Accepted Manuscript

Title: EXPERIMENTAL *STAPHYLOCOCCUS AUREUS* INFECTION OF THE MAMMARY GLAND INDUCES REGION-SPECIFIC CHANGES IN INNATE IMMUNE GENE EXPRESSION

Authors: Cormac J. Whelehan, Kieran G. Meade, P. David Eckersall, Fiona J. Young, Cliona O'Farrelly

PII: S0165-2427(10)00397-1
DOI: doi:10.1016/j.vetimm.2010.11.013
Reference: VETIMM 8414

To appear in: *VETIMM*

Received date: 31-5-2010
Revised date: 11-11-2010
Accepted date: 15-11-2010

Please cite this article as: Whelehan, C.J., Meade, K.G., Eckersall, P.D., Young, F.J., O'Farrelly, C., EXPERIMENTAL *STAPHYLOCOCCUS AUREUS* INFECTION OF THE MAMMARY GLAND INDUCES REGION-SPECIFIC CHANGES IN INNATE IMMUNE GENE EXPRESSION, *Veterinary Immunology and Immunopathology* (2010), doi:10.1016/j.vetimm.2010.11.013

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**EXPERIMENTAL *STAPHYLOCOCCUS AUREUS* INFECTION OF THE MAMMARY
GLAND INDUCES REGION-SPECIFIC CHANGES IN INNATE IMMUNE GENE
EXPRESSION**

Authors: Cormac J. Whelehan¹, Kieran G. Meade², P. David Eckersall³, Fiona J. Young^{3,4},
Cliona O'Farrelly¹

¹Comparative Immunology Group, School of Biochemistry and Immunology, Trinity College Dublin

²Animal Bioscience Department, Teagasc, Grange, Co Meath, Ireland

³Division of Animal Production and Public Health, Faculty of Veterinary Medicine, University of Glasgow,
Bearsden Rd, Glasgow G611QH, UK

⁴Current address: Agri-Food and Bioscience Institute, Hillsborough, Large Park, Hillsborough, Co. Down, UK,
BT26 6DR

Corresponding author: cliona.ofarrelly@tcd.ie (C. O'Farrelly).

Abstract

Staphylococcus aureus is a prolific mastitis-causing bacterium that resides naturally in the environment of the dairy cow. The aim of this study was to profile immune gene expression in tissue from the alveolar, ductal, gland cistern and teat canal regions of the bovine mammary gland following intra-mammary infection with *S. aureus*. Quantitative real-time PCR (qPCR) was used to profile expression of innate immune genes including pattern recognition receptors (PRRs), cytokines, chemokine, antimicrobial peptides (AMPs) and acute phase proteins (APPs). Consistent expression of Toll-like receptors (TLRs) 1-10 and NOD-like receptors (NODs) 1-2 was detected in all four tissue regions. Pro-inflammatory cytokines (*IL6*, *IL17A* and *IL8*) and anti-inflammatory cytokine (*IL10*) were induced in all 4 tissues. APP (*SAA3* and *HP*) and AMP (*DEFB4* and *DEFB5*) genes showed the greatest induction throughout the mammary gland in response to *S. aureus*, with particularly high expression in alveolar tissue (*SAA3* and *HP* >133- and >80-fold respectively, $P < 0.05$; *DEFB4* and *DEFB5* >9- and >27-fold respectively, $P < 0.05$). Collectively, our data show both sentinel and effector immune functions throughout the mammary gland in response to *S. aureus* challenge.

Keywords: Dairy cow; Mastitis; *Staphylococcus aureus*; qPCR; Pattern recognition receptors; Acute phase proteins; β -defensins

1. Introduction

Bovine mastitis, characterised by inflammation of the mammary gland, is a major disease affecting dairy cattle worldwide. The severity of the inflammation is dependent on the causative agent and the host response to it (Bannerman et al., 2004; Barkema et al., 2006; Burvenich et al., 2003; Petzl et al., 2008). Resident and recruited cells together play important roles in immediate defence against local infection (Rainard and Riollet, 2006). Extensive neutrophil recruitment from the circulation to the lumen of the mammary gland is a hallmark of the early immune response to mammary infection (Thomas et al., 1994). Intramammary infections with *S. aureus* predominantly cause subclinical mastitis resulting in chronic infection that can persist for the life of the animal (Bannerman et al., 2004; Riollet et al., 2000; Yang et al., 2008). Chronic, subclinical infections account for approximately 80% of mastitis related costs, due to reduced milk yield and product quality (Shim et al., 2004). Further understanding of the immune response throughout the mammary gland is a prerequisite to developing alternative strategies to combat this costly disease.

Once a pathogen enters the mammary gland, recognition through activation of pattern recognition receptors (PRRs) is essential for initiation of the immune response in the mammary gland (Elazar et al., 2010). For example, Toll-like receptor 2 is a PRR receptor that is activated by lipoteichoic acid (LTA), a cell wall pathogen-associated molecular pattern (PAMP) of gram-positive bacteria (Akira et al., 2006), including *S. aureus*. Ten TLRs have been identified in cattle to date and their expression profile determined in selected tissues and in subsets of antigen presenting cells (Menzies and Ingham, 2006; Werling et al., 2006). Increased expression of TLRs in mammary tissue and in mammary epithelia in response to bacterial challenge have been reported (Goldammer et al., 2004; Juliane et al., 2009; Petzl et al., 2008). Activation of the PRRs initiates signal transduction pathways that culminate in the transcription of a wide range of

immune genes including cytokines, which are synthesised by infiltrating cells (Lee et al., 2006) as well as resident cells in response to *S. aureus* infection (Lahouassa et al., 2007) and have been shown to orchestrate both the local and the systemic immune response (Elazar et al., 2010; Fitzgerald et al., 2007; Mitterhuemer et al., 2010).

Pathogen triggering of PRRs results in the expression of effector molecules of the innate immune response, these include host defense peptides (HDPs) and acute phase proteins (APPs). HDPs are a large family of innate immune effector molecules that are conserved across a wide range of species (Linde et al., 2008). Predominantly synthesised in neutrophils and epithelial cells, they have been shown to be important in the resolution of local infection through both antimicrobial and immune-regulatory properties (Lai and Gallo, 2009). Defensins are an important family of HDPs in cattle (Cormican et al., 2008). Several β -defensins, including lingual antimicrobial peptide (*LAP*), tracheal antimicrobial peptide (*TAP*) and β -defensin 5 (*DEFB5*) have been found to be expressed in mammary tissue in both a constitutive and in an inducible manner in response to bacterial challenge (Goldammer et al., 2004; Petzl et al., 2008; Swanson et al., 2004; Tetens et al., 2010). A broad spectrum of antimicrobial activity has been demonstrated for several bovine β -defensins, in particular, LAP and TAP have been shown to demonstrate high activity against *S. aureus* and *Escherichia coli* (Diamond et al., 1991; Isobe et al., 2009). Induction of the acute phase response (APR) is also prominent during intra-mammary infection with *S. aureus* (Bannerman et al., 2004; Eckersall et al., 2006). Orchestrated through hepatic and local synthesis of acute phase proteins (APPs; (Eckersall et al., 2006), it provides the animal with an early defence mechanism until specific immunity is achieved. Serum amyloid A (SAA) and haptoglobin (HP) are two important acute phase proteins in cattle whose expression in milk is elevated in response to experimentally induced mastitis (Eckersall et al., 2001; Eckersall et al., 2006). Of the bovine SAA family of proteins (Uhlir et al., 1994), SAA3 has been shown to be the main SAA protein present in bovine colostrum (McDonald et al., 2001).

