Original article

Innate immune response to intramammary infection with *Serratia marcescens* and *Streptococcus uberis*

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Abstract – *Streptococcus uberis* and *Serratia marcescens* are Gram-positive and Gram-negative bacteria, respectively, that induce clinical mastitis. Once initial host barrier systems have been breached by these pathogens, the innate immune system provides the next level of defense against these infectious agents. The innate immune response is characterized by the induction of pro-inflammatory cytokines, as well as increases in other accessory proteins that facilitate host recognition and elimination of the pathogens. The objective of the current study was to characterize the innate immune response during clinical mastitis elicited by these two important, yet less well-studied, Gram-positive and Gram-negative organisms. The pro-inflammatory cytokine response and changes in the levels of the innate immune accessory recognition proteins, soluble CD14 (sCD14) and lipopolysaccharide (LPS)-binding protein (LBP), were studied. Decreased milk output, induction of a febrile response, and increased acute phase synthesis of LBP were all characteristic of the systemic response to intramammary infection with either organism. Infection with either bacteria similarly resulted in increased milk levels of IL-1β, IL-8, IL-10, IL-12, IFN-γ, TNF-α, sCD14, LBP, and the complement component, C5a. However, the duration of and/or maximal changes in the increased levels of these inflammatory markers were significantly different for several of the inflammatory parameters assayed. In particular, *S. uberis* infection was characterized by the sustained elevation of higher milk levels of IL-1β, IL-10, IL-12, IFN-γ, and C5a, relative to *S. marcescens* infection. Together, these data demonstrate the variability of the innate immune response to two distinct mastitis pathogens.

cytokines / innate immunity / mastitis / *Serratia* / *Streptococcus*

1. INTRODUCTION

Mastitis is an inflammation of the mammary gland and remains one of the most costly diseases to animal agriculture [50].

Economic losses attributed to this disease are estimated to approach 2 billion dollars annually in the USA alone [35]. Mastitis most commonly results following the passage of pathogenic bacteria through the streak

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canal barrier of the teat where these organisms gain access to the gland and establish infection [47]. The ensuing inflammatory response that accompanies bacterial entry into the gland is highly variable and can dictate whether a pathogen is quickly eliminated or successfully establishes infection.

Serratia spp. have been implicated in bovine mastitis and in two reports have been estimated to account for approximately 9–12% of all naturally acquired Gram-negative bacterial infections [19, 56]. Of those species of *Serratia* that cause mastitis, *S. marcescens* is the most prevalent [55]. *Serratia* spp. have been isolated from water, soil, feed, and bedding materials [67], and in at least one report, herd outbreaks of *Serratia* have been attributed to contaminated teat dips [60]. Cows with *Serratia* intramammary infections (IMI) typically display mild clinical symptoms with the subclinical form of infection being more characteristic than for IMI's caused by other Gram-negative bacteria [55]. *Serratia* IMI also tend to become chronic with a mean duration of infection lasting > 160 days. The mild clinical symptoms displayed during *Serratia* IMI, as well as, the finding that this pathogen is shed in low numbers complicates the ability to identify *Serratia* as a causative agent of mastitis during outbreaks [6]. Of perhaps greater concern is that *Serratia* isolates from cases of mastitis are reportedly resistant to most approved antibiotics [6, 60].

Among the environmental *Streptococcus* spp. that cause mastitis, *S. uberis* is the most prevalent [62]. In one study, the percentage of all IMI's attributed to *S. uberis* was reported to range from 12–16% [21]. Other studies have implicated this pathogen as the causative agent of clinical mastitis in 13–20% of all cases [8, 64]. *S. uberis* has been recovered from soil, bedding materials, feces, as well as, various anatomical regions of the cow [7, 8]. Similar to *S. marcescens* infections, IMI's caused by *S. uberis* are predominantly subclinical and can persist for long periods of time in a chronic state [21, 22, 40]. Currently recommended antibiotic therapy in the USA for *S. uberis* IMI remains sub-optimal as two-day treatment with pirlimycin was recently reported to eliminate only 58% of infections [38].

Innate immune recognition of bacteria is mediated, in part, by Toll-like receptors (TLR's). TLR-2 recognition of peptidoglycan [66] and lipoteichoic acid [33, 48] and TLR-4 recognition of bacterial lipopolysaccharide (LPS) [16] contribute to the ability of the innate immune system to respond to Gram-positive and Gram-negative bacteria, respectively. Activation of these distinct TLR's by Gram-positive and Gram-negative cell wall products is characterized by differential gene expression and cellular responses [3, 36, 43].

Staphylococcus aureus and *Escherichia coli*, Gram-positive and Gram-negative pathogens, respectively, elicit differential mammary gland innate immune responses [5, 45]. Relative to *S. aureus*, *E. coli* IMI is accompanied by a heightened inflammatory response characterized by sustained production of IL-8 and TNF-α, complement activation, and enhanced mammary vascular permeability. Whether the lack of such a pronounced pro-inflammatory cytokine response during *S. aureus* IMI is specific to IMI's by *S. aureus* or is characteristic of all Gram-positive IMI's remains unknown. The objective of the current study was to characterize the innate immune response to IMI with either *S. marcescens* or *S. uberis* and to determine whether the inflammatory cytokine response elicited by either of these organisms is comparable to that previously reported for *E. coli* and *S. aureus* (i.e., whether there are conserved cytokine responses among Gram-positive and/or Gram-negative organisms). In addition, we studied changes in the intramammary concentrations of two accessory molecules, soluble CD14 (sCD14) and LPS-binding protein (LBP), which facilitate host innate recognition of bacterial cell wall products.

2. MATERIALS AND METHODS

2.1. Cows

A pool of 15 healthy, mid-lactating Holstein cows (185 ± 9 DIM) were selected from the USDA National Animal Disease Center's dairy herd (Ames, Iowa, USA) on the basis of milk somatic cell counts (SCC) of < 200 000 cells/mL and the absence of detectable bacteria growth from aseptically collected milk samples plated on blood agar. The use and care of all animals in this study was approved by the National Animal Disease Center's Animal Care and Use Committee.

2.2. Intramammary challenge with *S. uberis* or *S. marcescens*

Prior to intramammary challenge, 10 mL of brain heart infusion broth (Becton-Dickinson Diagnostic Systems, Inc., Sparks, MD, USA) were inoculated with either *S. marcescens* (gift of Dr K. Larry Smith; Ohio State University, Wooster, OH, USA) or *S. uberis* strain 0140 (gift of Dr A.J. Bramley, Institute for Animal Health, Compton Laboratory, Newbury, England) and incubated overnight at 37 °C. The bacteria were subsequently pelleted by centrifugation, resuspended in PBS, aliquotted, and frozen. Prior to challenge, representative aliquots were thawed, serially diluted, and spread on blood agar plates. Following an overnight incubation at 37 °C, the concentrations of the frozen stock aliquots were calculated. For preparation of the inoculum used for intramammary infusion, frozen aliquots were diluted in PBS to yield a final approximate concentration of 100 CFU/mL.

