Proteomic analysis of differentially expressed proteins in caprine milk during experimentally induced endotoxin mastitis

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ABSTRACT

The goal of the current study was to identify proteins in goat milk before and at 18 h following intramammary challenge with lipopolysaccharide (LPS). Initial evaluation of protein profiles generated using 2-dimensional gel electrophoresis on skim milk samples from a group of 6 goats collected before challenge and at 18, 24, and 48 h after LPS challenge revealed little change in the abundance of casein proteins, and minimal changes in the presence or abundance of the plasma protein serum albumin, which is known to leak into milk during coliform mastitis in dairy cattle. Proteins in baseline milk samples and in milk from the same goats 18 h post-LPS challenge were excised from the gels, and peptides were sequenced using nano-flow liquid chromatography coupled with tandem mass spectrometry. Despite the overwhelming presence of casein proteins and β -lactoglobulin, the lower abundance proteins β -2-microglobulin, fatty acid-binding protein, serum albumin, and retinol-binding protein were detected in skim milk samples from healthy goats. Skim milk samples 18 h postchallenge were characterized by the sustained presence and abundance of the casein proteins, and by the presence of haptoglobin, serum amyloid A, lactoferrin, cathelicidin-1, and cathelicidin-3. No marked differences in the intensity of the spot corresponding to serum albumin were observed in gels of skim milk samples 18 h postchallenge, which could indicate that the breakdown of the blood–milk barrier during endotoxin mastitis may not be as profound in goats as has been observed in dairy cattle. Nonetheless, the occurrence of an inflammatory response was supported by elevated somatic cell counts in the goat milk following inoculation with endotoxin, as well as by the presence of both antimicrobial and acute phase proteins. The results provide information about the composition of

proteins in goat milk as well as added knowledge of the host response during endotoxin mastitis in goats.

Key words: proteomic analysis, endotoxin mastitis, milk protein, caprine

INTRODUCTION

Sustained economic losses, limited treatment options, and poor efficacy of available preventative therapies explain why a continued focus of ruminant mastitis research remains the elucidation of mechanisms and mediators involved in the host response to intramammary bacterial pathogen invasion. Additionally, the use of antimicrobial agents for the treatment and prevention of mastitis infections has introduced concerns regarding the safety and quality of dairy products. Specifically, the sustained use of antibiotics in food animals can result in the presence of drug residues in edible tissues and milk products, and can affect the continued emergence of resistant strains of bacteria.

Escherichia coli, a gram-negative environmental pathogen, is a prominent cause of clinical mastitis that is problematic to treat. Earlier studies have indicated that antibiotics offer little or no efficacy in resolving clinical or subclinical cases of mastitis caused by environmental pathogens (Hogan et al., 1989; Erskine, 2000). Further, the outer cell wall of *E. coli* contains LPS, a toxin that stimulates the upregulation of several proinflammatory cytokines, including IL-1 β , IL-6, IL-8, and tumor necrosis factor α , that cause profound inflammation and potential tissue damage in the bovine mammary gland (Bannerman, 2009).

The soluble mediators of inflammation in bovine milk and plasma during clinical mastitis have been researched extensively (Bannerman, 2009), but most studies have been accomplished through the use of antibody-based strategies such as Western blots and ELISA. Although these methodologies are both quantitative and accurate, the use of such strategies can limit the detection of novel inflammatory mediators, as the techniques are highly dependent on the availability or development of specific antibodies. Conversely, mass spectrometry-based proteomics technologies allow for the simultaneous analysis of a larger number of proteins

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without reliance on antibodies. Accordingly, the use of such strategies to characterize the bovine inflammatory response to gram-negative pathogens and to profile changes in the bovine milk proteome during mastitis has gained popularity in recent years (Smolenski et al., 2007; Boehmer et al., 2008, 2010a,b; Danielsen et al., 2010).

Milk protein modulation during bovine mastitis has been evaluated using several common proteomic approaches, including 2-dimensional gel electrophoresis (**2D-GE**), matrix-assisted laser desorption-ionization mass spectrometry (**MALDI-MS**), and liquid chromatography coupled with tandem mass spectrometry (**LC-MS/MS**; Hogarth et al., 2004; Smolenski et al., 2007; Boehmer et al., 2008, 2010a,b; Danielsen et al., 2010). Likewise, recent studies have sought to quantify differential milk protein expression using milk from clinically healthy cows and cows with experimentally induced coliform mastitis (Boehmer et al., 2010a,b; Danielsen et al., 2010). However, although our knowledge of modulation in the bovine milk proteome during coliform mastitis continues to expand, very little comparative data exist on lactating dairy goats.

At present, goat milk accounts for a small percentage of global milk consumption, although the popularity of goat milk-derived products has increased in recent years because of the inherent nutritional factor of goat milk and its suitability as a substitute for bovine milk when intolerance, allergies, and gastrointestinal disorders are a factor (Bellioni-Businco et al., 1999; McCullough, 2003; Sanz Ceballos et al., 2009). Like other ruminant species that are managed for milk production, goats are also affected by mastitis; however, much less is known about the goat innate immune response to mastitis pathogens or the subsequent changes in goat milk protein expression over the course of a clinical infection. Although limited, previous research aimed at characterizing the host response during mastitis in goats has revealed that goats exhibit clinical symptoms similar to those evidenced in dairy cattle during experimental induction of endotoxin (LPS) and *E. coli* mastitis, including increased rectal temperature, elevated SCC, reduced appetite and milk production, increased heart rates, and changes in blood chemistry parameters (Anderson et al., 1991; Massart-Leen and Vandeputte-Van Messom, 1991). Similarly, prior reports of proteomic evaluations of goat milk have been limited to the analysis of casein protein expression in goat milk, and the determination of the molecular weights of major goat milk proteins (Roncada et al., 2002; Ham et al., 2012). To date, no studies have focused on the proteomic analysis of goat milk protein modulation during clinical mastitis.