Several studies have quantified changes in concentrations of cytokines, APP, and AMPs in bovine milk following experimentally induced intramammary infections with *E. coli* (Bannerman et al., 2004; Chockalingam et al., 2005; Shuster et al., 1997). Furthermore, recent studies have outlined transcriptome-wide changes in gene expression within mammary tissue during intramammary *E. coli* infection (Rinaldi et al., 2010). However, limited information is available on the local immune response to intramammary infection with *S. aureus*. To address this, the aim of this study was to examine the regional variation in the expression of genes encoding PRRs, pro- and anti-inflammatory cytokines, acute phase proteins and antimicrobial peptides in tissue from the alveolar, ductal, gland cistern and teat canal regions of control and *S. aureus* challenged mammary quarters.

2. Materials and methods

2.1 Animal infection model and tissue collection

The details of the infection model used in this study have been previously published (Eckersall et al., 2006). Briefly, 10 healthy, Holstein-Friesian cows had their udders sequentially inoculated with a low-virulence strain of *S. aureus* (strain NCTC13047) previously isolated from a case of subclinical mastitis (Young et al., 2001). On day zero, both the right fore (RF) and the right hind (RH) quarters were infused with 5×10^4 cfu of *S. aureus* in 10 ml Ringers solution. On day 28, the RH quarter was re-infused and the left hind (LH) quarter was infused with the same bacterium. The left-fore (LF) quarter, which had no exposure to the bacterial inoculum, acted as a control and is referred to as the non-challenged quarter. Eight healthy Holstein-Friesian cows from the same herd were also used as non-challenged controls and were infused with 10 ml Ringers solution (Fig. 1). All cows were euthanized on day 30. For each quarter, tissue was taken aseptically from four different tissue regions (alveolar, ductal, gland cistern and teat canal), snap frozen in liquid nitrogen then stored at -80°C . The study was performed under a Home Office Project Licence and with approval of the Ethics Committee of the Hannah Research Institute.

In this study, tissue samples from the LH quarter of the *S. aureus* challenged cows (alveolar, n=6; ductal, n=6; gland cistern, n=5 and teat canal, n=6) and tissue from the LH quarter of the non-challenged control (n=3 for all four regions) cows were used (Fig. 1). These tissue samples, both control and *S. aureus* challenged quarters, were categorised as 48 h post challenge. Tissue samples from the control quarter of challenged cows (LF) and tissue from the LF quarter of non-challenged controls were also used in this study.

2.2 RNA isolation and quality control

Tissue samples were removed from the -80°C and kept in liquid nitrogen to prevent thawing. Tissues were then sliced to a suitable volume (approximately 3 mm) and placed in a 2 ml microcentrifuge tube. RNA was extracted from the mammary tissue using the RNeasy® Mini Kit protocol (Qiagen Ltd., Crawley, UK), and DNase digested according to the manufacturer's instructions. RNA yield and quality were assessed using the ND-1000 NanoDrop® spectrophotometer (Labtech International Ltd., Sussex, UK) and Agilent 2100 Bioanalyzer (Agilent Technologies Ireland Ltd., Cork, Ireland), respectively. The ratio of absorbance at 260 nm and 280 nm averaged >1.8 and the RNA Integrity Number (RIN) averaged >7.0 for all tissue samples.

2.3 Quantitative real-time PCR (qPCR)

One µg of total RNA from each sample was reverse transcribed into cDNA using a OmniScript™ III first strand synthesis kit with oligo (dT) primers according to the manufacturer's instructions (Invitrogen Ltd., Paisley, UK). The cDNA was quantified using the ND-1000 NanoDrop® spectrophotometer and then diluted to a 20 ng/µl working concentration. Gene specific primers for quantitative real-time PCR were designed using Primer3 express software. These were designed, where possible, to traverse introns and then commercially synthesized (Invitrogen Ltd.). Details of these primer pairs are presented in supplementary files (Supplementary table 1). qPCR was performed using the Sybr Green-based fluorescent method (Roche Diagnostics Ltd., Sussex, UK) and the MX3000P® quantitative PCR system (Stratagene Corp., La Jolla, CA), as follows: 95 °C for 10 min, followed by 40 cycles of 95°C for 30 sec, 60°C for 1 min and 72°C for 30 sec, and finally amplicon dissociation at 95°C for 1 min, 55°C for 30 sec and 95°C for 30 sec. Each reaction had a total volume of 25 µl – 12.5 µl of 2 X master mix (Stratagene Corp.), 10.5 µl of the primer-H₂O and 2 µl of cDNA. Optimal primer

concentrations were determined by titrating 300, 600 and 900 nM final concentrations and dissociation curves were examined for the presence of a single product. Amplicons were also assessed on 1.5 % native agarose gels. To determine PCR primer efficiencies, qPCR was performed for each primer set using a dilution series (two-fold over 3 orders of magnitude, including working concentration) of pooled cDNA from all *S. aureus* challenged and non-challenged control samples as template. Standard curves were generated and efficiency determined with the formula $10^{(-1/\text{slope})}-1$. Primer efficiency for *H3F3A* and all significantly differentially expressed genes were considered comparable and within the recommended range for using the $2^{-\Delta\Delta C_q}$ method (90 to 100% efficiency).