In an initial experiment, 10 cows were infused with 2 mL of the prepared inoculum of *S. marcescens* in one quarter immediately following the morning milking. The contralateral quarter of each infected quarter was infused with 2 mL of PBS. Seven days after challenge, two cows were euthanized for the harvesting of tissue while the remainder of the cows were sampled for an additional two weeks. In a subsequent exper-iment, 12 cows were infused as above with either PBS or *S. uberis*. All *S. uberis*-infected cows developed severe clinical mastitis and distress resulting in the termination of the study at day 7 (168 h) and the initiation of palliative care. Post-plating of the final prepared inoculums that were infused confirmed that cows received 260 or 220 CFU/quarter of *S. marcescens* or *S. uberis*, respectively. Following challenge, aseptic milk samples were collected from all infused quarters at various time points, serially diluted, and plated on blood agar plates. Following a 16 h incubation at 37 °C, CFU/mL were determined. Colonies were initially identified as either *S. marcescens* or *S. uberis* based on morphological characteristics. Further biochemical tests and gas chromatography were performed by the Maryland Department of Agriculture Animal Health Section (College Park, MD, USA) to confirm initial identification.

2.3. Determination of milk somatic cell and total white blood cell (WBC) counts

To quantitate somatic cells, milk samples were heated to 60 °C and subsequently maintained at 40 °C until counted on an automated cell counter (Fossomatic model 90, Foss Food Technology, Hillerod, Denmark) as previously described [32]. For the determination of circulating WBC counts, jugular vein blood samples were collected in Vacutainer® glass tubes containing sodium heparin (Becton-Dickinson Corp., Franklin, Lakes, NJ, USA) and diluted 1:250 in Isoton II diluent (Beckman Coulter Corp., Fullerton, CA, USA). Six drops of Zap-oglobin II lytic (Beckman Coulter Corp.,) reagent were added to the diluted samples and cells counted using a Nova Celltrak 2 cell counter (Angel Engineering Corp., Trumbull, CT, USA) using a threshold setting of 6.8.

2.4. Whey and plasma preparation

For the preparation of whey, milk samples were centrifuged at 44 000 × *g* at 4 °C

for 30 min and the fat layer removed with a spatula. The skimmed milk was decanted into a clean tube and centrifuged again for 30 min as above and the translucent supernatant collected and stored at –70 °C. For the preparation of plasma, heparinized jugular vein blood samples were collected as above, centrifuged at $1\,500 \times g$ for 15 min, and the clear plasma supernatant collected, aliquotted, and stored at –70 °C.

2.5. Enzyme-linked immunosorbent assays (ELISA's)

ELISA's for BSA, C5a, IFN-γ, IL-1β, IL-8, IL-10, IL-12, LBP, sCD14, and TNF-α, were all performed as previously described [4, 5]. Milk and plasma samples collected from *S. marcescens*- and *S. uberis*-infected cows were all assayed by any given ELISA in parallel on the same day using the same known standards.

2.6. Statistical methods

Repeated measures ANOVA was performed using the PROC MIXED model (SAS 8.2; SAS Institute, Cary, NC, USA) to compare the mean responses between experimental groups and the pre-infused (time 0) groups. For statistical analysis of milk SCC, data were transformed to $\log_{10}$ values. An unpaired t-test (GraphPad Prism version 4.0 for Windows; GraphPad Software Inc., San Diego, CA, USA) was used to compare the maximal responses elicited by *S. uberis* and *S. marcescens* in a given experimental assay. A *P*-value of < 0.05 was considered significant.

3. RESULTS

3.1. Bacterial recovery from infected quarters

To determine whether initial infusion of *S. marcescens* (260 CFU/quarter) or *S. uberis* (220 CFU/quarter) resulted in the establish-

ment of infection, aseptic milk samples were collected at varying time points following infusion. Milk samples were collected from all challenged quarters throughout the first 7 days (168 h) of the study. After day 7, two cows that had originally been challenged with *S. marcescens* were euthanized for the harvesting of tissue for future analysis. Due to the development of severe clinical mastitis and distress in all cows challenged with *S. uberis*, antibiotic treatment was initiated in these cows at day 7 resulting in termination of the study.

Within 6 h of and up to 96 h following challenge, viable bacteria were recovered from all ten quarters infused with *S. marcescens* (Fig. 1A). Transient decreases in the number of quarters from which viable *S. marcescens* were recovered occurred at 168 and 336 h post-infection, however, by day 21 (504 h) viable bacteria were recovered from all challenged quarters. In contrast, viable bacteria were not recovered from all quarters infused with *S. uberis* until 24 h post-infection. Once viable *S. uberis* was recovered from an infected quarter, the quarter remained infected until the end of the study (i.e., 168 h post-infection).

Comparable numbers of *S. marcescens* ($3.82 \pm 0.18 \log_{10}$ CFU/mL) and *S. uberis* ($3.97 \pm 0.36 \log_{10}$ CFU/mL) were recovered from infected glands within 12 and 18 h, respectively, of challenge (Fig. 1B). After reaching a peak at 12 h post-inoculation, the number of CFU's of *S. marcescens* recovered from infected quarters declined until 24 h, after which the levels remained constant at ~ $2.5 \log_{10}$ CFU/mL. In contrast, the number of CFU's recovered from *S. uberis* infected quarters continued to increase throughout the study reaching a peak ($7.84 \pm 0.26 \log_{10}$ CFU/mL) at 168 h.

