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Distinct Local and Systemic Innate Immune Gene Expression in Response to *Staphylococcus aureus*-induced Subclinical Mastitis

This thesis is submitted to the University of Dublin for the degree of Doctor of

Philosophy

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October 2012

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DECLARATION

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Cormac Whelehan

October 2012

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Abbreviations

°C	Degrees celsius
Aa	Amino acid
ACTB	Actin, beta
AGP	Alpha 1 acid glycoprotein
AGP	α-1-acid glycoprotein
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APP	Acute phase protein
APR	Acute phase response
BIR	Baculovirus inhibitor of apoptosis protein repeat
BLAST	Basic local alignment search tool
BLAT	BLAST-like alignment tool
bp	Base pairs
CARD	Caspase-activating and recruitment domain
CATHL	Cathelicidin
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU	Cell forming units
CP	Ceruloplasmin
CRP	C-reactive protein
DC	Dendritic cell
DEFB	Defensin, beta
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides triphosphate
dsRNA	Double-stranded RNA

ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FPR	Formyl peptide receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	Hour
H3F3A	H3 histone, family 3A
HDP	Host defence peptide
HNP	Human neutrophil peptide
HP	Haptoglobin
ICAM	Intracellular adhesion molecule
IEL	Intestinal epithelial cell
IFN	Interferon
IGFG	Interferon, gamma
IKB	I-kappa-B
IKK	IκB kinase
IL	Interleukin
IL1β	Interleukin 1, beta
ILF	Isolated lymphoid follicle
IPAF	Ice protease-activating factor
IRAK	Interleukin-1 receptor associated kinase
IRF	Interferon regulatory factor
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
LAP	Lingual antimicrobial peptide
LBP	Lipopolysaccharide binding protein
LFA	Lymphocyte function-associated antigen
LOS	Lipooligosaccharide

LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LTA	Lipoteichoic acid
M	Molar
MAL	MyD88-adapter like
MAPK	Mitogen-activated protein kinase
Mb	Megabase
MCP	Monocyte chemotactic protein
MD2	Myeloid differentiation protein-2
MDA	Melanoma differentiation-associated
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene (88)
NALP	Nacht LLR protein
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa-B
ng	Nanogram
NK	Natural killer cell
NLR	Nod-like receptor
nM	Nanomolar
NOD	Nucleotide-binding oligomerization domain
ORF	Open reading frame
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PGN	Peptidoglycan
pH	Power of hydrogen
pi	Post infection
PMN	Polymorphonuclear
PPIA	Peptidylprolyl isomerase A
PRR	Pattern recognition receptor
qPCR	Quantitative real-time polymerase chain reaction
RANTES	Regulated upon activation, normally T-expressed, and presumably secreted
RLR	Rig-like receptor
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPS	Ribosomal protein Subunit
SAA	Serum Amyloid A
SC	Somatic cells
SCC	Somatic cell count
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
ssRNA	Single stranded RNA
TAK	Transforming growth factor activated protein kinase
TAP	Tracheal antimicrobial peptide
TGF	Transforming growth factor
TGFB1	Transforming growth factor, beta 1

TIR	Toll/interleukin-1 receptor
TLAK	Lymphokine-activated killer T-cell-originated protein kinase
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
Tollip	Toll interacting protein
TRAF	TNF-receptor associated factor
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
VCAM	Vascular cell adhesion molecule
µg	Microgram
µl	Microlitre
µM	Micromolar

Abstract

Both local and systemic innate immune activity has a critical role in maintaining immunological homeostasis in the mammary gland. Dysregulation of the homeostatic process leads to increased infection and related pathology, either of which will affect quality and quantity of milk. Infections and inflammations of the mammary gland (mastitis) represent the most frequent and costly disease in dairy cows. Chronic, subclinical infections are of particular relevance. They impair the well-being of the host and cause ~80% of all mastitis related costs in dairy industries. *Staphylococcus aureus* is the bacteria most commonly isolated from cases of subclinical mastitis. However, limited information has been published to date on the innate immune response to *S. aureus*-induced subclinical mastitis.

The initial aim of this study was to investigate both the local and systemic immune response in an experimentally-induced model of *S. aureus* subclinical mastitis. The expression of candidate innate immune genes was profiled in mammary tissue isolated from *S aureus* challenged quarters and in liver tissue from the same animals. Expression of the proinflammatory cytokine *IL6* was significantly increased in *S.aureus* challenged quarters (5.1-fold) 48 h post-challenge compared to control animals. In contrast, *IL1B* and *TNF* showed no significant differential expression in challenged quarters. The chemokine, *IL8*, showed a strong and significant increase in *S. aureus* challenged quarters (13-fold,) with no significant differential expression detected in neighbouring quarters. *TGFB* was significantly induced in unchallenged quarter (1.8-fold).

Expression of acute phase protein (APP) genes was profiled in liver tissue. Expression of serum amyloid A (*SAA*) and haptoglobin (*HP*) was significantly elevated 48 h after challenge compared with control animals (13- and 14.8-fold respectively). Expression of α-1-acid glycoprotein (*AGP*) and ceruloplasmin (*CP*) was significantly decreased (4.3- and 2.4-fold respectively).

From our initial observations, we hypothesised that the systemic response had an influence on neighbouring unchallenged quarters in animals with subclinical mastitis. To test this we profiled the expression of proinflammatory cytokines, antimicrobial peptides (AMPs) and APP genes in tissue from unchallenged quarters of infected animals. Expression of *IL6* was significantly increased in unchallenged quarters (19.2-fold) compared to control animals. The

APPs, *SAA3* and *HP* were significantly induced in unchallenged neighbouring quarters (9.4- and 9.7-fold respectively).

Following on from our initial study, a comprehensive quantification of innate immune genes was carried out on mammary tissue from the same infection model. Expression of genes encoding PRRs, pro- and anti-inflammatory cytokines, APPs and AMPs in tissue from the alveolar, ductal, gland cistern and teat canal regions of control and *S. aureus* challenged mammary quarters were profiled.

Consistent expression of Toll-like receptors (TLRs) 1-10 and NOD-like receptors (NODs) 1-2 was detected in all 4 tissue regions. Pro-inflammatory cytokines (*IL6*, *IL17* and *IL8*) and the anti-inflammatory cytokine (*IL10*) were induced in all 4 tissues in response to infection. Acute phase protein (*SAA3* and haptoglobin (*HP*) and defensin (*DEFB4* and *DEFB5*) genes showed the greatest induction throughout the mammary gland in response to *S. aureus*, with particularly high expression in alveolar tissue of *SAA3* and *HP* (>1000 and >500 fold respectively, $P < 0.05$) and the defensins *DEFB4* and *DEFB5*(>20 and >40 fold respectively, $P < 0.05$).

We proposed that the mammary epithelial cell was a primary contributor to early immune activity in response to *S. aureus* infection. We examined innate immune gene expression in primary cultures of mammary epithelial cells following challenge with heat inactivated *S. aureus*. TLRs 1-10 and NODs 1-2 were also expressed in *S. aureus* stimulated mammary epithelial cells. Expression of TLRs 2, 4 and 6 was moderately increased in response to *S. aureus*. Recognition was further confirmed by increased expression of the proinflammatory cytokines *TNFA*, *IL1B*, *IL6*, and chemokine *IL8*. *SAA3* and *HP* were also significantly induced.

Finally, we used a bioinformatics approach to characterise the entire repertoire of cathelicidin, important antimicrobial peptides, encoded in the bovine genome. Bioinformatic analysis revealed seven protein-coding cathelicidin genes and three pseudogenes clustered on chromosome 22. We profiled their expression in a range of bovine tissues. All tissues expressed at least one cathelicidin, and several expressed multiple protein-coding genes (spleen, lung and liver tissue). *CATHL4* and *CATHL5* expression was detected in mammary tissue. To further investigate the role of cathelicidins in the mammary gland, we profiled their expression in *S. aureus* stimulated mammary epithelial cells and in milk leukocytes from naturally occurring mastitis infection. *CATHL4* was significantly increased as early as 30 min post stimulation with

S. aureus (> 60-fold). Analysis of cathelicidin expression in milk leukocytes revealed a high level of expression of all seven protein-coding cathelicidins.

Our results show that intramammary infection of the bovine mammary gland with *S. aureus* induces both a local and systemic immune response. Furthermore, the immune response induced in *S. aureus* inoculated quarters has an influence on the expression of innate immune genes in unchallenged quarters which we believe to be mediated systemically. This has important implications for the design of appropriate models to study bovine mastitis. In contrast to *TNF* and *IL1B*, *IL-6* expression was significantly induced throughout the mammary gland. IL-6 is one of the key mediators of the acute phase response and may be a key cytokine involved in activation of the systemic response in our model. 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.

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 that these molecules are important in the pathogenesis of mastitis caused by *S. aureus* in cattle. Finally, our results suggest that cathelicidins may be useful biomarkers of infection.

Chapter 1: General Introduction

1.1 Introduction

Among the ailments that affect dairy ruminants, infection and inflammation of the mammary gland (mastitis) plays a prominent part. Mastitis causes major economic losses through reduction in milk yield and milk unfit for consumption and is a major cause of premature culling. In addition to the agricultural cost, continuous use of antibiotics is contributing to the emergence of antibiotic resistant strains of micro-organisms which can infect many species including humans. Milk and dairy products are an essential source of food for the majority of the world population. To meet the growing global demand and to keep the dairy farming profitable at the same time, prevention and treatment of mastitis are primary concerns of the dairy industry. In spite of the efforts made to control it, the rate of mastitis incidence has been quite stable for years and the highest of all the cattle diseases. Furthermore, as a result of the long-lasting feature of subclinical mastitis, the most common form of the mastitis, its prevalence in dairy herds is a major concern on an international scale.

Development of a vaccine against mastitis has long been an attractive option, but at present the panoply of mastitis vaccines are not very efficient (Middleton *et al.*, 2009). Earlier detection of mastitis will improve the well-being of animals by allowing timely and efficient treatment. The application of biomarker-based assays, developed within the last decade, has already shown considerable promise for mastitis detection (Viguier *et al.*, 2009). Selection of animals genetically resistant to mastitis for subsequent breeding is another area of research gaining momentum, with data suggesting that enhanced expression of certain antimicrobial genes increases resistance to mastitis (Compton *et al.* 2009). In fact, genetic selection for increased milk yield and better composition has resulted in increased susceptibility to mastitis (Fleischer *et al.*, 2001). Studies have also confirmed an antagonistic association between genetic merit for yield and fertility (Hageman *et al.*, 1991; Snijders *et al.* 2000).

Innate immunity is a target of choice for the development of new biomarkers and for selection against infectious diseases. The broad diversity of its mechanisms offers many potentialities. A preliminary to devising alternative management strategies for reversing the increase in susceptibility to mastitis lies in advancing our understanding of innate immune

responses during mastitis infection, in particular, subclinical mastitis, and thus forms the basis of this thesis.

1.2 Overview of the immune system

Living organisms are constantly exposed to a wide range of microbial species which seek to promote their proliferation by accessing the nutritional environment of host cells. As a result, all life forms have evolved strategies that are designed to limit the invasion of the host by microorganisms. These strategies, collectively called the immune system, work by distinguishing between 'self' and 'non-self', and consequently eliminating any substance found to be foreign.

The immune system can be divided into two broad categories – the innate immune system and the adaptive immune system. Plants, fungi and lower multicellular organisms rely exclusively on the innate immune system, whereas vertebrates rely on both innate and adaptive immunity to protect the host. While the precise characteristics of the innate immune systems differ between various species, they all share several core features: responce is rapidly induced and targets whole classes of invading pathogens through the expression of germ-line encoded pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). In contrast, the adaptive immune system, which selectively expands antigen-specific clones from a pool of T and B cells harboring unique antigen receptors, is highly specific, sustainable and able to form immunological memory which can efficiently deal with subsequent re-challenge by a previously encountered pathogen. Although the adaptive immune response is initiated and directed by specific mediators of the initial innate response, both innate and adaptive immunity are intricately entwined with both systems sharing many effector mechanisms.

Innate immunity itself is multifaceted and functional at all times. This includes, anatomical barriers, such as skin and mucous membranes that prevent the entry and colonisation of pathogens. In addition, certain bodily secretions, such as saliva, tears and sweat can wash away invading microbes. However, if microorganisms, such as bacteria breach these anatomical and physical barriers they are met immediately by cells and molecules that initiate a process known as inflammation.

1.3 Inflammation

Inflammation is the immune system's response to infection and injury. The acute phase of inflammation is characterized by the rapid influx of leucocytes, typically neutrophils, followed a short time later by monocytes that rapidly differentiate into macrophages upon entering the tissue. The migration of leukocytes into the tissue and their subsequent actions cause the chief signs of acute inflammation - *rubor* (redness), *calor* (heat), *tumor* (swelling), and *dolor* (pain). Typically, acute inflammation resolves following the removal of the initiating stimulus. During the resolution, neutrophils are eliminated; macrophages and lymphocytes return to pre-inflammatory numbers and function, and tissue damage is repaired. This inflammatory process requires extremely tight regulation as insufficient inflammatory responses can hinder pathogenic clearance or, in the other extreme, damage host tissues which can lead to scarring and loss of physiological function.

1.4 Cells of the innate immune response

The cells of the immune system, collectively known as leukocytes (white blood cells), originate in the bone marrow and are derived from hematopoietic stem cells, which also give rise to erythrocytes (red blood cells) and platelets (Fig. 1.1). The myeloid progenitor is the precursor of macrophages, neutrophils and dendritic cells (DCs), and the lymphoid progenitor gives rise to natural killer cells (NK). These cells can exert rapid effector functions through a limited repertoire of germ-line encoded receptors.

The cells of the innate immune system play a crucial part in the initiation and subsequent direction of adaptive immune responses, as well as participating in the removal of pathogens that have been targeted by an adaptive immune response. Moreover, because some time is required to achieve the specific adaptive immune response, typically 4-7 days, innate immunity provides the first line of defence during the critical period just after the host's exposure to a pathogen (Janeway, 2005).

