with *m/z* 1724, which was apparent only in the spectrum from mastitic whey. Marked differences were apparent in the MALDI-TOF spectra of variant forms of serum albumin from mastitic whey samples however (Fig. 5), as the peptide with *m/z* 1479, which exhibits a high intensity in intact serum albumin in both normal and mastitic whey, was absent from MS spectra of two of the serum albumin variants (Spots 6 and 15). Additionally, clear differences existed in the size and distribution of tryptic peptides between the peptide mass maps of the variant forms of

BSA. The peptide mass map of Spot 6 was characterized by the high intensity of the peptide with *m/z* 1511, as well as a peptide with an *m/z* 1166 that was not present in any other forms of BSA. The results indicate potential modifications of BSA in the mastitic whey samples that resulted in variance among the peptide mass maps.

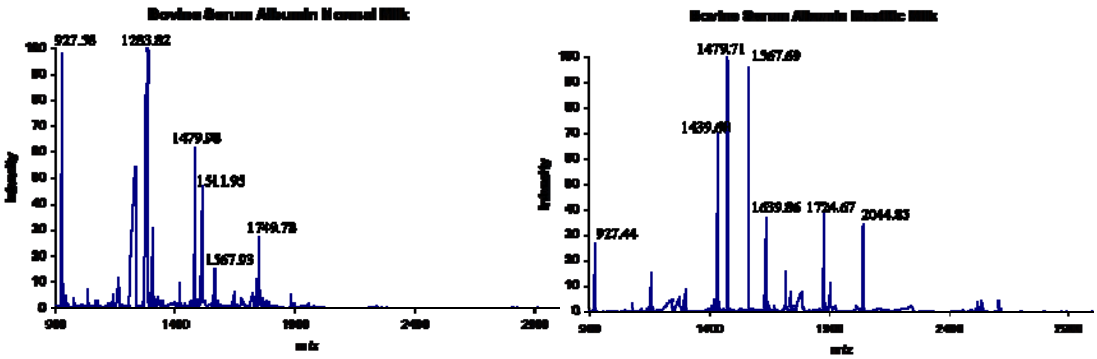


Figure 4 Comparison of mass spectra of the tryptic peptides generated from serum albumin in normal milk and in mastitic milk samples. Normal milk serum albumin tryptic peptides differ slighty in mass and in intensity from serum albumin peptides in mastitic milk.

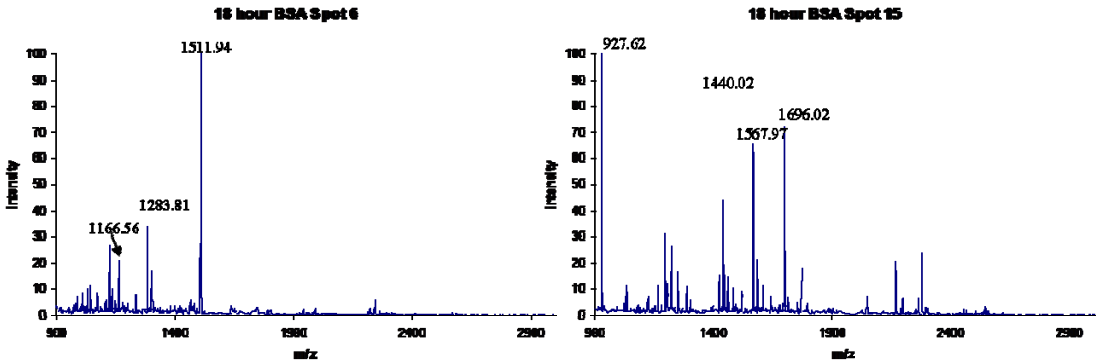


Figure 5 Mass spectra of variant forms of serum albumin in mastitic milk samples. Both spectra lack the abundant serum albumin peptide with *m/z* 1479. The spectra of spot 6 contains a peptide found only in that spot (*m/z* 1166) and the spectra of spot 15 contains a peptide (*m/z* 1696) from complement C3.

Alpha-1-Acid-Glycoprotein is Present in Both Normal and Mastitic Whey Samples

Alpha-1-acid glycoprotein was the only acute phase protein identified in both normal and mastitic whey samples. 2D-GE profiles of whey from milk collected from all of the cows in the study (n=8) prior to infection with *E. coli* revealed low-abundance of A1AG. In contrast, 2D-GE of whey from milk collected 18 h following infection with *E. coli* revealed A1AG to be in relatively high-abundance. The identification of A1AG in whey from cows suffering from coliform mastitis has not been previously reported.

Discussion

Results of the current study demonstrate that exposure to *E. coli* profoundly alters milk protein profiles. Additionally, the discovery of several low abundance proteins not previously reported in proteomic analyses of bovine milk (Yamada et al., 2002; Hogarth et al., 2004; Smolenski et al., 2007) indicates the success of the current methods in identifying differentially expressed milk proteins during coliform mastitis.

In addition to the caseins α-S1, β, and κ, the whey proteins β-lactoglobulin and α-lactalbumin, and PIGR were all identified in these analyses as well as in previous proteomic analyses. Several other proteins were identified in whey samples from healthy cows however, that were not reported in earlier proteomic investigations of normal milk (Yamada et al., 2002; Hogarth et al., 2004; Smolenski et al., 2007) including lactadherin, fatty-acid binding protein (FABP), β-2 microglobulin, transthyretin (TTR), and epididymal secretory protein E1. While lactadherin and FABP were found in the proteomic analysis of the milk fat globular membrane, neither of

these proteins were identified in proteomic studies of normal whey. Lactadherin and FABP are known to be abundant in the milk fat globular membrane (Mather, 2000), and thus it is reasonable to deduce that the identification of these proteins could be due to trace remnants of the fat layer present in the normal whey. Likewise, β-2 microglobulin was identified in colostrum (Smolenski et al., 2007), but not in normal whey. Also identified in normal whey samples was epididymal secretory protein E1, also known as NPC2. NCP2 is a small lysosomal glycoprotein secreted in milk that is involved in cholesterol uptake (Xu et al., 2007). NPC2 has not been reported in previous investigations into the bovine milk proteome. The definitive presence of NPC2 in these normal whey samples is speculative however, as only one peptide from the protein was successfully sequenced.

The presence of TTR in normal whey samples is compatible with previous proteomic analyses (Yamada et al., 2002; Hogarth et al., 2004; Smolenski et al., 2007); however, using the methods described in these analyses, TTR was identified in both the milk of healthy cows and in cows with coliform mastitis. Other proteins not previously identified in proteomic analyses of mastitic whey that were identified in the current study include the complement factors C3 and C4, and the apolipoproteins A-II and C-III. Complement C3 was previously identified in normal bovine whey (Yamada et al., 2002), but in the current study complement C3 was identified in both normal and mastitic whey samples. These findings are compatible with the previously well characterized role of the complement system in innate immunity (Rainard and Riolett, 2006). While the discovery of complement factors in both normal and mastitic whey is not novel, the identification of these proteins using 2D-GE and MALDI-TOF PSD is

supportive of the success of the methods used in the current study to identify low abundance proteins without the prior sample clean-up. Additionally, apolipoprotein A-II and C-III were not found in previous proteomic analyses (Yamada et al., 2002; Hogarth et al., 2004; Smolenski et al., 2007). Apolipoprotein A-II is known synonymously as bovine antimicrobial peptide-1 (BAMP-1) and has been shown to have antimicrobial properties (Motizuki et al., 1998). The acute phase protein serum amyloid A (SAA) belongs to the apolipoprotein family and SAA is known to be elevated during mastitis (Hirvonen et al., 1999; Eckersall et al., 2001; Grönlund et al., 2005). Whether or not the apolipoproteins identified in the current study are present in mastitic milk as the result of an acute phase response, leakage into the gland from systemic circulation, or the result of another mechanism entirely requires further analyses.

The appearance of serum albumin, serotransferrin, fibrinogen, fetuin (α-2-HS-glycoprotein), and α-1-antiproteinase in relatively high-abundance in the 2D-GE of mastitic whey in the current proteomic analyses supports the leakage of serum proteins into milk following cytokine production and altered vascular permeability of the mammary epithelium. Serum albumin and serotransferrin have been identified previously in mastitic whey (Hogarth et al., 2004; Smolenski et al., 2007) as well as fibrinogen and fetuin (Smolenski et al., 2007); however, identification of α-1-antiproteinase in mastitic whey has not been reported.

The identification of several bovine antimicrobial peptides (AMP) using MALDI-TOF/TOF PSD supports the influx of neutrophils into the mammary gland following *E. coli* invasion (Saad and Ostensson, 1990). The cytoplasmic granules of circulating

neutrophils are a well characterized source of AMP belonging to the cathelicidin family and are released upon neutrophil degranulation in the mammary gland (Tomasinsig and Zanetti, 2005). The identification of cyclic dodecapeptide (cathelicidin-1) in mastitic milk has been reported (Smolenski et al., 2007), but not indolicidin (cathelicidin-4), bactenecin-5 (cathelicidin-2), and bactenecin-7(cathelicidin-3), as reported in the current study. Previous research on members of the cathelicidin family of host defense peptides has indicated that cathelicidins could function in the neutralization of endotoxin, as well as the down-regulation of pro-inflammatory cytokine expression (Scott et al., 2002; Wiese et al., 2003; Zanetti et al., 2004). The identification of several members of the cathelicidin antimicrobial peptides in mastitic but not normal whey samples in these analyses support the notion that AMPs serve a role in host defense.

As in previous proteomic analyses comparing normal and mastitic bovine whey (Hogarth et al., 2004; Smolenski et al., 2007), serum albumin was detected in both normal and mastitic samples. However, in the current study, multiple forms of serum albumin were detected in mastitic whey. It is established that serum albumin levels increase dramatically in bovine milk during coliform mastitis, but there have not been previous reports of different isoforms of serum albumin in mastitic milk. Previous investigations have however, demonstrated that albumin is synthesized and secreted by the bovine mammary gland, and that local expression of albumin in the mammary gland was increased both in mastitic mammary tissue, and following exposure to LPS, when compared to expression in healthy mammary tissue (Shamay et al., 2005). Based on reported RT-PCR data, the sizes of mammary-derived albumin and albumin in bovine serum appear to be the same (Shamay et al., 2005), but the exact amino acid

sequence of the albumin expressed in the mammary gland was not determined. Differences in the peptide sizes, distribution, and relative intensities apparent in the MALDI-TOF spectra of tryptic fragments of albumin from normal and mastitic whey samples, as well as the variants of albumin detected in mastitic whey, could therefore have been due to potential structural differences between albumin produced in the mammary gland and albumin that leaked into the gland as a result of increased vascular permeability, but determination of such would require further investigation. Differences in the albumin MALDI spectra could also have been due to slight variance in conditions during the digestion stage, or the result of compounds bound to the serum albumin that might have affected tryptic digestion patterns. Serum albumin is known to bind and transport long chain fatty acids as well as other small molecules including bradykinin and interferons, and acute phase proteins including SAA and α-1-acid glycoprotein (Peters, 1977; Gundry et al., 2007). Bovine serum albumin is also known to have 3 isoforms with slightly differing isoelectric points. In a previous study, bovine serum albumin exhibited the same molecular weight but three different p*I*'s (5.4, 5.5, and 5.6) following 2D-GE (Chang et al, 2005). Results of the current study however, indicate shifts in both the size and the p*I* of bovine serum albumin. The most likely explanation for the multiple forms of BSA apparent on the 2D gels of mastitic whey is proteolysis, which is consistent with previous findings that treatment with trypsin yielded several fragmented forms of BSA (Peters et al., 1975). Fragments of BSA resulting from proteolysis did however retain the ability to bind ligands (Peters, 1977). Whether or not the obvious shift in mass and p*I* of the BSA fragments was in part due to binding of small molecules or proteins was not determined. In the current study, the

majority of the peptides sequenced from spot 16 were serum albumin peptides. Evidence of the potential binding of other proteins to serum albumin was evident in this spot in mastitic whey, as one highly intense peptide that was present in the spectrum from spot 16 and subsequently sequenced (*m/z* 1696) matched complement C3. The explanation could be close proximity and therefore incomplete separation of the two proteins, or a binding event either before or after leakage into the mammary gland from systemic circulation.

Perhaps the most promising discovery in these proteomic analyses, however, was the identification of A1AG in both normal and mastitic whey samples, and the apparent higher abundance of this acute phase protein (APP) in the mastitic samples. Previous analyses of APP expression during bovine mastitis have revealed increases in A1AG, SAA, LPS-binding protein (LBP), and haptoglobin (Hp) levels in serum (Hirvonen et al., 1999; Hiss et al., 2004; Bannerman et al., 2004; Grönlund et al, 2005), but the only acute phase proteins to be identified in mastitic milk were SAA, LBP, and Hp (Eckersall et al., 2001; Hiss et al., 2004; Bannerman et al., 2004). A more recent study, however, identified A1AG in colostrum and normal milk of cattle (Ceciliani et al., 2005), and determined that A1AG was expressed in mammary tissue. Likewise, there have been reports of the local expression of A1AG in human mammary epithelial cells (Gendler et al., 1982). The identification of A1AG in normal whey is compatible with previous analyses (Ceciliani et al., 2005). The presence of A1AG in mastitic whey however, has not been previously reported, but it is congruent with reports of increased hepatic production of APP during coliform mastitis, and the subsequent leakage of APP

into the milk as a result of altered vascular permeability (Eckersall et al., 2001; Hiss et al., 2004; Bannerman et al., 2004).

In the present study, 2D-GE performed on whey samples both prior to inoculation with *E. coli* and 18 hours following infection revealed protein profiles with marked differences. A greater number of differentially expressed proteins in mastitic versus normal whey were identified than in previous analyses that utilized 2D-GE followed by MS (Hogarth et al., 2004; Smolenski et al., 2007). The most likely explanation is that no sample clean-up steps were employed in the analyses reported here, which reduced protein loss and allowed for the identification of a greater number of proteins. The hypothesis regarding the number of proteins identified is supported by the success of a more recent proteomic analysis of several types of milk fractions (Smolenski et al, 2007) in identifying a greater number of milk proteins related to host defense despite the lack of sample clean-up prior to analysis. The number of cows sampled in the current study was much larger than previous analyses, and mastitis was experimentally induced so as to enable controlled analysis of changes in milk protein composition in response to infection. Both the larger sample size used and the experimental induction of mastitis improve the potential of the current study for discovering protein biomarkers, while reducing the risk of reporting a host defense response unique to only one or a small number of cows. Peptide identity scores are likewise an important parameter when considering the stringency of protein identification. Previous proteomic analyses failed to report scores associated with protein identification (Yamada et al., 2002; Smolenski et al., 2007) or the scores reported were based on peptide mass mapping (Hogarth et al., 2004). With few exceptions, the peptide identity

scores reported in the current study are robust and the protein identification criteria more stringent. The methods and experimental design of the present study have demonstrated success in producing a more complete proteomic profile of milk protein changes during coliform mastitis, and have provided candidates for future research into the effects of *E. coli* induced inflammation on protein expression in the bovine mammary gland.

Chapter 5: Proteomic Analysis of the Temporal Expression of Abundant Bovine Milk Proteins during Coliform Mastitis using a Label-Free Approach.

Abstract

Previous studies have used proteomic approaches to evaluate the differential expression of proteins in milk from clinically healthy cows and from those with experimentally induced coliform mastitis. Earlier studies, however, have been limited to the analysis of only one time point during infection and have lacked quantification of protein modulation. The objectives of the current study were to evaluate the use of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to analyze changes in the temporal expression of abundant bovine milk proteins during the course of *Escherichia coli* intramammary infection, and to utilize a label-free semiquantitative approach to evaluate change in relative abundance of identified proteins. An additional objective was to compare observed trends in protein modulation detected using LC-MS/MS with those detected by ELISA, so as to investigate the feasibility of using a non-antibody based approach to track changes in biomarker expression during mastitis. LC-MS/MS conducted on whey from milk samples, collected just prior to infusion with *E. coli* and at 12, 18, 24, 36, 48, and 60 hours following infection, resulted in the identification of the high to medium abundance proteins αS_1-, β-, and κ-casein, and the whey proteins serum albumin, β-lactoglobulin and α-lactalbumin. Additionally, a select number of low abundance markers of inflammation including lactoferrin, transferrin, α-1-acid glycoprotein

(A1AG), complement factor C3, apolipoproteins A-I and A-II, fibrinogen, glycosylation-dependent cell adhesion molecule-1 (Glycam-1), peptidoglycan recognition receptor protein (PGRP) and cathelicidin-1 were also identified. Two methods for determining relative protein abundance, spectral counts and number of unique peptides, were compared and the latter used to evaluate temporal changes in milk proteins in response to infection. For comparison with the proteomic-based methods of determining relative protein abundance, changes in serum albumin, lactoferrin, and transferrin in milk during mastitis were also measured using ELISAs. Label-free, proteomic-based quantification revealed relative changes in milk proteins that were comparable to expression profile changes detected by ELISA. The results indicate that label-free LC-MS/MS methods are a viable means of tracking changes in relative protein abundance in milk, and that LC-MS/MS can be used to evaluate mastitis-associated temporal changes in the expression of bovine proteins for which no antibody or ELISA currently exists.

Key Words: bovine milk proteome, liquid chromatography/tandem mass spectrometry (LC-MS/MS), coliform mastitis, label-free quantification.

Introduction

Characterization of the bovine milk proteome is a relatively new field in dairy cattle research, and to date has involved only a limited number of endeavors. The earliest attempt to identify bovine milk proteins via a proteomic approach involved the use of liquid chromatography coupled with mass spectrometry (LC-MS) (Léonil et al., 1995); however, the findings of this study were limited by the absence of peptide sequencing data to support the authors' identification of major milk proteins on the basis of molecular mass. Efforts to further characterize the bovine milk proteome have included studies of normal bovine whey using 2-dimensional gel electrophoresis (2D-GE) to fractionate milk proteins, and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to identify low abundance proteins (Fong et al., 2008), as well as 2D-GE coupled to both matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and LC-MS/MS (Yamada et al., 2002). Additionally, LC-MS/MS has been applied to the characterization of the bovine milk fat globule membrane (MFGM) proteome (Reinhardt and Lippolis, 2006; 2008). Proteomic approaches have also been applied to investigations into markers for bovine disease including mastitis. Recent attempts to identify differentially expressed proteins and potentially novel protein biomarkers of host response during mastitis have utilized several proteomic strategies including 2D-GE followed by either MALDI-TOF mass spectrometry (Hogarth et al., 2004; Smolenski et al., 2007), or LC-MS/MS (Smolenski et al., 2007).

The most reliable identifications made in nearly all prior bovine milk proteome analyses, whether in normal or mastitic whey, have included the caseins α-S_1-casein,

α-S_2-casein, β-casein, κ-casein, and the abundant whey proteins β-lactoglobulin, α-lactalbumin, and serum albumin. Efforts, however, have been focused on the identification of proteins in bovine milk, and not on monitoring changes in expression over time, or over the course of infection. Likewise, while efforts to elucidate milk protein expression during cases of clinical mastitis have expanded our knowledge of protein modulation in bovine milk during mastitis, analyses have been limited to: (1) identification of differentially expressed proteins at only one time point following experimental infection (Smolenski et al., 2007); (2) small sample sizes (Smolenski et al., 2007); or (3) cases of mastitis confirmed only by clinical parameters rather than the presence of a defined intramammary infection (Hogarth et al., 2004; Smolenski et al., 2007). Assessments of temporal changes in bovine milk protein expression during clinical mastitis have been limited to examination of proteolysis in milk, and changes in serum albumin, lactoferrin, the caseins, β-lactoglobulin, α-lactalbumin, and the proteose peptone during experimentally induced coliform mastitis (Shuster et al., 1991; Moussaoui et al., 2004). Changes in the concentration of lactoferrin, serum albumin, α-lactalbumin, and immunoglobin G (IgG) have also been monitored in milk from cows with both natural and experimental coliform mastitis (Harmon et al., 1976). Additionally, many efforts have been made to measure changes in cytokine expression during experimentally induced mastitis (reviewed in Bannerman, 2009).

Quantification of the abundant milk proteins has been accomplished using reverse-phase high performance liquid chromatography (RP-HPLC), but the methods developed were applied only to milk collected from healthy cows, and were not used to analyze mastitic milk (Bobe et al., 1998). To date, information is only available on

the actual temporal expression of a limited set of milk proteins following experimental induction of coliform mastitis with *Escherichia coli*.