The goals of the current study were to identify proteins in goat milk and to qualitatively evaluate potential

changes in protein expression in goat skim milk following experimental induction of endotoxin mastitis. Milk samples were obtained from a group of 6 lactating goats before and at 3 time points following intramammary challenge with LPS. Proteins present in baseline (0 h) and milk samples collected 18 h following LPS challenge were profiled using 2D-GE, and tryptic peptides resulting from in-gel digestion were identified by nano-flow (**n**)LC-MS/MS. The results of the study provide additional information regarding protein expression in goat milk, expand current knowledge of proteins present in both normal and mastitic goat milk, and may aid in the further characterization of soluble mediators of inflammation present in goat milk during disease.

MATERIALS AND METHODS

Goats

Six clinically healthy, mixed-breed dairy goats in their first lactation, approximately 100 to 150 DIM and ranging in weight from 49 to 63 kg, were used in these experiments. None of the goats had prior incidence or treatments for mastitis, and all had milk SCC <200,000 cells/mL at the onset of the study. Composite milk samples from all goats were cultured before the start of the study, and all cultures were negative for the presence of pathogens (Quality Milk Production Services, Cornell University, Ithaca, NY). The care and use of all animals in this study was approved by the Center for Veterinary Medicine Office of Research Institutional Animal Care and Use Committee.

Determination of Milk SCC

Goat milk can contain cytoplasmic masses, as well as a greater number of epithelial cells than are typically detected in bovine milk. Because electronic cell counters cannot accurately differentiate between cytoplasmic masses, epithelial cells, and white blood cells, goat milk SCC are routinely determined using the California Mastitis Test (**CMT**), which reacts with the genetic material of somatic cells, and provides a reasonably accurate SCC. A CMT score of 0 (negative) was used as an indicator to determine a SCC <200,000 cells/mL.

Intramammary Challenge with LPS

On the day of challenge, the right udder half of each of the 6 does was infused with 4 μ g of LPS/kg in 2 mL of pyrogen-free saline using a sterile disposable teat cannula. The left half of each udder was infused with 2 mL of sterile PBS to serve as a control to the challenged half and to validate that any inflammation or

modulation of protein expression observed was a result of the LPS infusion.

Sample Collection and Handling

Approximately 30 mL of milk was collected aseptically as outlined by the National Mastitis Council (Madison, WI). Briefly, a few streams of milk were discarded and the teat end scrubbed with 70% ethanol until clean. Milk samples were collected from both the saline-infused mammary half and the challenged half at milking for the 3 d before challenge for baseline milk samples, and at 18, 24, and 48 h following LPS challenge. After collection, milk samples were centrifuged at $4,000 \times g$ at 4°C for 15 min, and the resulting fat layer removed. The skim milk was transferred to sterile 1.5-mL microcentrifuge tubes and frozen at -20°C until further analysis.

2D-GE

The protein content of the skim milk fractions from each goat was determined using a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL) using BSA as a standard. Approximately 200 µg of total protein (25 to 35 µL in volume, depending on animal and time point) from each sample was then desalted by acetone precipitation. Acetone precipitation was performed by mixing each sample with 6 times the initial volume of ice-cold acetone, incubating on ice for 30 min, followed by centrifugation at $10,000 \times g$ at 4°C for 10 min. The resulting pellets were air-dried at room temperature (RT; approximately 23°C) for 15 min, and then resuspended in 200 µL of rehydration buffer {8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 50 mM dithiothreitol, 0.2% BioLyte 3/10 ampholyte, 0.001% bromophenol blue; BioRad Laboratories, Hercules, CA}. Accounting for losses due to acetone precipitation, approximately 150 µg of total protein was applied to 11-cm, pH 3–10 nonlinear immobilized pH gradient strips (Bio-Rad Laboratories), focused in a Bio-Rad Protean iso-electric focusing cell, and reduced and alkylated as previously described (Boehmer et al., 2008). The second-dimension SDS-PAGE was performed using Criterion Tris-HCl (8–16%) precast gels in 1× Tris-glycine-SDS buffer (Bio-Rad Laboratories) at 200 V for 1 h. Gels were fixed in 40% methanol:10% acetic acid for 1 h and then stained overnight in Sypro Ruby stain (Lonza, Walkersville, MD). Gels were destained twice in 10% methanol:7% acetic acid for 1 h at RT and then imaged on a Typhoon 8600 multimode image scanner (GE Healthcare Bio-Sciences, Piscataway, NJ) and saved as a TIFF file. All samples were prepared in duplicate for a total of 2 gels per goat

per time point for both baseline (0 h) and mastitic (18 h) skim milk samples.