3.2. Systemic response to intramammary infection with either *S. marcescens* or *S. uberis*

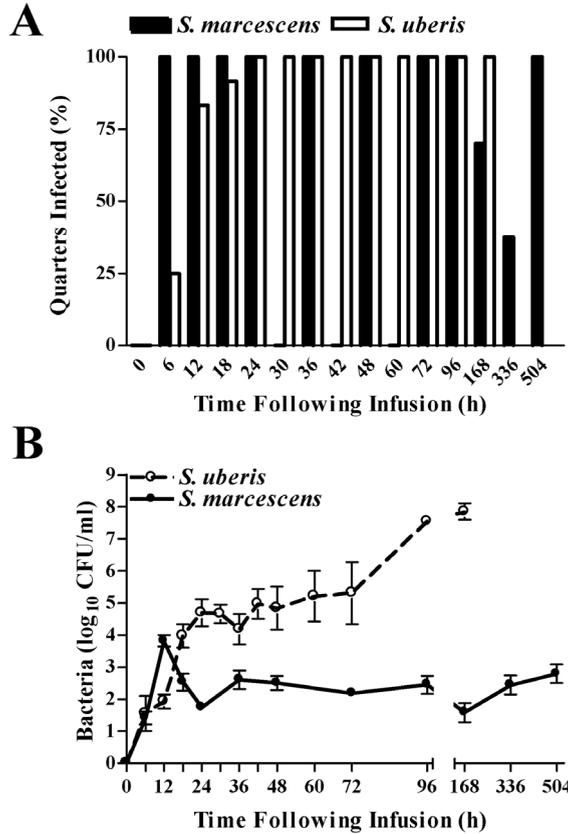Daily milk output consisting of the sum of both the morning and evening milk

Figure 1. Recovery of viable *Serratia marcescens* or *Streptococcus uberis* following experimental intramammary infection. Following intramammary infusion of one quarter on each of 10 cows with 260 CFU of *S. marcescens* or one quarter on each of 12 cows with 220 CFU of *S. uberis*, milk samples were aseptically collected from all infused quarters at various time points and spread on blood agar plates. *S. uberis* infected cows were followed out until 168 h, whereas, those infected with *S. marcescens* were followed out for a total of three weeks (504 h). The percent of quarters from which viable bacteria were recovered is indicated (A). In quarters from which bacteria were recovered, the mean (± S.E.) of log_{10} CFU/mL is shown (B).

weights of cows infected with *S. marcescens* dropped on day 0, reflecting a decrease in evening milk production 12 h after infusion (Fig. 2A). Within 1 day following *S. marcescens* or 2 days following *S. uberis* challenge, comparable decreases of ~ 35% in milk production relative to pre-challenge levels were observed. At > 2 days of challenge, milk production returned to normal in cows infected with *S. marcescens*, whereas, milk output remained depressed throughout

the study in those cows infected with *S. uberis*.

In cows challenged with *S. marcescens*, a febrile response was first observed at 12 h post-infection, peaked 6 h later, and returned to baseline levels by 36 h (Fig. 2B). In contrast, elevated temperatures were not detected until 30 h post-infection with *S. uberis*, after which the temperatures remained elevated throughout the study. The sustained elevation in
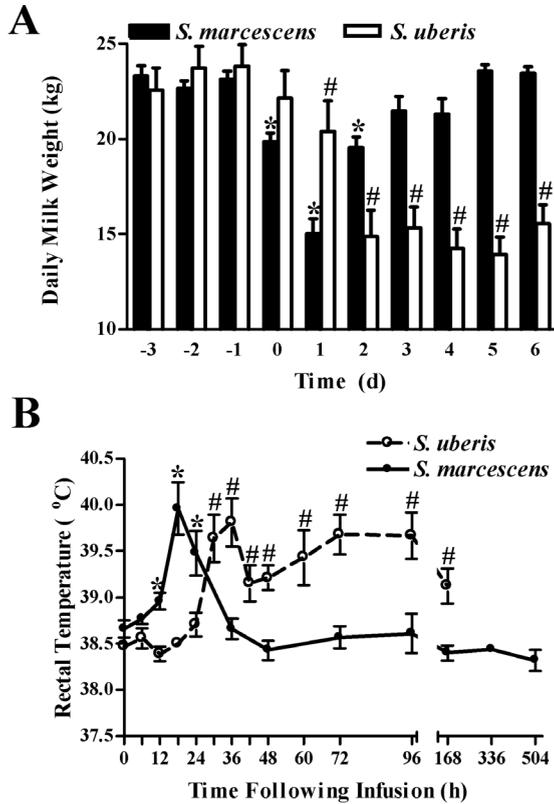
Figure 2. Effect of intramammary infection with *Serratia marcescens* or *Streptococcus uberis* on milk production and core body temperature. Total daily milk weights (sum of morning and evening outputs) were collected for 3 days prior to and until 6 days after intramammary infusion on day 0 (A). *, # Significantly decreased compared to pre-challenge levels (day –1) in cows challenged with *S. marcescens* or *S. uberis*, respectively ($P < 0.05$). As an indicator of a systemic response, rectal temperatures were measured immediately prior to and at various time points following intramammary infection (B). *, # Significantly increased compared to time 0 in cows challenged with *S. marcescens* or *S. uberis*, respectively ($P < 0.05$).

temperature over several days following IMI with *S. uberis* is consistent with that of a previous report [17]. Peak elevations in body temperatures were comparable among cows receiving either *S. marcescens* (39.96 ± 0.28 °C) or *S. uberis* (39.81 ± 0.26 °C). The ability of either pathogen to elicit a systemic response was further demonstrated by an increase in circulating levels of LBP, a hepatically-derived protein whose expression is upregulated during the acute phase

response to infection [49]. Circulating levels of LBP initially increased within 24 or 30 h of infection with either *S. marcescens* or *S. uberis*, respectively, and remained elevated for ≥ 7 days from the start of the study (Fig. 3). Peak levels of plasma LBP (99.34 ± 6.18 µg/mL) detected 72 h following *S. uberis* infusion, were significantly higher than the peak levels (77.55 ± 5.28 µg/mL) observed 36 h post-challenge with *S. marcescens* ($P = 0.016$).
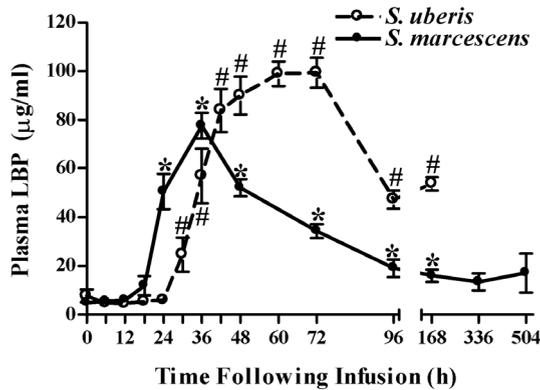
Figure 3. Changes in the circulating levels of LPS-binding protein (LBP) following intramammary infection with *Serratia marcescens* or *Streptococcus uberis*. LBP levels in plasma prepared from blood collected immediately prior to and at various time points following intramammary infection were assayed by ELISA. *, # Significantly increased compared to time 0 in cows challenged with *S. marcescens* or *S. uberis*, respectively ($P < 0.05$).