2.4 Data analysis

qPCR data (Cq values) was converted to gene expression fold changes for each sample after normalisation to a reference gene, using the $2^{-\Delta\Delta C_q}$ (Cq/Ct represents the quantification cycle) method (Schmittgen and Livak, 2008), and recorded relative to control samples. Determination of a stable reference gene was determined using the geNorm version 3.4 Microsoft Excel add-in software package (Vandesompele et al., 2002). For each tissue region (alveolar, ductal, gland cistern and teat canal) from both *S. aureus* challenged and non-challenged quarters (n=6), and from the LH quarter of non-challenged controls (n=3), seven putative normaliser genes peptidylprolyl isomerase A (*PPIA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β -actin (*ACTB*), h3 histone, family 3A (*H3F3A*), ribosomal protein S6, S9 and S15 (*RPS6*, *RPS9* and *RPS15*) were tested. The data was then analysed using the geNorm software to generate *M* stability values for specific comparison between control and *S. aureus* challenged regions (i.e. alveolar challenged vs alveolar control, ductal challenged vs ductal control, etc). GeNorm generates a gene expression stability measure (“*M*” value) and then ranks the values in order of decreasing *M* values which corresponds to increasing mRNA

expression stability. The recommended M value cut-off is 0.5 for homogeneous samples and can be higher for tissue samples. The optimal number of reference genes was then estimated by the pairwise variation “V” between two sequential normalization factors. Statistical analysis of qPCR results was carried out using the non-parametric Mann-Whitney U test as implemented in version 5.01 of GraphPad Prism. P values of <0.05 were considered statistically significant.

3. Results

3.1 Elevated levels of somatic cells in milk in response *S. aureus* challenge

Before infusion, a full bacteriological examination was carried out on milk samples from all 4 quarters of infused and control cows at several timepoints, and was found to be negative for *S. aureus* and other major mastitis-causing pathogens. Increased somatic cell count (SCC) from pre-infusion to post-infusion of $>2 \times 10^6$ cells/ml in the absence of clinical signs (swelling, redness, pain in the udder or clots in the milk) was taken as an indication of subclinical mastitis, and was detected in all *S. aureus* challenged animals. The mean SCC in milk from the LH quarter of non-challenged controls as well as the milk from the non-challenged quarter of challenged cows did not exceed 2×10^6 cells/ml. In this study, the mean somatic cell count in milk from *S. aureus* inoculated LH quarters ($n=6$) increased from pre-infusion levels of 2.3×10^4 cells/ml to 6.5×10^6 cells/ml by 12 h post-challenge and remained elevated over the 48 h period. The corresponding LH quarters of the non-challenged controls ($n=3$), did not exceed 2×10^6 cells/ml over the 48 h period (pre-infusion level of 1.6×10^4 cells/ml to 3×10^4 cells/ml at 12 h, 6.6×10^4 cells/ml at 24 h, 2.1×10^4 cells/ml at 36 h and 4.4×10^4 cells/ml at 48 h post-inoculation) (Fig. 2).

3.2 Selection of stable normaliser gene for qPCR and primer efficiency

The expression of seven genes, *PPIA*, *GAPDH*, *ACTB*, *H3F3A*, *RPS6*, *RPS9* and *RPS15*, was examined in cDNA from each animal sample, across all four regions (alveolar, ductal, gland cistern and teat canal) of both control and *S. aureus* inoculated quarters. *H3F3A* demonstrated the greatest stability throughout for all four tissue regions of the mammary gland (alveolar, $M < 0.25$; ductal, $M < 0.25$; gland cistern, $M < 0.25$ and teat canal, $M < 0.2$) and was therefore

selected as the most suitable gene for normalisation of qPCR data. In contrast to this, the most variable of the putative normaliser genes was *ACTB* and *GAPDH*.

3.3 Selection of control animals for qPCR

In a pilot experiment, expression of candidate cytokine, APP and AMP genes was profiled in tissue from *S. aureus* inoculated quarters (LH) and compared with expression levels in tissue from non-challenged quarters (LF) of challenged cows (Fig. 1). Large variation in the magnitude of expression was observed relative to control animals, resulting in no statistical significance between the tested comparisons (data not shown). The expression of these candidate immune genes was then profiled in tissue from control quarters and compared with the expression data from control cows. Expression of *SAA3*, *HP*, and *DEFB5* was significantly induced (data not shown). These results confirmed previous reports that udder quarters are influenced by infection of neighbouring quarters. As a result, it was decided that samples from non-challenged controls should be used for accurate quantification of relative gene expression changes due to *S. aureus* challenge (Fig. 1).

3.4 Consistent TLR and NOD gene expression across mammary gland

The expression of TLRs 1-10 and NODs 1 and 2 was detected in all four tissue regions from challenged and control quarters (Fig. 3), with *TLR 8* having the least expression in comparison to the other PRRs. Differential expression levels of each PRR were further profiled in the four tissue regions from challenged quarters relative to non-challenged controls 48 h post challenge with *S. aureus*. *TLR1* gene expression was significantly increased in ductal, gland cistern and teat canal tissue from challenged quarters relative to control quarters (6.1-, 6- and 4.2-fold respectively, $P < 0.05$; Fig. 4 B, C and D). *TLR3* showed a moderate fold increase in teat canal tissue from challenged quarters (2.5-fold, $P < 0.05$; Fig. 4 D). Similar moderate fold

increases were observed for *TLR6* and *TLR7* in gland cistern tissue from infected animals (3.7- and 3.6-fold respectively, $P < 0.05$; Fig. 4 C). *TLR5* and *TLR7* was also significantly increased in alveolar tissue from challenged animals (1.8 - and 2.3-fold respectively, $P < 0.05$; Fig. 4 A). In contrast, the genes encoding *TLR4*, *NOD1* and *NOD2* were significantly decreased in teat canal tissue (2.8- and 2.7- and 1.2-fold respectively, $P < 0.05$; Fig. 4 D), *TLR6* in ductal tissue (1.1-fold, $P < 0.05$; Fig. 4 B) and *TLR8* in gland cistern tissue (1.9-fold, $P < 0.05$; Fig. 4 C). *TLR2*, *TLR9*, and *TLR10* showed no significant differential expression across the four tissue regions (Supplementary table 2).

3.5 Regional variation in cytokine gene expression

Expression of the proinflammatory cytokine *IL6* significantly increased in three of the four tissue regions profiled, alveolar, ductal and gland cistern (5.1-, 2.1- and 2.9-fold respectively, $P < 0.05$; Fig. 4 A, B and C). In contrast, *IL1B* showed a slight decrease in gene expression in ductal tissue from challenged quarters (2.9-fold, $P < 0.05$; Fig. 4 B). *TNF* showed no significant differential expression across the four tissue regions (Supplementary table 2). *IL8* showed a strong and significant increase in alveolar tissue (13-fold, $P < 0.05$; Fig. 4 A) and a moderate fold increase in gland cistern tissue (2.1-fold, $P < 0.05$; Fig. 4 C). A similar fold increase in teat canal tissue was observed for the gene encoding *IFNG* (11-fold, $P < 0.05$; Fig. 4 D). The gene encoding proinflammatory cytokine *IL17A* showed significant increases in all four mammary regions - alveolar, 5.7-fold; ductal, 1.5-fold; gland cistern, 1.5-fold; teat canal, 9-fold ($P < 0.05$; Fig. 4 A, B, C and D). Gene expression of the proinflammatory antagonists *TGFB* and *IL10* were also profiled. *TGFB* showed a moderate increase in ductal tissue and a moderate decrease in teat canal tissue (1.4- and 1.8-fold respectively, $P < 0.05$; Fig. 4 B and D). *IL10* showed a strong increase in both ductal and teat canal tissue (8.4- and 6.3-fold respectively, $P < 0.05$; Fig. 4 B and D).