In-Gel Digestion

Spots of interest were excised from gels generated from 0- and 18-h skim milk samples with a sterile stainless steel scalpel blade, and transferred to a sterile 1.5-mL microcentrifuge tube containing 100 µL of deionized water. Only spots present in both replicate gels from each animal as well as in all gels for each time point (0 and 18 h) were excised and pooled for protein identification. The excised spots were destained in 100 µL of 50% acetonitrile:100 mM ammonium bicarbonate 2 to 3 times over 1 h at RT. For tryptic digestion, gel spots were dehydrated in acetonitrile for 15 min and dried to completeness in vacuum centrifuge for 30 min at RT (MiVac Module Series Centrifuge Vacuum Concentrator; Gardiner, NY). Spots were rehydrated in 10 ng/µL of sequencing-grade modified trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate to achieve an approximate enzyme:protein ratio of 1:100, and allowed to digest on ice for 15 min. Excess solution was replaced with 25 µL of 50 mM ammonium bicarbonate and digested overnight at 37°C. Peptides were extracted with 50 µL of 50% acetonitrile and water containing 5% trifluoroacetic acid (TFA) and then with 50 µL of acetonitrile containing 5% TFA. Extracted peptides were dried to completeness in a vacuum centrifuge and redissolved in 25 µL of 0.1% TFA in deionized water. Extracted peptides were stored at -20°C until further analysis.

Analysis by nLC-MS/MS

One-dimensional nLC-MS/MS analyses were carried out by injecting 5 µL (approximately 5 µg) of each tryptic digest into a nanoflow HPLC instrument (Eksigent, Dublin, CA), coupled to a linear ion trap LTQ Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Peptides were loaded onto a 100 µm × 100 mm nanoAcquity UPLC C18 reverse phase column (Waters, Milford, MA). The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Peptides were eluted with the following gradient: 5–20% B for 15 min, 20–60% B for 10 min, 60–80% B for 5 min, and 80–95% B for 5 min at a flow rate of 500 nL/min.

The eluate was electro-sprayed by applying 1.6 kV to the terminal PicoTip emitter (25 µm i.d., 30 µm i.d. tip; New Objective, Woburn, MA). The LTQ Velos mass spectrometer was operated in positive ion mode, and spectra were acquired for 35 min in data-dependent tandem MS mode. The 6 most intense ions in each MS

survey scan (over the range 400 to 2000 m/z) were subjected to MS/MS by collision-induced dissociation at a normalized collision energy of 35%. Dynamic exclusion was used to allow one data-dependent MS/MS scan for a given parent mass:charge ratio (m/z) in a 45-s time window. Mass spectral peak lists were generated using Proteome Discoverer software (Thermo Fisher Scientific) without processing. To minimize carryover, a 55-min blank injection was run between samples.

Database Searching

Mass spectral peak lists were submitted to the Mascot search engine (v. 2.3.0; Matrix Sciences, London, UK) using Proteome Discoverer (v. 1.2; Thermo Fisher Scientific), and searched against the UniprotKB Swiss-Prot protein sequence library (<http://www.uniprot.org/>). The following search parameters were applied: *other mammalia* species, trypsin enzyme, 3 allowed missed cleavages, carbamidomethylation modification to the cysteine residue as a static modification, methionine oxidation dynamic 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 64,838. The data were filtered by peptide ion score equal to or greater than the Mascot identity threshold (at $P < 0.05$) and number of peptides per protein ($n \geq 2$).

RESULTS

Clinical Signs of Endotoxin Mastitis in Dairy Goats

Rectal temperatures of each goat and milk SCC were monitored over the course of the study (48 h) at approximately 3-h intervals. Clinical signs of the host response to intramammary challenge with LPS were apparent as early as 3 h after experimental induction of endotoxin mastitis, as evidenced by increases in rectal temperatures, with maximal increases at 6 h (Figure 1A). Rectal temperatures declined by 9 h following induction of endotoxin mastitis and, despite slight increases at 12, 15, and 18 h following challenge, returned to baseline levels by 24 h and remained as such for the duration of the study. Similarly, SCC (as measured by CMT) were profoundly elevated by 9 h following challenge, with maximal increases apparent at 15 h following induction of endotoxin mastitis (Figure 1B). In contrast to rectal temperature, however, the SCC remained elevated for the duration of the study and did not return to baseline. No increases in SCC were apparent in milk collected from the saline-infused udder half (data not shown), indicating that the inflammation was localized to the challenged mammary half.

Figure 1. (A) Rectal temperature ($\pm$ SE) before and at time points following intramammary infusion with LPS, and (B) California Mastitis Test (CMT) scores before and at time points following intramammary infusion with LPS.

Protein Expression Profiles in Goat Milk Following Challenge with LPS

Skim milk from clinically healthy goats (collected before challenge with LPS) and mastitic skim milk samples collected at 18, 24, and 48 h following challenge with LPS were separated by 2D-GE and the resulting protein profiles evaluated. Variation in the protein profiles between goats was apparent but minimal; thus, representative gels from each time point evaluated were chosen to illustrate changes in milk protein expression across the time points (Figure 2A to D). The protein profiles of skim milk collected from healthy goats were characterized by high and qualitatively constant abundances of casein (Figure 2A, box 2) and whey proteins (Figure 2A, box 3) across the 4 time points sampled. Interestingly, very few changes were evident in the protein profiles generated from milk samples collected over

the course of the experimental mastitis. Although serum albumin was detected in normal skim milk (Figure 2A, box 1), no visible increases were apparent in goat milk at 18 (Figure 2B, box 1), 24 (Figure 2C, box 1), or 48 h (Figure 2D, box 1) after challenge. Similarly, the casein and whey proteins remained relatively constant in abundance after the onset of endotoxin mastitis. However, proteins in the lower molecular weight regions of the gels (regions 3 and 4) did appear to be modulated and follow-up analyses were thus performed to identify proteins present in baseline (0 h) and mastitic (18 h) skim milk samples.