3.3. Intramammary infection with *S. marcescens* or *S. uberis* is characterized by decreases in circulating WBC and elevations of milk SCC

Decreased numbers of total circulating WBC's were evident within 18 h of *S. marcescens* challenge and six hours later reached a minimum of $7\,070 \pm 650$ cells/μL (Fig. 4A). Within 36–48 h of infection, a transient increase in circulating WBC's relative to time 0 was observed in *S. marcescens* infected cows. In contrast, initial decreases in circulating WBC's in cows challenged with *S. uberis* were not observed until 36 h after infection and remained significantly decreased between 60 and 96 h following infection. There was no difference in the minimum levels of circulating WBC's detected following infection with either bacteria.

Increases in milk somatic cells, which are primarily composed of neutrophils during the acute phase of infection [46], were evident within 18 and 30 h of *S. marcescens* or *S. uberis* challenge, respectively (Fig. 4B). Maximal numbers of milk SCC were observed

at 36 or 60 h following *S. marcescens* or *S. uberis* challenge, respectively, and the magnitude of the peak SCC did not statistically differ between quarters infected with either organism. Milk SCC in all infected quarters remained elevated relative to pre-challenge levels throughout the study. Milk SCC in saline control quarters did not change over the course of the study (data not shown).

3.4. Intramammary infection with *S. marcescens* or *S. uberis* elicits an increase in mammary vascular permeability

As an indicator of changes in mammary vascular permeability, milk levels of BSA were quantified by ELISA. Milk from quarters infused with *S. marcescens* demonstrated an acute increase in levels of BSA within 18 h of infection and elevated levels persisted for an additional 18 h (Fig. 5). Increases in milk BSA levels in *S. uberis* infected quarters were not detected until 30 h after challenge and remained augmented throughout the study. Maximal increases in BSA levels did not statistically differ between quarters infected with either bacterium.
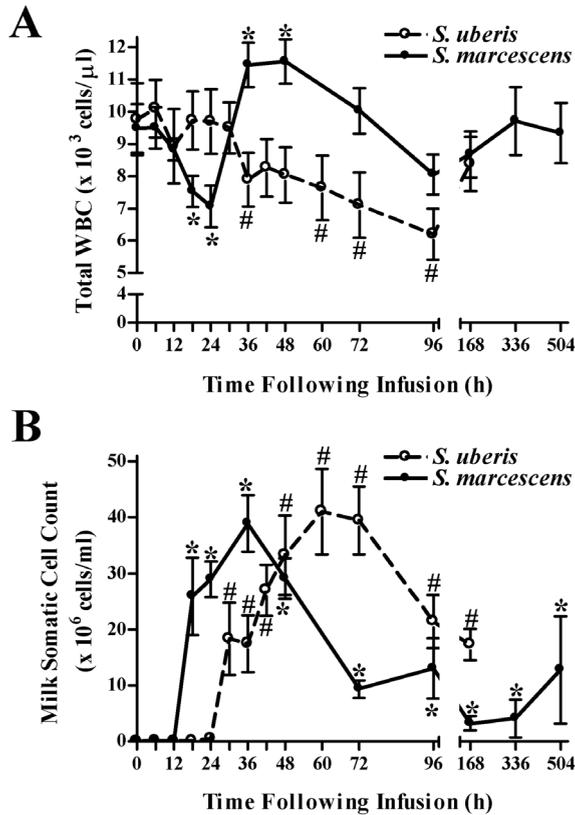
Figure 4. Effect of intramammary infection with *Serratia marcescens* or *Streptococcus uberis* on total circulating white blood cells (WBC) and milk somatic cell counts (SCC). Total WBC counts were determined from blood samples collected immediately prior to and at various time points following intramammary infection (A). *, # Significantly different compared to time 0 in cows challenged with *S. marcescens* or *S. uberis*, respectively ($P < 0.05$). Milk SCC were quantified in milk samples collected from infected quarters throughout the study (B). *, # Significantly increased in *S. marcescens*- or *S. uberis*-infected quarters, respectively, relative to time 0 ($P < 0.05$).

3.5. Differential changes in the levels of the chemoattractants IL-8 and C5a following intramammary challenge with either *S. marcescens* or *S. uberis*

Intramammary infection with *S. marcescens* induced transient increases in the milk concentrations of IL-8 and C5a, whereas *S. uberis* challenge resulted in a more sustained increase in milk IL-8 and C5a (Fig. 6). At time points in which IL-8 levels were significantly elevated relative to time 0, there was no statistical difference in the maximal levels of IL-8 detected in milk from quarters infected with either organism. However, the mean peak concentrations of C5a following *S. uberis* infection (45.56 ± 3.77 ng/mL) were significantly higher than those reached in *S. marcescens* infected quarters (5.92 ± 2.76 ng/mL) ($P < 0.0001$). Strikingly, the C5a levels in milk from *S. uberis* infected quarters continued to increase throughout the infection in contrast to the C5a levels in the *S. marcescens* quarters that quickly returned to baseline.
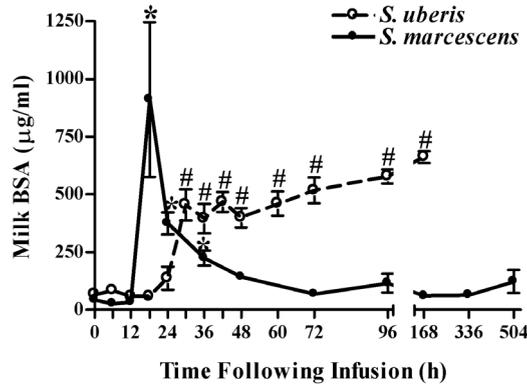
Figure 5. Intramammary infection with *Serratia marcescens* or *Streptococcus uberis* increases mammary vascular permeability. As an indicator of changes in mammary vascular permeability, milk BSA concentrations were assayed by ELISA in samples obtained from quarters immediately prior to and at varying time points following intramammary infusion with *S. marcescens* or *S. uberis*. *, # Significantly increased in *S. marcescens*- or *S. uberis*-infected quarters, respectively, relative to time 0 (*P* < 0.05).

3.6. Differential temporal induction of TNF-α and IL-1β following intramammary challenge with either *S. marcescens* or *S. uberis*

Intramammary infection with either *S. marcescens or S. uberis* induced increased levels of TNF-α and IL-1β in milk (Fig. 7). Increases in TNF-α in response to infection with either organism were transient and highly variable (Fig. 7A). Relative to time 0, increases in TNF-α were observed in *S. marcescens* challenged quarters at 18 and 72 h and in *S. uberis* infected quarters at 168 h. Peak increases in TNF-α in *S. marcescens* challenged quarters at 18h (11.24 ± 3.45 ng/mL) were significantly higher than those in *S. uberis* infected quarters at 168 h (2.54 ± 0.67 ng/mL) (*P* = 0.0127). A transient increase in IL-1β was initially observed 18 h following *S. marcescens* challenge and increased again 78 h later, after which the levels remained elevated until the end of the study. IL-1β levels in *S. uberis* infected quarters increased at 60 h and remained elevated throughout the study. Maximal increases in IL-1β following infection with either organ-

ism were observed 96 h post-challenge and those in *S. uberis* infected quarters (1.81 ± 0.55 ng/mL) were significantly higher than those in *S. marcescens* quarters (0.49 ± 0.053 ng/mL) (*P* = 0.0427).