The release of lipopolysaccharide (LPS) from gram negative bacteria in the mammary gland stimulates the release of cytokines and other inflammatory mediators that in turn induce dramatic changes in bovine milk protein profiles during coliform mastitis (Smolenski et al., 2007). Cytokines cause altered vascular permeability, a phenomenon that results in profound increases in levels of bovine serum albumin (BSA) in milk (Shuster et al., 1993; Bannerman et al., 2003; 2004), as well as other vascular derived proteins. Chemokines recruit polymorphonuclear neutrophils (PMN) to the site of infection in the gland, and upon degranulation these cells release cationic antimicrobial proteins into the milk (Tomasinsig and Zanetti, 2005), as well as acute phase proteins (Rahman et al., 2008). Additionally, cytokines stimulate the hepatic production of acute phase proteins (APP; Dinarello, 1996; Eckersall et al., 2001) as well as local production of APP in the mammary gland (Ceciliani et al., 2005).

To date, knowledge of cytokine expression in milk following *E. coli* infection or LPS challenge has been derived primarily from enzyme-linked immunosorbent assays (ELISA). The chief limitation of utilizing methodologies such as ELISAs in marker discovery analyses, however, is that ELISAs are designed to measure only one protein at a time, and are limited by antibody development and availability. Currently, the number of commercially available bovine-specific antibodies is minimal compared to the number of antibodies available for human and conventional lab animal species.

Conversely, proteomic methodologies are not reliant on antibodies and, thus, not restricted to the detection of only a select number of proteins (Lippolis and Reinhardt, 2008). Accurate quantification of the abundance of individual proteins identified in complex biological samples using proteomic strategies is, however, an emerging research area. A popular means of quantifying proteins in a complex biological sample has been 2D-GE, but the "top down" approach using electrophoresis and quantification via densitometry is limited in sensitivity and problematic for proteins that are insoluble or have either a very high or low molecular weight (Old et al., 2005). An alternative approach is "bottom up" or "shotgun" proteomics that involves the digestion of proteins in mixtures and subsequent analysis using LC-MS/MS.

Several labeling strategies have been utilized in conjunction with LC-MS/MS analyses including: metabolic labeling commonly known as stable isotope labeling by amino acids in cell culture or SILAC (Ong et al., 2002); the use of synthetic standards such as the commercially available AQUA™ peptides (Stemmann et al., 2001); proteolytic labeling with ^{18}O (Yao et al., 2003); or isotope incorporation by several means including chemical derivatization also known as isotope coded affinity tags or ICAT (Gygi et al., 1999), isobaric tags for relative and absolute quantitation (iTRAQ; Ross et al., 2004), or global internal standard technology (GIST; Chakraborty and Regnier, 2002). Labeling strategies are cost limiting, not amenable to all biological matrices, and frequently require pairwise comparisons analysis between samples that renders retrospective assessments impossible (Old et al., 2005). As a result, recent trends have been towards label-free relative quantification based on the comparison of: (1) the mean peak area of all peptides identified for each protein in a sample

(Bondarenko et al., 2002; Old et al., 2005); (2) number of unique peptides identified for each protein in a sample (Liu et al., 2004); or (3) number of spectral hits (spectral counting) for each protein in a sample (Liu et al., 2004; Old et al., 2005; Zybailov et al., 2005).

The goals of the current study were to profile temporal expression of abundant proteins in bovine milk samples collected over a time course following infection with *Escherichia coli* using LC-MS/MS, and to utilize a label-free approach to quantify relative changes in identified proteins. A further objective was to compare observed trends in protein modulation detected using LC-MS/MS versus ELISA to examine the accuracy of label-free quantification when used to track changes in the relative abundance of milk proteins during clinical infection.

Experimental Procedures

Cows

Eight clinically healthy multiparous Holstein cows in middle to late lactation (211 DIM ± 12 d) were selected on the basis of milk SCC (MSCC) of < 200,000 cells/mL and no history of treatment for mastitis over the past 6 months. The care and use of all animals in this study were approved by the University of Maryland's Animal Care and Use Committee.

Determination of MSCC

To quantitate somatic cells, milk samples were heated to 60°C for 15 min and subsequently maintained at 40°C until counted on an automated cell counter (Bentley Somacount 150; Bentley Instruments, Inc., Chaska, MN).

Escherichia coli challenge and whey preparation

Preparation of the inoculum and experimental infection were performed as previously described (Bannerman et al., 2004). Following overnight incubation, 1mL of inoculum was serially diluted in phosphate-buffered saline (PBS) and plated on trypticase soy agar plates. Plates were incubated overnight at 37°C and the stock solution maintained at 4°C. After determination of the stock culture concentration based on colony counts, the stock culture was diluted in PBS to a final concentration of approximately 40 CFU/mL.

Baseline milk samples were collected aseptically from the right front quarter of each of the 8 cows the evening before and the morning of challenge. Following morning milking, the right front quarter of each of the 8 cows was infused with 2mL of 40CFU/mL of *E. coli* (~80CFU). Milk samples were collected aseptically at 12, 18, 24, 36, 48, and 60 hours following challenge. For the preparation of whey, milk samples were centrifuged at 4,000 rpm at 4°C for 30 min and the fat layer removed. The skimmed milk was transferred to a clean tube, centrifuged again 20,000 x *g* at 4ºC for 60 min, and the translucent supernatant collected, aliquoted into sterile 1.5mL microcentrifuge tubes, and stored at –80 °C.

ELISAs for BSA, Lactoferrin, and Transferrin

Milk levels of bovine serum albumin (BSA) were determined using a commercially available ELISA kit (Bethyl Laboratories, Inc., Montgomery, TX) with slight modifications as previously described (Bannerman et al., 2003). Concentrations of lactoferrin, and transferrin in milk were determined using commercially available ELISA kits (Bethyl Laboratories, Inc., Montgomery, TX) just as previously described for BSA (Bannerman et al., 2003) with the slight modification that the wash and blocking buffers used were 50mM TBS with 0.05% Tween, pH 8.0, and 50mM TBS with 1 % BSA, pH 8.0 for both assays, respectively.

Absorbance for all ELISAs were read at 450nm on a microplate reader (SpectraMax Plus, Molecular Devices, Sunnyvale, CA), and the concentration of BSA, transferrin, and lactoferrin calculated by extrapolating from a standard curve using SoftMax Pro software (v. 5.2, Molecular Devices, Sunnyvale, CA).

In-Solution Digests

The protein content of all of the whey fractions from each cow (n=56) was determined using a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL) with BSA as a standard. Approximately 500 µg of each whey sample was diluted in 0.5M triethylammonium bicarbonate (TEAB; Sigma Aldrich, St Louis, Missouri)/ 6M urea buffer to a final volume of 100µl. Samples were reduced in a final concentration of 5mM tris(2-carboxyethyl)phosphine (TCEP; Promega, Madison, Wisconsin) for one hour at 37°C. Samples were alkylated in a final concentration of 10mM iodoacetamide for one hour at room temperature in the dark. Prior to the addition of

trypsin, samples were diluted with dH_2O to reduce urea to a final concentration of <1M. Approximately 5µg of sequencing grade modified trypsin (Promega, Madison, WI) was added to each sample to achieve a 1:100 enzyme-to-protein ratio, and digestion was carried out for 16 hours in a 37°C water bath. Samples were acidified following digestion by the addition of acetic acid to a final concentration of 0.5%.

LC-MS/MS analyses

One-dimensional LC-MS/MS analyses were carried out by injecting 10 µL of each whey digest into an ultra-pressure LC instrument (UPLC; Waters Acquity UPLC, Waters, Milford, Massachusetts) coupled to a quadrupole time-of-flight (Q-TOF) Premier mass spectrometer (Waters, Milford, Massachusetts). Peptides were loaded onto a Symmetry NanoEase 300µm x 150mm C_{18} reverse phase HPLC column (Waters, Milford, Massachusetts). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was 100% A for 5 min and then ramped linearly to 60% B over 70 minutes at a flow rate of 20µL/ min. The Q-TOF mass spectrometer was equipped with an electro-spray ionization source (ESI) and was operated in positive ion mode with a capillary voltage of 3.0 kV. Spectra were acquired for 70 minutes in data-dependent tandem MS mode. The five most intense ions in each MS survey scan were subjected to MS/MS by collision-induced dissociation (CID). Collision energies used to fragment each peptide ion were based on the mass to charge (*m/z*) value of each peptide. Following MS/MS, precursor ions were excluded from MS/MS for 10 seconds.

Database searching and protein identification

Mass spectral peak lists were extracted using Mascot Distiller and resulting peak lists were searched with the Mascot search engine (v.2.1.0; Matrix Sciences, London, UK) against the Swiss-Prot protein sequence library. The following search parameters were applied: *other mammalia* species, trypsin enzyme, one allowed missed cleavage, carbamidomethylation fixed modification, methionine oxidation variable modification, precursor ion mass tolerance of ± 0.3 Da, and a fragment ion mass tolerance of ± 0.3 Da. The number of sequences searched in the *other mammalia* taxonomy was 10635. Only peptides with a Mascot Ion Score equal or greater to the Identity Score (at $P < 0.05$) were included for identification.

Label-free Quantification

Label-free quantification was determined using both the number of unique peptides identified per protein and total peptides hits or spectral counts. Spectral counts are defined as the MS/MS sampling rate, or the total number of mass spectra assigned to peptides for a given protein (Liu et al., 2004). The number of unique peptides identified, and the spectral counts for each protein, were determined using the software program MassSieve (Slotta et al., 2007; McFarland et al., 2008). Number of unique peptides was normalized relative to the total number of peptides identified in samples from all 8 cows at each sampling time point, and spectral counts were normalized relative to total peptide hits for all proteins identified at each time point (Florens et al., 2006; Paoletti et al., 2006). The average of the normalized values for spectral counts and number of unique peptides across all 8 cows was calculated for each identified

protein, and were expressed graphically as mean ± standard error (SE). To measure differences in protein abundance across time points, the fold-change between normalized spectral counts or number of unique peptides before and after challenge was calculated. Changes in relative abundance of proteins across the time points following infection with *E. coli* were considered relevant if the difference between pre- and post-challenge normalized abundance factors was 2-fold or greater (Mosley et al., 2008). The 2-fold or greater level of significance was only applied to proteins that met the arbitrary threshold of greater than 5 peptide assignments per time point to avoid potential over-estimation of fold-changes in relative abundance of proteins with limited peptide assignments. For proteins not present in milk prior to challenge, peak expression was marked as the time point with the highest normalized abundance factor, and fold changes were measured relative to the time point at which the protein was first detected.

Statistical analysis

Repeated-measures analysis of variance was performed on ELISA data using the PROC MIXED model with Dunnett post hoc pairwise comparisons (SAS version 9.1; SAS Institute, Carey, N.C.) to compare changes in the proteins serum albumin, lactoferrin, and transferrin following infection to the pre-infection concentrations of each protein. Means and standard errors for all ELISA and label-free quantification data were calculated and graphed using SigmaPlot (v. 11.0 Systat Software, Inc., Chicago, IL). Means for normalized spectral counts and number of unique peptides identified for BSA were compared for significant differences using *t*-tests performed in SigmaStat (v. 3.5 Systat Software, Inc., Chicago, IL). Milk somatic cell counts

(MSCC) were log transformed to milk somatic cell scores (MSCS). For all statistical analyses, a value of $P < 0.05$ for the comparisons was considered to represent a significant difference between groups.

Results

Milk proteins identified in whey prior to and following infection with E. coli

Proteomic analyses of abundant bovine milk proteins before and after infection with *E. coli* revealed changes in the relative abundance of several milk proteins, as well as the appearance of proteins not present in milk prior to infection. Normal whey samples and whey samples collected at time points following experimental induction of coliform mastitis were both characterized by the presence of the abundant milk proteins α-S_1-casein, β-casein, κ-casein, β-lactoglobulin, α-lactalbumin, and serum albumin; however, the number of unique peptides identified and the number of spectral counts for each peptide differed for each protein at the separate time points (Table 1). Although at lower frequencies, lactoferrin and glycosylation-dependent cell adhesion molecule-1 (glycam-1) were also identified in the milk of healthy cows and cows with mastitis (Table 2). Proteins only identified in mastitic whey samples included transferrin, apolipoprotein A-I and A-II, and some extremely low abundance proteins detected transiently across different time points including fibrinogen, peptidoglycan recognition receptor protein (PGRP), fetuin (α-2-HS-glycoprotein), cyclic dodecapeptide (cathelicidin-1), indolicidin (cathelicidin-4), complement factor C3, and the acute phase proteins haptoglobin and α-1-acid glycoprotein.

Table 1 Average spectral counts and number of unique peptides of abundant milk proteins.

Swiss-Prot entry name	Protein	Pre-Challenge No. Peptides[a]	Pre-Challenge NP[b]	Post-Challenge Time Point	Post-Challenge No. Peptides[a]	Post-Challenge NP[b]	NP Fold Change[c]
ALBU_BOVIN	Serum albumin	2	0.00329	12h	6	0.01275	3.9
				18h	12	0.03011	9.2
				24h	10	0.02138	6.5
				36h	13	0.02130	6.5
				48h	7	0.01137	3.5
				60h	5	0.00885	2.7
LACB_BOVIN	B-lactoglobulin	8	0.05263	12h	7	0.04687	0.9
				18h	5	0.04257	0.8
				24h	5	0.03556	0.7
				36h	7	0.03917	0.7
				48h	8	0.04462	0.9
				60h	7	0.04290	0.8
LALBA_BOVIN	α- lactalbumin	3	0.02163	12h	2	0.02030	0.9
				18h	1	0.01413	0.7
				24h	1	0.01226	0.6
				36h	1	0.00982	0.5
				48h	1	0.01009	0.5
				60h	2	0.01295	0.6
CASA1_BOVIN	α-S_1-casein	4	0.02368	12h	4	0.02268	1.0
				18h	4	0.02812	1.2
				24h	4	0.02588	1.1
				36h	6	0.03021	1.3
				48h	6	0.02738	1.2
				60h	5	0.02709	1.1
CASB_BOVIN	β-casein	1	0.00617	12h	1	0.00677	1.1
				18h	2	0.01094	1.8
				24h	2	0.00989	1.6
				36h	2	0.00679	1.1
				48h	2	0.00930	1.5
				60h	2	0.00947	1.5
CASK_BOVIN	κ- casein	2	0.01132	12h	2	0.00958	0.9
				18h	2	0.01290	1.1
				24h	2	0.01499	1.3
				36h	3	0.01334	1.2
				48h	2	0.01234	1.1
				60h	3	0.01488	1.3

[a] Average number of unique peptides identified across all 8 cows; [b] Normalized number of unqiue peptides; [c] Fold-change relative to pre-challenge levels.

Table 2 Average spectral counts and number of unique peptides of low abundance milk proteins.

Swiss-Prot entry name	Protein	Pre-Challenge No. Peptides[a]	Pre-Challenge NP[b]	Post-Challenge Time Point	Post-Challenge No. Peptides[a,c]	Post-Challenge NP[b]	NP Fold Change[d]
TRFE_BOVIN	Transferrin	0	0.00000	12h	1	0.00151	n/a
				18h	1	0.00083	0.5
				24h	1	0.00090	0.6
				36h	1	0.00072	0.5
				48h	0	0.00000	n/a
				60h	0	0.00000	n/a
TRFL_BOVIN	Lactoferrin	<1	0.00022	12h	<1	0.00064	2.9
				18h	1	0.00138	6.3
				24h	1	0.00089	4.0
				36h	<1	0.00036	1.6
				48h	1	0.00184	8.4
				60h	3	0.00419	19.0
GLCM1_BOVIN	Glycam-1	1	0.00602	12h	1	0.00397	0.7
				18h	<1	0.00146	0.2
				24h	<1	0.00310	0.5
				36h	1	0.00331	0.6
				48h	1	0.00766	1.3
				60h	1	0.00462	0.8
CTHL1_BOVIN	Cathelicidin-1	0	0.00000	12h	0	0.00000	n/a
				18h	<1	0.00059	n/a
				24h	<1	0.00098	1.7
				36h	<1	0.00041	0.7
				48h	<1	0.00050	0.8
				60h	<1	0.00053	0.9
PGRP_BOVIN	Peptidoglycan recognition receptor protein	0	0.00000	12h	0	0.00000	n/a
				18h	0	0.00000	n/a
				24h	<1	0.00196	n/a
				36h	<1	0.00041	0.2
				48h	<1	0.00050	0.3
				60h	0	0.00000	n/a

[a] Average number of unique peptides identified across all 8 cows; [b] Normalized number of unique peptides; [c] Number of peptides <1 indicates only one peptide assignment in less than 8 cows; [d] Fold-change relative to pre-challenge levels, or the first time point detected.

ELISA quantification of BSA, Lactoferrin, and Transferrin

Increased permeability of the blood-milk barrier, a hallmark of coliform mastitis, was apparent by 18 h following infection as evidenced by increases in the vascular derived proteins BSA and transferrin. BSA measured in milk from quarters inoculated

with *E. coli* exhibited significant ($P < 0.05$) differences in concentration at 18 h and 24 h following infection when compared to levels detected in milk prior to infection (Fig. 1A). BSA levels in milk from cows with coliform mastitis peaked at 18 h following infection, but returned to near baseline levels by 60 h. Similarly, transferrin (Fig. 1B) was significantly increased ($P < 0.05$) when compared to baseline measurements at 18 h, 24 h, and 36 h following inoculation with *E. coli,* but returned to pre-challenge levels by 60 h. Lactoferrin concentrations in milk increased significantly ($P < 0.05$) when compared to pre-infection levels at 18 h, 24 h, 36 h, 48 h, and 60 h, but unlike BSA and transferrin, did not return to baseline levels during the course of the study (Fig. 1C).

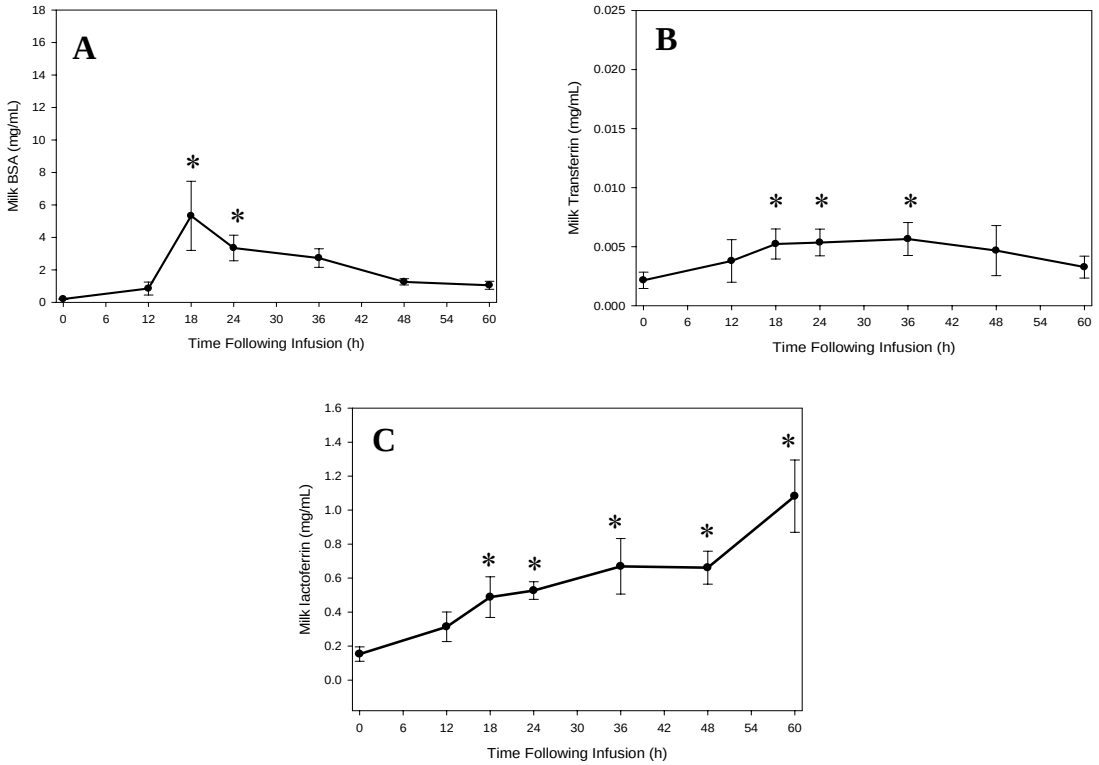


Figure 1 ELISA results for BSA (A), transferrin (B), and lactoferrin (C) concentrations measured in milk. Means concentration (± standard error) are shown for each time point. * denotes significant changes (P<0.05) relative to time zero.

LC-MS/MS label-free quantification of abundant milk proteins

The number of unique peptides that contributed to the identification of each milk protein, as well as the number of times each unique peptide was identified by MS/MS (spectral count), was determined for each protein using MassSieve (Table 1). A comparison of normalized values for spectral counts and number of unique peptides identified for BSA (Fig. 2A), transferrin (Fig. 2B), and lactoferrin (Fig. 2C), revealed nearly identical temporal expression patterns. No significant differences ($P > 0.05$) in the means for each label-free measure were detected at any time point for any of the proteins. Although both methods yielded similar results, normalized spectral counts were less robust overall than normalized number of unique peptides. Thus, the number of unique peptides identified was utilized for the label-free quantification of all proteins throughout the rest of the analyses.