Casein Proteolysis and Host Response Proteins in Goat Milk During Endotoxin Mastitis

To identify proteins present in goat milk and to evaluate possible differences in protein composition between goat skim milk samples collected before and following challenge with LPS, spots were excised from 0- and 18-h gels and in-gel digested, and the resulting tryptic peptides were analyzed by nLC-MS/MS. Attempts were made to excise all protein spots that were apparent on the gels, and to excise the same spots from both the 0-h and 18-h skim milk gels (same apparent mass and isoelectric point) to serve as a means of comparison. Additional protein spots were, however, present in the 2D gels of goat skim milk collected 18 h following LPS challenge that were not apparent in the baseline (0 h) skim milk samples. Protein identifications (inferences or inferred identifications) were considered robust if at least 2 unique peptide assignments had ion scores greater than Mascot identity scores at $P < 0.05$ (Supplemental File 1; <http://www.journalofdaairyscience.org/>).

Of the 72 spots excised from 2D gels of goat skim milk samples collected at before challenge (0 h), 11 unique proteins were identified from 32 different spots (Table 1). In many cases, nLC-MS/MS of tryptic peptides from the remaining spots also resulted in protein identifications, but the proteins were eliminated from the data set following the application of identification criteria ($n \geq 2$ peptides with ion score greater than identity score at $P < 0.05$). As noted earlier, the skim milk samples collected from clinically healthy goats were dominated by the presence of the casein variants, specifically β - and α_{s2} -caseins, which were detected in 13 and 6 separate spots at varying isoelectric points on the gel, respectively (Table 1, Figure 3A). Conversely, α_{s1} - and κ -casein were each detected in only 2 separate spots on the gel. Despite the overwhelming abundance of the caseins and the whey proteins β -LG and α -LA, the lower abundance proteins serum albumin, gelsolin, retinol binding protein-1, fatty acid binding protein,

Figure 2. Representative 2-dimensional (2D) gel electrophoresis goat skim milk protein profiles (A) before intramammary infusion with LPS and at (B) 18 h, (C) 24 h, and (D) 48 h following challenge with LPS. Boxes within the gel represent high-molecular-weight proteins, including serum albumin (1), region in the 2D gel where the caseins are typically detected (2), and regions where low-molecular-weight proteins, including whey proteins, antimicrobial proteins, and casein proteolysis products, are typically detected (3 and 4).

Figure 3. Two-dimensional gel profiles of goat skim milk (A) before intramammary infusion with LPS, and (B) at 18 h following challenge.

and β -2-microglobulin were likewise detected in goat skim milk samples collected just before challenge with LPS.

Of the 82 spots excised from 2D gels of goat skim milk samples collected at 18 h following challenge, 17 unique proteins were identified from 49 different spots (Table 2). Similar to baseline (0 h) skim milk samples,

nLC-MS/MS analysis of tryptic peptides from several remaining spots also resulted in protein identifications, but the proteins were eliminated from the data set following the application of identification criteria ($n \geq 2$ peptides with ion score greater than identity score at $P < 0.05$). Also, similar to the protein expression profiles generated from milk samples collected before infection, β - and α_{S2} -caseins dominated the profiles of milk samples collected after LPS challenge (Table 2, Figure 3B). The number of β -casein fragments detected was reduced from 13 spots in the 0-h samples to 9 spots in the 18-h samples, but the number of α_{S2} -casein spots detected on the gel increased from 6 spots in the 0-h samples to 16 in the 18-h samples. Similar to analyses of the caseins present in the 0-h skim milk samples, both α_{S1} - and κ -casein were each detected in only a limited number of spots.

Plasma proteins such as complement and serotransferrin were not robustly identified following the analysis of spots excised from the 2D gels of mastitic goat skim milk. Similarly, although serum albumin was detected, no profound change in the abundance of the plasma protein was apparent based on visual observation of spot intensities on the 2D gels. However, several antimicrobial proteins (**AMP**), including cathelicidin-1, cathelicidin-3, and lactoferrin, were detected in goat skim milk samples 18 h after induction of endotoxin mastitis. The induction of the acute phase response during endotoxin mastitis in goats was also apparent, as the acute phase proteins (**APP**), haptoglobin (**HPT**) and serum amyloid A (**SAA**) were likewise detected in the mastitic goat skim milk samples collected 18 h after intramammary infusion with LPS. Although differences in spot intensities were apparent for both the AMP and APP detected on the 2-dimensional gels of skim

Table 1. Proteins detected in milk from healthy goats

Spot number ¹	Protein name	Accession number	Species ²	Peptides ³ (no.)	Coverage ⁴ (%)
1	Gelsolin	Q28372	Equine	2	2
2, 3	Serum albumin	P85295	Caprine	6	64
4, 27	α_{S1} -Casein	P18626	Caprine	12	62
5, 7, 9, 11, 12, 17, 18, 20, 24, 28, 30–32	β -Casein	P11839	Ovine	3	18
6, 13–16, 19, 21	α_{S2} -Casein	P04654	Ovine	4	16
8, 10	κ -Casein	P02670	Caprine	6	32
22	β -Lactoglobulin	P02756	Caprine	22	53
23	Retinol-binding protein-1	P02694	Bovine	4	31
25	α -Lactalbumin	P00712	Caprine	3	21
26	Fatty acid-binding protein, heart	Q4TZH2	Bovine	5	29
29	β -2-Microglobulin	Q6QAT4	Ovine	3	18

¹Number corresponds to spot number on gel in Figure 3A.

²Species of highest scoring protein assignment from Swiss Prot sequence library (<http://www.uniprot.org/>).

³Number of peptide assignments or average number of peptide assignments if protein detected in more than one spot.

⁴Coverage of protein based on peptide assignments or average percentage coverage if protein detected in more than one spot.