3.6 Local mammary expression of acute phase protein genes

Of the 5 APPs genes profiled in mammary tissue, *SAA3* and *HP* expression was significantly increased in alveolar, ductal and gland cistern tissue of challenged quarters relative to non-challenged controls. *SAA3* expression was strongly induced in both alveolar and ductal tissue (133- and 14-fold respectively, $P < 0.05$; Fig. 4 A and B), with a moderate fold increase detected in gland cistern tissue (4.1-fold, $P < 0.05$; Fig. 4 C). Similarly *HP* expression was strongly induced in both alveolar and ductal tissue (80- and 30-fold respectively, $P < 0.05$; Fig. 4 A and B), with a moderate fold increase detected in gland cistern tissue (5.6-fold, $P < 0.05$; Fig. 4 C). Lesser fold increases were detected for LPS-binding protein (alveolar, ductal and gland cistern, 3.3-, 3.3- and 1.7-fold respectively; $P < 0.05$ – Fig. 4 A, B and C) and ceruloplasmin (gland cistern and teat canal, 3.2- and 1.9-fold respectively; $P < 0.05$ - Fig. 4 C and D). Expression of alpha-1 acid glycoprotein (*AGP*) was significantly decreased in alveolar tissue (54-fold, $P < 0.05$; Fig. 4 A) and increased in ductal and gland cistern tissue (7.6- and 7.6-fold respectively, $P < 0.05$; Fig. 4 B and C).

3.7 β -defensins gene expression

Expression of the β -defensins, *DEFB4* and *DEFB5*, was strongly induced in both alveolar (9.4- and 27-fold respectively, $P < 0.05$; Fig. 4 A) and gland cistern tissue (10- and 34-fold respectively; $P < 0.05$; Fig. 4 C) from challenged quarters. *DEFB5* showed similar strong induction in ductal tissue (22-fold, $P < 0.05$; Fig. 4 B). *DEFB1* was moderately induced in the alveolar region (4.9-fold, $P < 0.05$; Fig. 4 A) with no significant induction detected in the remaining three regions. Similarly the expression of the β -defensins *LAP* and *TAP* was not significantly induced in any of the regions profiled (Fig. 4 A, B, C and D).

4. Discussion

Understanding the innate immune response to the bacterial causes of mastitis in cattle is a prerequisite to the development of novel and effective diagnostics and therapeutics. Towards this end, recent studies have examined the immune response to *S. aureus*, *E. coli* (Bannerman et al., 2004; Lee et al., 2006), and *S. uberis* (Swanson et al., 2009), the predominant causal organisms. In this study, an experimental infection model of *S. aureus* was performed and comprehensive quantification of innate immune genes carried out to assess the regional variability in the tissue-specific expression of effector molecules and in order to examine the molecular basis for local acute phase protein production detected previously (Eckersall et al., 2006).

qPCR analysis confirmed expression of *TLRs 1-10* and *NODs 1-2* in all four tissue regions profiled. Expression of genes encoding extracellular PRRs, *TLRs 1, 2, 4, 6*, and intracellular PRRs, *NODs 1-2* was abundant throughout the mammary gland relative to the normaliser gene *H3F3A* (data not shown), suggesting that all four regions of the mammary gland profiled are sensitive to bacterial infection through the ability to recognise both gram positive and gram negative bacteria (Akira et al., 2006). Expression of intra-cellular receptors may be important in detection of *S. aureus* which has the potential to invade epithelial cells (Almeida et al., 1996; Buzzola et al., 2007; Hensen et al., 2000). Expression of the *TLR1* gene was significantly increased in three tissue regions from challenged quarters relative to non-challenged controls. TLR1 and TLR6 form heterodimers with TLR2 and can recognise gram-positive cell wall components (Kawai and Akira, 2010). Interestingly, significant increases in the expression of TLRs that recognise viral ligands were also observed (*TLR3* and *TLR7*). Similar results were observed in human monocytes in response to both *S. aureus* and IL6 treatment (Zarembek and Godowski, 2002). Additionally, TLR7 has been shown to recognise gram-positive bacteria (Mancuso et al., 2009). Of the PRRs profiled, *TLR8* showed the lowest levels of expression in the

four regions profiled. *TLR8* is expressed in various bovine tissues (Menzies and Ingham, 2006), with its highest expression in monocytes (Werling et al., 2006). Immunohistochemistry analysis on tissue from both *S. aureus* challenged and control animals reported low levels of immune cells (data not shown) and this could explain the low levels of expression observed in mammary tissue. Similar low levels of *TLR8* expression were observed in bovine endometrial tissue, with no transcript detected in purified stromal and epithelial cells (Davies et al., 2008). In contrast to the increased expression of the PRRs profiled, the expression of *NOD1*, *NOD2* and *TLR4* was significantly decreased in teat canal tissue.

Although not significantly differentially expressed, *TLR2* showed the highest constitutive expression in all four tissue regions compared to other TLRs (data not shown). Unchanged expression of *TLR2* has previously been reported in *S. aureus* inoculated quarters over long periods (72 h and 84 h) (Petzl et al., 2008). However, in contrast, host detection of *S. aureus* was evident through increased somatic cells from as early as 12 h post inoculation (Fig. 2), and through the induction of proinflammatory cytokines (Fig. 4); suggesting that modulation of immune genes is initiated through activation of these baseline expressed PRRs or other receptors.

The expression of the proinflammatory cytokines *IL1B* and *TNF* were not significantly increased across the mammary gland. A similar proinflammatory cytokine response has been observed with *S. aureus* strains that caused subclinical infections (Bannerman et al., 2004; Lee et al., 2006; Petzl et al., 2008). This is in contrast with results observed following intramammary infection with *E. coli* where increased expression of *TNF* has been observed in both mammary tissue and milk (Blum et al., 2000; Petzl et al., 2008). The lack of a *TNF* or *IL1B* response throughout the mammary gland may be a factor in the pathogenesis of *S. aureus* infection which commonly results in chronic infections that can persist for the life of the animal (Petzl et al., 2008; Sutra and Poutrel, 1994). Whereas, *E. coli* infection usually resolves spontaneously within a short period (Blum et al., 2000; Hoeben et al., 2000).