3.7. *S. marcescens* or *S. uberis* infection elicits IL-12, IFN-γ, and IL-10 production

Within 18 and 30 h of infection with either *S. marcescens* or *S. uberis*, respectively, initial and comparable increases in IFN-γ levels were detected (Fig. 8A). IFN-γ levels continued to increase in *S. uberis* infected quarters, whereas, those in *S. marcescens* infected quarters transiently decreased before increasing again at 96 h. Relative to time 0, maximal concentrations of IFN-γ that were significantly increased in *S. uberis* quarters were detected at 168 h (3.55 ± 1.36 ng/mL) and these peak levels were significantly higher than the maximal levels observed in *S. marcescens* quarters at 24 h (0.16 ± 0.11 ng/mL) (*P* = 0.0345).

In quarters infected with either bacteria, elevated levels of IL-12 (Fig. 8B) were observed temporally coincident with or
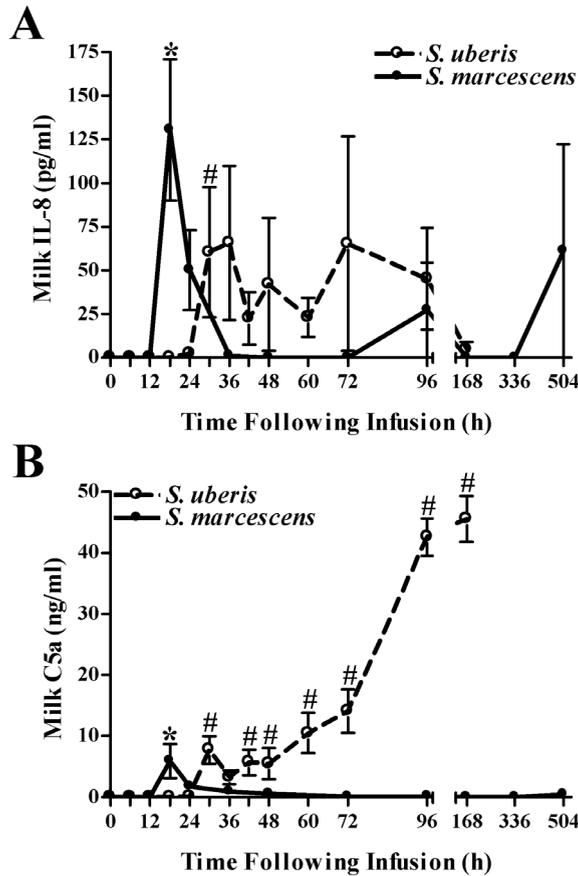
Figure 6. Effect of intramammary infection with *Serratia marcescens* or *Streptococcus uberis* on milk concentrations of IL-8 and C5a. Milk concentrations of IL-8 (A) and C5a (B) in samples collected from quarters immediately prior to and at varying time points following intramammary bacterial infection were assayed by ELISA. *, # Significantly increased in *S. marcescens*- or *S. uberis*-infected quarters, respectively, relative to time 0 ($P < 0.05$).

immediately following initial increases in IFN-γ. Changes in IL-12 levels correlated with those of IFN-γ in both *S. uberis* ($r = 0.8$) and *S. marcescens* ($r = 0.93$) infected quarters. Similar to IFN-γ, maximal levels of IL-12 measured in *S. uberis* infected quarters ($1\,169 \pm 117$ biological units/mL) exceeded those detected in quarters infected with *S. marcescens* (77.16 ± 21.27 biological units/mL) ($P < 0.0001$).

Initial increases in the anti-inflammatory cytokine IL-10 were observed in milk within 18 h of *S. marcescens* infection and returned to pre-challenge levels > 18 h later (Fig. 8C). In contrast, IL-10 levels initially increased in *S. uberis* infused quarters 30 h after infection and elevated levels persisted from 42 h until the end of the study. Peak levels of IL-10 in *S. uberis*-challenged quarters (160.12 ± 32.86 biological units/mL) were significantly higher than those detected in quarters challenged with *S. marcescens* (71.78 ± 14.64 biological units/mL) ($P = 0.0328$).
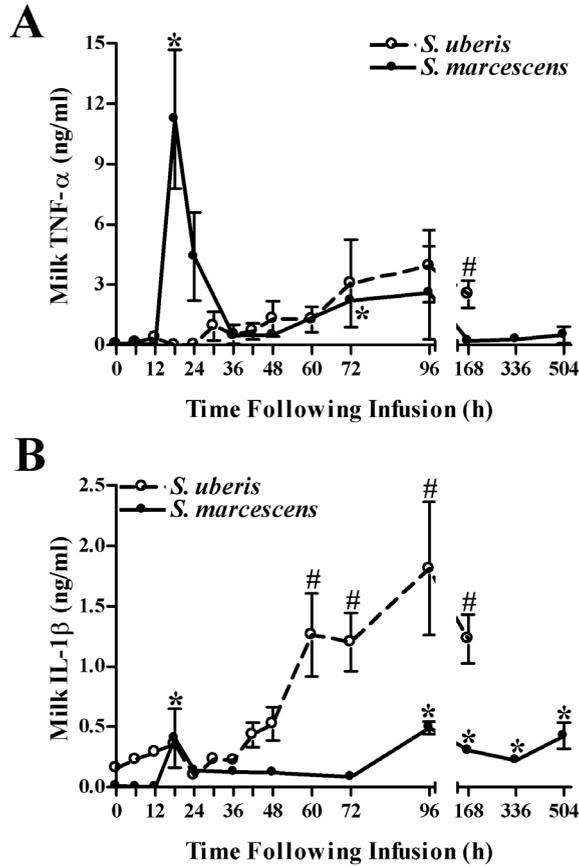
Figure 7. Effect of *Serratia marcescens* or *Streptococcus uberis* infection on TNF-α and IL-1β concentrations in milk. ELISA's were used to determine the concentrations of TNF-α (A) and IL-1β (B) in milk obtained from quarters infected with *S. marcescens* or *S. uberis*. *, # Significantly increased in *S. marcescens*- or *S. uberis*-infected quarters, respectively, relative to time 0 ($P < 0.05$).