Examination of the temporal expression of milk proteins before and after experimental induction of coliform mastitis revealed increases in the proteins albumin (Fig. 3A) and transferrin (Fig. 3B) at 18 h and 12 h, respectively, after infusion. Number of unique peptides identified for BSA at exhibited changes in relative abundance greater than 2-fold over pre-challenge levels at 12 h, 18 h, 24 h, 36 h, 48 h, and 60h following infection, but relative abundance at 60 h was approaching baseline. Less than five peptides were identified for transferrin, and thus no relevance was attributed to calculated fold-changes across time points. Label free quantification of lactoferrin (Fig. 3C) revealed a gradual increase in relative abundance with time. As with transferrin, less than 5 peptides were assigned to lactoferrin and thus no relevance of fold changes was assigned.

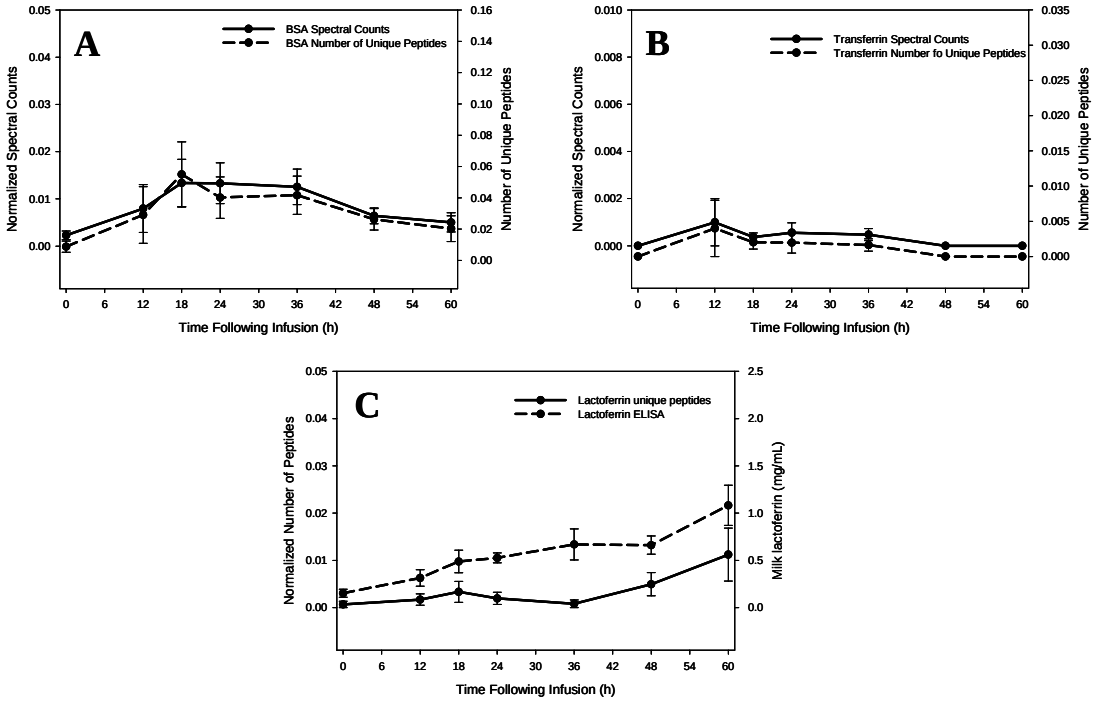


Figure 2 Comparison of normalized spectral counts and number of unique peptides identified at each time point for BSA (A), transferrin (B), and , lactoferrin (C). Means (± standard error) are shown for each time point.

Profiles of milk samples also revealed a reduced abundance of the whey proteins β-lactoglobulin and α-lactalbumin, with the largest decreases evident between 12 and 36 hours following infection, which corresponded with increases in serum albumin (Fig. 3D). Changes in relative abundance of β-lactoglobulin and α-lactalbumin, as determined by number of unique peptides identified, were not greater than 2-fold compared to pre-infection levels, however, and were thus not considered significant. Likewise, abundance of α-S1-casein, β-casein, and κ-casein remained relatively unchanged throughout the course of the mastitis infection and no significant differences in relative abundance across time points were detected (Fig. 3E).

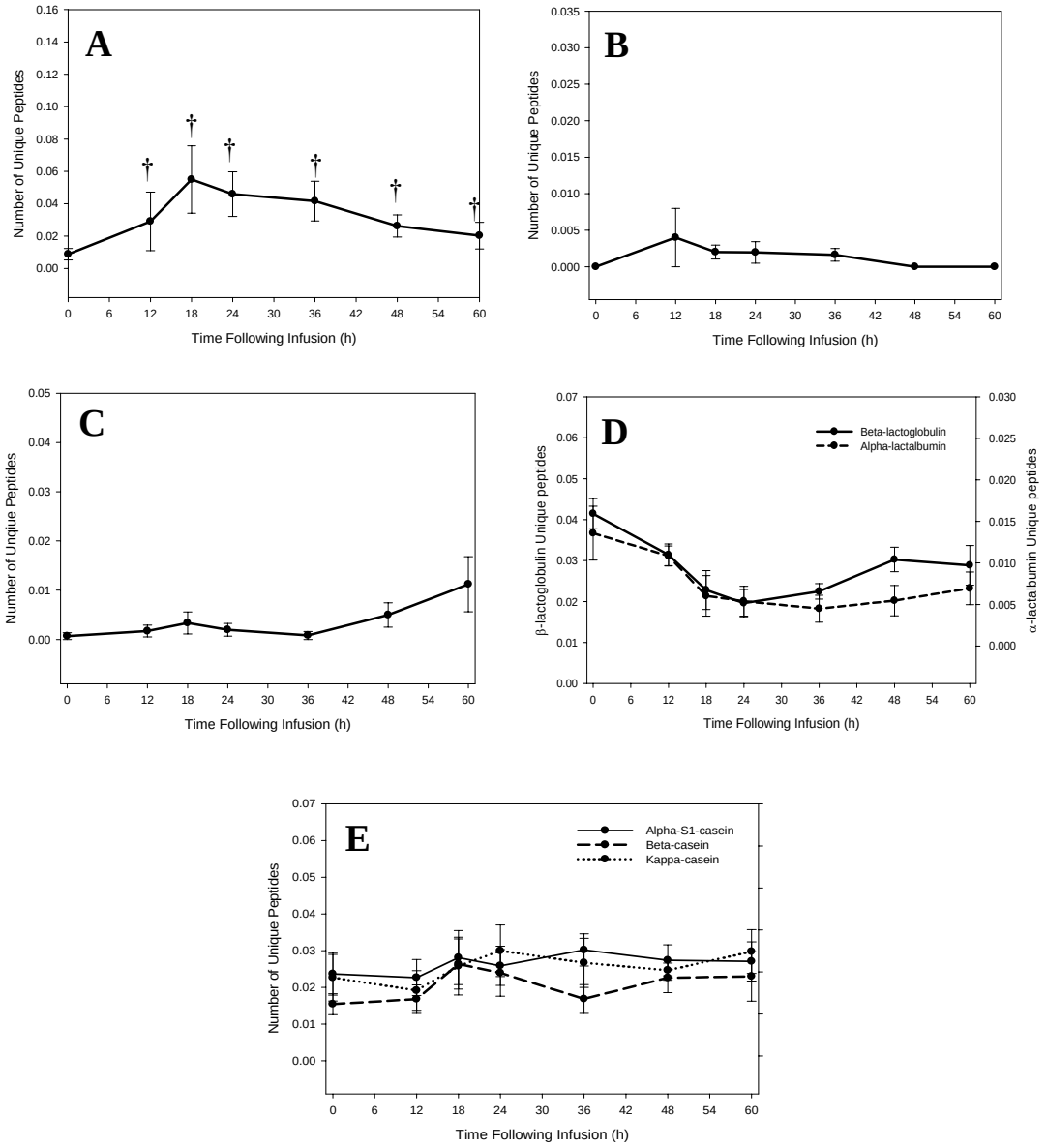


Figure 3 Figure 2 Normalized number of unique peptides identified at each time point for BSA (A), transferrin (B), lactoferrin (C), whey proteins (D), and caseins (E). Means (± standard error) are shown for each time point. † denotes 2-fold or greater changes in abundance relative to time zero.

LC-MS/MS Label free quantification is in accord with ELISA data

The comparison of changes in milk concentrations of BSA following experimental induction of coliform mastitis as measured by ELISA and label-free LC-MS/MS quantification levels revealed significant differences from pre-challenge

levels at 18 h and 24 h for ELISA data, and at 12, 18, 24, 36, 48, and 60 h for label-free quantification data (Fig. 4A). Additionally, temporal expression patterns of BSA determined using an ELISA assay and normalized number of unique peptides identified by LC-MS/MS were nearly identical. Similar to the results found with BSA, the comparison of graphic representations of the quantification of transferrin (Fig. 4B) and lactoferrin (Fig. 4C) by ELISA versus label free LC-MS/MS methods revealed similar trends.

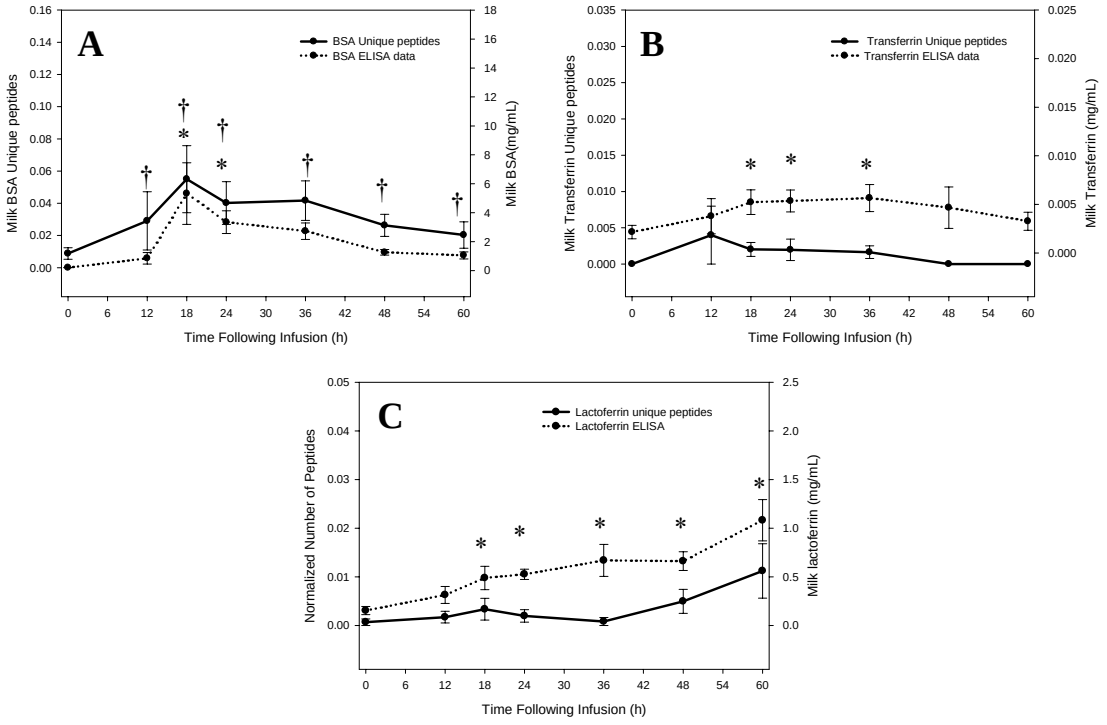


Figure 4 Normalized number of unique peptides identified compared to ELISA data across time points for BSA (A), transferrin (B), and lactoferrin (C). Means (± standard error) are shown for each time point. * denotes significant increases ($P < 0.05$) detected by ELISA, and † denotes greater than 2-fold increase in relative abundance of unique peptides.

LC-MS/MS label-free quantification of low abundance milk proteins comparess with clinical data.

Temporal expression patterns of the antimicrobial proteins cathelicidin-1 and peptidoglycan recognition receptor (PGRP), before and after infection with *E. coli* were evaluated using normalized number of unique peptides identified per time point. Because expression of both antimicrobial proteins was transient, however, and few peptides assignments were made, the identification of cathelicidin-1 and PGRP must be regarded as tentative. Nonetheless, peak expression of both cathelicidin-1 and PGRP occurred at 24 h following infection (Fig. 5A), which corresponded to peak increases in milk somatic cell scores (Fig. 5B). MSCS were significantly elevated ($P < 0.05$) at 24 and 48 h post infection.

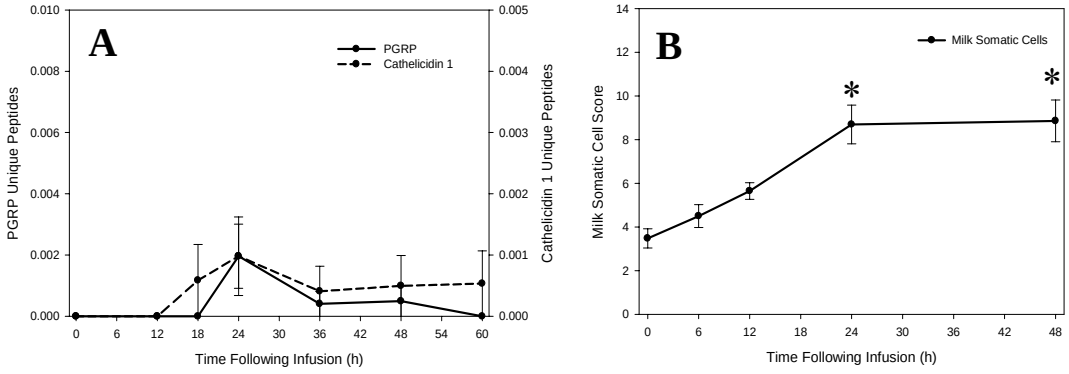


Figure 5 Comparison of the expression of antimicrobial proteins in milk with milk somatic cells scores. * denotes significant changes (P<0.05) relative to time zero.

Discussion

Whey from bovine milk samples collected prior to infection with *E. coli*, and whey from milk samples collected at 12, 18, 24, 36, 48, and 60 h following infection with *E. coli* were digested in-solution with trypsin, separated using reverse phase

liquid chromatography, and identified by peptide sequencing using tandem mass spectrometry followed by database searching. As the aim was to develop rapid throughput analyses of high to medium abundance milk proteins, no sample depletion was performed and only a one-dimensional separation was employed. In accord with previous proteomic analyses that did not involve sample clean-up, the abundant milk proteins BSA, β-lactoglobulin , α-lactalbumin, α-S1-casein, β-casein, and κ-casein were identified in whey samples at all time points from all 8 cows (Smolenski et al., 2007).

ELISAs were used to evaluate the temporal expression of the proteins BSA, transferrin, and lactoferrin. Consistent with previously published results, expression of all 3 proteins increased in milk during mastitis (Rainard, 1983; Bannerman et al, 2004). Compared to pre-challenge levels, significant increases in all 3 proteins were detected at 18 and 24 h following infection, and increases in transferrin and lactoferrin persisted until 36 and 60 h after infection, respectively. While increases in levels of BSA (Fig. 1A) and transferrin (Fig. 1B) gradually started to decrease back towards baseline levels ≥36 h after infection, levels of lactoferrin (Fig. 1C) continued to increase throughout the study. The most probable explanation is that lactoferrin is produced locally by mammary epithelial cells in response to LPS (Pareek et al., 2005; McClenehan et al., 2005), whereas BSA and transferrin are serum derived proteins produced primarily in the liver that leak into the milk due to increased vascular permeability in the bovine mammary gland during inflammation (Shuster et al., 1993; Bannerman et al., 2003; 2004). Expression levels of the key inflammatory cytokines known to affect vascular permeability, TNFα and IL-1β, have been shown to peak

around 16 hours following experimental induction of coliform mastitis and to then decline steadily back towards basal levels (Bannerman et al., 2004). Thus, the effects of cytokine expression on vascular permeability could have diminished significantly by 36 h following infection.

In-solution digests of whey proteins from milk collected prior to and following experimental induction of coliform mastitis were analyzed using LC-MS/MS, and data files generated from searching peak lists against the Swiss-Prot protein library using the Mascot search engine were distilled using MassSieve, an in-house software program (Slotta et al., 2007; McFarland et al., 2008). The number of unique peptides identified for each abundant protein, and affiliated spectral counts for each peptide, were normalized (Florens et al., 2006; Paoletti et al., 2006) and plotted over time to evaluate temporal expression of each protein. Comparison of expression profiles generated using the normalized number of unique peptides with normalized spectral counts for each peptide revealed no significant differences in the label-free methods for determining temporal expression profiles of BSA, transferrin, or lactoferrin. Despite the fact that no significant differences were found between the mean number of unique peptides identified and the spectral counts for each peptide, the unique peptide data appeared slightly more robust than the spectral count data after normalization.

Intramammary infection with *E. coli* resulted in changes in the temporal expression patterns of the abundant milk proteins BSA (Fig. 3A), β-lactoglobulin and α-lactalbumin (Fig. 3D), and in lesser abundant proteins, including lactoferrin (Fig. 3C) and transferrin (Fig. 3B). Increases in the vascular derived proteins BSA and

transferrin detected using LC-MS/MS label-free quantification, as well as the variable identification of other hallmarks of inflammation including fibrinogen, complement component C3, and the acute phase protein α-1-acid-glycoprotein only in whey samples following infection with *E. coli,* supported the findings of previous proteomic analyses of mastitic bovine milk (Hogarth et al., 2004; Smolenski et al., 2007).

Contrary to previous proteomic analyses that utilized 1D and 2D gel electrophoresis to profile changes in milk protein expression following experimental induction of coliform mastitis (Moussaoui et al., 2004), no apparent fluctuations in the relative abundance of casein proteins were detected in this study using LC-MS/MS to evaluate milk protein modulation during mastitis (Fig. 3E). Possible explanations are the limitations of the amount of protein that can be analyzed using gel electrophoresis, and the fact that the majority of the total protein concentration of mastitic milk samples is BSA. As a result, the accurate representation of the prevalence of other milk proteins on a 1 or 2D gel of mastitic bovine milk could be suppressed by the overabundance of BSA to the point that other proteins, such as the caseins, appear to be reduced in expression (Garbis et al., 2005). In all previous analyses, decreased casein expression during mastitis coincided with profound increases in levels of BSA in the milk (Moussaoui et al., 2004).

Relative abundance of the milk proteins BSA, transferrin, and lactoferrin, as determined by the number of unique peptides identified by LC-MS/MS analyses, exhibited nearly identical expression patterns when compared to quantitative changes determined by ELISAs for the time points following experimental induction of

mastitis. The primary difference between the two methods of elucidating temporal expression patterns of milk proteins during experimentally induced mastitis was the significance determined by statistical analyses of changes in abundance at the various time points following infection when compared to pre-challenge levels. BSA, when measured using an ELISA, was shown to increase significantly ($P< 0.05$) relative to pre-challenge levels at both 18 and 24 h following infection, while significant fold changes in normalized number of unique peptides were apparent relative to 0 h at all time points after infusion (Fig. 4A). In contrast, an ELISA for transferrin revealed significant changes ($P< 0.05$) relative to basal levels at 18, 24, and 36 h following infection, but significance of fold-changes in normalized number of unique peptides for the time points during the mastitis infection relative to pre-infection levels could not be inferred because too few peptide assignments were made for transferrin (Fig. 4B). Significant ($P< 0.05$) increases relative to 0 h were detected in lactoferrin at 18, 24, 36, 48 and 60 h using an ELISA, but like transferrin, no inferences could be made regarding the significance of fold-changes in relative abundance due to insufficient peptide numbers (Fig. 4C).

Label-free semiquantitative analyses are still considered an emerging research focus in the field of proteomics (reviewed in Mueller et al., 2008). Most label-free quantification strategies are based on the hypothesis that the number of peptides identified for a given protein, and the MS/MS sampling rate of a specific peptide (spectral count), corresponds to the relative abundance of a particular protein in a complex mixture (Liu et al., 2004; Old et al., 2005; Zybailov et al., 2005). Estimations of relative abundance based on label-free quantification strategies can be

skewed, however, by the size and number of theoretical peptides a protein can yield, the actual abundance of the protein in a given biological matrix, the resolution and sensitivity of the instrument system, the physiochemical properties of a given peptide that enhance or suppress ionization and peptide observability, and the complexity of the biological sample (reviewed in Mueller et al., 2008). Normalization of number of peptides identified and spectral counts can, however, account for some of the inherent drawbacks to label-free semiquantitative analyses, including the size and number of peptides for a given protein (Florens et al., 2006; Paoletti et al., 2006).