IL6, which regulates the APR through the synthesis of APP (Jensen and Whitehead, 1998), was significantly induced in three of the four tissue regions profiled. *IFNG*, *IL17A*, and *IL8* were also significantly increased. *IL8*, a major chemokine, was significantly increased in both alveolar and gland cistern tissue, with particularly high induction in alveolar tissue (13-fold) at 48 h post-infection. At 48 h post infection there was an extensive increase in somatic cell infiltration into the mammary gland (Fig. 2). These cells, which are predominantly neutrophils, are probably a major contributor to the increased levels of *IL8* expression (Rainard and Riollot, 2006). Interestingly, in humans *IL17A* has been shown to be involved in the recruitment of neutrophils through regulating *IL8* (Laan et al., 1999). Expression of molecules involved in the resolution of infection was also profiled. While the expression of *TGFBI* was similar to that of control animals, the anti-inflammatory gene *IL10* was increased in challenged animals, indicating an attempt to prevent tissue damage associated with excessive inflammation. Alternatively, induced expression of these anti-inflammatory genes may be a mechanism by which *S. aureus* can evade the host's innate immunity.

Two major classes of APPs have been previously studied in cattle: type I APPs including SAA are regulated by IL-1 and TNF whereas type II APPs such as HP are regulated by IL6 (Jensen and Whitehead, 1998). The increased cytokine gene expression, in conjunction with the APP expression supports a proinflammatory host response to *S. aureus* infection. *IL6* gene expression has also been demonstrated in milk somatic cells from cows infected with *S. aureus* (Lee et al., 2006). Previous studies indicated that APP detected in the mammary gland resulted from leakage from the serum in times of stress or infection (Salonen et al., 1996). In an earlier analysis of the same animals used in this study, intramammary infection with *S. aureus* induced both local and a systemic immune responses (Eckersall et al., 2006). In this study, we confirmed the expression of acute phase genes, *SAA3* and *HP* at the mRNA level reaffirming these findings of the local production of these two major APPs throughout the mammary gland in response to *S.*

aureus infection. *SAA3* and *HP* genes were particularly highly induced in alveolar tissue 48 h post challenge (Fig. 3 A), which, with increases of 133-fold and 80-fold respectively were the largest increases of any of the genes examined. Similar regional variation and marked induction of *SAA3* and *HP* in the alveolar region were observed in *E. coli* infected mammary quarters (Rinaldi et al., 2010). *SAA3* has been shown to have antibacterial activity against *E. coli*, *S. uberis* and *Pseudomonas aeruginosa* (Molenaar et al., 2009) and may therefore have an important antibacterial role in the mammary gland. An extramammary protective role has been suggested for *SAA3* in neonates, and possibly adults, against gastrointestinal infections by inducing mucin production in the intestine resulting in reduced bacterial adherence (Gillian et al., 2009; Larson et al., 2003). The large increase in the expression of the *HP* gene would suggest that this protein also has an important protective role in response to *S. aureus* infection. This protective role may be in the form of limiting the availability of iron (Schaer et al., 2002) that is necessary for *S. aureus* to proliferate (Skaar et al., 2004), or reducing the tissue damage caused by over production of reactive oxygen species as a result of infiltrating neutrophils (Lauzon et al., 2005; Tseng et al., 2004). Additionally, *HP* has also been shown to modulate neutrophil function through inhibiting neutrophil chemotaxis, phagocytosis and bactericidal activity (Rossbacher et al., 1999).

The present study also examined the gene expression of the APP molecules, LPS-binding protein, ceruloplasmin and alpha 1 acid glycoprotein throughout the mammary gland. Induction of the gene encoding LPS-binding protein was observed in all four tissues (Fig. 4). Increased concentration of LPS-binding protein has been previously reported in milk and serum after intramammary infection with *S. aureus* (Bannerman et al., 2004). Primarily associated with gram-negative bacterial infection, LPS-binding protein has been shown to bind to components of gram-positive bacteria cell wall, namely lipoteichoic acid (Schröder et al., 2003). Ceruloplasmin gene expression was significantly increased in gland cistern and teat canal tissue (Fig. 3 C and

D). Increased concentration of ceruloplasmin has been reported in the milk of cows with subclinical mastitis (Tabrizi et al., 2008). Interestingly, alpha-1 acid glycoproteins expression was significantly decreased in expression in the alveolar region alone (54-fold), whereas expression was induced in both ductal and gland cistern tissue (Fig. 3 A, B and C). Alpha-1 acid glycoproteins has been identified in bovine colostrum and milk and expression of mRNA localised to mammary tissue (Ceciliani et al., 2005). Immunomodulatory functions have also been demonstrated. In particular, it has been shown to inhibit bovine neutrophil respiratory burst activity while enhancing IL8 production. However, they report that this inhibitory effect did not affect the ability of neutrophils to phagocytosis or kill *S. aureus* (Rinaldi et al., 2008). Decreased expression in the alveolar region may inhibit the early recruitment of neutrophils to the mammary gland and could be a result of *S. aureus* modulating the host's immune response in order to enhance survival.

β -defensins have also been shown to be bactericidal against mastitis-causing pathogens (Diamond et al., 1991; Isobe et al., 2009). We profiled, *DEFB1*, *DEFB4*, *DEFB5*, *TAP* and *LAP* in all four regions of the mammary gland. Expression of these β -defensins was detected in all four regions of the mammary gland. However, only *DEFB4*, *DEFB5* and *DEFB1* were significantly differently expressed across the mammary gland. *DEFB5* demonstrated strong induction in the alveolar (27-fold), ductal (22-fold) and gland cistern tissue (34-fold) (Fig. 3 A, B and C). Similarly high differential expression was detected for *DEFB4* in alveolar and gland cistern tissue (Fig. 4 A and C). A previous study showed that following intramammary infection with *S. aureus*, expression of *DEFB5* was not detected until 84 h post-inoculation (Petzl et al., 2008). *S. aureus* pathogens have been shown to elicit a much weaker immune defence, in comparison to *E. coli* (Bannerman et al., 2004; Yang et al., 2008). Although our result may be in part due to the specific strain used, the time kinetics of the experiment (Fig. 1) must be considered when interpreting these results, since the expression of *DEFB5*, was significantly

increased in the control quarter of challenged cows compared to the non-challenged controls. This indicates that the innate immune response being observed is influenced by systemic effects induced as a result of inoculation of neighbouring quarters with the same strain *S. aureus* 28 days prior. As a result, the innate immune response being observed is influenced by a secondary adaptive immune response.

Similar results were reported for intramammary infection with *E. coli*, whereby inoculation in one quarter influenced the transcriptome in neighbouring uninfected quarters (Mitterhuemer et al., 2010). Having previously been shown to be inducible and constitutively expressed in mammary tissue (Petzl et al., 2008; Tetens et al., 2010), and considering the fact that they are in all four regions of the mammary gland profiled, β -defensins may represent an important arm of the local innate immune response to *S. aureus* infection. Thus, determining the regulation of these defensins, both inducible and constitutively expressed, requires further work.