3.8. Intramammary infection with *S. marcescens* or *S. uberis* augments milk levels of sCD14 and LBP

Changes in milk levels of sCD14 and LBP, two proteins involved in host cell recognition of bacterial wall products, were assayed by ELISA. Relative to pre-challenged quarters (time 0), increased levels of milk sCD14 were evident within 36 or 48 h of *S. marcescens* or *S. uberis* infusion, respectively (Fig. 9A). Increased levels of sCD14

persisted in *S. uberis* infected glands until the end of the study, whereas, those in *S. marcescens* challenged quarters returned to time 0 levels by 168 h. Maximal sCD14 concentrations observed 48 h after *S. marcescens* infection ($11.00 \pm 1.29 \, \mu g/mL$) were significantly higher than the peak levels detected in *S. uberis* infected quarters at 72 h ($5.29 \pm 0.81 \, \mu g/mL$) ($P = 0.0009$).

Increases in milk levels of LBP were detected within 24 or 30 h of *S. marcescens*
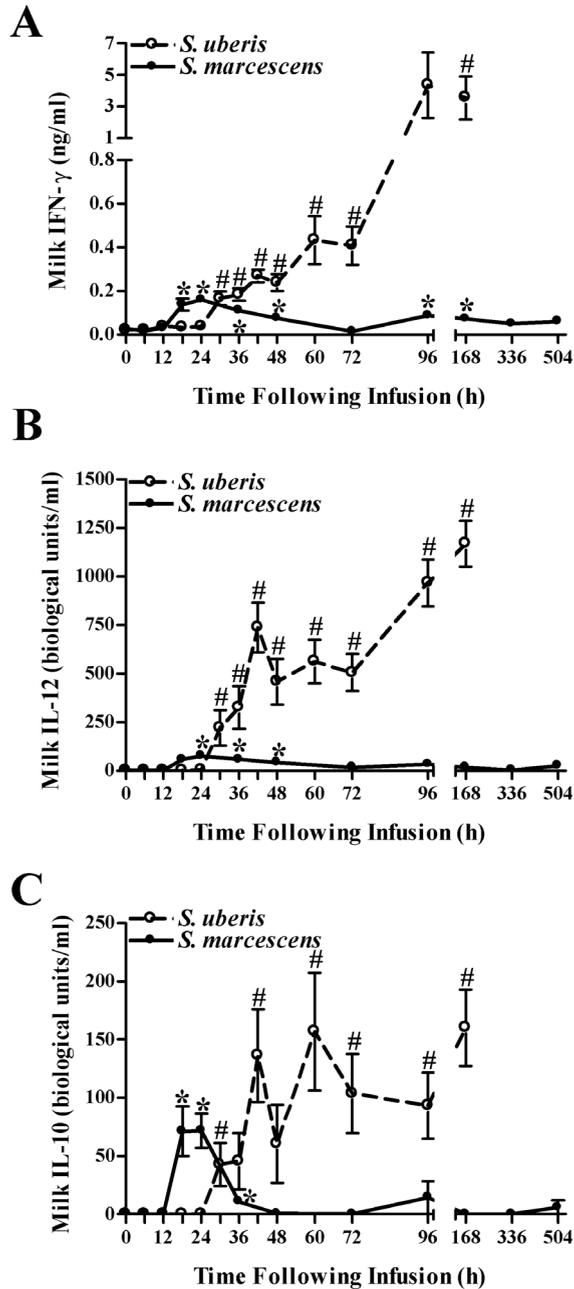
Figure 8. Effect of intramammary infection with *Serratia marcescens* or *Streptococcus uberis* on milk levels of IFN-γ, IL-12, and IL-10. Milk concentrations of IFN-γ (A), IL-12 (B), and IL-10 (C) in samples collected from quarters infused with *S. marcescens* or *S. uberis* were assayed by ELISA. *, # Significantly increased in *S. marcescens*- or *S. uberis*-infected quarters, respectively, relative to time 0 ($P < 0.05$).
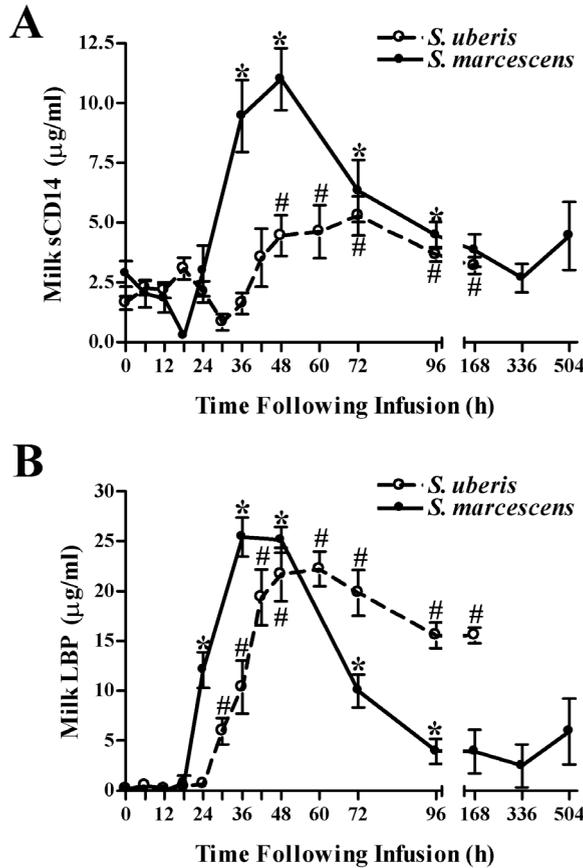
Figure 9. Effect of intramammary infection with *Serratia marcescens* or *Streptococcus uberis* on milk levels of sCD14 and LBP. sCD14 (A) and LBP (B) concentrations in milk obtained from quarters infused with either *S. marcescens* or *S. uberis* were quantified by ELISA. *, # Significantly increased in *S. marcescens*- or *S. uberis*-infected quarters, respectively, relative to time 0 ($P < 0.05$).

or *S. uberis* challenge, respectively (Fig. 9B). Similar to sCD14, augmented levels of LBP in *S. uberis* infected glands persisted throughout the study, whereas, those in *S. marcescens* challenged quarters returned to pre-challenge levels by 168 h. LBP concentrations peaked within 36 or 60 h of *S. marcescens* (25.44 ± 1.96 µg/mL) or *S. uberis* infection (22.22 ± 1.74 µg/mL), respectively, and were comparable in magnitude. Changes in LBP concentrations in milk correlated with those in plasma (Fig. 3) for both *S. marcescens* ($r = 0.9499$) and *S. uberis* ($r = 0.9726$) infected animals.