The number of peptides identified, and the number of times each peptide triggered an MS/MS event (spectral count), were nearly identical in the current protein data set There was, however, a large gap in the number of peptides identified for abundant whey proteins, and the number of peptide assignments for the remaining proteins identified. The most likely explanation for the potential over-estimation of fold-changes relative to pre-challenge levels for the protein BSA, when compared to significant increases in abundance detected using an ELISA, was the fact that the number of peptide assignments for BSA accounted for the majority of total peptides identified at each time point, with the exception of the pre-infection sample that was characterized by a greater abundance of β-lactoglobulin. Thus, normalized abundance factors were likely skewed towards the relative abundance of BSA, except in whey from normal milk. Results of the analyses support previous demonstrations that the biological complexity of bovine milk before and after challenge varies greatly (Smolenski et al., 2007), as well as the notion that the dynamic range of proteins in a

given sample, in addition to biological complexity, can affect the accuracy of semiquantitative analyses (Mueller et al., 2008; Simpson et al., 2009).

The intense dynamic range of proteins present in bovine milk (O'Donnell et al., 2004; Gagnaire et al., 2009), coupled with the yet unresolved method of accurately measuring changes in low abundance proteins present in complex biological matrices (reviewed in Mueller et al., 2008) presents a serious challenge for quantifying changes in low abundance proteins present in milk following infection. Temporal expression patterns of milk proteins determined using ELISAs and mass spectrometry were, however, very similar for BSA, transferrin, and lactoferrin. Since the aim was to investigate the feasibility of using semiquantitative label-free strategies to evaluate the expression patterns of bovine milk proteins without the reliance on antibody-based methodologies, label-free quantification was used to map the temporal expression of two transiently expressed low-abundance proteins involved in host response identified in whey following experimental induction of coliform mastitis.

PGRP is a highly conserved pattern recognition molecule involved in innate immunity that has also been shown to interact with LPS, and to exhibit antibacterial activity in cattle (Dziarski, 2003; Tydell et al., 2006). Cathelicidin-1 is a cationic antimicrobial peptide also involved in innate immunity that is known to inactivate and destroy invading pathogens by perturbing microbial membranes (Scocchi et al., 1997). Both PGRP and cathelicidin-1 were identified in previous proteomic analyses of mastitic bovine milk (Smolenski et al., 2007), but changes in expression level during coliform mastitis were not evaluated. Based on normalized number of unique peptides identified, increases in cathelicidin-1 were evident as early as 12 h following

infection, while PGRP did not exhibit increased abundance until 18 h after infusion (Fig. 5A). Neutrophils are an established source of both PGRP and cathelicidin-1 (Scocchi et al., 1997; Tydell et al., 2006), and are known to be the primary component of milk somatic cell counts (MSCC) during intramammary infection (Paape et al., 1981). Increases in MSCC (log transformed) were apparent starting 12 h following experimental induction of coliform mastitis, and counts peaked at 24 h after infusion (Fig. 5B). Thus, the increases in relative abundance of both PGRP and cathelicidin-1, determined using mass spectrometry, are in accord with clinical data.

The comparison of relative abundance determined using mass spectrometric label-free quantification to concentrations measured using an ELISA have not been reported for milk proteins. However, milk proteins have been identified using LC-MS/MS (Smolenski et al., 2007; Reinhardt and Lippolis, 2006; 2008), and differential expression of bovine milk proteins during mastitis has been investigated using proteomic strategies (Hogarth et al., 2004; Smolenski et al., 2007). Additionally, there have been numerous reports on the evaluation of the expression of cytokines and soluble inflammatory mediators in milk during coliform mastitis (reviewed in Bannerman, 2009). To date, quantification of milk protein and inflammatory marker expression during mastitis has been limited to antibody-based strategies such as ELISAs, and to polymerase chain reaction (PCR) measurements of mRNA levels (Bannerman et al., 2004; McClenahan et al., 2005; Vanselow et al., 2006).

The primary limitation of established quantification methods is, however, that most are designed to measure only one protein in a sample at a time. While proteomic methodologies provide the opportunity to measure an unlimited number of

proteins in any given sample, investigations into the quantification methods affiliated with mass spectrometry have not been initiated in studies of the bovine milk proteome. Although the focus of the current study was primarily on abundant milk proteins, the temporal expression of some low abundance proteins associated with host response was possible using LC-MS/MS label-free quantification. Biases were apparent, however, in the estimation of fold-changes in relative abundance of proteins across multiple time points following infection, despite normalization. While the current study does represent the first longitudinal LC-MS/MS analyses of bovine milk samples before and after experimental induction of coliform mastitis, further research is required to address the impact of the dynamic range of bovine milk proteins, and the apparent fluctuations in biological complexity across time points following infection, on quantification of protein modulation during mastitis using semiquantitative label-free methodologies. Nonetheless, the analogous trends in temporal expression determined by the comparison of ELISA and mass spectrometric data are promising indications that LC-MS/MS analyses could prove useful in the evaluation of the modulation of bovine milk proteins as well as other inflammatory mediators during the course of coliform mastitis. Of particular value would be the application of the label-free proteomic strategies to studies focused on the discovery of novel markers of disease in bovine milk using more sophisticated mass spectrometric analyses, and to the evaluation of milk proteins for which no bovine antibody currently exists.

Chapter 6: Label-free Quantification of Biomarkers of Host Response in Bovine Milk during Coliform Mastitis.

Abstract

Analyses of the bovine milk proteome following experimental induction of *Escherichia coli* mastitis have revealed profound changes in the relative abundance of milk proteins, as well as the presence of proteins related to host response. Identification of biomarkers of inflammation in bovine milk during intramammary infection has, however, been limited by the number of cows sampled, and the number of time points analyzed during a mastitis infection. The objectives of the current study were to examine the temporal expression of low abundance biomarkers of host response in 8 cows across 4 time points following *Escherichia coli* challenge using nano-LC in line with nano-spray ion trap tandem mass spectrometry (LC-MS/MS), and to compare label-free methods of semiquantitiative analysis to evaluate changes in relative abundance of identified proteins. Additionally, a goal was to compare trends in the modulation of select acute phase proteins (APP) in milk determined by ELISA and LC-MS/MS, to evaluate the accuracy of mass spectrometric-based label-free quantification in depicting relative changes in biomarker expression during mastitis. LC-MS/MS conducted on whey from milk samples, collected just prior to infusion with *E. coli* and at 12, 18, 24, and 48 hours following infection, resulted in the identification of the high abundance proteins αS_1-, αS_2-, β-, and κ-casein, and the whey proteins serum albumin (BSA), β-lactoglobulin and α-lactalbumin. Increased

vascular permeability, a hallmark of the inflammatory response associated with coliform mastitis, was evidenced by the presence of plasma-derived proteins in whey from mastitic milk including transferrin, complement factors C3, C4, and B, the apolipoproteins A1, A2, A4, and C3, fibrinogen -α, -β, and -γ chains, hemopexin, alpha fetoprotein, and alpha-2-HS-glycoprotein. Hepatic production of APP was apparent by the detection of serum amyloid A (SAA), haptoglobin (Hp), inter-alpha trypsin inhibitor heavy chain-4, and α-1-acid glycoprotein. Additional biomarkers of host response detected in bovine milk during mastitis included lactoferrin, kininogen 1 and 2, osteopontin, glycosylation-dependent cell adhesion molecule-1, and the antimicrobial proteins cathelicidin-1, -2, -3, -4, -5, -6, -7, and peptidoglycan recognition protein. Normalized spectral abundance factors were used to track temporal changes in milk proteins in response to infection. Changes in BSA, Hp, and SAA in the milk during mastitis were also measured using an ELISA for each protein. Label-free quantification revealed relative changes in milk proteins that were in accord with patterns observed by ELISA and with previously published data. The results indicate that LC-MS/MS can be used to detect low abundance milk proteins that are biologically relevant to disease, and that NSAF are a viable non-antibody based method of monitoring changes during coliform mastitis in milk proteins for which no antibody or ELISA currently exists.

Keywords: bovine milk proteome, label-free quantification, biomarkers of inflammation

Introduction

Mastitis remains a principal focus of veterinary research due to staggering associated economic losses, insufficient means of early disease detection, and the lack of efficacious treatment options for intramammary infections caused by gram negative pathogens. Specific obstacles associated with treating cases of coliform mastitis include: 1) inflammation caused by bacterial cell wall toxins for which there are no approved adjunctive therapies (Anderson, 1989; Wilson 1999); 2) vaccines against coliforms exhibit limited efficacy (Hogan et al., 1992); and 3) severe systemic complications that arise from the host inflammatory response (Eberhart et al., 1987). Attempts to better understand host response to gram negative pathogens have led to several investigations into the mediators of innate immunity in the bovine mammary gland (Paape et al., 1996; Lee et al., 2003; Bannerman et al., 2004; Petzl et al., 2008). Due to the prevalence of cases of coliform mastitis caused by *Escherichia coli* strains, *E. coli* has been used in several prior studies to experimentally induce coliform mastitis (Hirvonen et al, 1999; Lee at al., 2003; Bannerman et al., 2004).

The focus of previous studies of bovine innate immunity during experimentally induced *E. coli* mastitis has primarily been soluble mediators of inflammation in bovine milk (reviewed in Bannerman, 2009). Expression of inflammatory mediators during coliform mastitis is triggered by the release of lipopolysaccharide (LPS) from the cell wall of gram negative bacteria following intramammary infection (Shuster et al., 1993; Bannerman et al., 2003). Production of the cytokines IL-8 and TNFα by mammary epithelial cells (MEC) is an established response to LPS (Pareek et al., 2005; McClenehan et al., 2005). Likewise, previous studies have determined that IL-

1β, IL-6, IL-8, and TNFα are all upregulated by LPS (Shuster et al., 1993; Bannerman et al., 2003), while in vivo studies have demonstrated that the cytokines IL-1β, IL-8, and TNFα also increase in response to *E. coli* infection (Bannerman et al., 2004). The majority of previously published data detailing the innate immune response in the bovine mammary gland during coliform mastitis has, however, been based on enzyme-linked immunosorbent (ELISA) assays. Despite their specificity, ELISAs have limited application in biomarker discovery due primarily to the fact that ELISAs measure only one protein at a time, and are restricted by the development and availability of species-specific antibodies. Currently, the number of commercially available bovine-specific antibodies is limited compared to the number of antibodies available for human and conventional lab animal species. To overcome the reliance on antibody-based methodologies, recent efforts to elucidate protein modulation and mediators of inflammation in bovine milk during coliform mastitis have involved the application of proteomic methodologies to the evaluation of host response in the bovine mammary gland (Smolenski et al., 2007; Hogarth et al., 2004).

Biomarkers of host response identified thus far in bovine milk using proteomic strategies include: lipocalin-type prostaglandin D synthase (Baeker et al., 2002); the acute phase proteins (APP) serum amyloid A (SAA; Smolenski et al., 2007); and, the antimicrobial proteins (AMP) peptidoglycan recognition receptor (PGRP) and cathelicidin-1 (Smolenski et al., 2007). However, prior identifications of APP and AMP in bovine milk during mastitis have been in the milk fat globule membrane (MFGM) fraction (Smolenski et al., 2007). Major limitations surrounding previous efforts to identify protein markers of inflammation during cases of clinical mastitis

have been: identification of only a small number of proteins (Baeker et al., 2002; Hogarth et al., 2004); analyses of differentially expressed proteins at only one time point following experimental infection (Smolenski et al., 2007); a small number of cows sampled (Smolenski et al., 2007); or the incidence of mastitis confirmed only by clinical parameters (Smolenski et al., 2007; Hogarth et al., 2004). Despite inherent shortcomings, previous proteomic analyses of milk from cows with clinical mastitis have nonetheless contributed significantly to our current knowledge of changes in bovine milk proteins during intramammary infection, and have expanded the somewhat limited annotation of the bovine milk proteome.

Arguably, the most significant drawback to studies of the bovine milk proteome is the biological complexity of milk, an aspect made even more cumbersome by the numerous post-translational modifications (PTMs) that occur in milk proteins including glycosylation, phosphorylation, disulphide bond formation, and proteolysis (O'Donnell et al., 2004). Additionally, analyses of the bovine milk proteome are hampered by the dynamic range of proteins in milk, which have been shown to vary in concentration by as much as a factor of 10^6 (O'Donnell et al., 2004; Gagnaire et al., 2009). Conventional approaches to reducing sample complexity prior to analyses, such as the removal of high abundance proteins or the fractionation of proteins or peptides in complex matrices, have to date been unsuccessful in studies involving the use of proteomic techniques to analyze bovine milk. Attempts to remove high abundance proteins, including casein depletion by acid precipitation and the removal of immunoglobulins by immunoaffinity, have resulted in a rather drastic reduction in the number of milk proteins identified (Yamada et al, 2002; Hogarth et al., 2004).

Likewise, attempts to fractionate acidic and basic milk proteins using 2 dimensional gel electrophoresis (2D-GE) was hindered by carryover, and the identification of abundant proteins across multiple fractions (Fong et al., 2008). Endeavors to reduce the complexity of bovine milk and affiliated subcellular fractions have resulted in more thorough comprehension of the complete bovine milk proteome, however, a tremendous gap in knowledge still exists regarding temporal expression of bovine milk protein modulation during disease (O'Donnell et al., 2004).

A patent advantage of utilizing proteomic methodologies for biomarker discovery is that proteomics is not reliant on antibodies and can theoretically result in the detection of an unlimited number of proteins in any given sample (Lippolis and Reinhardt, 2008). However, there is still no gold standard for the accurate quantification of the abundance of individual proteins in complex biological samples using proteomic strategies, especially for proteins present in low abundance (Mueller et al., 2008). Several labeling strategies are available for the quantification of proteins in conjunction with LC-MS/MS analyses, but labeling strategies can be cost limiting, often require pair wise comparisons which can be problematic when quantifying proteins that are only present in a given physiological state, and labeling strategies do not allow for retrospective quantification (Old et al., 2005). Consequently, label-free methods for the semiquantitative analysis of proteins in complex biological samples have been investigated including the comparison of: (1) the mean peak area of all peptides identified for each protein in a sample (Old et al., 2005; Bondarenko et al., 2002); (2) number of unique peptides identified for each protein in a sample (Liu et al., 2004); and (3) number of spectral hits (spectral

counting) for each protein in a sample (Old et al., 2005; Zybailov et al., 2005; Liu et al., 2004). Label-free methods have gained popularity primarily because there are no associated costs, normalization allows for comparisons of protein abundance across a longitudinal set of samples, and analyses can be conducted retrospectively (Mueller et al., 2008).

The aim of the current study was to expand existing knowledge of the expression of proteins related to host response present in bovine milk over the course of infection by analyzing milk samples collected from a group of 8 Holstein cows at multiple time points following inoculation with *Escherichia coli.* A separate objective was to profile temporal expression of host response proteins in bovine milk by utilizing semiquantitative methods, and to compare trends in protein modulation detected using an LC-MS/MS label-free approach with changes in protein concentration detected using an ELISA for select proteins.

Experimental Procedures

Escherichia coli challenge and whey preparation

The care and use of all animals in this study were approved by the University of Maryland's Animal Care and Use Committee. Eight clinically healthy multiparous Holstein cows in middle to late lactation (211 DIM ± 12 d) were selected on the basis of milk SCC (MSCC) of < 200,000 cells/mL and no history of treatment for mastitis over the past 6 months. Preparation of the *E. coli* inoculum and experimental infection were performed as previously described (Bannerman et al., 2004). Baseline

milk samples were collected aseptically from the right front quarter of each of the 8 cows the evening before and the morning of challenge. Following morning milking, the right front quarter of each of the 8 cows was infused with 2mL of 40CFU/mL of *E. coli* (~80CFU). Milk samples were collected aseptically at 12, 18, 24, and 48 hours following challenge. For the preparation of whey, milk samples were centrifuged at 4,000 rpm at 4°C for 30 min and the fat layer removed. The skimmed milk was transferred to a clean tube, centrifuged again 20,000 x *g* at 4ºC for 60 min, and the translucent supernatant collected, aliquoted into sterile 1.5mL microcentrifuge tubes, and stored at –80 °C.

ELISAs for BSA, Haptoglobin, and Serum Amyloid A

Milk levels of BSA were determined using a commercially available ELISA kit (Bethyl Laboratories, Inc., Montgomery, TX) with slight modifications as previously described (Bannerman et al., 2003). Milk levels of Hp were measured using a commercially available bovine sandwich ELISA kit (Immunology Consultants Laboratory, Inc., Newberg, OR) according to the manufacturer's instructions, with modifications in the samples dilutions. The 0 and 12 hour whey samples were diluted 1:50, and the remaining samples were diluted 1:200. Milk levels of SAA were measured using a commercially available phase sandwich ELISA kit (Tridelta Development Ltd., Maynooth, Co. Kildare, Ireland) according to the manufacturer's instructions, with modifications in the samples dilutions. The 0 and 12 hour whey samples were diluted 1:50 and the remaining samples were diluted 1:100. Absorbance for all ELISAs were read at 450nm on a microplate reader (SpectraMax

Plus, Molecular Devices, Sunnyvale, CA), and the concentration of BSA, SAA, and Hp calculated by extrapolating from a standard curve using SoftMax Pro software (v. 5.2, Molecular Devices, Sunnyvale, CA).

In-Solution Digests

The protein content of all of the whey fractions from each cow (*n*=40) was determined using a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL) with BSA as a standard. Approximately 100 µg of each whey sample was diluted in 100mM ammonium bicarbonate (Sigma Aldrich, St Louis, Missouri) to a final volume of 50µl. Samples were reduced in a final concentration of 5mM tris(2-carboxyethyl)phosphine (TCEP; Promega, Madison, Wisconsin) for one hour at 37°C. Alkylation was performed using a final concentration of 10mM iodoacetamide for one hour at room temperature with the samples protected from light. Approximately 1µg of sequencing grade modified trypsin (Promega, Madison, WI) was added to each sample to achieve a 1:100 enzyme-to-protein ratio, and digestion was carried out for 16 hours in a 37°C water bath. Samples were diluted 1 to 1,000 in 0.5% acetic acid prior to analysis.

Chromatography and Mass Spectrometry

One-dimensional LC-MS/MS analyses were carried out by injecting 5µL of each diluted whey digest into an ultra-pressure nano-LC instrument (nano-UPLC; Waters Nano Acquity UPLC, Waters, Milford, Massachusetts) coupled to a linear ion trap LTQ XL mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts). Peptides were loaded onto a 100µm x 100mm C_{18} reverse phase HPLC column

(Waters, Milford, Massachusetts). The mobile phase consisted of 0.5% acetic acid in water (A) and 0.5% acetic acid in 80% acetonitrile (B). The gradient was 0 to 20%B for 1 min, 20-50% B in 42 min and 50-100% B in 5 min at a flow rate of 500nL/min. The LTQ mass spectrometer was equipped with a nano-electrospray ionization source (nano-ESI) and was operated in positive ion mode with a capillary voltage of 1.3V. Spectra were acquired for 80 minutes in data-dependent tandem MS mode. The ten most intense ions in each MS survey scan were subjected to MS/MS by collision-induced dissociation (CID). The collision energy used to fragment each peptide ion was 35kV. Following MS/MS, precursor ions were excluded from MS/MS for 15 seconds.

Informatics

Mass spectral peak lists were extracted using XCalibur and resulting lists searched with the Mascot search engine (v.2.1.0; Matrix Sciences, London, UK) against the Swiss-Prot protein sequence library. The following search parameters were applied: *other mammalia* species, trypsin enzyme, one allowed missed cleavage, carbamidomethylation fixed modification, methionine oxidation variable modification, precursor ion mass tolerance of ± 1.3 Da, and a fragment ion mass tolerance of ± 0.8 Da. The number of sequences searched in the *other mammalia* taxonomy was 10635. Only peptides with a Mascot Ion Score equal or greater to the Identity Score (at $P < 0.05$) were included for identification.

Label-free quantification was determined using total peptides hits and spectral counts, which are defined as the MS/MS sampling rate, or the total number of mass

spectra assigned to peptides for a given protein (Liu et al., 2004). Only proteins detected in 4 or more cows at a minimum of one time point following infection were included in the analyses. The number peptide hits (spectral counts) was determined for each protein using the software program MassSieve (Slotta et al., 2007; McFarland et al., 2008). Normalized spectral abundance factors (NSAF) were calculated based on spectral counts as previously described (Florens et al., 2006; Zybailov et al., 2006; Mosley et al., 2008), using the following formula:

$$(\text{NSAF})_i = \frac{(\text{Spectral Count / Length})_i}{\sum_{k=1}^{N}(\text{Spectral Count / Length})_k}$$

The average of the normalized values for spectral counts across all 8 cows was calculated for each identified protein and expressed graphically as mean ± standard error (SE). To measure differences in protein abundance across time points, the fold-change between NSAF before and after challenge was calculated. Changes in relative abundance of proteins across the time points following infection with *E. coli* were considered relevant if a 2-fold or greater NSAF were identified after challenge, or vice versa (McFarland et al., 2008; Mosley et al., 2008). The 2-fold or greater level of significance was only applied to proteins that met the arbitrary threshold of greater than 5 peptide assignments per time point to avoid potential over-estimation of fold-changes in relative abundance based on small NSAF values. For proteins not present in milk prior to challenge, peak expression was marked as the time point with the highest NSAF value, and fold changes were measured relative to the time point at which the protein was first detected. Alignment of protein sequences, to determine the

presence of two distinct protein isoforms, was carried out using the publicly available protein sequence alignment tool ClustalW (European Bioinformatics Institute, Cambridgeshire, UK).