Of the regions profiled (Fig. 1), chemokine and effector molecule expression was most significantly stimulated in alveolar tissue, in particular the expression of *SAA*, *HP*, *DEFB4* and *DEFB5*. A similar response was seen in response to *E. coli* infection (Rinaldi et al., 2010). It was suggested that regional differences are a result of the timeframe of the bacterial interaction with the host cells in a particular location, moving from the teat canal into the gland cistern and eventually entering the alveoli. However, in contrast, our experimental model involved the infusion of the pathogen directly into the gland cistern effectively bypassing the teat canal. The fact that the alveolar region appears more sensitive to pathogens in both infection models regardless of the point of entry of the pathogen, may be a result of differences in resident cell populations throughout the mammary gland (Nickerson and Pankey, 1983; Nickerson et al., 1984). The contribution of each cell population to the challenge-related changes in gene expression remains to be elucidated.

5. Conclusion

The potential of the mammary gland to sense a broad range of microbial products is evident through the expression of *TLRs 1-10* and *NODs 1-2* throughout the mammary quarter. We demonstrate the spatial and inducible expression of a panel of APPs and AMPs throughout the mammary gland in response to *S. aureus* infection indicating these molecules are important in the pathogenesis of mastitis caused by *S. aureus* in cattle. Their expression throughout the mammary gland suggests that they are part of the local inflammatory response and their quantification in milk might be suitable for the early detection and diagnosis of subclinical mastitis.

450 **Acknowledgements**

451

452   This work was supported by an Irish Department of Agriculture, Fisheries and Food
453   Research Stimulus Fund Grant (RSF 06-340) and the Biotechnology and Biological Sciences
454   Research Council (reference no: S15699).

455

456

456 **References:**

- 457 Akira, S., Uematsu, S., Takeuchi, O., 2006, Pathogen recognition and innate immunity. *Cell* 124,
458 783-801.
- 459 Almeida, R.A., Matthews, K.R., Cifrian, E., Guidry, A.J., Oliver, S.P., 1996, *Staphylococcus*
460 *aureus* invasion of bovine mammary epithelial cells. *J. Dairy Sci.* 79, 1021-1026.
- 461 Bannerman, D.D., Paape, M.J., Lee, J.-W., Zhao, X., Hope, J.C., Rainard, P., 2004, *Escherichia*
462 *coli* and *Staphylococcus aureus* elicit differential innate immune responses following
463 intramammary infection. *Clin. Diagn. Lab. Immunol.* 11, 463-472.
- 464 Barkema, H.W., Schukken, Y.H., Zadoks, R.N., 2006, Invited review: The role of cow,
465 pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus*
466 *aureus* mastitis. *J. Dairy Sci.* 89, 1877-1895.
- 467 Blum, J.W., Dosogne, H., Hoeben, D., Vangroenweghe, F., Hammon, H.M., Bruckmaier, R.M.,
468 Burvenich, C., 2000, Tumor necrosis factor- α and nitrite/nitrate responses during acute
469 mastitis induced by *Escherichia coli* infection and endotoxin in dairy cows. *Domest.*
470 *Anim. Endocrinol.* 19, 223-235.
- 471 Burvenich, C., Van Merris, V., Mehrzad, J., Diez-Fraile, A., Duchateau, L., 2003, Severity of *E.*
472 *coli* mastitis is mainly determined by cow factors. *Vet. Res.* 34, 521 - 564.
- 473 Buzzola, F.R., Alvarez, L.P., Tuchscher, L.P.N., Barbagelata, M.S., Lattar, S.M., Calvinho, L.,
474 Sordelli, D.O., 2007, Differential abilities of capsulated and noncapsulated
475 *Staphylococcus aureus* isolates from diverse agr groups to invade mammary epithelial
476 cells. *Infect. Immun.* 75, 886-891.
- 477 Cecilian, F., Pocacqua, V., Provati, E., Comunian, C., Bertolini, A., Bronzo, V., Moroni, P.,
478 Sartorelli, P., 2005, Identification of the bovine α 1-acid glycoprotein in colostrum and
479 milk. *Vet. Res.* 36, 735-746.
- 480 Chockalingam, A., Paape, M.J., Bannerman, D.D., 2005, Increased milk levels of transforming
481 growth factor- α , β 1, and β 2 during *Escherichia coli*-induced mastitis. *J. Dairy Sci.* 88,
482 1986-1993.
- 483 Cormican, P., Meade, K., Cahalane, S., Narciandi, F., Chapwanya, A., Lloyd, A., O'Farrelly, C.,
484 2008, Evolution, expression and effectiveness in a cluster of novel bovine β -defensins.
485 *Immunogenetics* 60, 147-156.
- 486 Davies, D., Meade, K.G., Herath, S., Eckersall, P.D., Gonzalez, D., White, J.O., Conlan, R.S.,
487 O'Farrelly, C., Sheldon, I.M., 2008, Toll-like receptor and antimicrobial peptide
488 expression in the bovine endometrium. *Reprod. Biol. Endocrinol.* 6, 53.
- 489 Diamond, G., Zasloff, M., Eck, H., Brasseur, M., Maloy, W., Bevins, C., 1991, Tracheal
490 antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide
491 isolation and cloning of a cDNA. *Proc. Natl. Acad. Sci. U. S. A.* 88, 3952 - 3956.
- 492 Eckersall, P., Young, F., McComb, C., Hogarth, C., Safi, S., Fitzpatrick, J., Nolan, A., Weber,
493 A., McDonald, T., 2001, Acute phase proteins in serum and milk from dairy cows with
494 clinical mastitis. *Vet. Rec.* 148, 35 - 41.
- 495 Eckersall, P.D., Young, F.J., Nolan, A.M., Knight, C.H., McComb, C., Waterston, M.M., 2006,
496 Acute phase proteins in bovine milk in an experimental model of *Staphylococcus aureus*
497 subclinical mastitis. *J. Dairy Sci.* 89, 1488 - 1501.
- 498 Elazar, S., Gonen, E., Livneh-Kol, A., Rosenshine, I., Shpigel, N., Yehuda, 2010, Neutrophil
499 recruitment in endotoxin-induced murine mastitis is strictly dependent on mammary
500 alveolar macrophages. *Vet. Res.* 41, 10.
- 501 Fitzgerald, D.C., Meade, K.G., McEvoy, A.N., Lillis, L., Murphy, E.P., MacHugh, D.E., Baird,
502 A.W., 2007, Tumour necrosis factor- α (TNF- α) increases nuclear factor- κ B (NF- κ B)
503 activity in and interleukin-8 (IL-8) release from bovine mammary epithelial cells. *Vet.*
504 *Immunol. Immunopathol.* 116, 59-68.