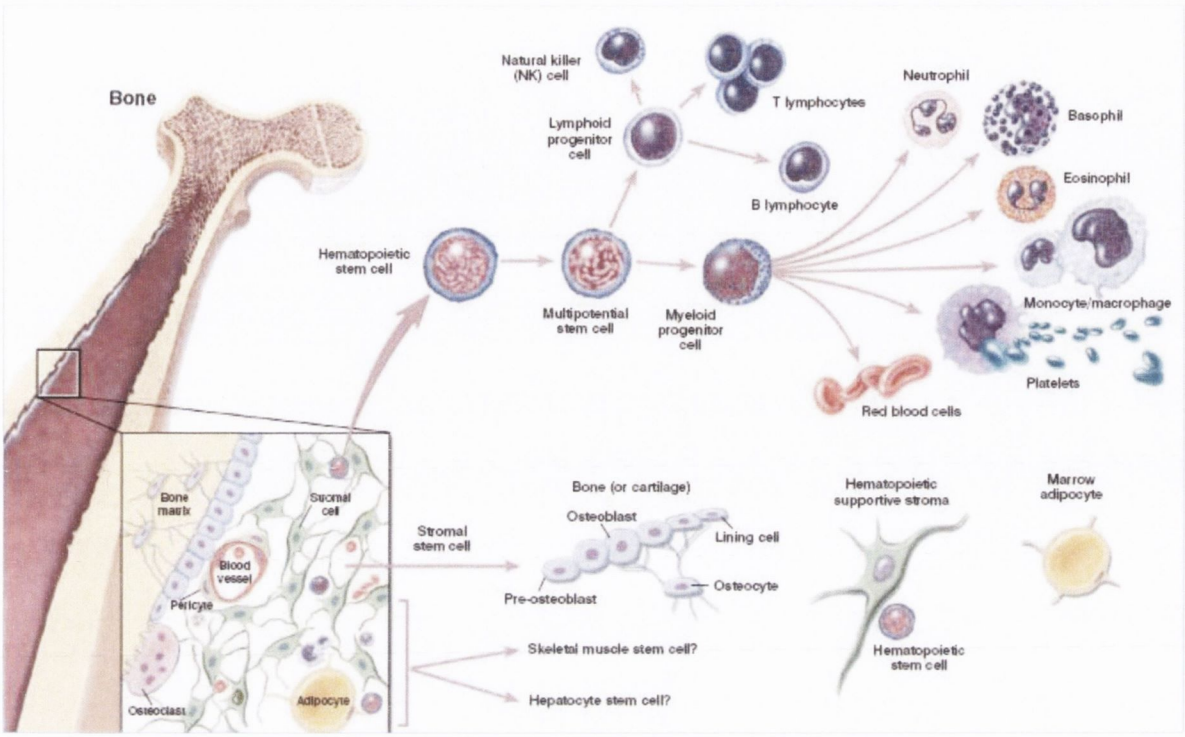


Figure 1.1 Derivation of circulating innate and adaptive immune cells from hematopoietic stem cells (HSC)

The HSC can give rise to the hematopoietic progenitor cells (HPC) which in turn give rise to the lymphoid progenitor cell and the myeloid progenitor cells. The lymphoid progenitor gives rise to both T and B lymphocytes and natural killer cells (NK), and the myeloid progenitor gives rise to macrophages, neutrophils, dendritic cells, eosinophils and basophils. Figure taken from (http://stemcells.nih.gov/).

1.4.1 Macrophages

Macrophages are phagocytic cells that are distributed widely in both lymphatic and non-lymphatic tissue where they play an essential role in innate immunity. They are constantly replenished by circulating monocytes that differentiate into macrophages upon migration into tissue (Mosser and Edwards, 2008). Large numbers are recruited by chemotaxis to sites of immune-cell signalling where they can engulf and destroy pathogens. This is mediated through the expression of a broad range of cell PRRs which, upon ligand engagement, initiate phagocytosis and production of pro-inflammatory cytokines. Once internalized, the pathogen is neutralized by the secretion of a wide variety of toxic substances including nitric oxide (NO), and hydrogen peroxide (H_2O_2) (Dale *et al.*, 2008). Macrophages also initiate adaptive immune responses by acting as antigen presenting cells (APCs) by presenting peptides generated from microbial proteins to T-lymphocytes (Hume, 2008). In addition to their important role as immune effector cell, macrophages are important in the maintenance of tissue homeostasis, via the clearance of apoptotic cells, and the production of growth factors (Mosser and Edwards, 2008).

1.4.2 Dendritic cells

Circulating monocytes can also give rise to dendritic cells (DCs). DCs are present in low numbers in a range of tissues and primarily function as antigen presenting cells (APCs) (Wan and Dupasquier, 2005). They are highly migratory cells that can move from tissues to the T cell and B cell zones of lymphoid organs. In their inactive immature form, they are highly phagocytic through their expression of high levels of PRRs (Geijtenbeek *et al.*, 2004). Following phagocytosis and the production of high levels of proinflammatory cytokines, DCs become activated, initiating migration from the tissue to the lymph node (Randolph *et al.*, 2005). Here they present their phagocytosed microbial components to naive T-lymphocytes, thus initiating the adaptive immune response (Itano and Jenkins, 2003).

1.4.3 Neutrophils

Neutrophils are granulocytic phagocytes, whose primary role is to engulf and eliminate pathogens, and are the major cell population in the acute inflammatory response (Dale *et al.*, 2008). Similar to macrophages, they possess mechanisms for microbial detection, phagocytosis and production of potent chemicals which play a key role in destroying the invading microorganisms (Segal, 2005). They circulate in the blood and rapidly move into tissues in response to chemotactic stimuli such as *N*-formyl peptide, an amino acid found in prokaryotic organelles (Mantovani *et al.*, 2011). Upon arrival at the site of inflammation, PRRs engage the pathogen and activate the effector functions of neutrophils. These include the production of reactive oxygen intermediates (ROI), lytic enzymes and antimicrobial peptides (Nathan, 2006). In addition, neutrophils augment the inflammatory response through the production of chemokines and cytokines that attract activated monocytes, other neutrophils and T cells, and also to support wound healing (Mantovani *et al.*, 2011).

1.4.4 Natural killer (NK) cells

Another important cell in innate immunity is the NK cell. NK cells are derived from the same lymphoid progenitor cell as T and B lymphocytes (Fig. 1.1) However, in contrast to T and B cells, they lack the antigen specificity that is the hallmark of the adaptive immune response. NK cells circulate in the blood and like other leukocytes, are recruited to sites of inflammation by chemotaxis. Although neutrophils and macrophages are well equipped to seek out and eliminate extracellular pathogens, NK cells are essential in the eradication of intracellular pathogens, including viral, bacterial and parasitic infections. Additionally, they recognize and kill abnormal cells, for example tumor cells (Vivier *et al.*, 2011). Their activation is regulated by a series of inhibiting or activating signals that result in specific killing of undesirable cells. Cells that do not express self markers, such as virus-infected and tumour cells are killed by the release of cytotoxic proteins. In contrast, normal cells expressing self markers are recognised by inhibitory molecules expressed on NK cells, and are therefore protected from NK cell-mediated killing (Yokoyama and Plougastel, 2003).

1.4.5 Mucosal epithelium

Mammalians comprise several mucosal systems, including the respiratory tract, the gastrointestinal tract, the oral cavity, the genitourinary tract, the skin, eye and the mammary gland. These systems have evolved to carry out their job of allowing exchange with the external environment, while protecting us from pathogenic assault. Epithelia, originally perceived as mere physical barriers at these sites, are now known to play an integral, multi-faceted role in mucosal immunity through the expression of PRRs, inflammatory mediators and antimicrobial peptides and have essential roles in both innate and adaptive immune responses (Maldonado-Contreras and McCormick, 2011). A representation of the role of epithelial cells in innate immunity is presented in figure 1.2.

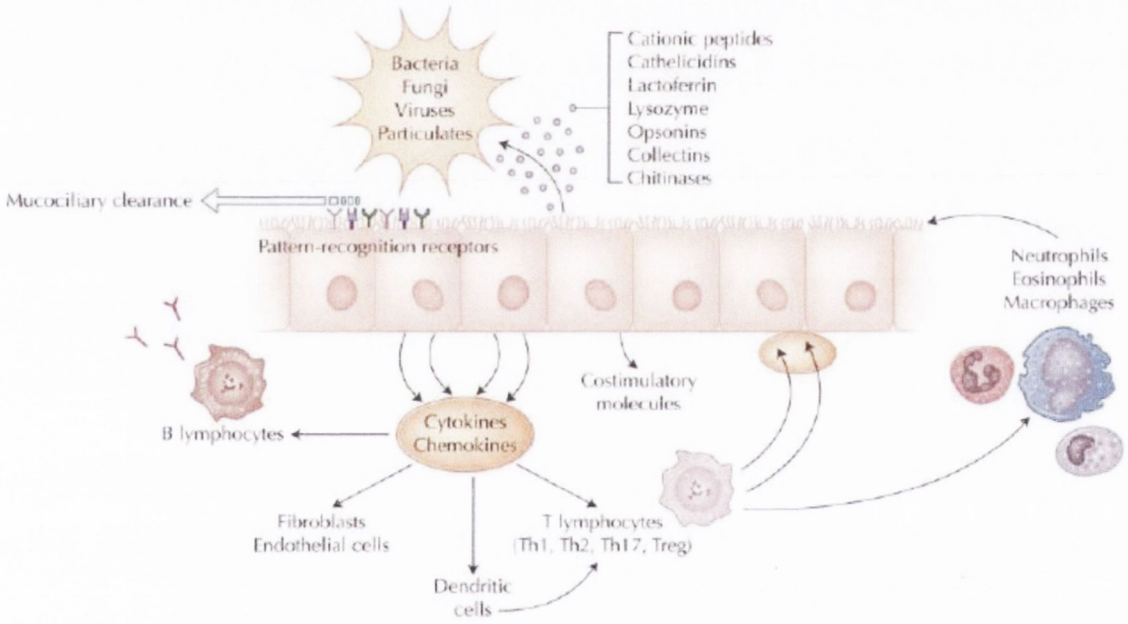


Figure 1.2 Innate immunity of the sinonasal tract

In addition to mucociliary clearance, sinonasal epithelial cells actively participate in innate immunity using pattern-recognition receptors (PRRs) to detect luminal pathogens and respond directly with selective expression of targeted antimicrobial effectors. At the same time, epithelial cells signal to adaptive immune cells through cytokines and costimulatory molecules to coordinate a vigorous defense of the mucosal surface. Figure taken from (Lane, 2009).

1.5 Pattern recognition receptors

Phagocytic cells, NK cells and epithelial cells sense pathogens through the use of cell-surface proteins called pathogen recognition receptors (PRRs). These receptors bind to conserved microbial components, known as pathogen-associated molecular patterns (PAMPs) and induce innate immune responses. There are several functionally distinct classes of PRRs, which includes the toll-like receptors (TLRs) and NOD-like receptors (NLRs). Once engaged, these PRRs can induce the production pro-inflammatory cytokine, chemokines and antimicrobial peptides (Takeuchi and Akira, 2010).

1.5.1 Toll-like receptors

The TLR family is one of the best-characterized PRR families to date. They are characterized by N-terminal leucine-rich repeats (LRRs), which confers ligand recognition, and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain which propagates internal signalling (Kawai and Akira, 2010). Being evolutionary conserved, they represent an important host defense mechanism of invertebrates and vertebrates. So far, 13 TLRs have been identified in mammals with 10 TLR genes present in most mammals. Ten TLRs have been identified in the chicken, three of which are unique to birds (Higgs *et al.*, 2006). Seventeen TLR genes are present in all bony fishes investigated to date (Rebl *et al.*, 2010) and 222 TLRs have been identified in the sea urchin genome (Rast *et al.*, 2006). Although primarily expressed in immune cells, including both lymphocytes and APCs such as macrophages and DCs, TLRs are also expressed in nonimmune cells, including epithelial cells.

TLRs can be broadly divided in two groups based on their cellular location. In humans, TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface and mainly detect bacterial molecules. The other group is composed of TLR3, TLR7, TLR8 and TLR9 which are expressed in endosomes where they recognise nucleic acids (Takeuchi and Akira, 2010).

The TLR4 receptor, the founding member of the TLR family, binds lipopolysaccharide (LPS) which is a component of the outer membrane of gram-negative bacteria (Poltorak *et al.*, 1998). TLR4 forms a complex with MD2 on the cell surface were they form a TLR4-MD2-LPS complex (Park *et al.*, 2009) and upon interaction, the resulting conformation changes initiate intracellular signalling transduction through the recruitment of adaptor molecules (Fig. 1.3). The proteins, CD14 and LBP, are also involved in the formation of the TLR4-MD2-LPS complex.

LBP binds LPS and CD14 binds LBP and delivers LBP-LPS to the TLR4 complex (Park *et al.*, 2009). As well as binding LPS, TLR4 has been proposed to be a functional receptor for various ligands both exogenous and endogenous, some of which include respiratory syncytial virus fusion proteins and the acute phase protein serum amyloid A (Klouwenberg *et al.*, 2009; Sandri *et al.*, 2008).

In most mammals, TLR2 forms heterodimers with TLR1 or TLR6 and as a result can recognise a broad range of pathogen-associated molecular patterns (PAMPs) derived from diverse microorganisms including bacteria, virues, parasites and fungi (Takeuchi and Akira, 2010). The TLR2-TLR1 heterodimer recognizes triacylated lipopeptides from Gram-negative bacteria and the TLR2-TLR6 heterodimer recognizes diacylated lipopeptides from Gram-positive bacteria (Fig. 1.3) (Takeuchi *et al.*, 2001; Takeuchi *et al.*, 2002). In bovine, TLR2 and TLR1 complexes respond to both triacylated and diacylated lipopeptides (Werling *et al.*, 2009). TLR5 detects the flagellin protein component of bacterial flagella from both gram-positive and gram-negative bacteria (Hayashi *et al.*, 2001).