4. DISCUSSION

The innate immune system via TLR's and other pattern recognition receptors is poised to rapidly respond to the earliest stages of infection [18]. The capability of the innate immune system to respond to a vast number of pathogens, which it may or may not have previously encountered, is mediated by its ability to recognize highly conserved motifs shared by diverse pathogens. Among the motifs recognized are the bacterial cell wall components, LPS, peptidoglycan, and lipoteichoic acid [1]. Recognition

of these bacterial cell wall components often leads to the induction of a pro-inflammatory cytokine response that promotes elimination of the infectious pathogen [13, 23, 53].

TNF-α and IL-1β are well-described pro-inflammatory cytokines that mediate the inflammatory response at both the local and systemic levels [13, 54]. Locally, these cytokines promote neutrophil recruitment to the site of infection by acting as chemoattractants and by inducing the upregulation of vascular adhesion molecules necessary for neutrophil transendothelial migration. Neutrophil recruitment to the site of infection is further mediated by the chemoattractants IL-8 and C5a, a cytokine and complement cleavage product, respectively [10]. Systemically, TNF-α and IL-1β are potent inducers of fever and the acute phase response, the latter of which is characterized by increased hepatic synthesis of proteins such as LPS-binding protein (LBP) and C-reactive protein (CRP) [59]. LBP and CRP facilitate host detection of bacterial wall products and complement activation, respectively. Other important cytokines that are upregulated during the inflammatory response to bacterial infection include IL-12 and IFN-γ, which contribute to both the innate and adaptive immune responses by activating neutrophils and macrophages and promoting a T_h1-type immune response [57]. Resolution of the inflammatory process is mediated by IL-10 which downregulates pro-inflammatory cytokine production [44, 52]. Together, cytokines mediate several aspects of the innate immune response including immune cell recruitment, activation, differentiation, and downregulation. In the present study, the cytokine response elicited by two important mastitis pathogens, *S. marcescens* and *S. uberis*, was characterized.

Cows infected with either *S. marcescens* or *S. uberis* developed clinical mastitis characterized by the development of a febrile response (Fig. 2B), induction of acute phase hepatic synthesis (Fig. 3), changes in the appearance of milk, and a marked decrease in milk production (Fig. 2A). The changes in milk appearance were consistent with the detection of increased SCC in milk (Fig. 4B) and the leakage of vascular components into the gland (Fig. 5). Relative to *S. uberis*-infected cows, the onset of systemic and localized changes was more rapid in cows challenged with *S. marcescens*. Equivalent concentrations of viable bacteria were recovered from *S. marcescens* and *S. uberis* infected quarters within 12 and 18 h of infusion, respectively (Fig. 1B). This 6 h lag in *S. uberis* growth necessary to reach comparable levels detected in *S. marcescens* quarters may explain the delay in the onset of systemic and localized changes. However, initial changes in several parameters, including increases in core body temperature, decreases in total circulating WBC's, increases in milk SCC, breakdown of mammary vascular barrier function, and increases in milk levels of IL-8, TNF-α, IL-1β, IFN-γ, IL-10, and sCD14 were delayed by ≥ 12 h in *S. uberis* cows relative to those infected with *S. marcescens*. The more rapid induction of the innate immune response to *S. marcescens* may be attributed to LPS, a component of the outer envelope of all Gram-negative bacteria. LPS is a highly pro-inflammatory molecule that is able to elicit a rapid innate immune response in the mammary gland [4]. Thus, the presence of LPS on *S. marcescens* and the TLR-4-mediated recognition of this molecule may contribute to the rapid mobilization of host defense mechanisms against this bacterium.

TNF-α and IL-1β are potent inducers of fever and acute phase hepatic synthesis [13]. Maximal increases in TNF-α and initial transient increases in IL-1β at 18 h (Fig. 7) were temporally coincident with maximal increases in core body temperature in cows infected with *S. marcescens* (Fig. 2B). Similarly, initial increases in TNF-α and IL-1β immediately preceded induction of acute phase hepatic synthesis as evidenced by increases in circulating LBP (Fig. 3), an acute phase protein. Both the rapid induction in the expression of TNF-α and IL-1β and the concentrations expressed are

consistent with studies on IMI's caused by another Gram-negative bacterium, *E. coli* [45]. In contrast to cows challenged with *S. marcescens*, elevations in core temperature and heightened levels of circulating LBP in *S. uberis*-infected cows preceded detectable increases in these cytokines. Increases in the production of TNF-α and IL-1β in these cows at ≥ 60 h are consistent with the findings of the only other report to assay for these cytokines during *S. uberis* IMI [42]. The induction of fever and acute phase hepatic synthesis prior to increases in TNF-α and IL-1β may be due to the upregulation of other cytokines such as IL-6, which can similarly elicit these responses [28, 31].

Increases in milk SCC (Fig. 4B) were first detected 18 or 30 h after infusion of *S. marcescens* or *S. uberis*, respectively, and were temporally coincident with initial increases in IL-8 and the complement cleavage product C5a (Fig. 6), both of which are chemoattractants [10]. The temporal changes and elevated concentrations of IL-8 detected in *S. marcescens*-infected quarters are similar to those reported following *E. coli* infection [27, 51]. The transient increase in C5a following challenge with *S. marcescens* was of briefer duration than that reported following *E. coli* IMI [45]. In comparison to the only other study to directly measure milk levels of IL-8 following *S. uberis* infection [42], the present study observed earlier and enhanced increases in IL-8. These differences may be explained by the different strains (0140 vs. UT888) used in the two studies.

Changes in milk C5a levels (Fig. 6B) correlated with increased mammary vascular permeability, as evidenced by elevated milk BSA levels (Fig. 5), in *S. marcescens*- ($r = 0.99$) and *S. uberis*-infected ($r = 0.76$) quarters. Since physiological levels of complement proteins in milk are relatively low [41], the current findings suggest that serum-derived complement components leak into infected quarters where complement becomes activated.

During the acute stages of mastitis, WBC's can constitute > 90% of the milk somatic cells present in infected quarters [46]. Initial increase in milk SCC in *S. marcescens* infected cows paralleled decreases in total WBC's (Fig. 4). Similarly, the delayed increases in milk SCC in *S. uberis* infected quarters relative to those infected with *S. marcescens* paralleled delayed decreases in circulating total WBC's. The initial increase in milk SCC in *S. uberis*-infected quarters at 30 h, however, was not accompanied by a detectable decrease in circulating WBC's. Relative to *S. marcescens*-infected quarters, the initial delay in recruitment of neutrophils to the mammary gland following *S. uberis* infection may enable recruitment from bone marrow stores of WBC's over a longer period of time, thus, precluding immediate corresponding decreases in circulating neutrophils.