Table 2. Proteins detected in milk from goats with experimentally induced endotoxin mastitis

Spot number ¹	Protein name	Accession number	Species ²	Peptides ³ (no.)	Coverage ⁴ (%)
1	Lactoferrin	Q29477	Caprine	19	27
2	Gelsolin	Q3SX14	Bovine	6	8
3	Serum albumin	P14639	Ovine	9	15
4	Apolipoprotein A-IV	Q32PJ2	Bovine	9	26
5	α_{S1} -Casein	P18626	Caprine	15	52
6, 19, 36, 37, 39, 40, 44, 45, 49	β -Casein	P11839	Ovine	4	23
7, 10–17, 20, 29, 31, 32, 43, 46, 47	α_{S2} -Casein	P33049	Caprine	5	21
8, 9	κ -Casein	P02670	Caprine	6	26
18	Haptoglobin	B6E141	Ibex	3	11
21	Cathelicidin-3	P19661	Bovine	2	11
22	β -Lactoglobulin	P02756	Caprine	10	54
23, 25	Cathelicidin-1	P22226	Bovine	3	23
24	Retinol-binding protein-1	P02694	Bovine	2	13
26	α -Lactalbumin	P00711	Bovine	2	11
27, 28, 30, 33–35, 38	Serum amyloid A	P42819	Ovine	4	33
41, 42	β -2-Microglobulin	Q6QAT4	Bovine	4	27
48	Profilin-1	P02584	Bovine	2	21

¹Number corresponds to spot number on gel in Figure 3B.

²Species of highest scoring protein assignment from Swiss Prot sequence library (<http://www.uniprot.org/>).

³Number of peptide assignments or average number of peptide assignments if protein detected in more than one spot.

⁴Coverage of protein based on peptide assignments or average percentage coverage if protein detected in more than one spot.

milk collected 18 h following challenge for all 6 goats (Figure 4), the overall patterns of protein expression were similar, and the proteins were detected in milk samples from all goats. The apparent distinctions in spot intensities for the proteins only detected in skim milk collected from the 6 goats 18 h after challenge did imply some measure of animal-to-animal variation in the host response to infection. However, the goal of the study was the identification of proteins present in the milk of goats before and following LPS challenge, not quantification of protein abundance.

DISCUSSION

Endotoxin mastitis was induced in a group of 6 lactating goats via intramammary infusion with LPS, and clinical parameters and changes in goat milk protein expression were evaluated at time points following challenge. Although comparative data are limited, the increases in rectal temperature and milk SCC are in accord with prior reports of changes in clinical parameters following experimental induction of clinical mastitis in goats via intramammary infusion with either LPS or *E. coli* (Anderson et al., 1991; Massart-Leen and Vandeputte-Van Messom, 1991) and confirmed a host response to LPS.

Milk protein profiles in samples collected from the goats before intramammary infusion with LPS, as well as at 18, 24, and 48 h following challenge, were likewise evaluated for visible changes in overall expression patterns using 2D-GE. Unlike published profiles of changes in bovine milk proteins during coliform

mastitis (Hogarth et al., 2004; Boehmer et al., 2008), no profound changes in protein expression patterns of goat milk were apparent following LPS challenge. Prior proteomic analyses of milk collected from cows with experimentally induced coliform mastitis indicated drastic reductions in casein and whey protein expression levels, as well as the presence and increased abundance of several large vascular-derived proteins

Figure 4. Region on 2-dimensional gels of goat skim milk collected 18 h following challenge that represents proteins within the 10 to 15 kDa mass range and with isoelectric points between 3 and 10 (lower region of gel). Segments of gels generated from all 6 goats (1–6) are represented, and the areas highlighted correspond to spots that were identified as acute phase proteins or antimicrobial proteins (Figure 3B).

in milk samples collected after infection including serum albumin, APP, complement, and serotransferrin (Boehmer et al., 2008). In contrast, the apparent relative abundance levels of the casein proteins remained rather unchanged in goat skim milk following challenge with endotoxin. Despite the increased presence of some low molecular weight proteins as time progressed, no high-molecular-weight APP such as α -1-acid glycoprotein (~37 kDa) were detected on the gels as reported in prior proteomic analysis of bovine milk (Boehmer et al., 2008). Likewise, there were no marked visible increases, based on changes in spot intensity apparent on the gels, in the abundance of large plasma proteins such as serum albumin (~75 kDa).

To further characterize and verify the identity of proteins expressed in normal versus mastitic goat milk, spots from 0- and 18-h gels were excised, in-gel digested with trypsin. The resulting tryptic peptides were separated using 1-dimensional reverse phase nano-flow liquid chromatography (RP-nLC), and identified by peptide sequencing using tandem mass spectrometry followed by database searching. Results further confirmed that intramammary infusion with LPS does not alter goat skim milk protein profiles as profoundly as has been reported in prior proteomic analyses of both naturally occurring and experimentally induced coliform mastitis in dairy cattle (Hogarth et al., 2004; Smolenski et al., 2007; Boehmer et al., 2008). As stated earlier, the serum albumin detected in 2D gels of both normal and mastitic goat milk did not appear to increase in abundance, based on relatively little change in the visible intensity of corresponding spots on the 2D gels, over the course of clinical mastitis. The observation of little change in the intensity of the spots corresponding to serum albumin on the 2D gels of goat milk collected 18 h following challenge was in contrast to similar analyses conducted using bovine milk (Hogarth et al., 2004; Boehmer et al., 2008). Increases in milk serum albumin levels, as well as other vascular-derived proteins such as complement, APP, and serotransferrin, are established hallmarks of coliform mastitis in dairy cattle, as their presence in bovine milk is a known secondary effect of the breakdown of the blood-milk barrier following cytokine induction. In contrast, the only plasma protein detected in the mastitic goat milk samples that was not present in milk collected before challenge was apolipoprotein A-IV (APOA4), which could indicate that the sensitivity to LPS and the cytokine-mediated responses are different in cattle and goats. However, prior proteomic analyses of mastitic bovine milk that used the same analytical strategies as those in the current study were on milk samples collected from cows with naturally occurring infections or following experimental induction of mastitis using *E. coli*, and not puri-