Statistical analysis

Repeated-measures analysis of variance was performed on ELISA data using the PROC MIXED model with Dunnett post hoc pairwise comparisons (SAS version 9.1; SAS Institute, Carey, N.C.) to compare changes in select proteins related to host response identified in samples collected following infection to the pre-infection concentrations of each protein. Means and standard errors for all ELISA and label-free quantification data (NSAF) were calculated and graphed using SigmaPlot (v. 11.0 Systat Software, Inc., Chicago, IL). For all statistical analyses, a value of $P < 0.05$ for the comparisons was considered to represent a significant difference between groups.

Results

Bovine Milk Protein Modulation Following Infection with E. coli

The two-fold aim of the study was to identify proteins related to host response in bovine milk samples from a group of 8 cows across multiple time points following experimental induction of coliform mastitis, and to quantify relative changes in protein abundance over the course of a clinical infection. Whey samples prepared from milk collected from 8 cows prior to infection (0 hour) and at 12, 18, 24, and 48 hours following inoculation with *E. coli* were analyzed using one-dimensional nano-LC followed by tandem MS (LC-MS/MS). A total of 275 proteins were identified in

bovine milk across all sample time points. Of the proteins identified, 67 appeared in milk samples from multiple cows at more than one time point (Table 1), 20 appeared in multiple cows at only one time point (Supplemental Table 1), and 33 proteins appeared in only one cow across multiple time points. The remaining proteins identified were based on only one peptide assignment. Proteins identified in only one cow across multiple time points or by only one peptide were culled, because protein identifications were not considered robust, or were indicative of a response unique to only one animal.

The most profound modulation in the composition of proteins present in the milk samples collected at time points following infection with *E. coli* was the presence of numerous vascular derived proteins including BSA, alpha-fetoprotein, transferrin, fibrinogen, several apolipoproteins, and complement factors. Another notable change in the protein content of the milk samples was the presence of the acute phase proteins SAA, Hp, and A1AG, as well as proteins with established antimicrobial properties including a number of cationic proteins belonging to the cathelicidin family, lactoferrin, lactoperoxidase, and peptidoglycan recognition receptor protein (PGRP). Additionally, proteins related to cell adhesion or lymphocyte trafficking including glycosylation-dependent cell adhesion molecule-1 (Glycam-1), alpha-2-HS-glycoprotein, and osteopontin-K were detected in the whey from milk samples collected during the course of infection.

Table 1 Proteins identified in bovine whey from milk samples collected across time points following experimental induction of coliform mastitis.

Swiss-Prot entry name	Primary accession number	Protein	Avg No. Cows Present	No. Time Points Present	Avg Spec Count[a]	Avg No. Peptides	Ref
ALBU_BOVIN	P02769	Serum ablumin	8	5	30	18	1,2,3,
LACB_BOVIN	P02754	β-lactoglobulin	8	5	37	9	1,2,3
CASA1_BOVIN	P02662	α-S1-casein	8	5	18	5	1,2,3
CASA2_BOVIN	P02663	α-S2-casein	8	5	8	4	1,2
CASB_BOVIN	P02666	β-casein	8	5	5	3	1,2,3
CASK_BOVIN	P02668	κ-casein	8	5	10	3	1,2,3
GLCM1_BOVIN	P80195	Glycam-1 (Lactophorin)	8	5	7	3	1,2
TRFL_BOVIN	P24627	Lactoferrin	8	5	20	15	1
LALBA_BOVIN	P00711	α-lactalbumin	7	5	13	5	1,3
OSTP_BOVIN	P31096	Osteopontin	7	5	2	2	
TRFE_BOVIN	Q29443	Transferrin	7	4	10	9	1,2,3
APOA1_BOVIN	P15497	Apolipoprotein A1	7	4	9	8	
CO3_BOVIN	Q2UVX4	Complement C3	7	4	9	9	1
PIGR_BOVIN	P81265	Polymeric immunoglobulin receptor	6	5	6	5	1,2
FETA_BOVIN	Q3SZ57	Alpha-fetoprotein	6	5	1	1	
FIT2_BOVIN	A4FIN5	Fat-inducing protein 2	6	5	1	1	
BT1A1_BOVIN	P18892	Butyrophylin	5	5	3	3	1
LSM4_BOVIN	Q3ZBK6	U6 snRNA-associated SM-like protein	5	5	1	1	
SYMC_BOVIN	Q2T9L8	Methionyl-tRNA synthetase	5	5	1	1	
CC060_BOVIN	Q2HJ12	Uncharacterized protein C3orf60 homolog	5	5	1	1	
PGRP_BOVIN	Q8SPP7	Peptidoglycan recognition receptor protein	5	4	4	4	
CLUS_BOVIN	P17697	Clusterin	5	4	3	3	1
CTHL1_BOVIN	P22226	Cathelicidin-1	5	3	4	3	
SAA_BOVIN	P35541	Serum amyloid A	5	2	2	2	
ACTG_BOVIN	P63258	Actin, cytoplasmic 2	5	2	4	4	
CRF_BOVIN	Q95MI6	Corticoliberin	4	5	1	1	
VTDB_BOVIN	Q3MHN5	Vitamin D binding protein	4	4	2	2	1
APOA2_BOVIN	P81644	Apolipoprotein A2	4	4	2	2	
KNG2_BOVIN	P01045	Kininogen 2	4	4	2	2	
FETUA_BOVIN	P12763	Alpha-2-HS-glycoprotein	4	4	3	3	1
CFAB_BOVIN	P81187	Complement factor B	4	4	2	2	
FIBA_BOVIN	P02672	Fibrinogen alpha chain	4	4	7	6	
FIBB_BOVIN	P02676	Fibrinogen beta chain	4	4	4	3	
FIBG_BOVIN	P12799	Fibrinogen gamma chain	4	4	2	2	1
ITIH4_BOVIN	Q3T052	Inter-alpha trypsin inhibitor heavy chain 4	4	4	2	2	
PERL_BOVIN	P80025	Lactoperoxidase	4	4	1	1	1,2
HEMO_BOVIN	Q3SZV7	Hemopexin	4	3	2	2	
ACTB_BOVIN	P60712	Actin, cytoplasmic 1	4	3	6	5	2
CTHL2_BOVIN	P19660	Cathelicidin-2	4	3	2	2	
HPT_BOVIN	Q2TBU0	Haptoglobin	4	3	2	2	
CTHL4_BOVIN	P33046	Cathelicidin 4	4	2	4	3	
MFGM_BOVIN	Q95114	Lactadherin	3	5	2	2	
APOC3_BOVIN	P19035	Apolipoprotein C3	3	4	1	1	
B2MG_BOVIN	P01888	Beta-2-microglobulin	3	4	2	2	1,2
GELS_BOVIN	Q3SX14	Gelsolin	3	4	1	1	1
CTHL6_BOVIN	P54228	Cathelicidin-6	3	3	2	2	
CTHL7_BOVIN	P56425	Cathelicidin-7	3	3	2	2	
THRB_BOVIN	P00735	Prothrombin	3	3	1	1	
CTHL3_BOVIN	P19661	Cathelicidin 3	3	2	2	2	
CTHL5_BOVIN	P54229	Cathelicidin 5	3	2	2	2	
A1AG_BOVIN	Q3SZR3	Alpha-1-acid glycoprotein	2	4	1	1	
BT3L4_BOVIN	Q2KIY7	Transcription factor BTF3 homolog 4	2	4	1	1	
KNG1_BOVIN	P01044	Kininogen 1	2	3	2	2	1
ITIH1_BOVIN	Q0VCM5	Inter-alpha trypsin inhibitor heavy chain 1	2	3	1	1	
APOE_BOVIN	Q03247	Apolipoprotein E	2	3	3	3	
UBC12_BOVIN	A3KN22	NEDD8-conjugating enzyme Ubc12	2	5	1	1	
GPC5C_BOVIN	Q2YDG0	G-protein coupled receptor family group 5-C	2	2	1	1	
ITIH5_BOVIN	A2VE29	Inter-alpha trypsin inhibitor heavy chain 5	1	4	1	1	
CENPH_BOVIN	Q3T0L1	Centromere protein H	3	4	1	1	
NUCB1_BOVIN	Q0P569	Nucleobindin-1	2	2	1	1	
B4GT1_BOVIN	P08037	Beta-1,4-galactosyltransferase 1	2	3	1	1	
XDH_BOVIN	P80457	Xanthine dehydrogenase	2	2	2	2	
OSTK_BOVIN	P31098	Osteopontin-K	3	3	2	2	
APOA4_BOVIN	Q32PJ2	Apolipoprotein A-IV	2	3	2	2	
APOH_BOVIN	P17690	Beta-2-glycoprotein 1	2	2	1	1	
PTGDS_BOVIN	O02853	Prostaglandin-H2 D-isomerase	1	3	1	1	4
ANT3_BOVIN	P41361	Antithrombin-III	2	2	1	1	

References: 1= Fong et. al.(2008); 2= Smolenski et. al.(2007); 3= Hogarth et. al. (2004); 4=Baeker et. al. (2002)

Novel acute phase and inflammatory mediators were likewise identified. Two isoforms of kininogen, the precursor to bradykinin, were identified in milk following infection with *E. coli,* as was the APP inter-alpha trypsin inhibitor heavy chain 4 (ITIH4). Additional proteins related to host response not identified in earlier proteomic analyses that were identified in the current study included osteopontin, SAA, Hp, α-1-acid-glycoprotein, complement factor B, apolipoproteins A-I, A-II, A-IV, and C-III, several cathelicidins, fibrinogen β-chain, and prothrombin (Table 2). In summation, a total of 48 proteins directly related to host response, or that are known to leak into milk from circulation as a secondary effect of cytokine production during coliform mastitis, were identified in milk following experimental induction of mastitis (Table 3).

Table 2 Proteins not previously identified in bovine milk using proteomic strategies.

Swiss-Prot entry name	Primary accession number	Protein	Avg No. Cows Present	No. Time Points Present
OSTP_BOVIN	P31096	Osteopontin	7	5
APOA1_BOVIN	P15497	Apolipoprotein A1	7	4
FETA_BOVIN	Q3SZ57	Alpha-fetoprotein	6	5
SAA_BOVIN	P35541	Serum amyloid A	5	2
CRF_BOVIN	Q95MI6	Corticoliberin	4	5
FIBB_BOVIN	P02676	Fibrinogen beta chain	4	4
APOA2_BOVIN	P81644	Apolipoprotein A2	4	4
KNG2_BOVIN	P01045	Kininogen 2	4	4
CFAB_BOVIN	P81187	Complement factor B	4	4
ITIH4_BOVIN	Q3T052	Inter-alpha trypsin inhibitor heavy chain 4	4	4
CTHL2_BOVIN	P19660	Cathelicidin-2	4	3
HPT_BOVIN	Q2TBU0	Haptoglobin	4	3
CTHL4_BOVIN	P33046	Cathelicidin 4	4	2
APOC3_BOVIN	P19035	Apolipoprotein C3	3	4
CTHL6_BOVIN	P54228	Cathelicidin-6	3	3
CTHL7_BOVIN	P56425	Cathelicidin-7	3	3
THRB_BOVIN	P00735	Prothrombin	3	3
OSTK_BOVIN	P31098	Osteopontin-K	3	3
CTHL3_BOVIN	P19661	Cathelicidin 3	3	2
CTHL5_BOVIN	P54229	Cathelicidin 5	3	2
A1AG_BOVIN	Q3SZR3	Alpha-1-acid glycoprotein	2	4
APOA4_BOVIN	Q32PJ2	Apolipoprotein A-IV	2	3
APOE_BOVIN	Q03247	Apolipoprotein E	2	3
ITIH5_BOVIN	A2VE29	Inter-alpha trypsin inhibitor heavy chain 5	1	4

Table 3 Proteins related to host response identified in bovine milk following infection with *E.coli*

Swiss-Prot entry name	Protein	Function
ALBU_BOVIN	Serum albumin	Main plasma protein; binds Ca2+, Na+, K+, fatty acids
GLCM1_BOVIN	Glycam-1 (Lactophorin)	Mediates trafficking of lymphocytes to lymph nodes
TRFL_BOVIN	Lactoferrin	Iron-binding protein; has antimicrobial activity
OSTP_BOVIN	Osteopontin	Enhances production of interferon-gamma and interleukin 12
TRFE_BOVIN	Transferrin	Iron-binding protein
APOA1_BOVIN	Apolipoprotein A1	Major plasma HDL protein
CO3_BOVIN	Complement C3	Major protein in complement activation
FETA_BOVIN	Alpha-fetoprotein	Plasma protein; binds copper, nickel, and fatty acids
PGRP_BOVIN	Peptidoglycan recognition receptor protein	Involved in innate immunity; microbicidal for gram(+) and gram(-) bacteria
CLUS_BOVIN	Clusterin	Function unclear: may be involved in programmed cell death
CTHL1_BOVIN	Cathelicidin-1	Potent microbicidal activity against *E. coli*
SAA_BOVIN	Serum amyloid A	Major acute phase protein
VTDB_BOVIN	Vitamin D binding protein	Associates w/immunoglobulins on lymphocytes
APOA2_BOVIN	Apolipoprotein A2	Stablizes HDL; has antimicrobial activity
KNG2_BOVIN	Kininogen 2	Produces active peptide bradykinin; inflammatory mediator
FETUA_BOVIN	Alpha-2-HS-glycoprotein	Promotes endocytosis; has lymphocyte stimulting properties
CFAB_BOVIN	Complement factor B	Part of the alternative complement pathway
FIBA_BOVIN	Fibrinogen alpha chain	Yields monomers that polymerize into fibrin; acts a cofactor in platelet aggregation
FIBB_BOVIN	Fibrinogen beta chain	Yields monomers that polymerize into fibrin; acts a cofactor in platelet aggregation
FIBG_BOVIN	Fibrinogen gamma chain	Yields monomers that polymerize into fibrin; acts a cofactor in platelet aggregation
ITIH4_BOVIN	Inter-alpha trypsin inhibitor heavy chain 4	Involved in acute phase reactions
PERL_BOVIN	Lactoperoxidase	Antimicrobial agent; protects udder from infection
HEMO_BOVIN	Hemopexin	Binds heme and transports it to the liver
CTHL2_BOVIN	Cathelicidin-2	Potent antimicrobial activity
HPT_BOVIN	Haptoglobin	Combines w/free plasma hemoglobin; acute phase protein
CTHL4_BOVIN	Cathelicidin 4	Potent microbicidal activity against *E. coli*
APOC3_BOVIN	Apolipoprotein C3	Inhibits lipoprotein lipase and hepatic lipase
B2MG_BOVIN	Beta-2-microglobulin	Beta chain of major histocompatibility complex class I molecules
CTHL6_BOVIN	Cathelicidin-6	Potent antimicrobial activity
CTHL7_BOVIN	Cathelicidin-7	Potent antimicrobial activity
THRB_BOVIN	Prothrombin	Converts fibrinogen to fibrin; functions in inflammation
CTHL3_BOVIN	Cathelicidin 3	Potent antimicrobial activity
CTHL5_BOVIN	Cathelicidin 5	Potent antimicrobial activity
A1AG_BOVIN	Alpha-1-acid glycoprotein	Modulates activity of immune system during acute phase reaction
KNG1_BOVIN	Kininogen 1	Produces active peptide bradykinin; inflammatory mediator
ITIH1_BOVIN	Inter-alpha trypsin inhibitor heavy chain 1	May act as a carrier for hyaluronan in serum
APOE_BOVIN	Apolipoprotein E	Mediates binding, internalization, and catabolism of lipoprotein particles
ITIH5_BOVIN	Inter-alpha trypsin inhibitor heavy chain 5	May act as a tumor suppressor
OSTK_BOVIN	Osteopontin-K	Involved in cell adhesion
APOA4_BOVIN	Apolipoprotein A-IV	Involved in chylomicron and VLDL secretion and catabolism
APOH_BOVIN	Beta-2-glycoprotein 1	Prevents blood coagulation cascade by binding to phospholipids on damaged cells
PTGDS_BOVIN	Prostaglandin-H2 D-isomerase	Prostaglandin; potent inhibitor of platelet aggregation
ANT3_BOVIN	Antithrombin-III	Serin protease; regulates blood coagulation cascade
CD36_BOVIN	Platelet glycoprotein 4	May function as cell adhesion molecule; binds collagen and anionic phospholipids
LBP_BOVIN	Lipopolysaccharide-binding protein	Binds LPS; interacts with CD14
DFB11_BOVIN	Beta-defensin 11	Potent microbicidal activity against *E. coli*
A2MG_BOVIN	Alpha-2-macroglobulin	Inhibits proteinases
CH3L1_BOVIN	Chitinase-3-like protein 1	May play a role in tissue remodeling and defense against pathogens

LC-MS/MS Label-free quantification using number of unique peptides versus spectral counts

A comparison of label-free quantification performed using normalized number of unique peptides and normalized spectral counts revealed differences in the semiquantitative measures of protein abundance at early time points for the proteins serum albumin (Fig. 1A) and β-lactoglobulin (Fig. 1B), but better uniformity of quantitative measures at later time points. In contrast, nearly identical expression patterns were observed between number of unique peptides and spectral counts for the low abundance proteins osteopontin (Fig. 1C) and complement factor B (Fig. 1D).

Normalized spectral abundance factors correspond with total ion intensity data

A comparison of relative abundance determined using spectral counts versus total ion current intensity was performed using NSAF and extracted ion chromatograms (XIC) for both the highly abundant protein BSA (Fig. 2A) and the low abundance protein haptoglobin (Fig. 2B). XIC were carried out on 2 BSA peptides and 1 Hp peptide. Only 1 Hp peptide was used because despite the multiple peptides that were assigned to Hp, only one peptide consistently contributed to the identification of Hp across multiple samples and time points. Relative abundance of BSA across all time points, as determined using both spectral counts and XIC, were nearly identical in pattern. Furthermore, peak increases in the abundance of BSA were apparent at 24 h following challenge relative to pre-infection levels using both XIC and NSAF. Similarly, temporal expression patterns of Hp in milk before and after infection

determined using XIC and NSAF exhibited patterns of estimated relative abundance that were nearly uniform.

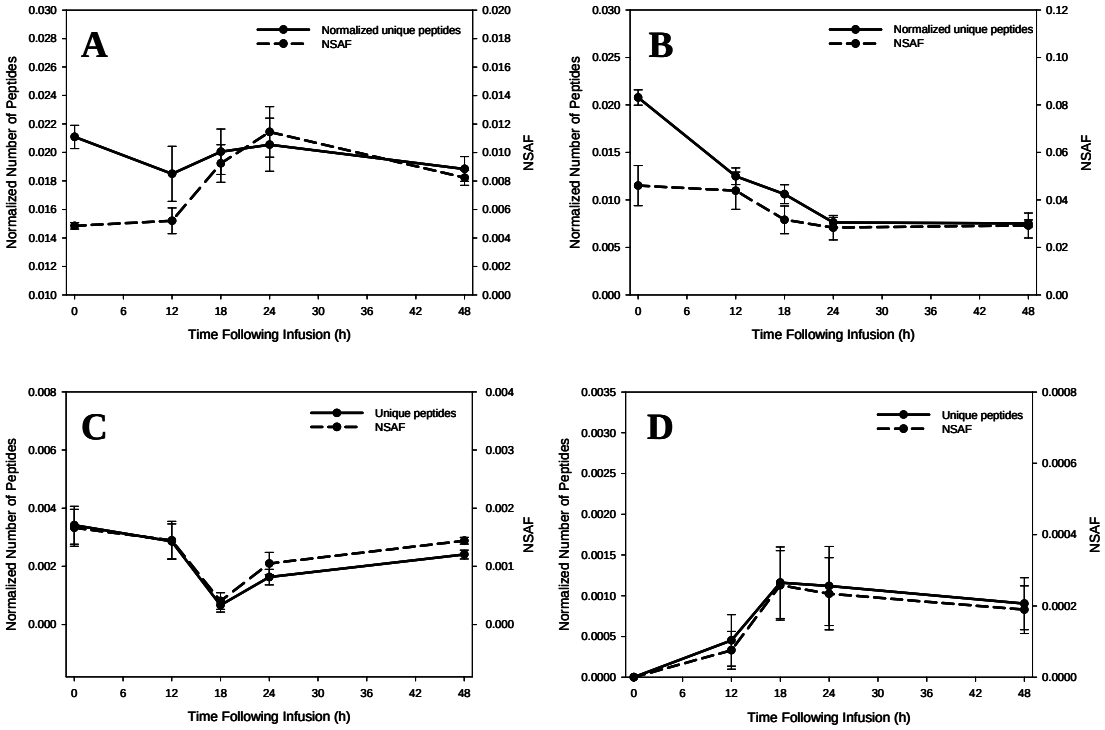


Figure 1 Comparison of label-free quantification using normalized number of unique peptides and normalized spectral abundance factors for the high abundance proteins serum albumin (A) and β-lactoglobulin (B) and for the low abundance proteins osteopontin (C) and complement factor B (D).