The intracellular TLRs (Fig 1.4) are involved in the recognition of microbial nucleic acids. TLR3 recognises double-stranded RNA (dsRNA) resulting in the activation of antiviral immune responses through the production of type I interferons such as IFNα and IFNβ. Originally identified as recognising a synthetic dsRNA poly (I:C), it is now known to be involved in the recognition of reoviruses, dsRNA produced during the replication of single-stranded RNA (ssRNA) viruses and certain small interfering RNAs. Thus, TLR3 has an essential role in the prevention of viral infection (Kawai and Akira, 2010). TLR7 recognises ssRNA derived from viruses such as influenza A and the human immunodeficiency virus. TLR8 is phylogenetically most similar to TLR7 and also recognises viral dsRNA. TLR8 is expressed in various tissue, with the highest expression levels detected in monocytes (Heil *et al.*, 2004). TLR9 recognizes unmethylated 2′-deoxyribocytidine-phosphateguanosine (CpG) DNA motifs that are present in bacteria and viruses but are rare in mammalian cells (Fig. 1.4) (Hemmi *et al.*, 2000; Hochrein *et al.*, 2004). It is highly expressed in plasmacytoid dendritic cells (pDC), thus allowing for heightened detection of circulating DNA viruses.

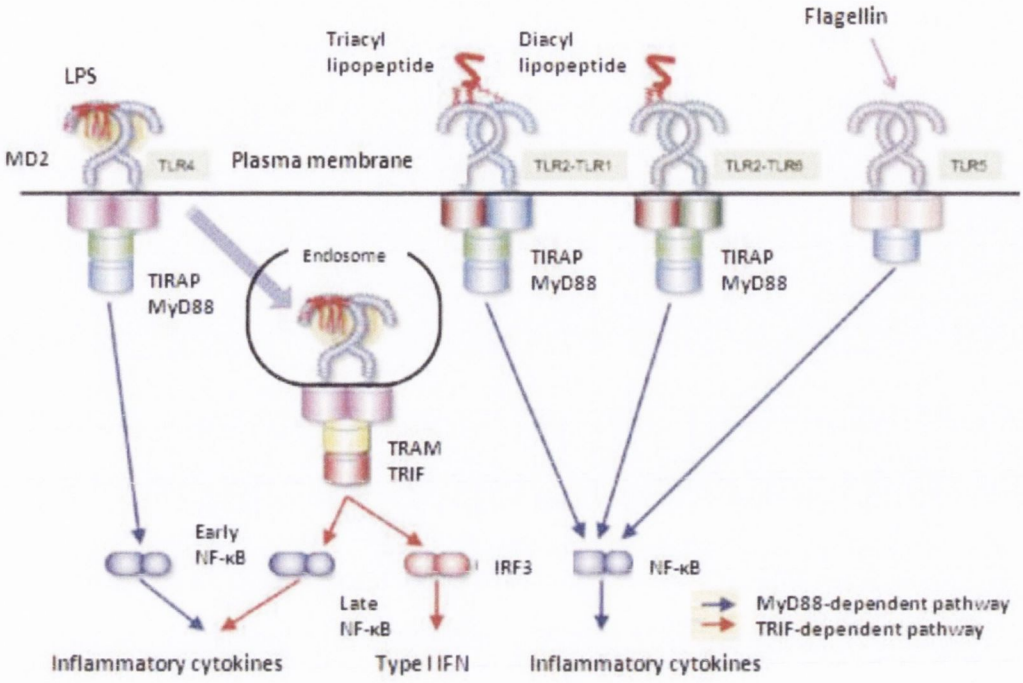


Figure 1.3 PAMP recognition by cell surface TLRs

TLR4-MD2-LPS complex initiates signals that culminate in induction of type I interferons and inflammatory cytokines. TLR2-TLR1 and TLR2-TLR6 heterodimers recognize triacylated and diacylated lipopeptide respectively to induce inflammatory cytokines. TLR5 recognizes flagellin resulting in the activation of inflammatory cytokines. Figure taken from (Kawai and Akira, 2010).

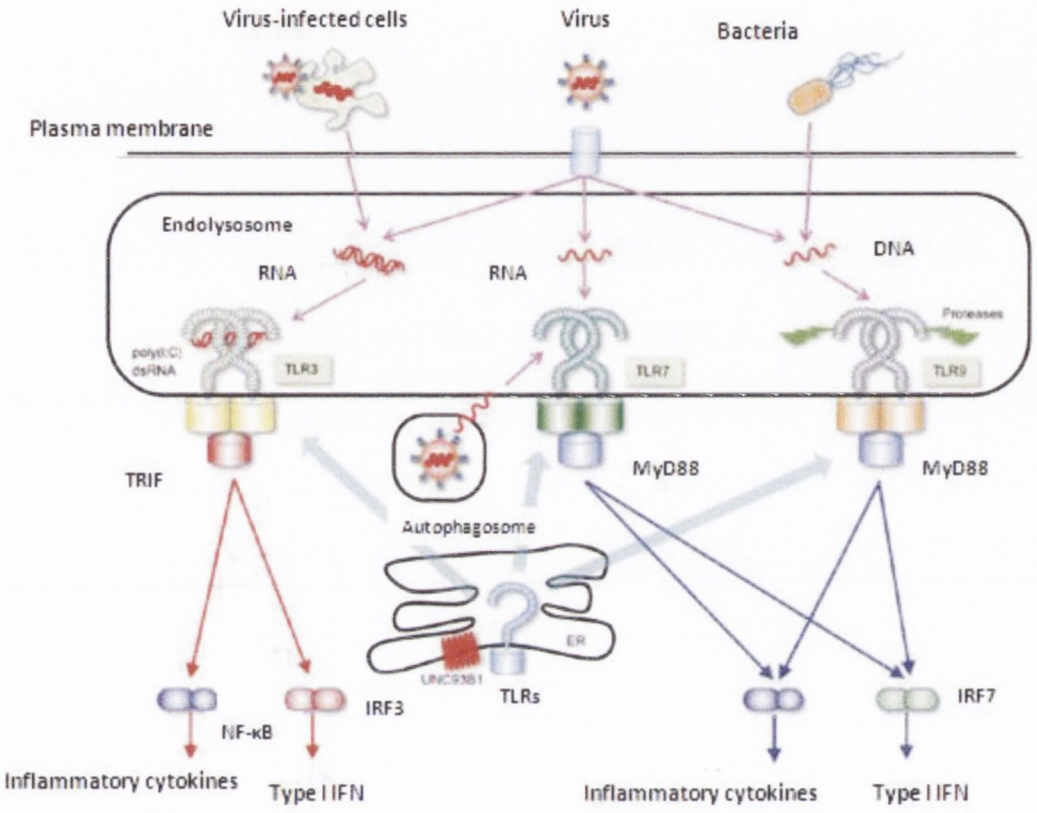


Figure 1.4 PAMP recognition by intracellular TLRs

TLR3 recognizes dsRNA derived from viruses or virus-infected cells, resulting in activation of type I interferon and inflammatory cytokines. TLR7 recognizes ssRNA derived from ssRNA viruses in endolysosomes to induce inflammatory cytokines and type I interferon and TLR9 recognizes DNA derived from both DNA viruses and bacteria. Figure taken from (Kawai and Akira, 2010).

1.5.2 TLR signalling

Ligand recognition results in a structural change in the TLR's TIR region, allowing the recruitment of TIR domain-containing adaptor molecules. At least five TIR domain-containing adaptor molecules are involved in the signal transduction of TLRs: myeloid differentiation primary response gene (88) (MyD88), TIR domain-containing adaptor inducing IFN-b (TRIF), TIR domain-containing adaptor protein (TIRAP), TRIF-related adaptor molecule (TRAM) and Sterile-alpha and Armadillo motif-containing protein (SARM) (Takeuchi and Akira, 2010). With the exception of TLR3 which uses the TRIF pathway, all TLRs use the MyD88 pathway. TLR4 also has a MyD88 independent pathway which uses the adaptor molecule TRAM and TRIF sequentially (O'Neill, 2006). Following engagement of TLRs by their associated PAMPs, MyD88 recruits the IL-1 receptor–associated kinase (IRAK) family members (Fig. 1.5). IRAK activation results in an interaction with TRAF6, which subsequently activates the transforming growth factor activated protein kinase 1 (TAK1) complex. TAK1 is involved in two different downstream responses. Firstly, TAK1 activates the I-kappa-B kinase (IKK) complex which leads to nuclear factor–kappa B (NF-κB) activation via phosphorylation and subsequent degradation of IκB proteins, which allows NF-κB to enter the nucleus to begin transcriptional regulation through binding to its cognate promoter region sites. TAK1 simultaneously activates the mitogen-activated protein kinase (MAPK) transcription factors, extracellular signal–regulated protein kinase 1 (ERK1), ERK2, p38 and c-Jun N-terminal kinase (JNK) which then activate various transcription factors resulting in transcriptional upregulation of pro-inflammatory cytokines.

The TRIF-dependent pathway (Fig. 1.5) culminates in the activation of interferon regulatory factor 3 (IRF3) and NF-κB, resulting in the transcriptional upregulation of inflammatory cytokine and type I interferon genes (Kumar *et al.*, 2009a)

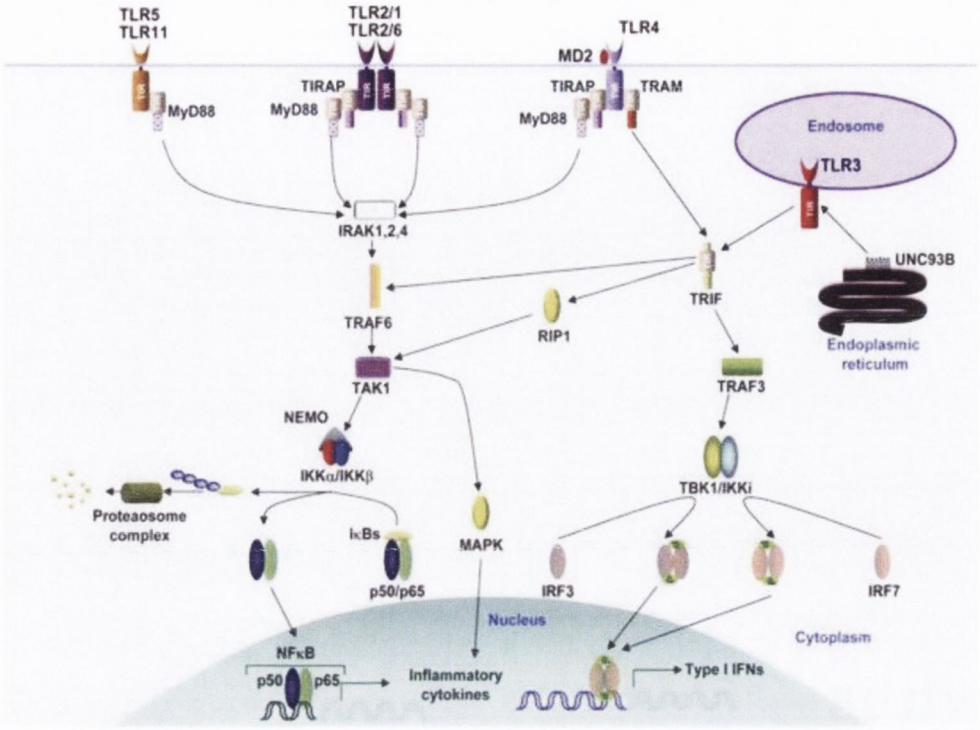


Figure 1.5 TLR signalling pathways

TLR activation may initiate MyD88 or MyD88-independent signalling pathways. The former pathway results in the activation of NF-κB and MAPK, which results in proinflammatory cytokine gene expression upregulation. The latter pathway primarily results in the activation of IRF transcription factors, resulting in type I IFN gene expression upregulation. Figure adapted from (Kumar *et al.*, 2009b).

1.5.3 NOD-like receptors

Another important family of PRRs are the cytosolic nucleotide binding oligomerization domain (NOD)-like receptors (NLR) (Takeuchi and Akira, 2010). In humans, the NLR family is composed of 22 proteins and there are at least 33 NLR genes in mice (Chen *et al.*, 2009). Similar to TLRs, they are expressed not only in macrophages and DCs but also in various non-professional immune cells. NLRs are generally composed of three separate domains, a central NOD domain, a C-terminal LLR domain that is required for ligand recognition, and a variable N-terminal domain that mediates the activation of downstream signalling. The caspase recruitment domain (CARD) containing NLRs, NOD1 and NOD2, were the first intracellular NLRs to be reported and thus are the best characterised NLRs to date. NOD1 is activated by γ-D-glutamyl-

meso-diaminopimelic acid (meso-DAP/iE-DAP) and NOD2 by muramyl dipeptide (MDP), both of which are derived from the bacterial cell wall component peptidoglycan (PGN) (McDonald *et al.*, 2005). Recognition results in the activation of NF-κB and MAPK via RIP-like interacting CLARP kinase (RICK; also known as RIP2) adaptor molecule, culminating in the upregulation of proinflammatory cytokines (Chen *et al.*, 2009). In addition, NOD2 can also induce type I interferon in response to viral infections. TLRs also recognize bacterial peptidoglycan components suggesting that both TLRs and NODs synergistically activate proinflammatory cytokines (Kanneganti *et al.*, 2007).

NLRs harbouring a pyrin domain or a baculovirus inhibitory repeat (BIR) domain in their N terminus, are not involved in the transcriptional activation of inflammatory mediators but are components of large multimeric complexes, known as inflammasomes, which regulate caspase-1 activation. Caspase-1 activation, in turn, is required to generate and secrete interleukin-1-beta (IL-1β), a highly proinflammatory cytokine (Fig 1.7 B) (Chen *et al.*, 2009).