fied LPS. Therefore, differences in protein modulation apparent in the goat milk during endotoxin mastitis versus bovine milk during coliform mastitis could also be ascribed to differences in the host response to invading bacteria compared with purified toxin.

Drawbacks to the current analyses were the lack of available translational data on goat milk proteins in the Swiss-Prot protein sequence library and the detection of only one tryptic peptide (with ion score greater than identity) for several of the lower intensity spots excised from the gels. More than 100 spots were detected in each of the 2D gels after SYPRO Ruby staining (Figures 2 and 3). However, despite excision and analysis of more than 80 of the protein spots from gels from each of the 2 time points, only 18 different proteins were successfully identified; 11 proteins in the 0-h samples and 17 in the 18-h samples. For many of the low intensity spots on the gels, the nLC-MS/MS analysis of tryptic peptides resulted in the detection of only a limited number of high-quality MS/MS spectra, many corresponding to the same peptide. Consequently, many of the protein identifications were eliminated from the data set following the application of inclusion criteria. As well, the Swiss-Prot *other mammalia* taxonomy, which was used for database searching, included only a limited number of goat sequences. Although some goat-specific proteins were identified, the majority of the protein identifications were assigned to other species, the most predominant being cow and sheep. Despite the limited number of proteins identified, however, the results demonstrated that some measure of homology exists between goat, cow, and sheep proteins.

The only previous reports of goat milk proteomic analyses have pertained solely to the analysis of caseins and major whey proteins (Roncada et al., 2002; Ham et al., 2012). Although only minor comparisons can be drawn, the expression patterns of the casein and major whey proteins on the 2D-GE, and the approximate molecular weights of the major milk proteins detected in the current analyses were in accord with prior proteomic analyses of goat milk (Roncada et al., 2002; Ham et al., 2012). Further, the apparent preferential proteolysis of β - and α_{S2} -casein compared with α_{S1} -casein is in agreement with earlier reports of the rather rapid hydrolysis of β - and α_{S2} -casein (Le Bars and Gripon, 1993) in the presence of proteases. Likewise, the proteolysis of β - and α_{S2} -casein correlated with the apparent increases in milk SCC and presumably the number of neutrophils present in the gland, which are the predominant source of proteases known to act on the caseins, such as elastase, cathepsin B, and plasmin (Faurischou and Borregaard, 2003). The data presented in the current analyses are likewise only partial, but do expand our knowledge of the goat milk proteome,

which to date has been very limited. The proteins gel-solin, fatty acid-binding protein, APOA4, HPT, SAA, cathelicidin-1, cathelicidin-3, retinol-binding protein 1, β -2-microglobulin, and profilin-1 have never before been detected in goat milk using proteomic strategies. Although the presence of these proteins in goat milk is consistent with prior reports of the proteomic analysis of normal and mastitic bovine milk (Smolenski et al., 2007; Boehmer et al., 2008, 2010a,b; Danielsen et al., 2010), no entries exist in the Swiss-Prot sequence library for these proteins in the caprine taxonomy. Nonetheless, several of these proteins are associated with the host response to infection, including APOA4, HPT, SAA, lactoferrin, cathelicidin-1, and cathelicidin-3 (Bannerman, 2009; Boehmer et al., 2010a; Danielsen et al., 2010).

Apolipoproteins are typically associated with high density lipoproteins, but other roles for the apolipoproteins during inflammation have been investigated (Cockerill et al., 2001; Burger and Dayer, 2002). Although several apolipoproteins were previously identified in bovine milk (Smolenski et al., 2007; Boehmer et al., 2008, 2010a; Danielsen et al., 2010), the role of the apolipoproteins during inflammation, and ruminant mastitis specifically, remains unclear.

The acute phase protein SAA also belongs to the apolipoprotein family and is known to be elevated during mastitis (Eckersall et al., 2001; Grönlund et al., 2005). Proteomic analyses of bovine milk following either LPS or *E. coli* challenge that involved LC-MS/MS analyses of in-solution milk digests have reported the detection of SAA (Boehmer et al., 2010a,b; Danielsen et al., 2010), but SAA has not been detected in bovine milk using 2D-GE (Hogarth et al., 2004; Boehmer et al., 2008). In the 18-h mastitic goat milk samples, SAA was detected in 6 spots, with different molecular weights and isoelectric points, which indicated the possible posttranslational modification of SAA in goat milk during endotoxin mastitis. Further, despite apparent differences in the intensity of the spots corresponding to SAA on the 2D gels of milk collected 18 h following challenge from each of the 6 goats, SAA was detected in milk from all 6 goats. The primary site of SAA production is the liver, but mammary-specific isoforms of SAA have been reported (McDonald et al., 2001; Rodriguez et al., 2009). Because both serum albumin and SAA are produced in the liver and thought to leak into the mammary gland from systemic circulation following the cytokine-induced breakdown of the blood–milk barrier, it is intriguing that SAA would be so abundant in mastitic goat milk while serum albumin levels in the milk remained relatively unchanged. However, the molecular weights of serum albumin and SAA differ substantially, which could indicate some leakage across

the blood–milk barrier during endotoxin mastitis in goats, but the breakdown of the barrier does not allow for the passage of the larger plasma proteins. Whether the apolipoproteins identified in the current study were present in mastitic milk as the result of an acute phase response and subsequent leakage into the gland from systemic circulation, or were the result of another mechanism entirely merits follow-up analyses. Nonetheless, the results indicate a potentially important role of SAA in the caprine mammary gland during the host response to infection.