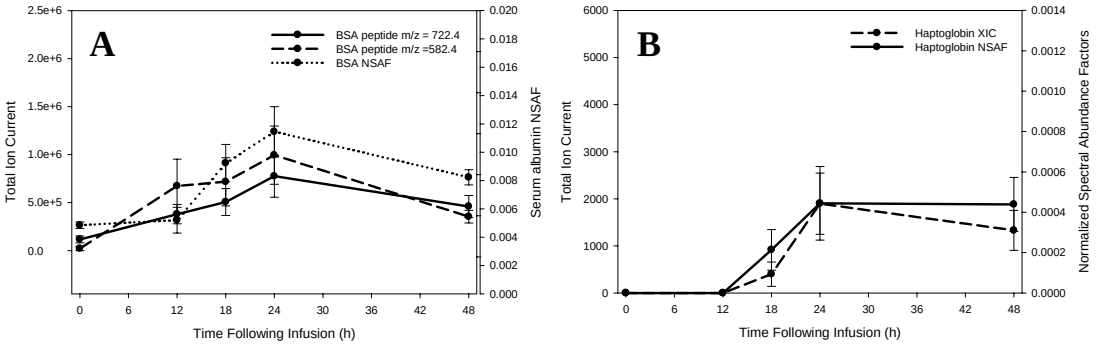


Figure 2 Comparison of relative quantification and determined using normalized spectral abundance factors (NSAF) and total ion intensity of the high abundance protein BSA (A) and the low abundance protein haptoglobin (B).

LC-MS/MS Label free quantification corresponds with ELISA data

To assess the feasibility of using NSAF to track changes in the relative abundance of proteins detected in bovine milk following infection with *E. coli*, NSAF for milk BSA, Hp, and SAA were compared to quantification of each protein determined using commercially available ELISAs. Overall, temporal expression patterns determined using an ELISA assay and NSAF were nearly identical for all 3 proteins. The comparison of changes in milk concentrations of BSA following experimental induction of coliform mastitis as measured by ELISA and NSAF revealed significant differences from pre-challenge levels at 18 h and 24 h for ELISA data, and greater than or approaching 2-fold changes in NSAF at the same time points (Fig. 3A). Increases in milk Hp were evident as early as 12 h following infection in both the NSAF and ELISA data, with peak increases apparent at 24 h (Fig. 3B). SAA was detected in milk as early as 12 h following infection using an ELISA, but not until 18 h using LC-MS/MS (Fig. 3C). Levels of SAA continued to rise throughout the duration of the study, and did not return to baseline. SAA and Hp were much lower in abundance than BSA, however, and were not present in milk prior to infection. Thus, detecting meaningful fold-changes in the APP was somewhat biased due to the limited number of peptides detected, the subsequently lower MS/MS sampling rates of assigned peptides, and the fact that baseline abundance values for each protein were zero.

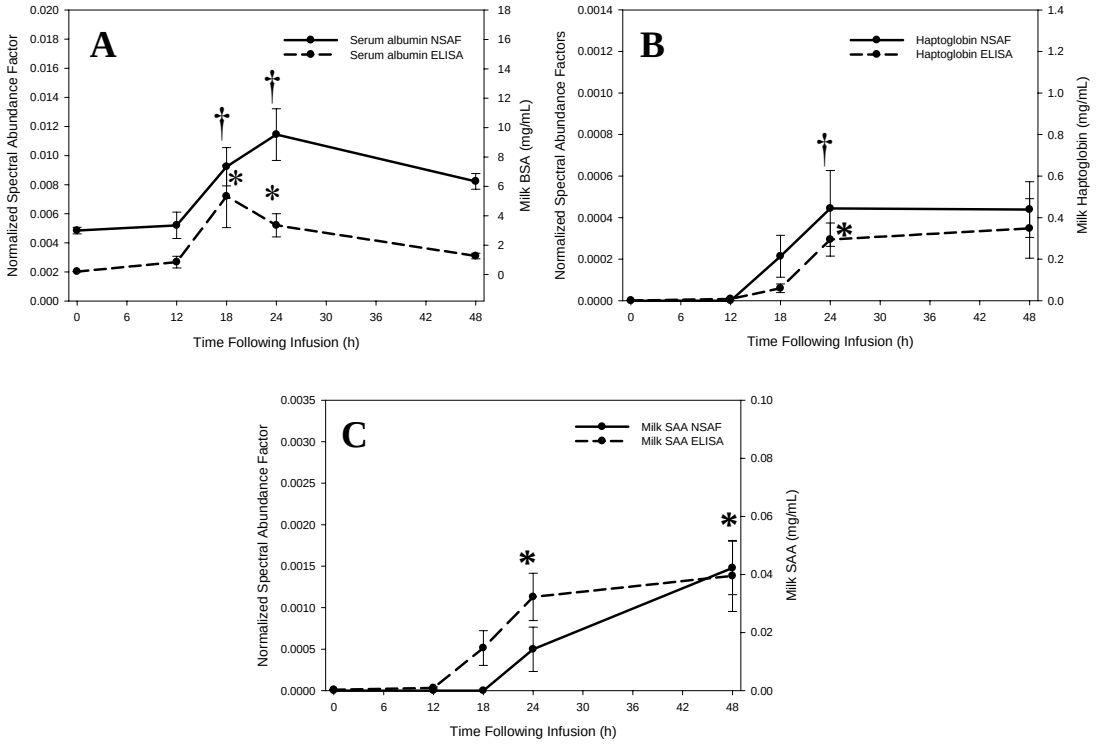


Figure 3 Comparison of quantification by ELISA and normalized spectral abundance factors (NSAF) for serum albumin (A), haptoglobin (B), and serum amyloid A (C). * denotes a significant increase ($P < 0.05$) in abundance determined using ELISA, † denotes a 2-fold or greater increase determined using NSAF.

Semiquantitative Analysis of Abundant Milk Proteins during Mastitis

Relative abundance of the whey and casein proteins in milk was determined using NSAF. Profiles of whey proteins across all time points revealed increases in BSA beginning at 12 h, with greater than 2-fold changes at 18 and 24 h following infusion (Tables 4 & 5). Reduced abundance of β-lactoglobulin and α-lactalbumin were evident between 12 h and 24 h following infection. Fold changes were not significant, but decreased expression of β-lactoglobulin and α-lactalbumin corresponded to peak increases in BSA in the milk (Fig. 4A). In contrast, abundance of α-$_{S1}$-casein, α-$_{S2}$-casein β-casein, and κ-casein fluctuated between 12 h and 24 h

but remained relatively unchanged after 24 h. No significant fold changes in relative abundance across time points were detected for any of the casein proteins (Fig. 4B).

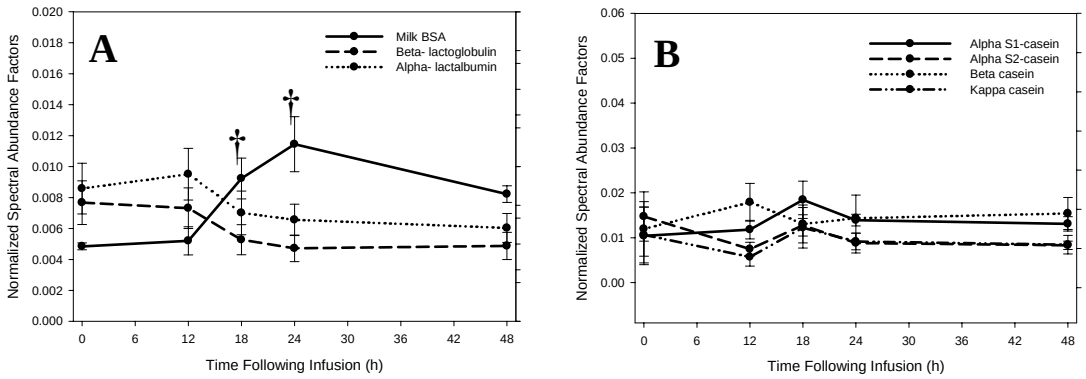


Figure 4 Temporal expression of normalized spectral abundance factors for whey and casein proteins. † denotes a 2-fold or greater increase determined using NSAF.

Semiquantitative Analysis of Proteins related to Host Response in Milk during Mastitis

Relative protein quantification of select proteins related to host response was determined using NSAF. Stringent thresholds were applied to the decision of which proteins to evaluate for temporal expression. Only proteins identified in 4 or more cows at multiple time points and with greater than one peptide observation were included in the analysis; proteins identified based on only one peptide assignment or in fewer than 4 cows were not considered. The types of host response proteins evaluated for temporal abundance patterns during clinical mastitis were vascular-derived (Table 5), acute phase, and antimicrobial (Table 6). NSAF for the plasma proteins complement C3 and complement factor B (Fig. 5A), fibrinogen α, β, and γ chains (Fig. 5B), and the most abundant apolipoproteins, A-I and A-II (Fig. 5C), revealed peak increases in relative abundance at 18 hours following experimental induction of coliform mastitis for all of the proteins.

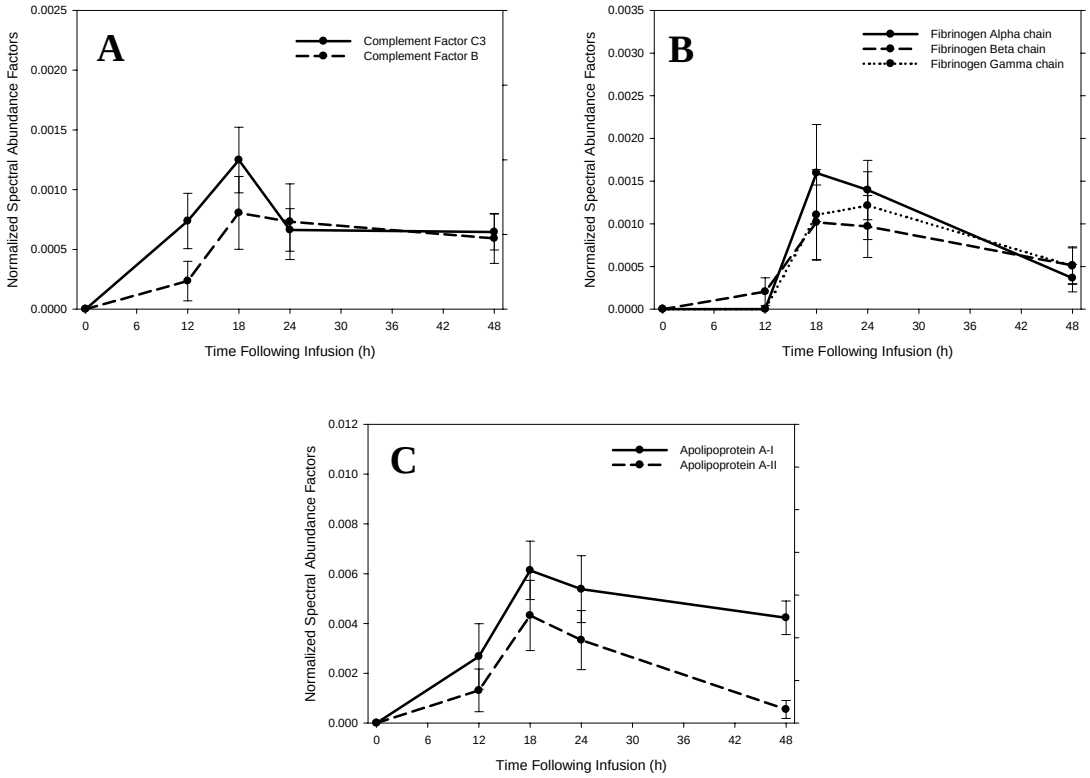


Figure 5 Normalized spectral abundance factors for vascular-derived proteins.

Fold changes in plasma-derived proteins could not be calculated relative to pre-challenge levels because none of the proteins that presumably leaked into the milk as a result of increased vascular permeability, with the exception of BSA, were present in milk prior to infection. Fold changes were calculated using NSAF between the first time point the protein was identified and later time points, but for lower abundance proteins with fewer peptide assignments, fold changes were not regarded as accurate.

Table 4 Normalized spectral abundance factors for whey and casein proteins before and after challenge.

Protein	Pre-Challenge Spectral Counts[a]	Pre-Challenge NSAF[b]	Post-Challenge Time Point	Post-Challenge Spectral Counts[a]	Post-Challenge NSAF[b]	NSAF Fold Change[c]
B-lactoglobulin	24	0.04603	12h	51	0.04388	1.0
			18h	29	0.03163	0.7
			24h	39	0.02829	0.6
			48h	41	0.02922	0.6
α-lactalbumin	7	0.01716	12h	18	0.01901	1.1
			18h	10	0.01401	0.8
			24h	14	0.01311	0.8
			48h	14	0.01206	0.7
α-S_1-casein	8	0.01041	12h	17	0.01181	1.1
			18h	20	0.01846	1.8
			24h	23	0.01392	1.3
			48h	22	0.01309	1.3
α-S_2-casein	6	0.00908	12h	7	0.00491	0.5
			18h	9	0.00795	0.9
			24h	10	0.00569	0.6
			48h	9	0.00541	0.6
β-casein	3	0.00300	12h	6	0.00436	1.5
			18h	4	0.00325	1.1
			24h	6	0.00354	1.2
			48h	7	0.00379	1.3
κ-casein	7	0.00995	12h	9	0.00746	0.8
			18h	10	0.01076	1.1
			24h	14	0.0092	0.9
			48h	13	0.00884	0.9

[a] Average across all 8 cows; [b] Normalized spectral abundance factors; [c] Relative to pre-challenge levels.

Relative quantification of the abundance of APP in milk during the course of clinical mastitis revealed increases in SAA, Hp, and the novel acute phase protein ITIH4. SAA was initially detected in milk 24 hours following inoculation, and levels continued to increase for the duration of the study (Fig. 6A). Increases in milk Hp were first detected 18 h after infection with *E. coli,* with peak abundance evident at 24 h that remained elevated through the 48 hr time point. The novel acute phase protein ITIH4 exhibited increases in abundance as early as 12 h following infection,

and peak expression at 18 h. In contrast to levels of SAA and Hp, the expression of ITIH4 in milk returned to near baseline levels by 48 h after infection. The presence of A1AG was likewise detected in milk (Table 1), but expression was transient and thus no meaningful quantification could be performed.

Proteins possessing antimicrobial activity were also evaluated for changes in relative abundance using NSAF. The most abundant antimicrobial proteins (AMP) in bovine milk during the course of clinical mastitis were PGRP and several members of the cathelicidin family of cationic AMP including cathelicidin-1, 2, and 4 (Fig. 6B). Peak abundance of PGRP was apparent in milk at 18 h following experimental induction of mastitis. Cathelicidin-1 and -2 both began to increase in abundance in milk at 18 h after infusion, while Cathelicidin-4 exhibited a slightly altered expression pattern with increases not apparent until 24 h following infection. Expression of all of AMP continued to increase throughout the duration of the study and did not return to baseline. Similarly, MSCC (log transformed) exhibited significant increases ($P<0.05$) at 24 h following induction of clinical mastitis and remained elevated through the course of the time points evaluated.

Table 5 Normalized spectral abundance factors for vascular-derived proteins before and after challenge.

Protein	Pre-Challenge Spectral Counts[a]	Pre-Challenge NSAF[b]	Post-Challenge Time Point	Post-Challenge Spectral Counts[a]	Post-Challenge NSAF[b]	NSAF Fold Change[c]
Serum albumin	9	0.00485	12h	21	0.00520	1.1
			18h	29	0.00923	1.9
			24h	54	0.01144	2.4
			48h	39	0.00823	1.7
Transferrin	0	0.00000	12h	8	0.00114	-
			18h	9	0.00247	2.2
			24h	12	0.00225	2.0
			48h	9	0.00171	1.5
Apolipoprotein A1	0	0.00000	12h	5	0.00267	-
			18h	8	0.00613	2.3
			24h	13	0.00538	2.0
			48h	9	0.00423	1.6
Complement C3	0	0.00000	12h	9	0.00074	-
			18h	11	0.00125	1.7
			24h	10	0.00066	0.9
			48h	8	0.00065	0.9
Apolipoprotein A2	0	0.00000	12h	2	0.00077	-
			18h	2	0.00252	3.3
			24h	2	0.00194	2.5
			48h	1	0.00032	0.4
Complement factor B	0	0.00000	12h	2	0.00008	-
			18h	2	0.00026	3.2
			24h	3	0.00023	2.9
			48h	2	0.00019	2.4
Fibrinogen alpha chain	0	0.00000	12h	0	0.00000	-
			18h	9	0.00138	-
			24h	8	0.00144	1.1
			48h	4	0.00027	0.2
Fibrinogen beta chain	0	0.00000	12h	3	0.00020	-
			18h	4	0.00102	5.1
			24h	6	0.00097	4.8
			48h	3	0.00051	2.6
Fibrinogen gamma chain	0	0.00000	12h	0	0.00000	-
			18h	3	0.00063	-
			24h	3	0.00069	1.1
			48h	2	0.00029	0.5

[a] Average across all 8 cows; [b] Normalized spectral abundance factors; [c] Relative to pre-challenge levels or the time point at which the protein was first detected.

Table 6 Normalized spectral abundance factors for proteins related to host response before and after challenge.

Protein	Pre-Challenge Spectral Counts[a]	Pre-Challenge NSAF[b]	Post-Challenge Time Point	Post-Challenge Spectral Counts[a]	Post-Challenge NSAF[b]	NSAF Fold Change[c]
Glycam-1 (Lactophorin)	5	0.01044	12h	10	0.00976	0.9
			18h	7	0.00732	0.7
			24h	7	0.00624	0.6
			48h	7	0.00586	0.6
Lactoferrin	8	0.00344	12h	17	0.00360	1.1
			18h	15	0.00423	1.2
			24h	27	0.00489	1.4
			48h	34	0.00613	1.8
Peptidoglycan recognition receptor protein	0	0.00000	12h	0	0.00000	-
			18h	6	0.00324	-
			24h	6	0.00281	0.9
			48h	5	0.00303	0.9
Clusterin	0	0.00000	12h	3	0.00057	-
			18h	3	0.00064	1.1
			24h	4	0.00041	0.7
			48h	1	0.00040	0.7
Cathelicidin-1	0	0.00000	12h	0	0.00000	-
			18h	2	0.00072	-
			24h	5	0.00303	4.2
			48h	5	0.00361	5.0
Serum amyloid A	0	0.00000	12h	0	0.00000	-
			18h	0	0.00000	-
			24h	1	0.00050	-
			48h	2	0.00148	3.0
Kininogen 2	0	0.00000	12h	3	0.00019	-
			18h	3	0.00068	3.6
			24h	2	0.00029	1.5
			48h	0	0.00000	-
Inter-alpha trypsin inhibitor heavy chain 4	0	0.00000	12h	3	0.00010	-
			18h	2	0.00028	2.8
			24h	2	0.00014	1.4
			48h	1	0.00007	0.7
Cathelicidin-2	0	0.00000	12h	0	0.00000	-
			18h	1	0.00080	-
			24h	2	0.00083	1.0
			48h	2	0.00100	1.3
Haptoglobin	0	0.00000	12h	0	0.00000	-
			18h	1	0.00021	-
			24h	3	0.00044	0.2
			48h	2	0.00044	0.2
Cathelicidin 4	0	0.00000	12h	0	0.00000	-
			18h	0	0.00000	-
			24h	3	0.00112	-
			48h	4	0.00245	2.2

[a] Average across all 8 cows; [b] Normalized spectral abundance factors; [c] Relative to pre-challenge levels or the time point at which the protein was first detected.