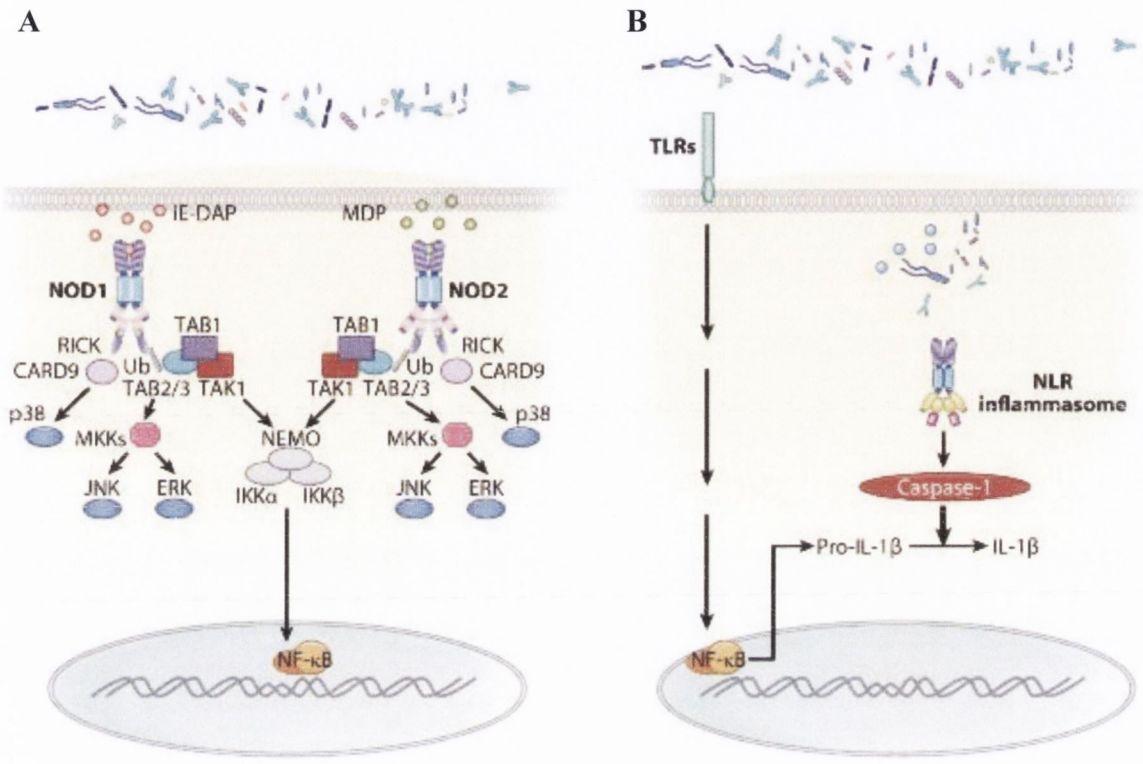


Figure 1.6 PAMP recognition by intracellular NLR and PRR synergy

(A) The NLR proteins NOD1 and NOD2 sense intracellular γ-D-glutyamyl-meso-DAP (iE-DAP) and muramyl dipeptide (MDP), respectively, leading to recruitment of the adaptor proteins RICK and caspase recruitment domain 9 (CARD9). Subsequently, both TLRs and NOD1/NOD2 signaling pathways recruit TAK1, which mediates the activation of nuclear factor–kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs), resulting in the transcriptional upregulation of proinflammatory genes. (B) Activation of NLRs by microbial or endogenous molecules in the cytosol results in the formation of caspase-1-activating inflammasomes. Activation of caspase-1 induces processing of the (IL-1β) precursor and secretion of the mature cytokine. Figure adapted from (Chen *et al.*, 2009).

1.6 Mediators of the immune response

In response to recognition of a microbe, innate immune cells are activated to secrete a variety of proteins capable of directing an appropriate immune response. These proteins are collectively called cytokines. Cytokines are soluble proteins that are released by cells and have an effect on other cells that bear receptors for them (Janeway, 2005). Most cytokines have more than one function and often have redundant effects with other cytokines. Because of the high affinity of their receptors, cytokines are highly potent and can elicit biological responses when present in small concentrations (Gouwy *et al.*, 2005). One of the primary mechanisms by which they exert their effects is through the activation of intracellular signalling pathways resulting in the regulation of specific gene expression which affects an array of biological processes. These include chemotaxis, cellular activation and proliferation of cells, and expression of molecules that are involved in antimicrobial activity (Janeway, 2005). Although cytokines play an essential role in the host response to infection, they can have deleterious effects on the host. Some of the major cytokines that are key to the inflammatory response include, tumour necrosis factor-α (TNF-α), IL1-β, interleukin-6 (IL-6), interferon gamma (IFN-γ), IL-8, interleukin-10 (IL-10) and transforming growth factor beta (TGF-β).

1.6.1 Tumour Necrosis Factor-α

TNF-α is the best-known member of the TNF-family. It is produced by multiple cell types, including macrophages, lymphocytes, neutrophils, and epithelial cells. Inducers of TNF-α include viral, fungal, and parasitic pathogens, bacterial wall products and toxins, cytokines and complement components (Papadakis and Targan, 2000). The primary role of TNF-α is the regulation of immune cells. At the site of infection, TNF-α promotes endothelial activation and the recruitment of leukocytes to the site of infection, with subsequent activation of these cells on arrival (Dale *et al.*, 2008). The systemic effects of TNF-α include the induction of fever and acute phase protein synthesis (Jensen and Whitehead, 1998). Although these local and systemic effects are beneficial to the host's immune defense against infection, TNF-α is associated with heightened inflammatory responses that can threaten the life of the host. A means of inhibiting these deleterious effects is through the TNF-α mediated production of the anti-inflammatory

cytokine, IL-10, which has a suppressive effect on TNF-α production (Papadakis and Targan, 2000).

1.6.2 Interleukin-1 beta

The proinflammatory cytokine IL-1β mediates both local and systemic inflammatory responses. Similar to TNF-α, the biological effects of IL-1β include up-regulation of E-selectin and ICAM-1 (endothelial activation), activation of leukocytes, and systemic induction of fever and acute phase protein synthesis (Jensen and Whitehead, 1998). Several cell types have been identified as sources of IL-1β, including monocytes, macrophages, dendritic cells, lymphocytes, fibroblasts, and both endothelial and epithelial cells (Papadakis and Targan, 2000). The release of mature IL-1β from these cells requires the co-activation of NF-κB and the inflammasome (Fig 1.6) (Schroder and Tschopp, 2010) in response to stimuli such as bacterial, viral, fungal, and parasitic components, as well as endogenous stimuli, including TNF-α. Secreted IL-1β is regulated by several decoy receptors such as IL-1-receptor antagonist (IL-1Ra) which binds to IL-1β neutralising its proinflammatory deleterious effects (Möller and Villiger, 2006).

1.6.3 Interleukin-6

IL-6 is a cytokine with both pro- and anti-inflammatory properties and is involved in modulating aspects of both innate and adaptive immunity, via its ability to enhance the proinflammatory response of neutrophils, and through its ability to induce fever, B-cell differentiation and T-cell activation (Naugler and Karin, 2008). It is expressed by a variety of cell types in response to both exogenous and endogenous ligands (Möller and Villiger, 2006). It is a major inducer of hepatic synthesis of acute phase proteins which are involved in the restoration of physiological homeostasis following inflammation. It also has the capacity to limit the deleterious effects of pro-inflammatory cytokines through its ability to inhibit expression of IL-1β and TNF-α, and to stimulate expression of IL-1Ra and IL-10 (Papanicolaou *et al.*, 1998).

1.6.4 Interferon gamma

IFN-γ is critical for host immune response against viral and intracellular bacterial infections. Cellular sources include NK cells, cells of the monocytic lineage and T lymphocytes. Interferon-γ upregulates major histocompatibility complex (MHC) class I and class II molecule expression, thus promoting the induction of cell mediated immunity through T-cell recognition of presented foreign peptides. In addition, IFN-γ enhances the microbicidal activity of both macrophages and neutrophils by increasing receptor-mediated phagocytosis, inducing respiratory burst activity, and priming nitric oxide production (Schroder *et al.*, 2004).

1.6.5 Interleukin-17A

The recently discovered IL-17 cytokines are emerging as key players in both adaptive and innate immunity (Pappu *et al.*, 2011). The IL-17 family members include IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. IL-17A is the best characterised of IL-17 family to date and has been shown to be produced by innate immune cells, including NK cells and neutrophils, in response to infection and injury (Cua and Tato, 2010). IL-17A is a proinflammatory cytokine that has an important role in promoting neutrophil mobilisation and cytokine production by epithelial cells (Pappu *et al.*, 2011). Neutrophil recruitment is indirectly induced by IL-17A through triggering cells that bear the IL-17 receptor A (IL-17RA) to secrete chemotactic cytokines such as IL-8 (Laan *et al.*, 1999).

1.6.6 Interleukin-8

IL-8 is a chemotactic cytokine that stimulates leukocyte movement (Gouwy *et al.*, 2005). It is produced by an array of cell types, including cells of monocytic lineage, endothelial and epithelial cells, fibroblasts, neutrophils, and T-lymphocytes by both exogenous and endogenous proinflammatory stimuli, such as LPS, TNF-α, IL-1β and serum amyloid A (SAA) (He *et al.*, 2003; Hoffmann *et al.*, 2002). The primary role of IL-8 is recruitment of neutrophils to the source of its induction (Baggiolini *et al.*, 1994) through binding to its cell surface receptors, CXCR1

and CXCR2 (Hay and Sarau, 2001). In addition to its chemotactic role, IL-8 can enhance the function of neutrophils through augmentation of respiratory burst activity (Gouwy *et al.*, 2005).

1.6.7 Interleukin-10

IL-10 is the founding member of a family of cytokines that include IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29 (Commins *et al.*, 2008). It is an important cytokine which functions by inhibiting components of the innate immune response (Mosser and Zhang, 2008). It is produced by various cell types, including lymphocytes, eosinophils, mast cells and cells of monocytic lineage, the latter being a major source of the cytokine *in vivo* (Moore *et al.*, 2001) . IL-10 inhibits the production of proinflammatory cytokines, including IL-1β, IL-6 and IL-8, from monocytes, macrophages, and neutrophils. IL-10 can also induce the expression of cytokine antagonists, such as IL-1Ra and soluble TNF receptors (Cassatella *et al.*, 1994).

1.6.8 Transforming growth factor-β

TGF-β is a cytokine that has well-described effects on cell growth and differentiation as well as a role in modulating inflammation. Although mainly regarded as a suppressor of immune responses, TGF-β does exert some proinflammatory properties. The immunoregulatory effects of TGF-β include 1) inhibiting macrophage cytokine and nitric oxide production and respiratory burst activity; 2) limiting IFN-γ production; 3) increasing IL-1Ra expression; and 4) enhancing macrophage clearance of injured parenchymal cells, inflammatory cells, and bacterial debris (Li *et al.*, 2006).

1.7 Acute phase response (APR)

As well as their important roles at the site of induction, cytokines, upon entering the circulation, induce a systemic response that adds to host defence. One of the most important of these effects is the initiation of the acute-phase response (APR), which is characterised by a shift in proteins (acute phase proteins (APP)) secreted by the liver into the blood in response to inflammatory stimuli, such as IL-1β, IL-6, and TNF-α that bind to their receptors on hepatocytes (Fig. 1.7) . The APR can be described as the systemic arm of the innate immune response, with the goal of re-establishing homeostasis and promoting healing.

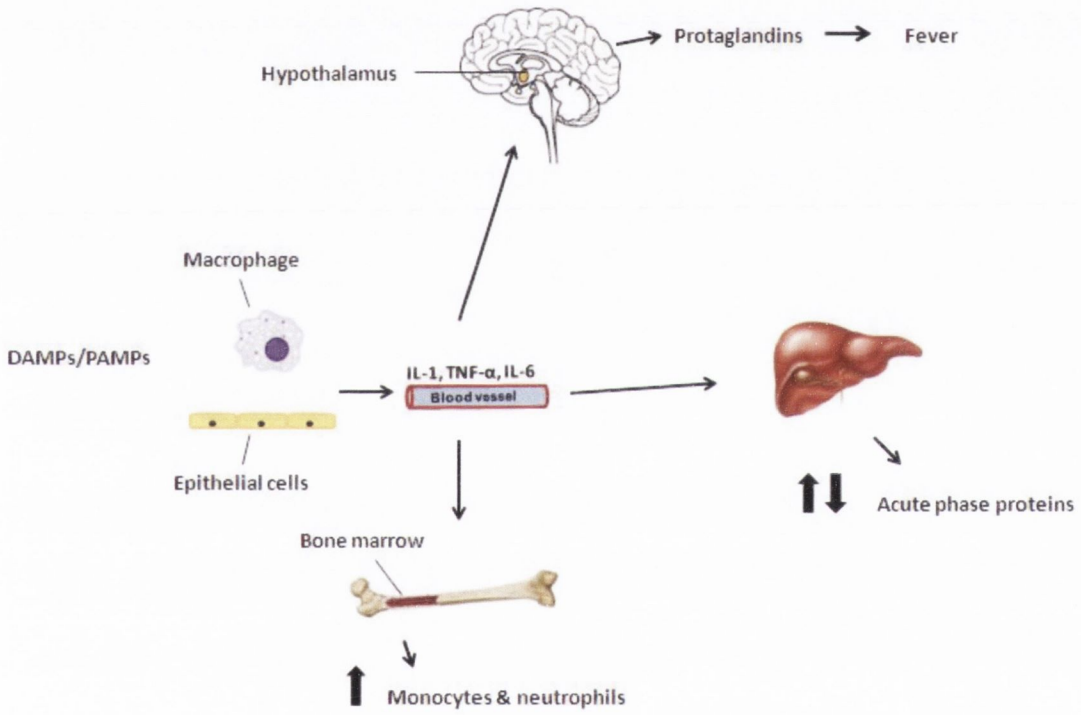


Figure 1.7 Cytokine-mediated activation of both the local and systemic immune response
Following exposure to the damage associated molecular pattern (DAMP) molecules or pathogen-associated molecular pattern (PAMP) molecules, cytokines produced by macrophages and epithelial cells act on neighbouring cells, e.g. leucocytes (monocytes, neutrophils and lymphocytes) and endothelial cells, in the vicinity of the inflamed site. Cytokines released to the circulation induce the hepatic acute phase response (APR), which involves the increased synthesis of acute phase proteins (APP). Stimulation of the central nervous system by cytokines induces fever. Image constructed by Cormac Whelehan.