The cytoplasmic granules of circulating neutrophils are a well-characterized source of AMP that are released upon neutrophil degranulation in the mammary gland (Tomasinsig and Zanetti, 2005). The identification of the AMP cathelicidin-1, cathelicidin-3, and lactoferrin in goat milk following challenge with endotoxin thus supports the hallmark influx of neutrophils into the mammary gland following LPS infusion, which is typically evidenced by increases in SCC in bovines (Saad and Ostensson, 1990; Paape et al., 2003). The detection of several members of the cationic family of cathelicidins in mastitic bovine milk has been reported (Smolenski et al., 2007; Boehmer et al., 2008, 2010a,b; Danielsen et al., 2010), but never in goat milk. Reports indicate that members of the cathelicidin family of host defense peptides could function in the neutralization of endotoxin, as well as in the downregulation of pro-inflammatory cytokine expression (Scott et al., 2002; Wiese et al., 2003; Zanetti, 2004).

Haptoglobin is also known to be synthesized and released from the granules of neutrophils (Tomasinsig and Zanetti, 2005; Lai et al., 2009). Recently, the production and release of HPT into milk has also been ascribed to increases in SCC during mastitis in cattle (Lai et al., 2009). The precise function of HPT during inflammation in the mammary gland remains unclear, but it is thought to scavenge hemoglobin-induced free radicals during oxidative stress and serve as a powerful antioxidant (Lai et al., 2009). The identification of cathelicidin-1, cathelicidin-3, as and HPT in mastitic but not normal goat skim milk samples in these analyses supports the notion that both AMP and APP serve a role in the caprine host defense.

In the present study, 2D-GE performed on skim milk samples before intramammary inoculation with LPS and 18 h following challenge revealed only moderate changes in protein profiles between normal and mastitic goat milk. However, the analyses represent the first report of proteomic analysis of goat milk following induction of endotoxin mastitis in goats and contribute valuable information not only to current knowledge of the goat milk proteome, but to the mechanisms of host defense in the caprine mammary gland. Despite

the lower abundance of plasma proteins in the goat milk following challenge compared with prior reports in bovine milk, important proteins related to host defense, including several AMP and APP, were detected. Whether the differences in proteomic profiling of goat milk over the course of clinical endotoxin mastitis differ from similar analyses conducted using bovine milk due to differences in the sensitivity of ruminant species to LPS or to differences in species-specific innate immune responses will require further research. Nonetheless, additional data were obtained on goat milk proteins, which will aid in future proteomic endeavors involving the analysis of both normal and mastitic goat milk fractions.