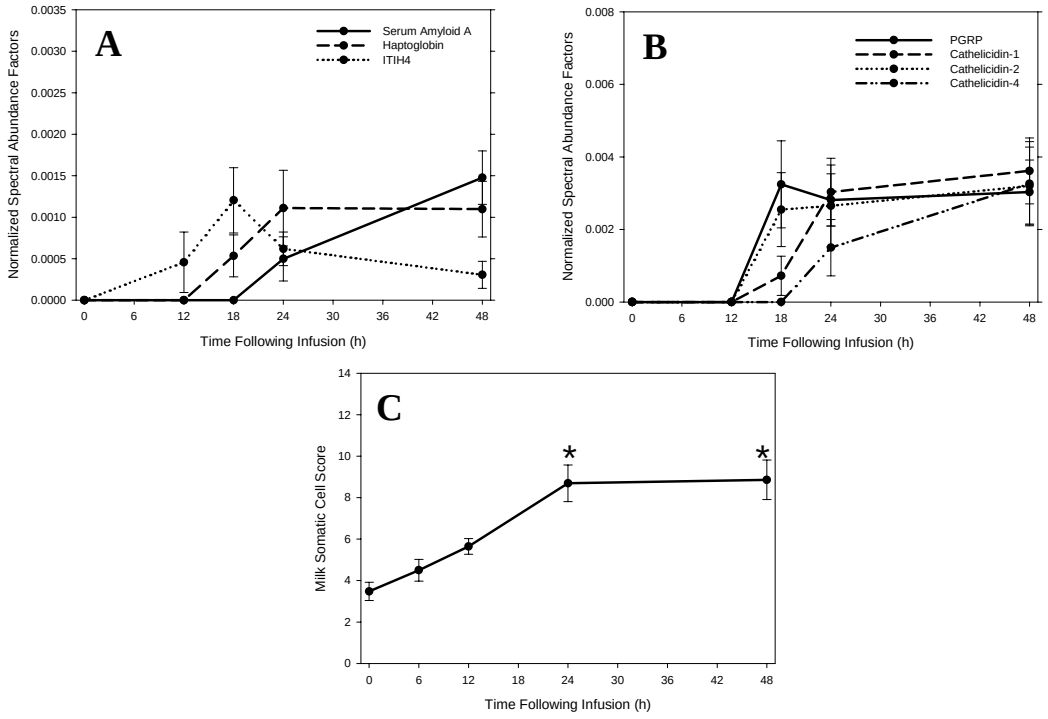


Figure 6 Normalized spectral abundance factors of low abundance acute phase (A) and antimicrobial proteins (B). * denotes significant changes ($P<0.05$) relative to time zero.

Temporal expression of novel markers of coliform mastitis

The abundance and temporal expression patterns of novel biomarkers of coliform mastitis detected in bovine milk during the course of an experimentally induced clinical infection were evaluated using NSAF. Comparisons made included ITIH4 and kininogen-2 (Fig. 7A), relative abundance of apolipoprotein A-I and A-II and the adhesion molecule Glycam-1 (Fig. 7B), and the expression patterns of apolipoprotein A-I and the relatively uncharacterized protein apolipoprotein J, or clusterin (Fig. 7C).

Despite the low relative abundance of the novel biomarkers of host response, similarities in the expression patterns of the host response proteins were detected. Temporal expression of the kallikrein-sensitive acute phase protein ITIH4 and the kallikrein kininogen-2 were nearly identical, with increases in relative abundance

apparent by 12 h following infection and peak expression at 18 h. Similarly, levels of ITIH4 and kininogen-2 returned to baseline by 48 h following inoculation. Both kininogen-1 and kininogen-2 were identified in mastitis milk in the current analyses, but kininogen-2 was found in higher abundance. Because the two kallikrein proteins have nearly homologous sequences, an alignment was performed using the alignment tool ClustalW, and differences in the amino acids sequences investigated. Two peptides that mapped to single amino acid differences between kininogen-1 and kininogen-2 were assigned to kininogen-2, which verified the presence of both isoforms of the bradykinin precursor in the mastitic milk samples (Fig. 8).

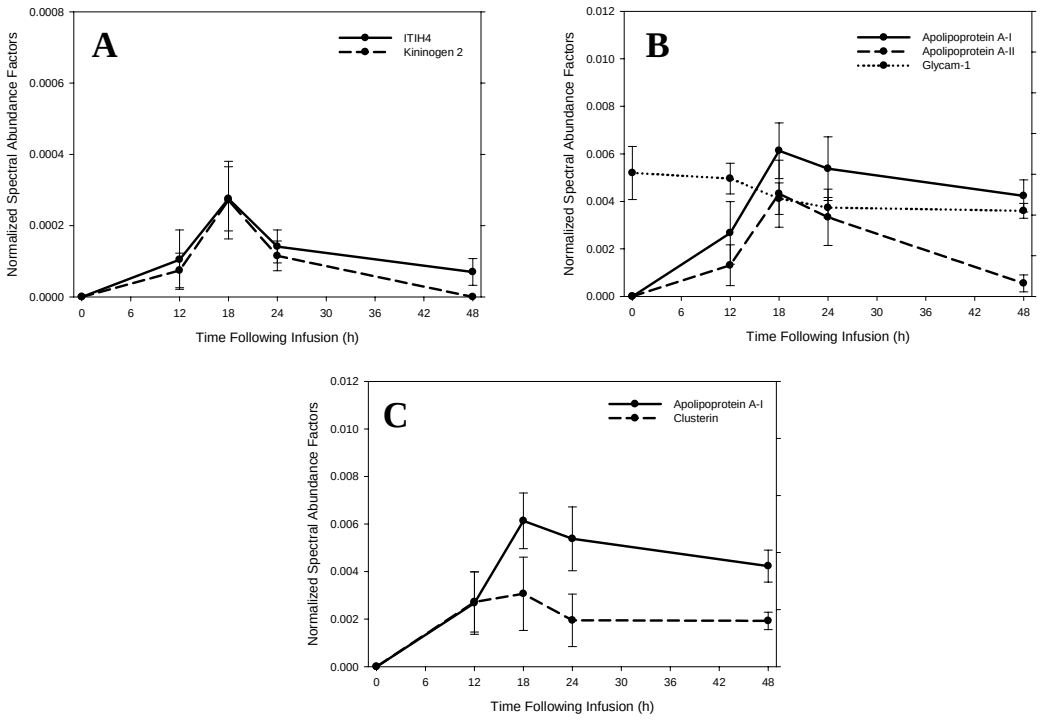


Figure 7 Evaluation of potential protein interactions over the course of experimental infection using NSAF.

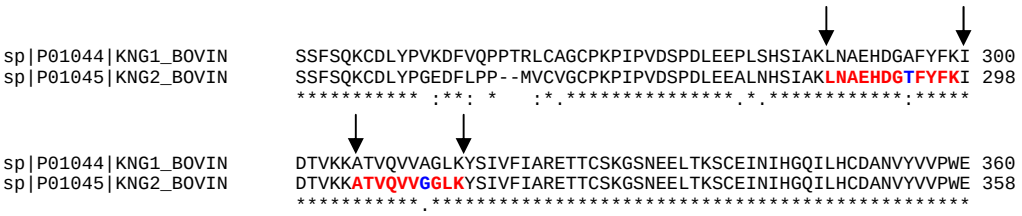


Figure 8 Portion of sequence alignment of kininogen 1 and 2. The two differentiable peptides assigned to kininogen-2 are flanked by arrows; single amino acid differences are marked with a ":" and "." for each peptide, respectively. Amino acids that are in accord for both protein sequences are marked by a "*".

Levels of apolipoprotein A-I and A-II peaked at 18 h during infection which corresponded to maximal decreases in the adhesion molecule Glycam-1. Abundance of apolipoprotein A-II returned to baseline by 48 h following infection, but levels of apolipoprotein A-I and Glycam-1 were still elevated at the conclusion of the analyses. Comparison of apolipoprotein A-I and clusterin revealed patterns of temporal expression that were similar despite the apparent lower abundance of clusterin.

Discussion

Whey from bovine milk samples collected prior to infection with *E. coli* (0 h), and at 12, 18, 24, and 48 following infection with *E. coli* were digested in-solution with trypsin, separated using nano-reverse phase liquid chromatography (nano-RP-LC), and identified by peptide sequencing using data dependent nano-spray tandem mass spectrometry followed by database searching. No sample depletion or removal of high abundance proteins was performed prior to analysis and only a one-dimensional LC separation was employed. In accord with previous proteomic analyses that did not involve sample clean-up, the abundant milk proteins BSA, β-lactoglobulin, α-lactalbumin, α-S1-casein, β-casein, and κ-casein were identified in the whey samples

of all 8 cows collected at each time point (Smolenski et al., 2007). Additionally, the proteins lactoferrin and Glycam-1 were identified in milk from all cows included in the study, and at all the time points analyzed (Table 1).

A total of 275 proteins were identified, but stringent criteria were applied to the selection of proteins to be considered for evaluation of temporal expression including: identification in milk samples from more than one cow, identity based on more than one peptide, and peptide assignments identified with Ion Scores greater than Identity (McFarland et al., 2008). Proteins identified by a single peptide did not meet inclusion criteria and were culled. Though a large number of proteins were discarded, a total of 68 proteins were successfully identified in more than one cow at two or more time points following infection. The results of the current study represent the first longitudinal analyses of milk samples over the course of a clinical infection to yield robust identification of biologically relevant proteins related to host response, including nearly 20 proteins not previously identified in whey from mastitic milk using proteomic strategies (Hogarthe et al., 2004: Smolenski et al., 2007).

Proteins related to host response identified in bovine milk included: the plasma-derived proteins BSA, transferrin, fibrinogen α, β, and γ chains, apolipoprotein A-I and A-II, A-IV, and C-3, and complement factor C3 and B, as well as several cationic AMP, osteopontin, bradykinin precursors, cell adhesion proteins, and the APP SAA, Hp, ITIH4, and A1AG (Table 3). While some of the protein identifications were in accord with previous proteomic analyses of mastitic milk fractions, (Hogarth et al., 2004; Smolenski et al., 2007), several host response-related proteins that were identified in the current analyses were not identified in earlier analyses of the bovine

milk proteome including: SAA, Hp, apolipoprotein A-I, A-II, C3, and E, complement factor B, osteopontin, cathelicidin-2, -3, -5, -6, -7, and prothrombin (Table 2). Additionally, novel markers of bovine mastitis were detected including ITIH4, a novel APP (Pineiro et al., 2004) and kininogen-2, a precursor to the eicosanoid bradykinin (Sharfstein et al., 2007).

Label- free quantification of two high abundance proteins, BSA and β-lactoglobulin, and two low abundance proteins, complement factor B and osteopontin, was performed using NSAF as previously described (Florens et al., 2006; Zybailov et al., 2006; Mosley et al., 2008). Additionally, normalized number of unique peptides identified was used to evaluate the relative abundance of the same proteins. For low abundance proteins, the difference in temporal expression patterns determined using NSAF versus number of unique peptides was negligible (Fig. 1C, 1D). The most plausible explanation is that due to the overabundance of a select number of proteins in milk, only a limited number of peptides were identified for proteins related to host response, and the MS/MS sampling rates of low abundance peptides were equivalent to the number of peptide assignments. Greater differences existed between the number of peptides identified and the number of times each precursor peptide triggered an MS/MS event for the more abundant proteins, however, particularly at the early time points following infection (Fig. 1A, 1B). Proteins related to host response were absent or in very low abundance at 0h and 12 h, thus the composition of the milk was primarily characterized by high abundance whey and casein proteins. The large differences between number of peptides identified and spectral counts at 0 h and 12 h were largely due to the fact that

abundant proteins, including BSA and β-lactoglobulin, yielded tryptic peptides with MS/MS sampling rates that exceeded the peptide assignments for each protein (Mueller et al., 2008). Normalization of number of unique peptides assigned to abundant proteins at earlier time points in the analyses resulted in estimates that were skewed towards the abundant proteins, and elevated relative to other proteins present in the milk. Thus, using number of unique peptides as a measure of relative abundance was not an accurate means of modeling temporal expression of abundant milk proteins over the entire time course of clinical infection in the current protein data set.

Spectral counts proved to be more sensitive measures of protein abundance, however, and thus NSAF were more accurate for semiquantitiative analysis of milk protein modulation during clinical mastitis for the protein data set generated using nano-LC coupled to a nano-spray ionization ion-trap mass spectrometer. Explanations include the fact that NSAF account for the higher MS/MS sampling rates of peptides assigned to abundant proteins. NSAF also reflect the size of the protein, and, indirectly the number of potential tryptic peptides the protein can theoretically yield (Florens et al., 2006; Zybailov et al., 2006; Mosley et al., 2008). The disparity between measures of relative abundance based on spectral counts and number of peptides supported the hypothesis that the dynamic range of proteins present in a given biological matrix will effect the accuracy of label-free quantification, and that spectral counts are better suited for the analysis of moderate mass resolution LC-MS/MS data generated on an ion-trap instrument (Mueller et al., 2008). Likewise, the apparent differences between evaluations based on spectral

counts and number of unique peptides supported previous reports that spectral count data is more accurate when applied to the semiquantitiative analysis of medium to high abundance proteins (Mueller et al., 2008).

Label- free quantification remains a topic of debate in terms of suitability for measuring changes in the relative changes of low abundance proteins (Mosley et al., 2008; Mueller et al., 2008). To date, there is no universal agreement on the most appropriate means to evaluate changes in relative abundance of proteins that are transient in expression, or that are not present in all samples included in a longitudinal study. Likewise, when dealing with the comparison of complex biological matrices in healthy versus diseased states, the challenge remains to accurately quantify changes in proteins that are only present in the diseased state, and to differentiate between changes in relative abundance that are biologically relevant versus statistically significant. Used as a screening tool to identify biomarkers that exhibit differential expression, however, label-free quantification based on NSAF could prove useful as a filter for distinguishing biomarkers that merit more rigorous follow-up validation studies and absolute quantification.

Extracted ion chromatograms (XIC) have been used to quantify proteins in complex mixtures by utilizing the mean peak area, or the sum of total ion intensity, of all peptides identified for each protein in a sample as a measure of abundance (Old et al., 2005; Bondarenko et al., 2002) To compare trends in temporal expression determined using the two different label-free methods, NSAF and XIC for the proteins BSA (Fig. 2A) and Hp (Fig. 2B) were evaluated. Changes in relative abundance detected using NSAF and XIC were in accord for both the high abundance

protein BSA and the low abundance protein haptoglobin. Using both methods of label-free semiquantitative analysis, peak abundances of both BSA and Hp were detected at 24 h following infection with *E. coli*, which was in agreement with previously published results for both proteins (Bannerman et al., 2008; Suojala et al., 2008), and with abundance determined using NSAF. Normalized spectral abundance factors appeared to accurately model changes in relative abundance of the proteins detected in milk both before and after infection with *E. coli* and thus NSAF were used throughout the remainder of the analyses to evaluate changes in the temporal expression of differentially expressed bovine milk proteins during coliform mastitis.

In the previous analyses, normalized number of unique peptides was compared to ELISA data generated on the proteins BSA, lactoferrin, and transferrin to evaluate the similarity of trends detected in the modulation of relative protein abundance across various times following infection using label free quantification and an antibody-based methodology. In the protein data set generated using HPLC coupled to the Q-TOF mass spectrometer, however, lactoferrin and transferrin represented low abundance proteins as fewer peptides were detected for each protein than in the current protein data set. To evaluate the accuracy of NSAF in detecting changes in relative abundance of high and low abundance proteins using nano-LC in-line with nano-spray-MS/MS, comparisons were made to ELISA data for BSA, Hp, and SAA.

As in the previous analyses, temporal expression patterns determined using ELISA and NSAF were in accord for both high and low abundance proteins. ELISA measurements of the concentration of BSA (Fig. 3A) across the time points before and after infection were likewise supported by previously published ELISA results

(Bannerman et al., 2004; 2008). Increased concentrations of milk BSA is a well established hallmark of coliform mastitis known to result from increased vascular permeability following the induction and action of pro-inflammatory cytokines following pathogen invasion (reviewed in Bannerman 2009). Additionally, there is evidence for the local production of serum albumin in the bovine mammary gland following exposure to LPS (Shamay et al., 2005). Similar to BSA, results of ELISA measurements of Hp (Fig. 3B) and SAA (Fig. 3C) concentrations following experimental induction of coliform mastitis were also in accord with previous analyses of milk APP levels in milk during clinical mastitis (Eckersall et al., 2001; Hiss et al., 2004; Grönlund et al., 2005). The present study does, however, mark the first instance of the identification of APP in the whey fraction of milk from cows with experimentally-induced clinical mastitis using proteomics. The comparison of semiquantitative measures to concentrations of proteins in milk during coliform mastitis determined using ELISAs is an additional novel aspect of the current analyses. The identification of APP using proteomics and the outcome of the comparison of quantitative measures indicate the applicability of LC-MS/MS in the analysis of complex biological matrices and the identification of low abundance proteins, and the feasibility of using NSAF to model protein modulation in bovine milk during disease.

Temporal expression of abundant whey and casein proteins using NSAF yielded results that were in accord with the previous analyses carried out using HPLC in line with a Q-TOF mass spectrometer. Peak increases in milk BSA were detected at 24 h following infection, which corresponded to maximal decreases in the whey proteins

β-lactoglobulin and α-lactalbumin (Fig. 4A). No significant fold-changes were detected in either β-lactoglobulin or α-lactalbumin, but 2-fold changes in abundance, as determined by the comparison of pre-challenge NSAF to abundance factors at later time points, were apparent in BSA at 18 h and 24 h following infection, which was in accord with significant differences ($P<0.05$) determined using an ELISA. Apparent decreases in the whey proteins β-lactoglobulin and α-lactalbumin agreed with previous reports of reduced expression during clinical mastitis (Hogarth et al., 2004), but in the prior proteomic analysis, temporal expression was not evaluated. In accord with previous proteomic analyses of the temporal expression of abundant casein proteins before and during clinical mastitis, no significant fold-changes were detected in casein expression across the time course analyzed (Fig. 4B). A reduction in αS_1-casein mRNA levels has been reported following experimental induction of coliform mastitis using *E. coli,* but no significant decreases in protein synthesis were detected, which supports the results of the current study (Vanselow et al., 2006).

Previous *E. coli* challenge studies have characterized cytokine expression during coliform mastitis (reviewed in Bannerman, 2009). Classic hallmarks of coliform mastitis following cytokine induction include fever, complement activation, hepatic production of APP (Dinarello, 1996; Grönlund et al., 2005; Suojala et al., 2008), and the leakage of plasma proteins and complement factors into the milk (Shuster et al., 1997; Riollet et al., 2000; Bannerman et al., 2004; 2008). The cytokines IL-1β and TNFα are known to induce pyrexia and vascular leak during coliform mastitis, and peak expression of both cytokines has been reported between 16 and 24 h following infection (Bannerman et al., 2004; 2008: Bannerman, 2009). In

accord with previously published reports on cytokine expression and the secondary effects of cytokine induction, vascular derived proteins were detected in bovine milk following experimental induction of coliform mastitis (Table 5). Peak expression of plasma proteins including complement factors C3 and B (Fig. 5A), fibrinogen –α, -β, and –γ chains (Fig. 5B), and the apolipoproteins A-I and A-II (Fig. 5C) was apparent between 18 h and 24 h after infusion, which corresponded to established cytokine expression patterns.

Another hallmark of intramammary infection is the rapid influx of polymorphonuclear neutrophils (PMN) into the gland following pathogen invasion. PMN are known to be the primary component of milk somatic cell counts (MSCC) during intramammary infection (Paape et al., 1981), and are also a well established source of AMP including PGRP, and members of the cathelicidin family of cationic AMP (Scocchi et al., 1997; Tomasinsig and Zanetti, 2005; Tydell et al., 2006). Increases in MSCC (log transformed) were evident 12 h following experimental induction of coliform mastitis, with peak increases in milk somatic cells at 24 h after infusion that remained elevated for the duration of the study (Fig. 6C). Elevation of MSCC in the current analysis modeled patterns detected in previous challenge studies (Bannerman et al., 2008). Corresponding to peak influx of PMN were increases in several AMP including cathelicidin-1, -2, and-4, as well as PGRP (Fig. 6B). Both the cathelicidins and PGRP are known to be involved in innate immunity, and to exhibit antibacterial activity in cattle (Tydell et al., 2006; Dziarski, 2003). The cathelicidins in particular inactivate and destroy invading pathogens by perturbing microbial membranes (Scocchi et al., 1997). Additionally, previous research has indicated that

cathelicidins might function in endotoxin neutralization, as well as down-regulation of cytokine expression (Scott et al., 2002; Wiese et al., 2003; Zanetti et al., 2004). Both PGRP and cathelicidin-1 were identified in the 2D-GE MALDI-TOF-MS analyses of whey from mastitic bovine milk, but the current proteomic analysis marks the first robust evaluation of the temporal expression of AMP during coliform mastitis. While AMP represented a low abundance component of the milk samples analyzed, the temporal expression patterns determined using NSAF were based on a greater number of peptide assignments than in earlier evaluations, and exhibited a better fit to clinical data.