- Gillian, E.G., Sarah, O.F., Pat, G.C., Annika, W., Thomas, L.M., Michael, C., Colin, H., Reynolds, P.R., Cormac, G.M.G., Fergus, S., 2009, Evaluation of colostrum-derived human mammary-associated serum amyloid A3 (M-SAA3) protein and peptide derivatives for the prevention of enteric infection: *in vitro* and in murine models of intestinal disease. *FEMS Immunol. Med. Microbiol.* 55, 404-413.
- Goldammer, T., Zerbe, H., Molenaar, A., Schuberth, H.-J., Brunner, R.M., Kata, S.R., Seyfert, H.-M., 2004, Mastitis increases mammary mRNA abundance of β -defensin 5, toll-like-receptor 2 (TLR2), and TLR4 but not TLR9 in cattle. *Clin. Diagn. Lab. Immunol.* 11, 174-185.
- Hensen, S.M., Paviccaronicacute, M.J.A.M.P., Lohuis, J.A.C.M., Poutrel, B., 2000, Use of bovine primary mammary epithelial cells for the comparison of adherence and invasion ability of *Staphylococcus aureus* strains. *J. Dairy Sci.* 83, 418-429.
- Hoeben, D., Burvenich, C., Trevisi, E., Bertoni, G., Hamann, J., Bruckmaier, R.M., Blum, J.W., 2000, Role of endotoxin and TNF- α in the pathogenesis of experimentally induced coliform mastitis in periparturient cows. *J. Dairy Res.* 67, 503-514.
- Isobe, N., Nakamura, J., Nakano, H., Yoshimura, Y., 2009, Existence of functional lingual antimicrobial peptide in bovine milk. *J. Dairy Sci.* 92, 2691-2695.
- Jensen, L.E., Whitehead, A.S., 1998, Regulation of serum amyloid A protein expression during the acute-phase response. *Biochem. J.* 334, 489 - 503.
- Juliane, G., Dirk, K., Wei, Y., Gerd, N., Dirk, R., Hans-Joachim, S., Zaneta, P., Nauman, M., Adrian, M., Hans-Martin, S., 2009, Assessment of the immune capacity of mammary epithelial cells: comparison with mammary tissue after challenge with *Escherichia coli*. *Vet. Res.* 40, 31.
- Kawai, T., Akira, S., 2010, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 11, 373-384.
- Laan, M., Cui, Z.-H., Hoshino, H., Lotvall, J., Sjostrand, M., Gruenert, D.C., Skoogh, B.-E., Linden, A., 1999, Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J. Immunol.* 162, 2347-2352.
- Lahouassa, H., Moussay, E., Rainard, P., Riollot, C., 2007, Differential cytokine and chemokine responses of bovine mammary epithelial cells to *Staphylococcus aureus* and *Escherichia coli*. *Cytokine* 38, 12-21.
- Lai, Y., Gallo, R.L., 2009, AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol.* 30, 131-141.
- Larson, M.A., Wei, S.H., Weber, A., Mack, D.R., McDonald, T.L., 2003, Human serum amyloid A3 peptide enhances intestinal MUC3 expression and inhibits EPEC adherence. *Biochem. Biophys. Res. Commun.* 300, 531-540.
- Lauzon, K., Zhao, X., Bouetard, A., Delbecchi, L., Paquette, B., Lacasse, P., 2005, Antioxidants to prevent bovine neutrophil-induced mammary epithelial cell damage. *J. Dairy Sci.* 88, 4295-4303.
- Lee, J.W., Bannerman, D.D., Paape, M.J., Huang, M.K., Zhao, X., 2006, Characterization of cytokine expression in milk somatic cells during intramammary infections with *Escherichia coli* or *Staphylococcus aureus* by real-time PCR. *Vet. Res.* 37, 219 - 229.
- Linde, A., Ross, C.R., Davis, E.G., Dib, L., Blecha, F., Melgarejo, T., 2008, Innate immunity and host defense peptides in veterinary medicine. *J. Vet. Intern. Med.* 22, 247-265.
- Mancuso, G., Gambuzza, M., Midiri, A., Biondo, C., Papasergi, S., Akira, S., Teti, G., Beninati, C., 2009, Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. *Nat Immunol* 10, 587-594.
- McDonald, T.L., Larson, M.A., Mack, D.R., Weber, A., 2001, Elevated extrahepatic expression and secretion of mammary-associated serum amyloid A 3 (M-SAA3) into colostrum. *Vet. Immunol. Immunopathol.* 83, 203-211.

- Menzies, M., Ingham, A., 2006, Identification and expression of Toll-like receptors 1-10 in selected bovine and ovine tissues. *Vet. Immunol. Immunopathol.* 109, 23-30.
- Mitterhuemer, S., Petzl, W., Krebs, S., Mehne, D., Klanner, A., Wolf, E., Zerbe, H., Blum, H., 2010, *Escherichia coli* infection induces distinct local and systemic transcriptome responses in the mammary gland. *BMC Genomics* 11, 138.
- Molenaar, A.J., Harris, D.P., Rajan, G.H., Pearson, M.L., Callaghan, M.R., Sommer, L., Farr, V.C., Oden, K.E., Miles, M.C., Petrova, R.S., Good, L.L., Singh, K., McLaren, R.D., Prosser, C.G., Kim, K.S., Wieliczko, R.J., Dines, M.H., Johannessen, K.M., Grigor, M.R., Davis, S.R., Stelwagen, K., 2009, The acute-phase protein serum amyloid A3 is expressed in the bovine mammary gland and plays a role in host defence. *Biomarkers* 14, 26-37.
- Nickerson, S., Pankey, J., 1983, Cytologic observations of the bovine teat end. *Am. J. Vet. Res.* 44, 1433.
- Nickerson, S., Pankey, J., Boddie, N., 1984, Distribution, location, and ultrastructure of plasma cells in the uninfected, lactating bovine mammary gland. *J. Dairy Sci.* 51, 209.
- Petzl, W., Zerbe, H., Günther, J., Yang, W., Seyfert, H.-M., Nürnberg, G., Schuberth, H.-J., 2008, *Escherichia coli*, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. *Vet. Res.* 39, 18.
- Rainard, P., Riollot, C., 2006, Innate immunity of the bovine mammary gland. *Vet. Res.* 37, 369-400.
- Rinaldi, M., Cecilian, F., Lecchi, C., Moroni, P., Bannerman, D.D., 2008, Differential effects of α 1-acid glycoprotein on bovine neutrophil respiratory burst activity and IL-8 production. *Vet. Immunol. Immunopathol.* 126, 199-210.
- Rinaldi, M., Li, R., Bannerman, D., Daniels, K., Evock-Clover, C., Silva, M., Paape, M., Van Ryssen, B., Burvenich, C., Capuco, A., 2010, A sentinel function for teat tissues in dairy cows: dominant innate immune response elements define early response to *E. coli* mastitis. *Funct. Integr. Genomics* 10, 21-38.
- Riollot, C., Rainard, P., Poutrel, B., 2000, Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*. *Clin. Diagn. Lab. Immunol.* 7, 161 - 167.
- Rosbacher, Wagner, Pasternack, 1999, Inhibitory Effect of Haptoglobin on Granulocyte Chemotaxis, Phagocytosis and Bactericidal Activity. *Scand. J. Immunol.* 50, 399-404.
- Salonen, M., Hirvonen, J., Pyörälä, S., Sankari, S., Sandholm, M., 1996, Quantitative determination of bovine serum haptoglobin in experimentally induced *Escherichia coli* mastitis. *Res. Vet. Sci.* 60, 88-91.
- Schaer, D., Boretti, F., Schoedon, G., Schaffner, A., 2002, Induction of the CD163-dependent haemoglobin uptake by macrophages as a novel anti-inflammatory action of glucocorticoids. *Br. J. Haematol.* 119, 239-243.
- Schmittgen, T., Livak, K., 2008, Analyzing real-time PCR data by the comparative CT method. *Nat. Protoc.* 3, 1101-1108.
- Schröder, N.W.J., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zähringer, U., Göbel, U.B., Weber, J.R., Schumann, R.R., 2003, Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J. Biol. Chem.* 278, 15587-15594.
- Shim, E.H., Shanks, R.D., Morin, D.E., 2004, Milk loss and treatment costs associated with two treatment protocols for clinical mastitis in dairy cows. *J. Dairy Sci.* 87, 2702-2708.