REFERENCES

- Anderson, K. L., E. Hunt, and B. J. Davis. 1991. The influence of anti-inflammatory therapy on bacterial clearance following intramammary *Escherichia coli* challenge in goats. *Vet Res. Commun.* 15:147–161.
- Bannerman, D. D. 2009. Pathogen-dependent induction of cytokines and other soluble inflammatory mediators during intramammary infection of dairy cows. *J. Anim. Sci.* 87(13 Suppl.):10–25.
- Bellioni-Busincio, B., R. Paganelli, P. Lucenti, P. G. Giampietro, H. Perborn, and L. Businco. 1999. Allergenicity of goat's milk in children with cow's milk allergy. *J. Allergy Clin. Immunol.* 103:1191–1194.
- Boehmer, J. L., D. D. Bannerman, K. J. Shefcheck, and J. L. Ward. 2008. Proteomic analysis of differentially expressed proteins in bovine milk during experimentally induced *Escherichia coli* mastitis. *J. Dairy Sci.* 91:4206–4218.
- Boehmer, J. L., J. A. DeGrasse, M. A. McFarland, E. A. Tall, K. J. Shefcheck, J. L. Ward, and D. D. Bannerman. 2010a. The proteomic advantage: Label-free quantification of proteins expressed in bovine milk during experimentally induced coliform mastitis. *Vet Immunol. Immunopathol.* 138:252–266. <http://dx.doi.org/10.1016/j.vetimm.2010.10.004>.
- Boehmer, J. L., J. L. Ward, R. R. Peters, K. J. Shefcheck, M. A. McFarland, and D. D. Bannerman. 2010b. Proteomic analysis of the temporal expression of bovine milk proteins during coliform mastitis and label-free relative quantification. *J. Dairy Sci.* 93:593–603.
- Burger, D., and J.-M. Dayer. 2002. High-density lipoprotein-associated apolipoprotein A-I: The missing link between infection and chronic inflammation? *Autoimmun. Rev.* 1:111–117.
- Cockerill, G. W., T. Y. Huehns, A. Weerasinghe, C. Stocker, P. G. Lerch, N. E. Miller, and D. O. Haskard. 2001. Elevation of plasma high-density lipoprotein concentration reduces interleukin-1-induced expression of E-selectin in an in vivo model of acute inflammation. *Circulation* 103:108–112.
- Danielsen, M., M. C. Codrea, K. L. Ingvarsen, N. C. Friggens, E. Bendixen, and C. M. Røntved. 2010. Quantitative milk proteomics—Host responses to lipopolysaccharide-mediated inflammation of bovine mammary gland. *Proteomics* 10:2240–2249.
- Eckersall, P. D., F. J. Young, C. McComb, C. J. Hogarth, S. Safi, A. Weber, T. McDonald, A. M. Nolan, and J. L. Fitzpatrick. 2001. Acute phase proteins in serum and milk from dairy cows with clinical mastitis. *Vet Rec.* 148:35–41.
- Erskine, R. J. 2000. Antimicrobial use in bovine mastitis. Pages 712–734 in *Antimicrobial Therapy in Veterinary Medicine*. 3rd ed. J. F. Prescott, J. D. Baggot, and R. D. Walker, ed. Iowa State Univ. Press, Ames.
- Faurschou, M., and N. Borregaard. 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect.* 5:1317–1327.
- Grönlund, U., C. Hallén Sandgren, and K. Persson Waller. 2005. Haptoglobin and serum amyloid A in milk from dairy cows with chronic sub-clinical mastitis. *Vet Res.* 36:191–198.
- Ham, J.-S., G.-S. Han, S.-G. Jeong, K.-H. Seol, A.-R. Jang, M.-H. Oh, D.-H. Kim, and Y. W. Park. 2012. Determination of molecular weights of caprine milk proteins by matrix-assisted laser desorption/ionization mass spectrometry. *J. Dairy Sci.* 95:15–19.
- Hogan, J. S., K. L. Smith, K. H. Hoblet, P. S. Schoenberger, D. A. Todhunter, W. D. Hueston, D. E. Pritchard, G. L. Bowman, L. E. Heider, B. L. Brockett, and H. R. Conrad. 1989. Field survey of mastitis in low somatic cell count herds. *J. Dairy Sci.* 72:1547–1556.
- Hogarth, C. J., J. L. Fitzpatrick, A. M. Nolan, F. J. Young, A. Pitt, and P. D. Eckersall. 2004. Differential protein composition of bovine whey: A comparison of whey from healthy animals and from those with clinical mastitis. *Proteomics* 4:2094–2100.
- Lai, I.-H., J. H. Tsao, Y. P. Lu, J. W. Lee, X. Zhao, F. L. Chien, and S. J. T. Mao. 2009. Neutrophils as one of the major haptoglobin sources in mastitis affected milk. *Vet Res.* 40:17 <http://dx.doi.org/10.1051/vetres:2008055>.
- Le Bars, D., and J. C. Gripon. 1993. Hydrolysis of α_{S1} -casein by bovine plasmin. *Lait* 73:337–344.
- Massart-Leen, A. M., and G. Vandeputte-Van Messom. 1991. Role of prostaglandin-mediated mechanisms during experimentally induced endotoxin fever in the lactating goat. *Verh. K. Acad. Geneeskd. Belg.* 53:241–279.
- McCullough, F. S. W. 2003. Nutritional evaluation of goat's milk. *Br. Food J.* 105:239–251.
- McDonald, T. L., M. A. Larson, D. R. Mack, and A. Weber. 2001. Elevated extrahepatic expression and secretion of mammary-associated serum amyloid A 3 (M-SAA3) into colostrum. *Vet Immunol. Immunopathol.* 83:203–211.
- Paape, M. J., D. D. Bannerman, X. Zhao, and J.-W. Lee. 2003. The bovine neutrophil: Structure and function in blood and milk. *Vet Res.* 34:597–627.
- Rodriguez, B. de J., C. Chevalere, G. Henry, D. Mollé, I. Virlogeux-Payant, M. Berri, F. Boulay, J. Léonil, F. Meurens, and H. Salmon. 2009. Identification in milk of a serum amyloid A peptide chemoattractant for B lymphoblasts. *BMC Immunol.* 10:4. <http://dx.doi.org/10.1186/1471-2172-10-4>.
- Roncada, P., A. Gaviraghi, S. Liberatori, B. Canas, L. Bini, and G. F. Greppi. 2002. Identification of caseins in goat milk. *Proteomics* 2:723–726.
- Saad, A. M., and K. Ostensson. 1990. Flow cytometric studies on the alteration of leukocyte populations in blood and milk during endotoxin-induced mastitis in cows. *Am. J. Vet Res.* 51:1603–1607.
- Sanz Ceballos, L., M. R. Sanz Sampelayo, F. Gil Extremera, and M. Rodriguez Osorio. 2009. Evaluation of the allergenicity of goat milk, cow milk, and their lactosera in a guinea pig model. *J. Dairy Sci.* 92:837–846.
- Scott, M. G., D. J. Davidson, M. R. Gold, D. Bowdish, and R. E. Hancock. 2002. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J. Immunol.* 169:3883–3891.
- Smolenski, G., S. Haines, Y.-S. Kwan, F. Bond, J. Farr, V. Davis Sr., K. Stelwagen, and T. T. Wheeler. 2007. Characterization of host defense proteins in milk using a proteomic approach. *J. Proteome Res.* 6:207–215.
- Tomasinsig, L., and M. Zanetti. 2005. The cathelicidins—Structure, function and evolution. *Curr. Protein Pept. Sci.* 6:23–34.
- Wiese, A., T. Gutschmann, and U. Seydel. 2003. Towards antibacterial strategies: Studies on the mechanisms of interaction between antibacterial peptides and model membranes. *J. Endotoxin Res.* 9:67–84.
- Zanetti, M. 2004. Cathelicidins, multifunctional peptides of the innate immunity. *J. Leukoc. Biol.* 75:39–48.