IL-12 and IFN-γ play key roles in promoting T_h1 differentiation, activating macrophages, and conferring overall resistance to bacterial infections [2, 30]. Similar to IL-8 and TNF-α, increases in IL-12 and IFN-γ were detected at earlier time points in *S. marcescens*-infected cows than in those infused with *S. uberis* (Fig. 8). In contrast to IL-8 and TNF-α, maximal levels of IL-12 and IFN-γ in *S. uberis*-infected quarters exceeded those challenged with *S. marcescens*. In response to infection with either bacteria, increases in IL-12 and IFN-γ correlated with one another, consistent with reports that each cytokine is capable of stimulating production of the other [11, 30, 34].

IL-10 is an anti-inflammatory cytokine that downregulates pro-inflammatory cytokine production, thus, contributing to the resolution of the inflammatory response to infection [12]. Resolution is essential in the mammary gland as prolonged inflammation can elicit injury to the epithelial lining of the gland resulting in permanent scarring and decreased milk output [29, 39]. Relative to quarters infused with *S. marcescens*, elevated levels of IL-10 persisted in *S. uberis*-infected quarters throughout the study. This

latter finding may reflect continued, but unsuccessful attempts of the host to downregulate the exuberant and possibly injurious inflammatory response elicited by *S. uberis* IMI.

Pathogen recognition by TLR's initiates a signal transduction cascade leading to inflammatory and immune responses that can potentially control and/or eliminate infectious agents [58]. Recently, TLR-2 and TLR-4 have been reported to be upregulated in cows with mastitis induced by Gram-positive and Gram-negative infections [15]. TLR-2 and TLR-4 recognition of Gram-positive and Gram-negative cell wall products is facilitated by the accessory molecules CD14 and LBP. CD14 exists in both a membrane-associated and soluble form [61]. LBP is a hepatically-derived acute phase protein that enhances CD14/TLR recognition of bacterial cell wall products [49]. An array of experimental approaches, including the use of LBP$^{-/-}$ [14, 20] or CD14$^{-/-}$ [65] mice, CD14 neutralizing antibodies [25, 63], and administration of exogenous LBP [24] have established a protective role for CD14 and LBP in mediating host responses to LPS and Gram-negative bacterial infection. We have shown that exogenous recombinant bovine sCD14 enhances intramammary clearance of *E. coli* in both mice [26] and cattle [27]. CD14 and LBP have further been shown to facilitate TLR-2 activation by lipoteichoic acid and peptidoglycan derived from Gram-positive bacteria [48, 66]. Thus, sCD14 and LBP contribute to host recognition of and responses to both Gram-positive and Gram-negative bacteria.

Intramammary challenge with either *S. marcescens* or *S. uberis* resulted in increased milk levels of both sCD14 and LBP (Fig. 9). Similar to other responses, initial changes in the concentrations of both of these proteins were observed at earlier time points in *S. marcescens*-infected animals. The increase in the concentrations of both sCD14 and LBP following infection would be expected to be optimally beneficial since these proteins act in concert to facilitate host recognition of bacterial wall products. Initial

increases in IL-1β, IL-8, IL-10, IFN-γ, and TNF-α in *S. marcescens*-infected quarters preceded initial increases in LBP and sCD14 at 24 and 36 h, respectively. This finding suggests that the induction of initial responses to *S. marcescens* may occur in the presence of physiological levels of sCD14 and LBP. Interestingly, increases in sCD14 and LBP in *S. uberis*-infected quarters preceded initial increases in milk levels of IL-1β and TNF-α. Whether elevated levels of sCD14 and LBP are necessary for the induction of these pro-inflammatory cytokines during Gram-positive infection remains unknown.

Increased concentrations of milk LBP (Fig. 9B) in quarters infected with either bacterium correlated with elevated plasma levels of LBP (Fig. 3) and were temporally coincident with increased mammary vascular permeability (Fig. 5). This finding is compatible with acute phase induction of hepatic synthesis of LBP and flux of LBP from the vascular compartment into the infected quarters. sCD14 concentrations in plasma were unable to be determined presumably due to assay interference from plasma components. Plasma component interference has been described for other ELISA's as well [37]. Therefore, whether the increases in sCD14 are a result of vascular leakage remains unknown. Increases in intramammary sCD14 during the course of mastitis have been proposed to be the result of CD14 shedding from the neutrophil surface [27]. Consistent with this hypothesis, increases in sCD14 levels (Fig. 9A) following a lag time paralleled elevations in milk SCC (Fig. 4B) in quarters infected with either bacteria.

To our knowledge, the present report is the first to characterize the innate immune response to IMI with *S. marcescens*. A recent report by Rambeaud et al. [42] characterized a limited set of innate immune responses to *S. uberis* strain UT888 including milk SCC, TNF-α, IL-1β, and IL-8. The present study examined all of those factors following IMI with *S. uberis* strain 0140 and expanded upon that initial report by

studying an array of additional responses including: complement activation; IL-10, IL-12, and IFN-γ production; changes in the levels of the host innate recognition accessory molecules sCD14 and LBP; and changes in mammary vascular permeability. The reports also differ in the strain of *S. uberis* used to experimentally establish IMI. Further, Holstein cows used in the study by Rambeaud et al. [42] were infused in 2 quarters with 10 500 CFU of *S. uberis* whereas those in the present study were infused in only one quarter with an ~ 50-fold lower inoculating dose of 220 CFU.

Despite the highly conserved nature of the innate immune system, the current study establishes that the temporal responses and the level of responses elicited by Gram-positive and Gram-negative bacteria are variable. The systemic and local innate immune response to *S. marcescens* is similar to that reported for another Gram-negative organism, *E. coli* [27, 51]. IMI with either organism was characterized by rapid and transient increases in body temperature and the pro-inflammatory cytokines TNF-α and IL-8. In contrast, the innate immune response to *S. uberis* differed greatly with that reported for another Gram-positive organism, *S. aureus*. In the context of a previous study demonstrating a complete absence of an IL-8, TNF-α, and IL-1β response to *S. aureus* IMI [45], the current finding that *S. uberis* induces the production of these cytokines suggests that the innate immune response to distinct Gram-positive bacteria is highly variable. Because the successful control and/or elimination of bacterial pathogens is mediated, in part, by the nature of the innate immune response [9], differential host responses to distinct mastitis-causing pathogens may dictate whether IMI's are acute and limited or develop into a chronic and prolonged infectious state.

ACKNOWLEDGEMENTS

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