The temporal expression of a select number of proteins related to host response identified in the mastitic milk samples were examined in further depth because the markers were novel, or there were previous reports of related expression. A protein of particular interest was the novel APP ITIH4. Prior reports of ITIH4 in cattle are limited to isolation of the positive APP from the serum of heifers with experimentally induced summer mastitis (Pineiro et al., 2004). ITIH4 has never been identified in bovine milk, and has never been associated with coliform mastitis in lactating dairy cattle. ITIH4 has, however, been studied in models of acute inflammation in the pig (Gonzalez-Ramon et al., 2000), and was recently reported to be a novel marker of acute ischemic stroke in humans (Kashyap et al., 2009). Previous research and similarity to a human homolog led to the classification of ITIH4 as a plasma kallikrein-sensitive glycoprotein, but the exact role and function of ITIH4 in the bovine mammary gland during inflammation associated with coliform mastitis is not yet clear (Nishimura et al., 1995). Plasma kallikreins are serine proteases that are

known to play keys roles in blood coagulation (Furie and Furie, 1988), fibrinolysis (Ichinose et al., 1984), activation of complement (Discipio, 1982), and the release of bradykinin from its precursor, kininogen (Sharfstein et al., 2007). Recently, both macrophages and neutrophils were demonstrated to possess binding sites for the kininogens (Barbasz et al., 2008; Kahn et al., 2009), and thus MSCC, which were elevated in milk at nearly the same time kininogen increases were detected, could represent a potential source of the bradykinin precursors.

The kinin peptides, including bradykinin, are potent mediators of vasodilation, pain, and udder edema during clinical mastitis (Eshraghi et al., 1999). The relationship between elevated levels of milk bradykinin and increased severity of clinical symptoms of mastitis has been demonstrated, but bradykinin levels in milk from cows with experimentally induced coliform mastitis have not yet been investigated (Eshraghi et al., 1999). Kininogen-1 has been identified in prior proteomic analyses of normal bovine milk (Fong et al., 2008), and the presence of bradykinin in normal milk has likewise been previously reported (Leach et al., 1967), but there are no previous reports of kininogen-2 discovery in bovine milk. Furthermore, while the activation of complement and the cleavage of complement proteins into active peptides that further enhance the inflammatory response have been documented during coliform mastitis (Shuster et al., 1997; Riollet et al., 2000; Bannerman et al., 2004), less is known about the contributions of the kallikrein-kinin system to inflammation in the bovine mammary gland. Previous studies of inflammation in the murine model have shown, however, that exogenous bradykinin

serves as a potent inducer of dendritic cell (DC) maturation, and could be a potential link between innate and adaptive immune responses (Aliberti et al., 2003).

Both kininogen-1 and -2 were identified in the current analyses, but kininogen-2 was identified in a greater number of cows, and had more peptide assignments on average across the time points sampled than kininogen-1 (Table 1). Though kininogen-1 and -2 are highly conserved and share a great deal of sequence homology, alignment of the protein sequences confirmed single amino acid differences that mapped to 2 distinct peptides assigned to kininogen-2, which confirmed the identification of both isoforms in the milk samples (Fig. 8). However, whether the kininogen isoforms possess distinct physiological roles during inflammation associated with coliform mastitis cannot be inferred from the results of the current study.

Temporal expression of ITIH4 and kiningen-2 revealed nearly identical patterns across the course of mastitis with peak increases in both proteins evident at 18 h following infection with *E. coli* (Fig. 7A). Changes in relative abundance were greater than 2-fold for both ITIH4 and kiningen-2, but the relevance of the fold increases should be regarded as biologically relevant as opposed to statistically significant, because a limited number of peptides were identified for each of the proteins (Mueller et al., 2008). Both ITIH4 and kininogen-2 were, however, detected in nearly all of the time points following infection, and relative to other low abundance proteins identified, had a greater number of peptide assignments at each sampling. Likewise, maximal increases in both ITIH4 and kininogen-2 were in accord with previously established peaks in cytokine expression during experimentally

induced coliform mastitis (reviewed in Bannerman 2009). Though the calculated abundance factors are tentative due to the limited number of identified peptides, kininogen-2 and ITIH4 are clearly linked to host response following infection in the bovine mammary gland, and merit further investigation as potential biologically relevant biomarkers of coliform mastitis.

Proteins that were consistently identified in higher abundance across all analyses conducted following infection with *E. coli* were the apolipoproteins. In all, 5 separate isoforms of apolipoprotein were identified in the current analysis, though apolipoprotein A-I and apolipoprotein A-II were the most abundant. Apolipoproteins are typically associated with high density lipoproteins (HDL), but other roles for the apolipoproteins during disease, and inflammation in particular, have been investigated (Bausserman et al., 1988; Cockerill et al., 2000; Burger and Dayer, 2002). The relationship between SAA and apolipoprotein A-I was investigated following induction of the acute phase response in human subjects (Bausserman et al., 1988). SAA, like the apolipoproteins, is transported in association with HDL. Increases in apolipoprotein A-I in particular have been associated with chronic inflammatory diseases such as rheumatoid arthritis, but at an undefined threshold SAA displaces apolipoprotein A-I from HDL and as a result, peak increases in SAA lead to a decline in the apoliproteins (Bausserman eta l., 1988; Burger and Dayer, 2002). Though the temporal expression patterns of the apolipoproteins and SAA were not specifically investigated in these analyses, examination of the changes in relative abundance of SAA (Fig. 3C) and apoliopoprotein A-I and A-II (Fig. 6C) indicate that as SAA increased in abundance, apolipoprotein A-I and apolipoprotein A-II begin to

decline, which is in accord with previous reports (Bausserman et al., 1988; Burger and Dayer, 2002).

A relationship that was specifically investigated was the potential reduction in adhesion molecules associated with leukocyte recruitment due to increases in abundance of apolipoproteins. Previous research has indicated that HDL, of which apolipoproteins are a major constituent, inhibit the cytokine-induced upregulation of E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in vitro as well as in vivo (Cockerill et al., 2000). Glycam-1 is a lymphocyte trafficking protein produced in the bovine mammary gland as well as in high endothelial venules (HEV) in lymph nodes (Nishimura, 2003). Glycam-1 is expressed by endothelial cells in the lactating mammary gland and secreted into milk, but the glycosylation state of the mammary form of Glycam-1 is different from the form of Glycam-1 detected in lymph nodes, and the mammary form of the protein is not thought to be involved in lymphocyte trafficking (Nishimura, 2003). The actual function of glycam-1 in the mammary gland thus remains undefined.

Glycam-1 was one of the only proteins detected in milk samples collected from all 8 cows included in the study both before and at all the time points following infection with *E. coli*. To investigate potential a relationship between the Glycam-1 protein detected in milk and the apolipoproteins A-I and A-II, temporal expression of all 3 proteins was examined graphically (Fig. 7B). The relationship was not profound, but increases in apolipoproteins A-I and A-II did appear to coincide with decreases in milk glycam-1. However, the glycosylation state of the glycam-1 protein present in milk during mastitis was not determined. Thus, no inferences could be made from the

current analyses whether or not the apolipoproteins had any down-regulatory effects on the expression of glycam-1 in bovine milk during coliform mastitis, or whether the form of glycam-1 detected in the milk was involved in lymphocyte trafficking.

Clusterin is another poorly characterized protein identified only in milk samples following experimental induction of coliform mastitis. In previous research, clusterin has also been referred to as apolipoprotein J, and has been shown to bind apolipoprotein A-I (McLaughlin et al., 2000; Jones and Jomary, 2002; Rosenburg et al., 2002). While the absolute function of clusterin remains unclear, the involvement of clusterin in several inflammatory diseases including myocarditis, and glomerulopathy has been implicated from previous research (McLaughlin et al., 2000; Rosenburg et al., 2002). Results of prior research likewise indicate that clusterin could possess anti-inflammatory properties (McLaughlin et al., 2000; Rosenburg et al., 2002). In terms of the role of clusterin in the bovine mammary gland, all that has been inferred is that clusterin could be associated with mammary gland involution, the clearance of cellular debris, and apoptotic cell death (Jones and Jomary, 2002). Because of the prior association with apolipoproteins and apolipoprotein A-I in particular, the temporal expression pattern of clusterin was compared to that of apolipoprotein A-I (Fig. 7C). Though no actual direct associations could be inferred from the graphic representation, comparison of changes in relative abundance of the two proteins revealed nearly identical patterns, though clusterin was in lower abundance than apolipoprotein A-I. A possible role for clusterin during inflammation associated with coliform mastitis cannot be deduced from the outcome of the current study, but similar to ITIH4 and kininogen, results support the notion that the protein

clusterin could merit further investigation as a potential biomarker of coliform mastitis.

Clearly, several challenges still remain regarding the identification and accurate quantification of biomarkers of host response in bovine milk following experimental induction of coliform mastitis. Nonetheless, the results of the current proteomic analysis provide information that could prove useful in the design and execution of future studies of inflammatory biomarkers in bovine milk. Contributions of the present study include the robust identification of several biologically relevant proteins related to host response in the bovine mammary gland including APP, AMP, vascular-derived proteins, bradykinin precursors, and cell adhesion factors. Additionally, an evaluation of semiquantitative methods for assessing changes in relative abundance of milk proteins during coliform mastitis evidenced that despite inherent flaws and the current lack of supporting statistical analysis, NSAF appear to effectively model protein modulation during clinical infections. Likewise, the number of proteins identified using nano-LC in line with nano-spray MS/MS indicated the suitability of the instrument system for identifying low abundance proteins in bovine milk, despite the biological complexity of the samples, and the profound variation in protein dynamic range across time points following challenge. Finally, the results of the current study represent the first longitudinal analysis of bovine milk samples over the course of a controlled clinical infection to include quantitative assessments of protein modulation, and to provide robust identification of several biomarkers of host response, including novel and potentially diagnostically relevant biomarkers of inflammation associated with coliform mastitis.

Chapter 7: Conclusions

Proteomic analysis of bovine milk and attempts to identify biomarkers of inflammation which were indicative of coliform mastitis posed several challenges. Specific hindrances included the lack of established methods for the robust identification of proteins in milk using proteomic methodologies, and the inherent difficulties associated with analyzing milk, which is a very complex biological matrix. Additionally, quantification of protein modulation over the course of clinical mastitis posed numerous additional obstacles due primarily to the extreme dynamic range of proteins present in bovine milk, but also due to profound fluctuations in protein content during infection. Nonetheless, the outcomes of the analyses conducted have afforded several insights into the dynamics of milk protein expression during disease, and have established useful parameters and methods for future analyses. Based on the propounded research, the following deductions can me made:

1. Proteomics is a viable and accurate means of identifying and assessing milk protein expression in bovine milk during an altered physiological state.

2. Detection of cytokines involved in the innate immune response during coliform mastitis is most accurately assessed using available antibody-based methodologies, including ELISAs, and could not be accomplished using 2D-GE followed by MALDI-TOF-MS, or LC-MS/MS using the methods employed in the current series of analyses, regardless of instrumentation. Nonetheless, proteins identified in bovine milk during

clinical mastitis using proteomics could enhance current knowledge of the effects and actions of cytokines during innate immunity, elucidate pathways or mechanisms of host response not previously known, and serve as valuable and diagnostically relevant biomarkers of coliform mastitis.

3. Proteins related to host response that are most amenable to identification using proteomics include vascular-derived proteins, acute phase proteins, antimicrobials proteins, and a select number of cell adhesion factors. Despite the presence of several highly abundant proteins, and the fact that no sample clean-up or fractionation strategies were applied, several low abundance proteins, including some proteins not previously identified in milk, were identified using 2D-GE followed by MALDI-TOF-MS, and by LC-MS/MS.

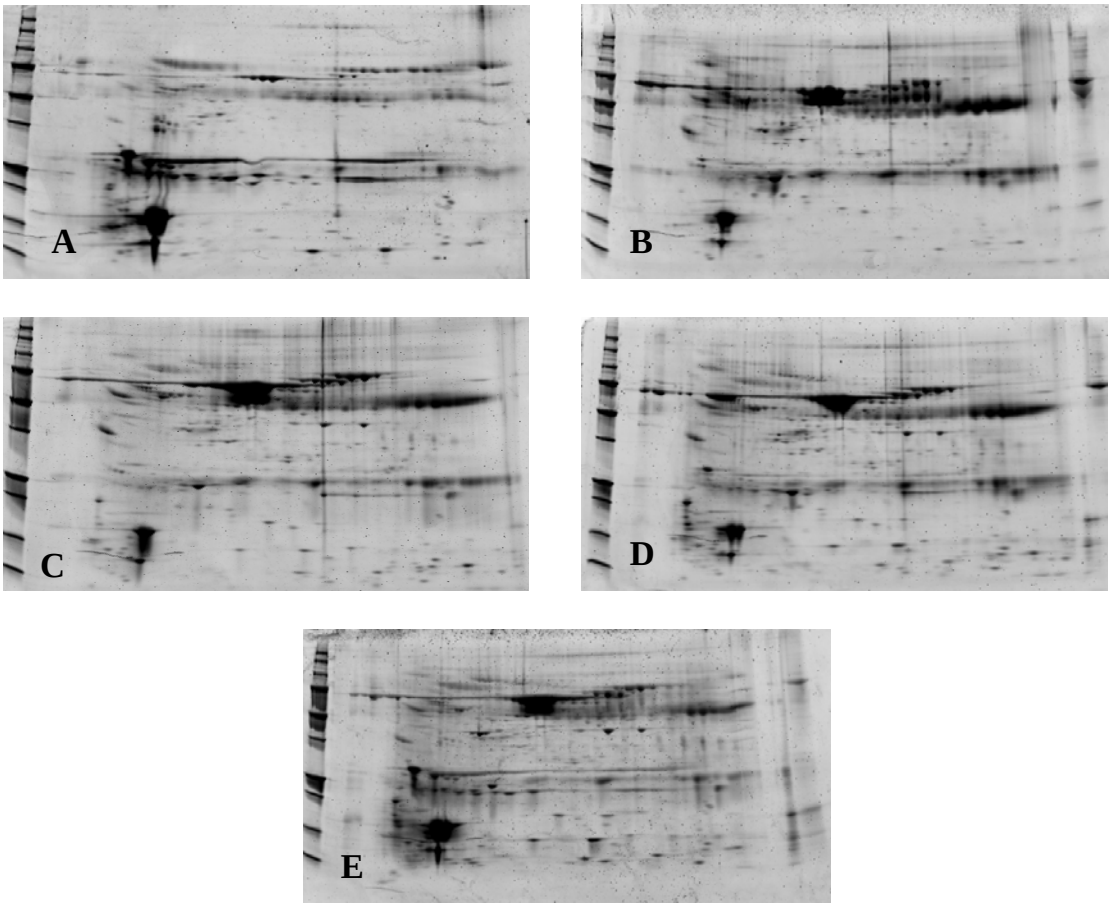
4. Despite inherent deficiencies in label-free semiquantitative analyses, evidence based on comparisons to ELISA data and an accepted and widely used means of label-free quantification demonstrated that NSAF is a potentially valuable screening tool for the assessment of changes in relative abundance of novel biomarkers.

5. Applying proteomic methodologies to the analysis of bovine milk following experimental induction of coliform mastitis resulted in the identification of potentially novel biomarkers of coliform mastitis not previously detected using antibody or PCR-based techniques, including: 1)

the acute phase protein ITIH4; 2) two isoforms of kininogen, the precursors to bradykinin; and 3) the relatively uncharacterized protein, clusterin.

In conclusion, the results of the current series of proteomic analyses of bovine milk have enhanced current knowledge of the bovine milk proteome, established more accurate methods for identifying milk proteins, and provided information on previously uncharacterized biomarkers in bovine milk during coliform mastitis which could serve as the foundation for future studies.

Appendices



Supplemental Figure 1. 2D-GE on a 0 h (A), 12 h (B), 18 h (C), 24 h (D), and 36 h (E) whey from mastitic milk samples.

Supplemental Table 1. Proteins present in more than one cow at only one time point

Swiss-Prot entry name	Primary accession number	Protein	Number Cows Present	Avg Spec Count[a]	Avg No. Peptides[a]	Time Point Present
KCMF1_BOVIN	Q1LZE1	E3 ubiquitin-protein ligase KCMF1	2	1	1	12 h
A2MG_BOVIN	Q7SIH1	Alpha-2-macroglobulin	3	1	1	18 h
LONM_BOVIN	Q59HJ6	Lon protease homolog, mitochondrial	3	1	1	18 h
PLCB4_BOVIN	Q07722	1-phosphatidyinositol-4,5-biphosphate phosphodiesterase beta-4	3	1	1	18 h
EF1G_BOVIN	Q3SZV3	Elongation factor 1-gamma	2	1	1	18 h
SNX17_BOVIN	Q5EA77	Sorting nexin-17	2	1	1	18 h
ANG1_BOVIN	P10152	Angiogenin-1	3	1	1	24 h
HBA_BOVIN	P01966	Hemoglobin subunit alpha	2	1	1	24 h
LIPL_BOVIN	P11151	Lipoprotein lipase	2	1	1	24 h
SBP1_BOVIN	Q2KJ32	Selenium binding protein 1	2	1	1	24 h
PLMN_BOVIN	P06868	Plasminogen	3	1	1	24 h
OMA1_BOVIN	Q3SZN3	Metalloendopeptidase OMA1	2	1	1	24 h
KGUA_BOVIN	P46195	Guanylate kinase	2	1	1	24 h
PROF1_BOVIN	P02584	Profilin-1	5	3	2	48 h
CD36_BOVIN	P26201	Platelet glycoprotein 4	2	1	1	48 h
TPST2_BOVIN	Q3SYY2	Protein-tyrosine sulfotransferase 2	2	1	1	48 h
LBP_BOVIN	Q2TBI0	Lipopolysaccharide-binding protein	2	1	1	48 h
DFB11_BOVIN	P46169	Beta-defensin 11	2	1	1	48 h
FOLR1_BOVIN	P02702	Folate receptor alpha	2	1	1	48 h

[a] Average across all 8 cows sampled.

Glossary

2D-GE	Two-dimensional gel electrophoresis
A1AG	Alpha-1 acid glycoprotein
AMP	Antimicrobial peptides
APP	Acute phase protein
BAMP-1	Bovine antimicrobial protein-1
BSA	Bovine serum albumin
CD-14	Cluster of differentiation -14
CFU	Colony-forming units
CHCA	α-cyano-4-hydroxycinnamic acid
CID	Collision-induced dissociation
COX	Cyclooxygenase
CVM	Center for Veterinary Medicine
DC	Dendritic cell
DHB	2,5-dihydroxybenzoic acid
DIM	Days in milk
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FABP	Fatty acid binding protein
FDA	Food and Drug Administration
FPLC	Fast protein liquid chromatography

GIST	Global internal standard
Glycam-1	Glycosylation-dependent cell adhesion molecule-1
HDL	High density lipoprotein
HEV	High endothelial venules
Hp	Haptoglobin
HPLC	High pressure liquid chromatography
ICAM-1	Intercellular adhesion molecule-1
ICAT	Isotope coded affinity tags
IEF	Isoelectric point
IFN-γ	Inteferon gamma
IgG	Immunoglobulin-G
IgM	Immunoglobulin-M
ITIH4	Inter-alpha trypsin inhibitor heavy chain-4
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IPG	Immobilized pH gradient
iTRAQ	Isobaric tags for relative and absolute quantitation
LBP	Lipopolysaccharide-binding protein
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LPS	Lipopolysaccharide

MALDI	Matrix-assisted laser desorption/ionization
MFGM	Milk fat globular membrane
MEC	Mammary epithelial cells
MS	Mass spectrometry
MSCC	Milk somatic cell counts
MS/MS	Tandem mass spectrometry
MSCS	Milk somatic cell score
m/z	Mass-to-charge ratio
NFκB	Nuclear factor kappa beta
NSAF	Normalized spectral abundance factor
NSAID	Non-steroidal anti-inflammatory drug
ONADE	Office of New Animal Drug Evaluation
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E2
PGRP	Peptidoglycan recognition receptor protein
PIGR	Polymeric immunoglobin receptor
PMF	Peptide mass fingerprinting
PMN	Polymorphonuclear neutrophils
PSD	Post source decay
PTM	Post translational modification
Q-TOF	Quadrupole time-of-flight

RP-LC	Reverse phase liquid chromatography
RT-PCR	Reverse transcriptase polymerase chain reaction
SAA	Serum amyloid A
SE	Standard error
SILAC	Stable isotope labeling by amino acids in cell culture
SCC	Somatic cell counts
SPA	Sinapinic acid
TEAB	Triethylammonium bicarbonate
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoroacetic acid
TGF-α	Transforming growth factor-alpha
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-alpha
TOF	Time-of-flight
TTR	Transthyretin
UPLC	Ultra-pressure liquid chromatography
VCAM-1	Vascular cell adhesion molecule-1
XIC	Extracted ion chromatogram

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