- Shuster, D., Kehrli Jr, M., Rainard, P., Paape, M., 1997, Complement fragment C5a and inflammatory cytokines in neutrophil recruitment during intramammary infection with *Escherichia coli*. *Infect. Immun.* 65, 3286.
- Skaar, E.P., Humayun, M., Bae, T., DeBord, K.L., Schneewind, O., 2004, Iron-Source Preference of *Staphylococcus aureus* Infections. *Science* 305, 1626-1628.
- Sutra, L., Poutrel, B., 1994, Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*. *J Med Microbiol* 40, 79-89.
- Swanson, K., Gorodetsky, S., Good, L., Davis, S., Musgrave, D., Stelwagen, K., Farr, V., Molenaar, A., 2004, Expression of a β -Defensin mRNA, lingual antimicrobial peptide, in bovine mammary epithelial tissue is induced by mastitis. *Infect. Immun.* 72, 7311-7314.
- Swanson, K.M., Stelwagen, K., Dobson, J., Henderson, H.V., Davis, S.R., Farr, V.C., Singh, K., 2009, Transcriptome profiling of *Streptococcus uberis*-induced mastitis reveals fundamental differences between immune gene expression in the mammary gland and in a primary cell culture model. *J. Dairy Sci.* 92, 117-129.
- Tabrizi, A., Batavani, R., Rezaei, S., Ahmadi, M., 2008, Fibrinogen and ceruloplasmin in plasma and milk from dairy cows with subclinical and clinical mastitis. *Pak. J. Biol. Sci.* 11, 571-576.
- Tetens, J., Friedrich, J., Hartmann, A., Schwerin, M., Kalm, E., Thaller, G., 2010, The spatial expression pattern of antimicrobial peptides across the healthy bovine udder. *J. Dairy Sci.* 93, 775-783.
- Thomas, L., Haider, W., Hill, A., Cook, R., 1994, Pathologic findings of experimentally induced *Streptococcus uberis* infection in the mammary gland of cows. *Am. J. Vet. Res.* 55, 1723-1728.
- Tseng, C.F., Lin, C.C., Huang, H.Y., Liu, H.C., Mao, S.J.T., 2004, Antioxidant role of human haptoglobin. *Proteomics* 4, 2221-2228.
- Uhlir, C.M., Burgess, C.J., Sharp, P.M., Whitehead, A.S., 1994, Evolution of the serum amyloid A (SAA) protein superfamily. *Genomics* 19, 228-235.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3, research0034.
- Werling, D., Piercy, J., Coffey, T.J., 2006, Expression of TOLL-like receptors (TLR) by bovine antigen-presenting cells--Potential role in pathogen discrimination? *Vet. Immunol. Immunopathol.* 112, 2-11.
- Yang, W., Zerbe, H., Petzl, W., Brunner, R.M., Gunther, J., Draing, C., von Aulock, S., Schuberth, H.J., Seyfert, H.M., 2008, Bovine TLR2 and TLR4 properly transduce signals from *Staphylococcus aureus* and *E. coli*, but *S. aureus* fails to both activate NF- κ B in mammary epithelial cells and to quickly induce TNF α and interleukin-8 (CXCL8) expression in the udder. *Mol. Immunol.* 45, 1385-1397.
- Young, F., Platt, D., Logue, D., Ternent, H., Fitzpatrick, J., 2001, Bovine *Staphylococcus aureus* mastitis: strain recognition and dynamics of infection. *J. Dairy Res.* 68, 377-388.
- Zarembek, K.A., Godowski, P.J., 2002, Tissue expression of human toll-like receptors and differential regulation of toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J. Immunol.* 168, 554-561.

648 Figure legends

649

650 Fig. 1 Experimental setup of intra-mammary infection with a low-virulent strain of *S. aureus*
651 isolated from a natural case of subclinical mastitis. qPCR was performed on tissue isolated from
652 the LH quarter of both *S. aureus* challenged and saline non-challenged controls (both quarters
653 are circled).

654

655 Fig. 2 Somatic cell counts in milk from *S. aureus* inoculated (n=6) LH and saline inoculated
656 (n=3) control quarters. Mean values (Error bars = SEM) are presented. Statistical significance is
657 denoted as: * = $P < 0.05$.

658

659 Fig. 3 Tissue expression profile of bovine pattern recognition receptors from mammary tissue 48
660 h post infection with *S. aureus* (a representative result is shown of n = 6).

661

662 Fig. 4 A, B, C and D. Differential gene expression profiles from qPCR analysis on bovine
663 mammary tissue isolated from alveolar, ductal, gland cistern and teat canal regions of *S. aureus*
664 challenged quarters and non-challenged controls. Median fold changes are shown for *S. aureus*
665 challenged animals (n=6) relative to non-challenged controls (n=3). Statistical significance is
666 denoted as: * = $P < 0.05$ and ** $P < 0.01$.

Fig. 1

*S. aureus*Saline solutionChallengedNon-challenged control

Fig . 2

Fig. 3

Fig 4