1.7.1 Acute phase proteins

APPs are defined as proteins whose plasma concentration increases or decreases by at least 25 percent during an inflammatory response and as such has led to them being identified as positive APPs and negative APPs respectively (Gabay and Kushner, 1999). In addition to hepatic production following infection or injury, APPs are also induced at the site of infection where they augment the local immune response (Cheng *et al.*, 2008). These findings suggest a key role for APPs in the establishment and maintenance of both local and system inflammation.

APP expression can vary from moderately induced, ceruloplasmin 2-fold, to 1000-fold serum amyloid A (SAA) and C-reactive protein (CRP) in response to inflammatory stimuli (Steel and Whitehead, 1994). Other important positive APPs are haptoglobin (HP), lipopolysaccharide-binding protein (LBP) and α 1-acid glycoprotein (AGP) (Gabay and Kushner, 1999).

SAA is a generic term for a family of acute phase proteins coded for by different genes with a high allelic variation and a high degree of homology between species. In humans, the SAA family consists of four SAA genes (Steel and Whitehead, 1994). *SAA1* and *SAA2* are inducible in response to inflammatory stimuli, whereas *SAA4* is constitutively expressed and *SAA3* is a pseudo gene (Jensen and Whitehead, 1998).

Bacterial products, such as LPS, and inflammatory cytokines, including IL-1β, TNF-α, and IL-6, induce SAA expression in hepatocytes as well as in tissue macrophages and epithelial cells. In circulation, the newly synthesized SAA is incorporated into high-density lipoprotein (HDL) and is involved in cholesterol transport. SAA produced locally at the site of inflammation does not associate with HDL and has numerous proinflammatory functions. Several receptors have been identified that mediate these functions including the G protein-coupled formyl peptide receptor 2 (FPR2) for the chemotactic activity of SAA, TLR2 for the induction of proinflammatory cytokines, and $P2X_7$ receptor ($P2X_7R$) for the activation of the inflammasome (Cheng *et al.*, 2008; Niemi *et al.*, 2011; Su *et al.*, 1999).

Another important positive acute phase protein is C-reactive protien (CRP). It binds bacterial phosphocholine resulting in the activation of phagocytic cells and removal of pathogens (Ceciliani *et al.*, 2002). CRP has also shown to induce the expression of inflammatory cytokines in monocytes and neutrophil (Tebo and Mortensen, 1990).

LBP interacts with bacterial LPS and transfers it to CD14, a receptor on the surface of innate immune cells. Together with TLR4, a complex is formed, which initiates the TLR

signaling pathway and the activation of several inflammatory and immune-response genes, including pro-inflammatory cytokines (Aderem and Ulevitch, 2000). α 1-acid glycoprotein has anti-inflammatory properties, in that it has been shown to inhibit neutrophil activation and increase the expression of IL-1 receptor antagonist by macrophages (Fournier *et al.*, 2000). Additionally, it has also been reported to bind LPS (Moore *et al.*, 1997).

APPs involved in antioxidant activities, include HP and ceruloplasmin (Cp). In plasma, HP binds free haemoglobin in plasma forming a haptoglobin-haemoglobin complex (HP-Hb). The Hp–Hb complex is recognised by a specific surface receptor on macrophages and is phagocytised, reducing the oxidative damage associated with haemolysis (Schaer *et al.*, 2002). HP has also been shown to modulate neutrophil function through inhibiting neutrophil chemotaxis, phagocytosis and bactericidal activity (Rossbacher *et al.*, 1999).

Cp has also be shown to have anti-inflammatory properties by disrupting neutrophil attachment to the endothelium and by acting as an extracelluar scavenger of peroxide (Broadley and Hoover, 1989).

1.8 Antimicrobial peptides

Antimicrobial peptides (AMPs) are evolutionarily conserved molecules, expressed in a wide range of organisms including bacteria, insects, plants and vertebrates (Zasloff, 2002). In humans some are expressed constitutively and/or induced rapidly in innate immune cells such as macrophages, neutrophil and epithelial cells to counteract assault from a wide range of pathogens including bacteria, virues, fungi and parasites (Lai and Gallo, 2009).

1.8.1 Classification

Although categorized on their ability to kill microbes, AMPs are hugely diverse with more than 1200 identified or predicted to date (Antimicrobial peptide database: http://aps.unmc.edu/AP/main.php.) However, these peptides do share some common features, such as small size and positively charged residues. Furthermore, based on their amino acid composition, size and conformation, they can be divided into several categories, such as α-helix and β-sheet peptides, and peptides with extended or loop structures (Fig. 1.8).

Defensins and cathelicidins are two of the most studied AMP gene families in humans.

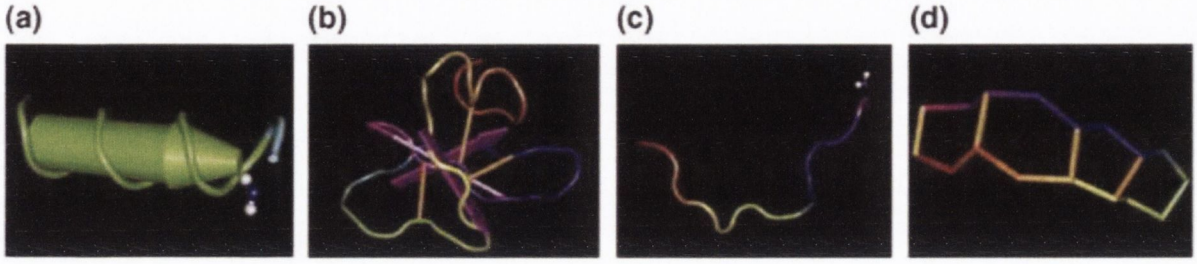


Figure 1.8 Structure of selected AMPs

A) α-helix structure the human LL-37 cathelicidin peptide. B) β-sheet structure of the human defensin hBD2. C) Extended structure of the bovine HDP indolicidin. D) Loop structure of the θ-defensin isolated from rhesus macaques. Figure adapted from (Lai and Gallo, 2009)

1.8.1.1 Defensins

Defensins are a comprehensively studied class of AMPs. They have been identified in vertebrates and invertebrates as well plants (Boman, 2003). Defensins are small cationic peptides with a molecular weight ranging from 3.5 to 4 kDa. They contain six highly conserved cysteine residues which form three pairs of stabilising disulfide bridges (Fig. 1.9). Based on the alignment of these six conserved cysteine residues, these defensins can be classified as α-defensins, β-defensins and θ-defensins.

α-defensins are 29 to 35 amino acids in length, with characteristic disulfide bridges between cysteines 1–6, 2–4, and 3–5 (Fig. 1.9). Six alpha-defensins have been identified in human, four of which are produced by neutrophils and as such are referred to as human neutrophil peptides (HNP-1, HNP-2, HNP-3 and HNP-4). α-defensins 5 and 6 (HD5 and HD6) are mainly produced by Paneth cells of the small intestine and in epithelial cells from the female reproductive tract (Doss *et al.*, 2010). The disulfide pairing of β-defensins is characterized by a 1–5, 2–4, and 3–6 cysteine pairing (Fig. 1.9), with the length of these peptides ranging from 36 to 42 amino acids (White *et al.*, 1995).

Six β-defensins have been studied to date in humans, although genomic analyses suggest there may be more (Schutte *et al.*, 2002). Human β-defensin-1 (hBD-1) is constitutively expressed in epithelial cells of the respiratory and urinary tracts. hBD-2 is expressed in the skin as well as in urinary, gastrointestinal, and respiratory epithelia. Also isolated from psoriasis-affected skin, hBD-3 is found in high concentration in saliva and vaginal fluids (Doss *et al.*, 2010). hBD-4 has been found in human testicles, stomach, and uterus (Doss *et al.*, 2010), while hBD-5 and hBD-6 are specifically found in the human epididymis (Yamaguchi *et al.*, 2002).

The third defensin sub-type, the θ-defensins, is specific to nonhuman primates. This class of peptide, which are expressed in leukocytes, appears to have evolved relatively recently as a result of the merging of two α-defensin-like precursors (Tang *et al.*, 1999; Tran *et al.*, 2002).

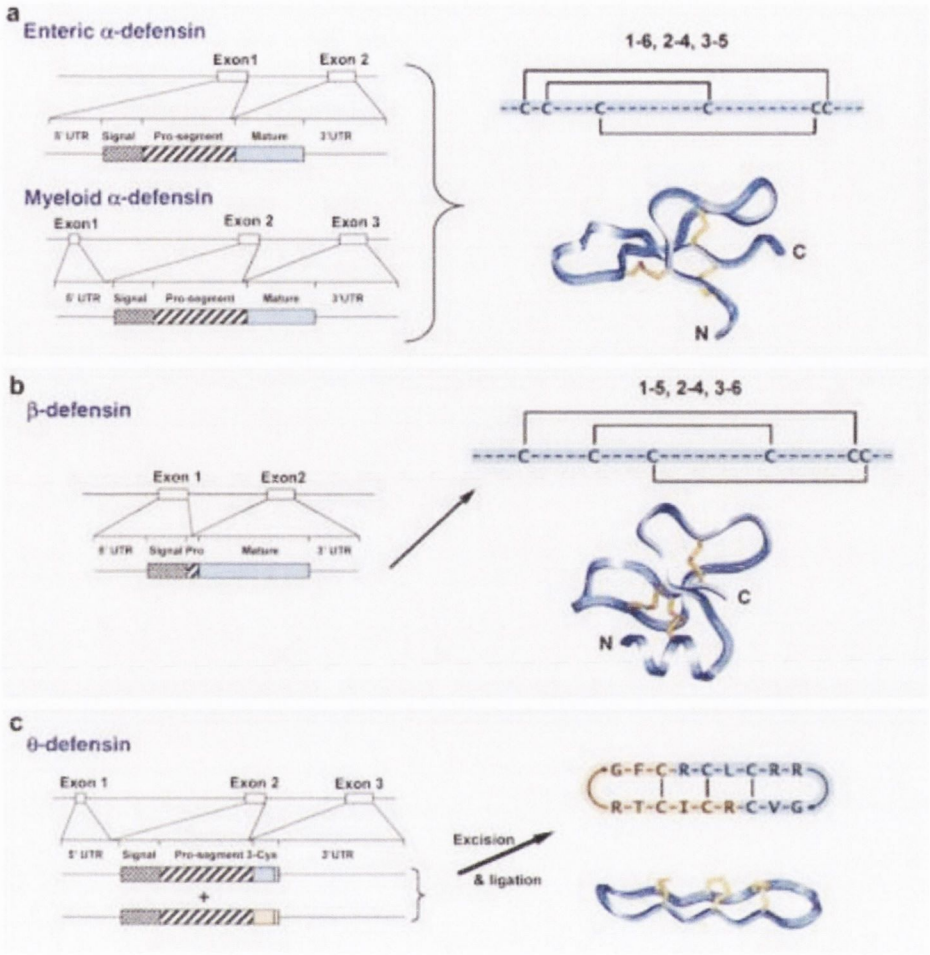


Figure 1.9 Defensin gene and peptide structure

On the left: α-defensin, β-defensin and θ-defensin genes are depicted. Signal peptide (crosshatching), Pro-segment (strips) and mature defensin (blue).

On the right: three different disulfide binding patterns. Numbers above diagrams indicate the disulfide connections in each. The three-dimensional structures are of rabbit α-defensin (RK-1), human β-1 (defensin-1) and θ-defensin (RTD-1), top to bottom. Figure taken from (Selsted and Ouellette, 2005).

1.8.1.2 Cathelicidins

Cathelicidins are so named based on the highly conserved N-terminal region known as the cathelin domain (Fig. 1.10). This protein contains two disulfide bonds between cysteine residues C85-C96 and C107-C124 and was given its name based on its ability to inhibit the protease cathepsin-L (Sanchez *et al.*, 2002; Storici *et al.*, 1996). The cathelicidins are only considered to be a gene family because of the conservation seen in this region of the gene.

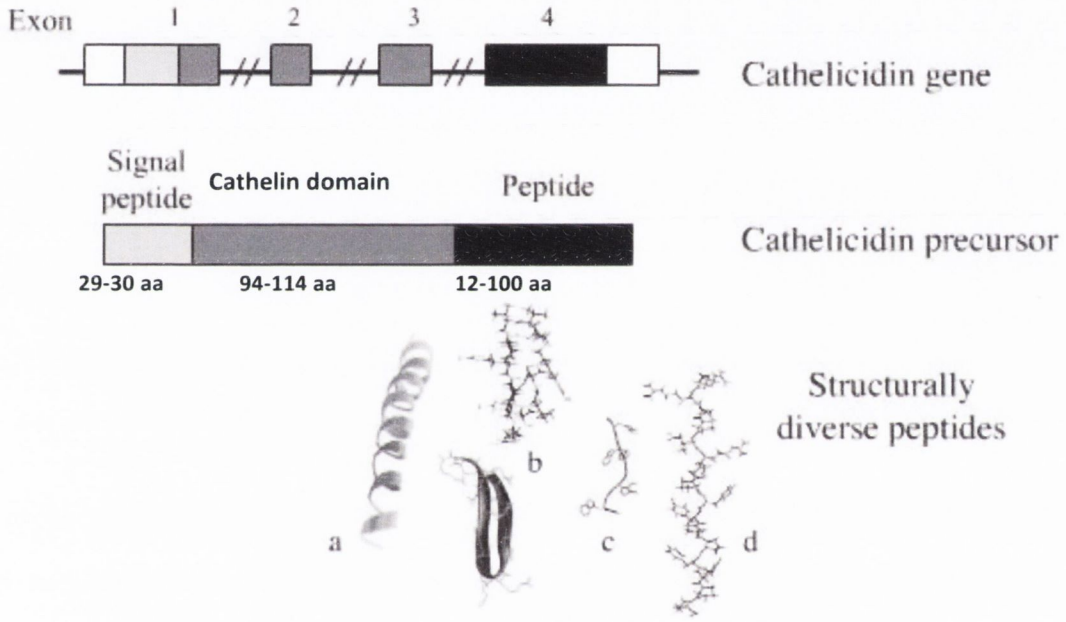


Figure 1.10 Schematic representation of the gene and protein structure of cathelicidins.
The mature cathelicidin peptides include (a) α–helical, (b) cysteine-rich, (c) tryptophan-rich and (d) proline-rich peptides. Figure taken and adapted from (Zanetti, 2005).

Cathelicidins have been identified in every mammalian species investigated to date (Fig. 1.11). The presence of at least one α-helical cathelicidin peptide in mammals suggests that an α-helical cathelicidin was the original from which the family expanded and evolved resulting in a variety of peptides in selected species.

Although found in several species, the peptides generated by cathelicidin genes found between species show little similarity to each other and are grouped solely on the similarity of the precursor protein which is largely made up of a cathelin propiece domain. The active peptides vary in length both within and between species and range from 12–100 amino acid residues (Fig. 1.10). Cathelicidins have been found in neutrophils and macrophages, as well as epithelial cells of the testis, skin, respiratory tract and gastrointestinal tract (Lai and Gallo, 2009).

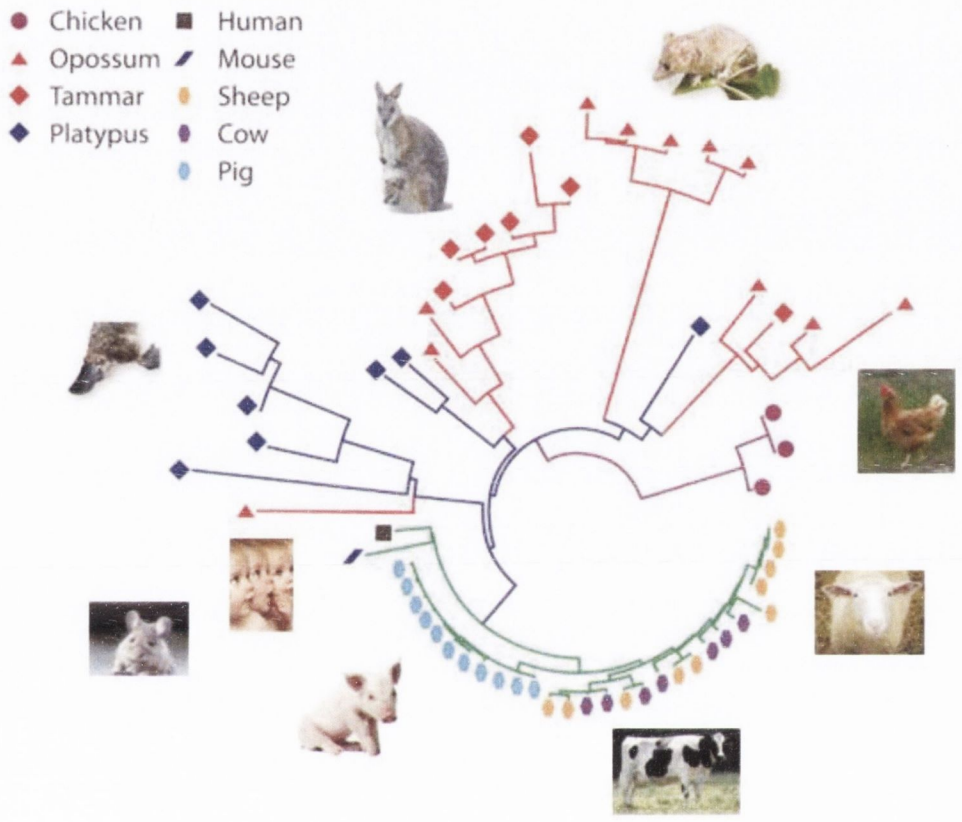


Figure 1.11 Phylogenetic relationships between known mammalian cathelicidin genes
The phylogenetic tree was constructed with the cathelin domain peptide sequences using the neighbour-joining method implemented by MEGA4. Figure taken and adapted from (Wang *et al.*, 2011).

1.8.2 Mode of action

The functions of AMPs can be broadly divided into two categories: direct antimicrobial killing and immune regulation.

Antimicrobial activity of AMPs is one of most important countermeasures that animals have evolved to counteract microbial invasion. *In vitro*, AMPs have been shown to be antimicrobial against a wide variety of pathogens including gram-negative and gram-positive bacteria, protozoa, fungi and some viruses, with maximal effectiveness against specific groups of organisms relevant to the tissue where the AMPs are expressed. For example, β-defensins expressed in skin are antimicrobial against *S. aureus* and *P. aeruginosa* which are major causative agents of skin infections (Huang *et al.*, 2007), whereas, intestinal-expressed α-defensins are antimicrobial against certain viruses and bacteria pathogens such as Salmonella (Cunliffe, 2003). *In vivo*, β-defensin knockout mice exhibit delayed clearance of pathogens associated with lung and bladder infection (Morrison *et al.*, 2002; Moser *et al.*, 2002). Similarly, cathelicidin knockout mice (*camp*) have been shown to be susceptible to *S. pyogenes*, herpes simplex virus, *E. coli* and vaccinia virus (Chromek *et al.*, 2006; Howell *et al.*, 2004; Howell *et al.*, 2006; Nizet *et al.*, 2001).

It is generally believed that cationic AMPs interact with bacterial membranes through electrostatic forces resulting in bacterial membrane disruption. Several models for mechanism of action have been put forward, for example the "barrelstave" model, and the "carpet model" (Fig. 1.12) (Brogden, 2005). In general, cationic AMPs are attracted by electrostatic forces to the negative phospholipid head groups present on the membrane surface of Gram-negative and Gram-positive bacteria (Fig. 1.12). Once AMPs gain access to the cytoplasmic membrane they interact with the lipid bilayer, followed by displacement of lipids, alteration of membrane structure and the creation of a physical hole causing cellular contents to leak out (Fig. 1.12). One mechanism as to why AMPs produced by mammals will not disrupt eukaryotic cell membrane is because of the presence of cholesterol in the eukaryotic cell membrane. Cholesterol is known to cause condensation of phospholipid bilayers and thus may prevent cationic AMPs from penetrating into the cytoplasmic membrane of eukaryotic cells (Brogden, 2005).

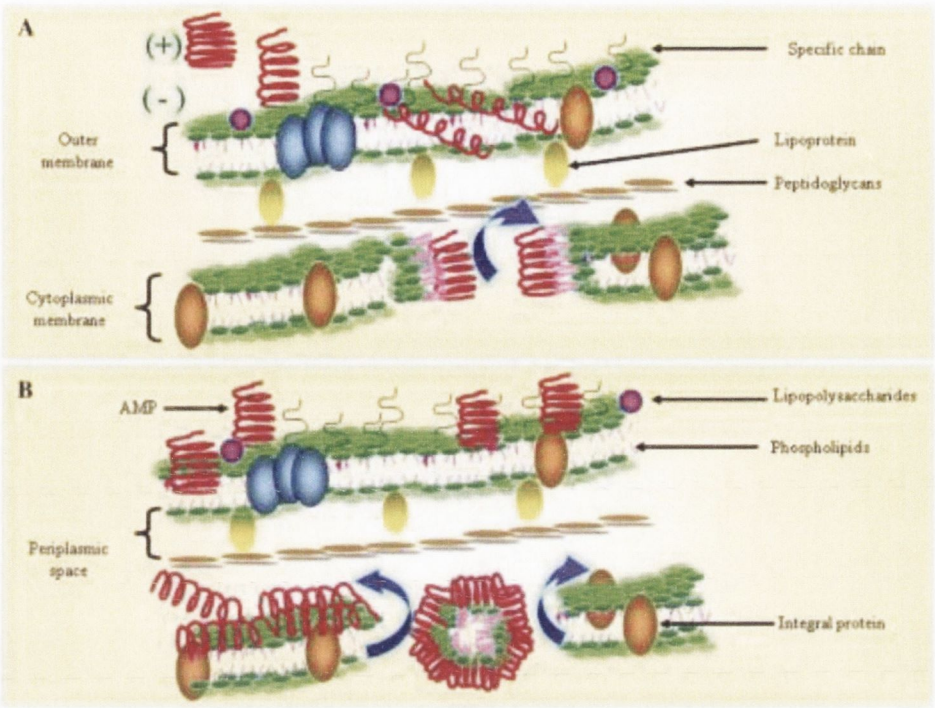


Figure 1.12 Schematic presentation of the mode of action of cationic peptides
A) Barrel stave model: The peptides bind to the cell membrane, then inserts into the hydrophobic core of the membrane forming a pore, causing leakage of cytoplasmic material and death of the cell. B) Carpet model. Figure taken from (Guaní-Guerra *et al.*, 2010)

1.8.2.1 Alternative physiological roles of AMPs

Initially categorized by their ability to act as endogenous antibiotics as a result of microbial cell membrane disruption (Brogden, 2005), it is now becoming clear that their biological activity is multifunctional and extends beyond microbial killing. These include neutralisation of endotoxin (Bowdish *et al.*, 2005; Mookherjee *et al.*, 2006), modulation of the immune response through induction of proinflammatory cytokines (Elssner *et al.*, 2004) and induction of angiogenesis and wound healing (Baroni *et al.*, 2009; Koczulla *et al.*, 2003). Thus HDPs are important effector molecules of the immune response.

Direct antimicrobial activity of most AMPs *in vitro* and in *vivo can* be muted by the physiological concentration of salt and serum proteins (Maisetta *et al.*, 2008). In addition, the

minimal inhibitory concentrations (MIC) of AMPs against microbes *in vitro* are typically much higher than the physiological concentrations of AMPs *in vivo*. Increasing evidence suggests that some AMPs protect the host from pathogens by mechanisms other than direct killing (Bowdish *et al.*, 2005a). They can function as potent immunoregulatory peptides (Fig. 1.13).

Human β-defensins 1-3 act as chemokines for different cell types, such as T cells and immature dendritic cells and mast cells through direct binding to the chemokine receptors (Niyonsaba *et al.*, 2002; Yang *et al.*, 1999). Additionally, β-defensins have been shown to induce the production of both chemokines and cytokines such as MCP-1, RANTES, IL6, IL10, TNF –α and IL1(Niyonsaba *et al.*, 2005; Niyonsaba *et al.*, 2006).

Similar to defensins, the human cathelicidin LL37 exerts a direct chemoattractive action on neutrophils, monocytes and T cells through binding of the formyl-peptide receptor 2 (FPR2) expressed in these cells (Wang *et al.*, 2000). Furthermore, LL37 induces the transcription of chemokines such as IL-8, MCP-1 and MCP-3 (Bowdish *et al.*, 2005b). Cathelicidins can also inhibit LPS and LTA induced transcription of proinflammatory cytokines such as TNF-α (Mookherjee *et al.*, 2006a; Mookherjee *et al.*, 2006b).

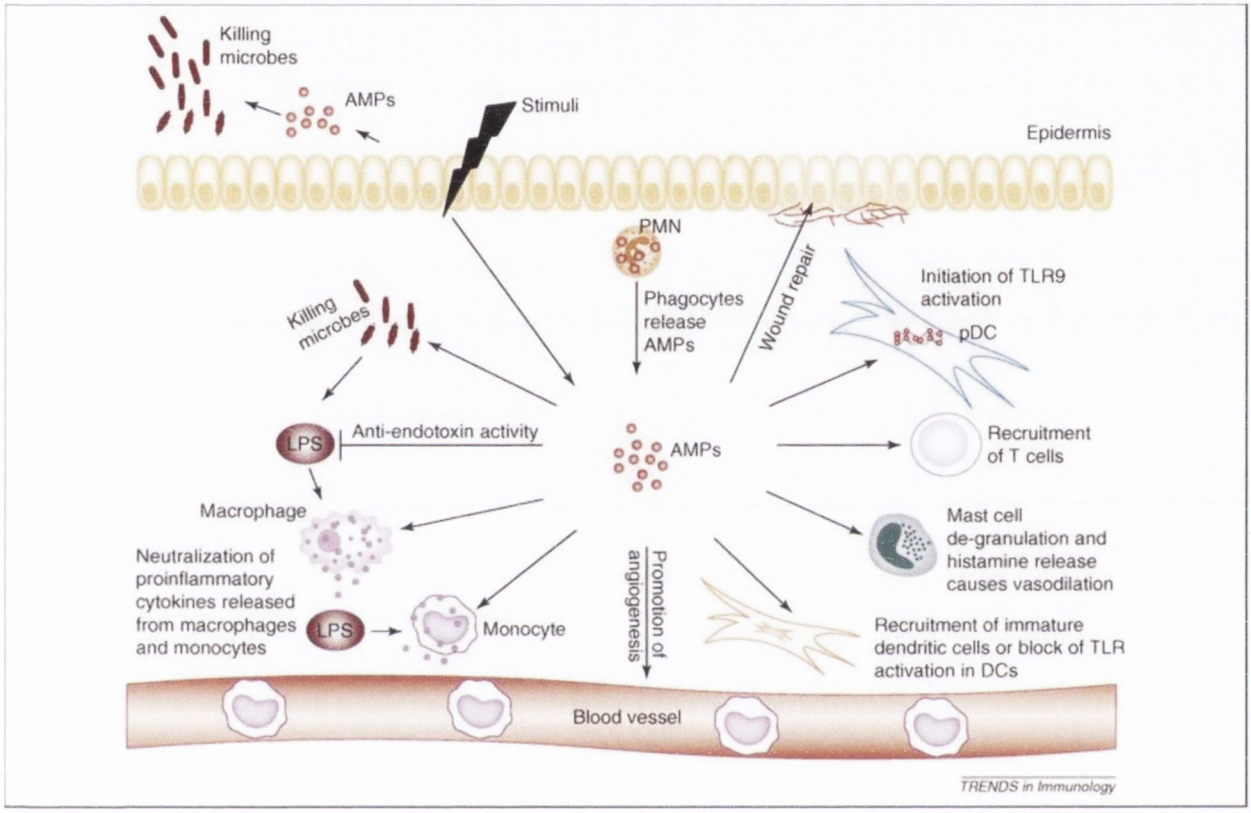


Figure 1.13 Multiple functions of antimicrobial peptides in host defence
Figure taken from (Lai and Gallo, 2009b).

1.9 Mammary gland

The mammary gland is a transformed dermal gland that most likely evolved from the innate immune system (Vorbach *et al.*, 2006). The mammary gland provides the newborn with milk, which has both nutritional and immunological functions. The macroscopic anatomy of the mammary gland varies between animal species and is influenced by the age, gestation stage and number of prior lactations. However, the microscopic structure is relatively similar in all species with no difference in milk producing cells or secretory cells

The mammary glands of cattle, sheep, goats, horses, and camel are located in the inguinal region; those of primates and elephants, in the thoracic region; and those of pigs, rodents and carnivores, along the ventral structure of both the thorax and the abdomen (Fig.1.14).

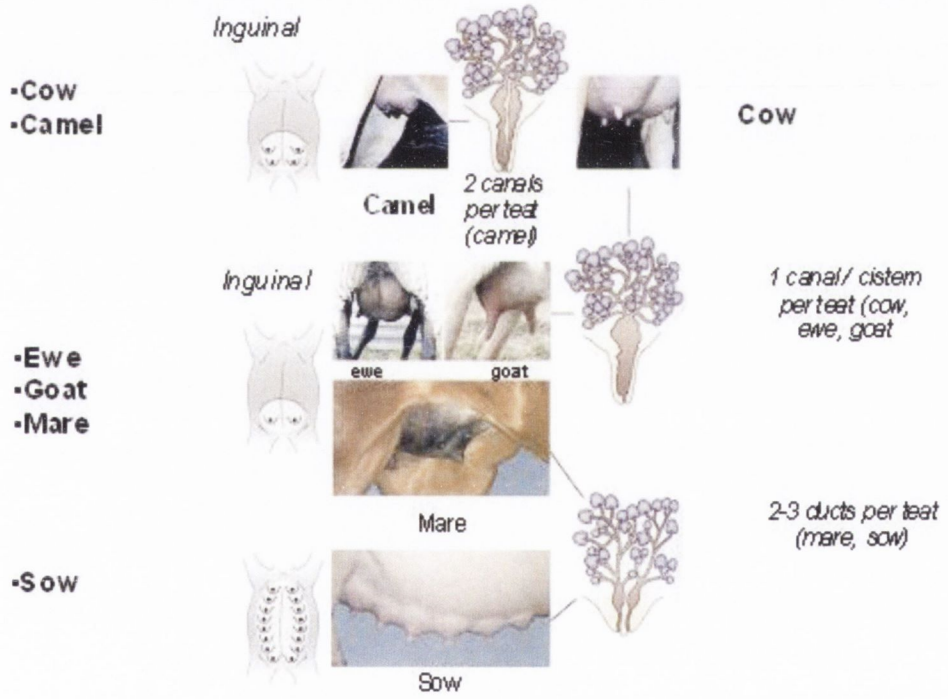


Figure 1.14 Diversity in anatomic position, number and teat morphology among mammals
Figure taken from (Tanhuanpaa, 1995).

1.9.1 Bovine mammary gland

The gland consists of 2 halves with each half having a front and rear quarter. The quarters are separated by connective tissue and each has separate milk collecting system (Fig.1.15A). The majority of the mammary gland consists of the lobule-alveolar system which is the large, milk-producing tissue above the teats. This region of the gland consists of lobes containing ducts that terminate in secretory alveoli (Fig. 1.15B). The alveoli are the functional unit of the mammary gland and contain the milk-producing epithelial cells (Fig. 1.15C). The alveolus consists of a simple layer of gland cells on a basal membrane. Between the basal membrane and the epithelium there are flat, branched myoepithelial cells which surround the alveolus. These cells can contract to expel secretion from the alveolus epithelial cells. The milk from the lumen of these alveoli drains into the interlobular ducts, followed by the gland cistern and eventually into the teat (Fig. 1.15B).

The duct system can be divided anatomically into primary ducts (which come from the alveoli), collecting ducts (intra-lobular, inter-lobular and intra-lobal) and inter-lobal (which leads to the gland cistern). The ducts initially comprise epithelium, but eventually incorporate smooth muscle tissue. Similarly, in the gland cistern there are intermittent fibres of smooth muscle tissue. In both the ducts and in the gland cistern there are myoepithelial cells between the basal membrane and the epithelium. The total volume of the intra-lobal ducts (largest ducts) and the gland cistern is a small fraction of the total volume of the udder. Most of the milk is stored in the in the alveoli and in the smaller ducts.

Each quarter terminates in a cylindrical teat. The lining of the teat is a double layer of multi-functional cuboidal and columnar epithelial cells. Under the epithelium there is a layer of connective tissue which contains randomly located smooth muscle fibres. Between the epithelium and the skin there are numerous veins, arteries and lymph vessels (Fig. 1.15)

Epithelial cells of the bovine mammary gland bear much similarity to those of other mucosal sites. They are polar and share tight junctional complexes, gap junctions and desmosomes with neighbouring cells which facilitate the exchange of molecules between neighbouring cells while the desmosomes maintain cell adhesion. The principal function of mammary epithelial cells is the synthesis of milk components during lactation.

Under normal conditions the mammary gland is sterile. However, at times of stress, for example, during lactation the organ is particularly susceptible to infection.

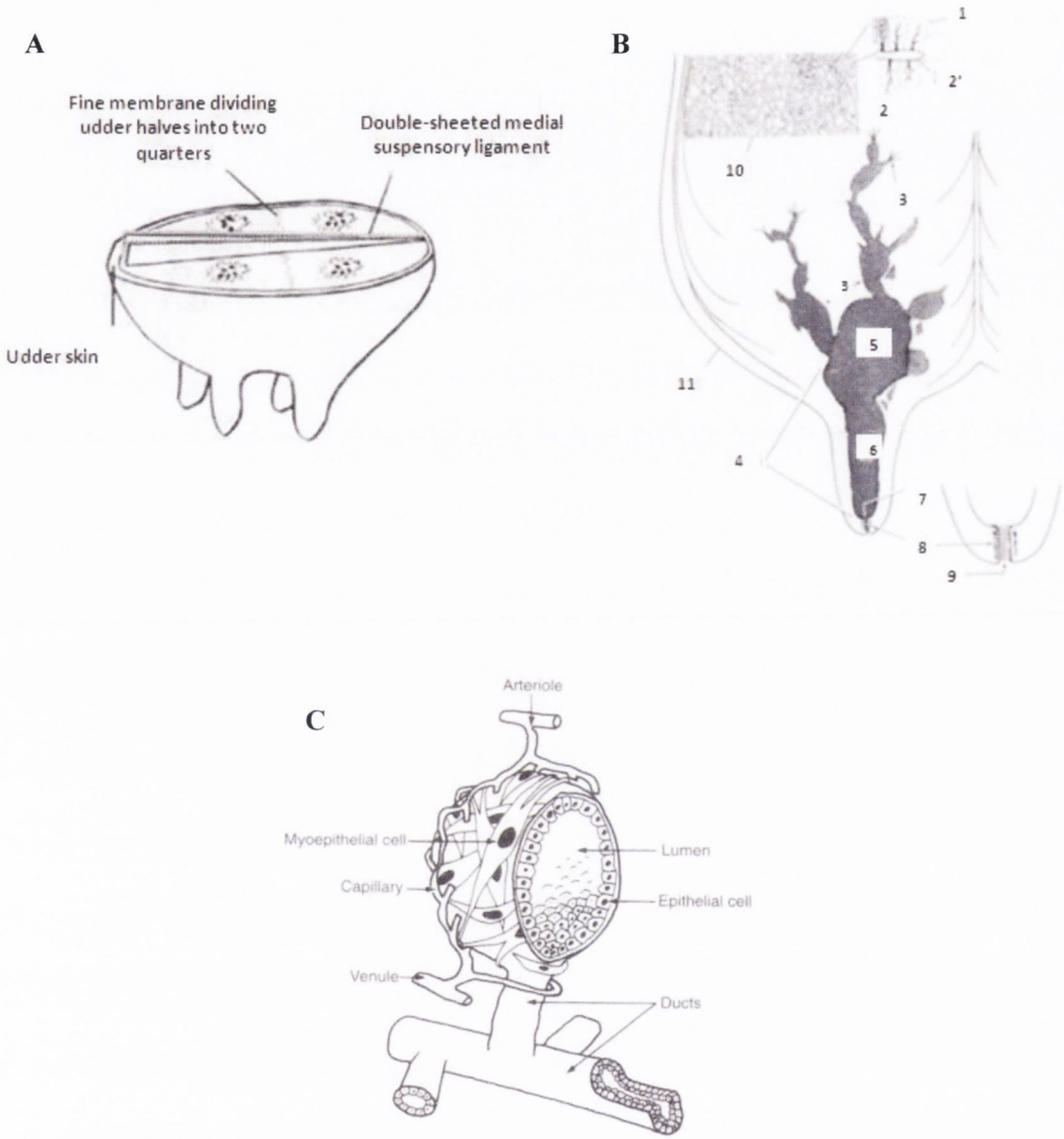


Figure 1.15 Schematic of cross-section of an udder and vertical section of teat, and schematic of an individual alveolus

A) The two halves of the bovine udder separated by the medial suspensory ligament. B) Duct system of the bovine mammary gland: 1) lobule, 2) intra-lobular duct, 2') inter-lobular duct, 3) lactiferous ducts of various diameter, 4) lactiferous sinus, 5) gland sinus, 6) teat sinus, 7) papillary duct, 8) teat sphincter, 9) teat orifice, 10) parenchyma of gland, 11) skin. C) An alveolus surrounded by blood vessels and myoepithelial cells in the mammary gland. Figure taken and adapted from (Tanhuanpaa, 1995).

1.10 Bovine mastitis

Bovine mastitis is an inflammatory response in the mammary gland (*mast* = breast, *itis* = inflammation), which is predominantly a result of infectious challenge and is the most frequent and costly disease of dairy cows (Seegers *et* al., 2003). The severity of the inflammation is dependent on the causative agent and the host's immune response to it, which is shaped by the breed, immunological health and lactation stage of the animal (Burvenich *et al.*, 2003). The severity of the inflammation can be categorised as either clinical or sub-clinical and further subdivided into environmental or contagious depending on the identity of the invading pathogen. Bacteria are the main mastitis-causing pathogens.

1.10.1 Clinical and subclinical mastitis

Clinical mastitis is diagnosed by an increased milk somatic cell count (SCC) accompanied by visible changes in the milk, udder or animal. There are three grades of clinical mastitis. Grade one or sub-acute mastitis is the mildest form and presents as milk consistency alteration such as clots. Grade-two mastitis can be further subdivided into acute or chronic. Acute cases will present with obvious symptoms of udder inflammation such as swelling, heat and pain and the milk consistency will be altered. Chronic grade two mastitis shows permanently reduced yield and a lumpy, distorted and often cold quarter due to scar tissue. Grade three or per-acute mastitis is the most serious threat to immediate animal health. Obvious systemic symptoms such as fever, loss of appetite and general depression are seen and require immediate veterinary treatment. An elevated SCC in the absence of clinical signs is diagnosed as subclinical mastitis. It can only be identified by analysing SCC and/or culturing bacteria from milk (Crilly, 1988).

Mastitis can also be categorised as contagious or environmental based on the source of infection. The most common pathogens associated with environmental mastitis are *Escherichia coli, Streptococcus dysgalactiae, Streptococcus uberis* and *Klebsiella spp* (Smith and Hogan, 1993), the source of which includes housing, bedding, pasture and milking parlours. Contagious mastitis is generally transmitted from infected quarters to other quarters or cows and the most common time of transmission is during milking. The pathogens most usually responsible for

contagious mastitis are, *Streptococcus agalactiae*, *Mycoplasma bovis* and *Staphylococcus aureus* (Fox and Gay, 1993).

1.10.2 Detection

In 1963 the term 'somatic cells' was introduced to describe cells of host origin in milk (Silva *et al.*, 2001). A healthy cow sheds a nearly constant amount of somatic cells (SC) daily. However, factors such as breed, lactation status and season can have an impact on cell number (Kelly *et al.*, 2000). Monocytes, macrophages and lymphocytes have all been shown to be present in milk. The number of these cell populations varies according to udder health. The counting of these SC from individual quarters of cows can be utilised to indicate the presence of infection. The SCC regulatory cut-off for healthy *vs.* mastitic milk varies from country to country. In general, SCC of $>2 \times 10^5$ cells/ml is considered mastitic in Ireland. The SC present in milk from a healthy quarter consists mainly of macrophages (58-84 % of the total SCC) (Kelly *et al.*, 2000) whereas inflammation results in the massive influx of neutrophils that can constitute for over 90% of the SC present in the milk (Paape *et al.*, 2003).

1.10.3 Pathogenesis

Upon entering the teat canal (Figure 1.16), bacteria must overcome local cellular and humoral defense mechanisms. If this is achieved, the bacteria begin to multiply in the mammary gland. They release toxins and trigger epithelial cells and leukocytes to release proinflammatory cytokines, such as TNF-α, IL-1β and IL-8 which facilitate the recruitment of circulating immune effector cells to the site of interaction (Elazar *et al.*, 2010; Griesbeck-Zilch *et al.*, 2008). These effector cells are predominantly made up of neutrophils, which act by engulfing and destroying the invading pathogen (Paape *et al.*, 2003). Killing is achieved through the release of molecules that are stored in intracellular granules, including bactericidal peptides, enzymes and acidic proteases (Paape *et al.*, 2003). As well as killing bacteria, these molecules can be toxic to mammary epithelial cells (Zhao and Lacasse, 2008). Once their task is fulfilled, neutrophils are destroyed through apoptosis and engulfed by macrophages (Fox *et al.*, 2010). Dead neutrophils

and the sloughed-off mammary epithelial cells are secreted into milk resulting in increased milk somatic cell count.

If the infection persists, the mammary gland becomes damaged and starts losing its anatomical integrity (Zhao and Lacasse, 2008). As a result, the blood–milk barrier is breached, causing extracellular fluid components, such as chloride, sodium, hydrogen, potassium and hydroxide ions, to enter the gland and mix with the milk. This marks the initial stage of clinical symptoms, which include visible change to the udder, such as enhanced external swelling and reddening of the gland. Changes also occur in the milk, including increased conductivity, increased pH, raised water content and the presence of visible clots and flakes, with blood occasionally present in the milk.

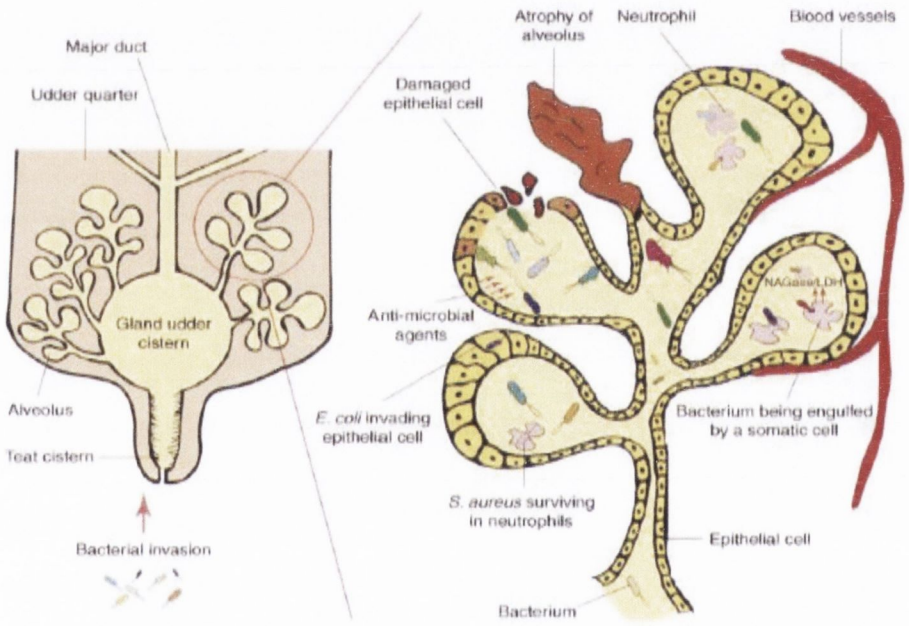


Figure 1.16 Schematic depiction of mastitis development in the infected udder. Microorganisms invade the udder through the teat cistern. They then multiply in the udder where they are attacked by both local and recruited defence mechanisms. If the infection persists, the mammary gland becomes damaged and starts losing its anatomical integrity. Figure taken from (Viguier *et al.*, 2009)

1.10.4 Methods currently used for the treatment and prevention of mastitis

Mastitis is the single most costly disease of the dairy industry (Kossaibati and Esslemont, 1997). The financial loss is contributed to by short and long term reduced milk yield, direct cost of treatment products and services, increased labour costs and culling. In the US, the projected annual losses caused by mastitis are $2 billion; in the UK, mastitis causes an annual loss of approximately £300 million to dairy farmers and in the Republic of Ireland, the cost of clinical mastitis is approximately 693 euro per year for every infected cow (Bradley, 2002; Hillerton and Berry, 2005; Huijps *et al*, 2008). In addition to the agricultural cost, one must also consider the potential human public health costs associated with mastitis. Continuous use of antibiotics is contributing to the emergence of antibiotic resistant strains of micro-organisms which can infect many species including humans (Kaszanyitzky *et al*., 2003).

The majority of mastitis cases are the result of bacterial infection. Therefore the most common treatment is the delivery of appropriate antibiotics by intramammary infusion. Penicillin and cloxacillin are commonly used in staphylococcal and streptococcal mastitic infections, whereas, streptomycin and neomycin are more suitable for coliform infections. Additionally antibiotics such as tetracycline and cephalosporins are useful against most mastitis-causing pathogens (Pyörälä, 2009). However, due its limited success and with ongoing international pressure to decrease antibiotic use in agriculture due to increasing antibiotic resistance, and the fact that mastitis incidence has remained stable for decades, preventative measures remain central to the most economically effective mastitis control programmes. Of particular relevance is, chronic, subclinical infection which causes ~80% of all mastitis related costs in dairy industries (Shim *et al*., 2004).

1.10.5 *Staphylococcus aureus*

S. aureus is a member of the Micrococcaceae family. It is a facultatively anaerobic, gram positive coccus that appears as grape-like cluster when viewed under magnification. *S. aureus* can exist as a commensal, inhabiting the skin and mucous membranes, but it can also survive in various tissue sites and the blood causing various diseases. In humans, it is responsible for a wide variety of community- and hospital-acquired infections (Lowy, 1998). It is also one of the most

contagious pathogenic bacteria causing bovine mastitis (Tollersrud *et al.*, 2000). It is difficult to control and can quickly invade all types of cells in the mammary gland (Buzzola *et al.*, 2006).

S. aureus expresses a wide array of secreted and cell surface virulence factors (Fig. 1.17), which promote adherence to the surface of host cells and facilitates the avoidance of the host's immune response (Foster and Höök, 1998; Foster, 2005). The ability of *S. aureus* to escape from the immune response and survive inside different cells is fundamental for this bacterium to persist for longer periods in the mammary gland (Rooijakkers *et al.*, 2005).

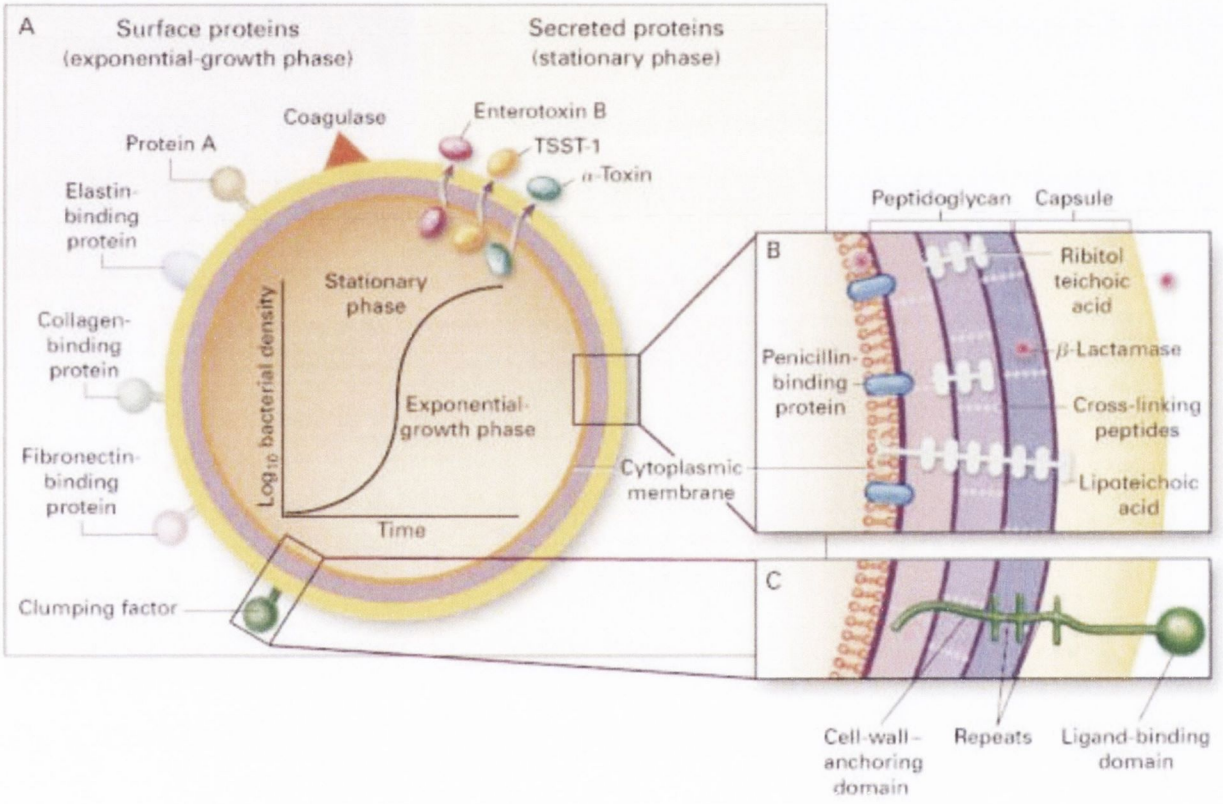


Fig 1.17 Structure of *S. aureus*.
Panel A shows the surface and secreted proteins. The synthesis of many of these proteins is dependent on the growth phase, as shown by the graph, and is controlled by regulatory genes such as *agr*. Panels B and C show cross sections of the cell envelope.Many of the surface proteins have a structural organization similar to that of clumping factor, including repeated segments of amino acids (Panel C). TSST-1 denotes toxic shock syndrome toxin 1. Figure taken from (Lowy, 1998).

1.10.6 Mammary gland innate immunity

Innate immunity predominates in the early stage of infection and is mediated by macrophages, neutrophils, natural killer cells (NK) and cytokines. It recognizes and responds to different pathogens, even if they are invading the mammary gland for the first time. In particular, bacteria have different cell wall structures that are recognized by specific plasma membrane receptors. These structures are lipopolysaccharide (LPS), peptidoglycan (PGN), and lipoteichoic acid (LTA), which constitute the pathogen-associated molecular patterns (PAMPs) (Bannerman *et al.*, 2004; Han *et al.*, 2003) These PAMPs are recognized by Toll like receptors (TLRs), which are located on the cell and endosomal membranes (Takeuchi and Akira, 2010). Interaction between the PAMPs and TLRs of immune cells induces production of cytokines and other endogenous mediators that are essential in protection of the mammary gland against pathogenic microorganisms (Rainard and Riollet, 2006). TLR4 recognizes the LPS of Gram-negative bacteria (e.g. *E. coli*) and molecules such as fibrinogen, heat shock proteins, and polypeptides (Kumar *et al.*, 2009b), whereas TLR2 is implicated in recognition of LTA and PGN from Gram-positive bacteria (e.g. *S. aureus*) (Takeuchi *et al.*, 2000). Apart from TLRs, recognition of *S. aureus* chemical structures can be mediated through formylated peptide receptors, mannose-binding lectins (MBL), ficolins, and complement molecules (Oviedo-Boyso *et al.*, 2007).

1.10.7 Innate immune response of the bovine mammary gland to *S. aureus* infection

Mastitis caused by *S. aureus* tends to become subclinical and chronic, and has a low response to conventional antibiotic treatment (Erskine *et al.*, 2002; Kerro *et al.*, 2002; Leitner *et al.*, 2003). This persistent infection has been related to an absence of the immune response in which various host and bacterial factors are involved (Barkema *et al.*, 2006; Petzl *et al.*, 2008; Riollet *et al.*, 2001; Yang *et al.*, 2008; Zecconi *et al.*, 2005). Mammary gland innate immunity is characterized by cytokine production and neutrophil recruitment. Therefore, to evaluate the innate immune response to *S. aureus* in the mammary gland different inflammatory markers such as TNF-α, IL-1β, IL-8, IFNγ and SAA have been studied (Griesbeck-Zilch *et al.*, 2008; Lahouassa *et al.*, 2007; Lee *et al.*, 2006).

Proinflammatory cytokines play an important role in the immune response against *S. aureus* infection. TNF-α and IL-1β are important cytokines produced in inflammatory reactions

such as mastitis (Yang *et al.*, 2008). Gene expression of these cytokines have been detected in milk from cows with *S aureus*-induced mastitis, however, the corresponding proteins have not been reported (Lee *et al.*, 2006; Riollet *et al.*, 2001).

Studies have shown that *S. aureus*, in contrast to *E.coli* intramammary infection which predominately results in an acute immune response leading to clearance of the pathogen, did not increase the SCC or the expression of innate immune gene such as TLRs and the defensins during the first 24 h after pathogen inoculation (Petzl *et al.*, 2008). The same strains have also been shown to induce in mammary epithelial cells IL-8 and TNF-α gene expression, but *S. aureus* to less than 5% of the degree caused by *E. coli*. (Yang *et al.*, 2008). These results suggest that TNF-α plays an important role in the early stages of mastitis, and the limited expression might explain the inefficiency of the mammary gland to eliminate *S. aureus*.

1.11 Thesis objectives

Several studies have quantified changes in concentrations of cytokines, APPs and AMPs in bovine milk following experimentally induced intramammary infection 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 *S. aureus*-induced subclinical mastitis. In addition to the local response, an effective systemic response is essential for the control of bacterial infections. Limited information is published on the systemic immune response to *S. aureus*-induced mastitis, in particular, the influence on distant organs such as the liver. The interplay of both local and systemic reactions may determine the course of infection in an individual animal. Increased knowledge of this may contribute to a greater understanding of the disease and pinpoint the most promising direction of research to identify effective biomarkers for mastitis-causing pathogens. For these reasons, we analysed both the local and systemic immune response in an animal model of *S. aureus*-induced subclinical mastitis.

Secondly, the udder is a complex organ composed of several cell types, all likely to contribute differentially to the immune competence of the udder. Although the principal function of mammary epithelial cells is milk synthesis during lactation, the immune significance of these cells has only recently been recognized (Griesbeck-Zilch *et al.*, 2008; Lahouassa *et al.*, 2007; Wellnitz and Kerr, 2004). These cells are the dominant cell type in healthy, uninfected mammary quarters and, therefore, are most likely the first cells to encounter the pathogen after entering the udder. These studies have demonstrated the enhanced expression of a variety of immune genes in response to mastitis-causing stimuli. However, little information is available describing the early immune response in mammary epithelial cells after pathogen challenge, in particular, the early expression of bactericidal molecules such as antimicrobial peptides and acute phase proteins. The host response induced early in mammary epithelial cells during infection may be a key event in the pathogenesis of subclinical mastitis. To address this, qPCR was used to measure the expression of genes encoding PRRs, pro- and anti-inflammatory cytokines, acute phase proteins and antimicrobial peptides in primary mammary epithelial cells at early time points following stimulation with a low-virulence strain of *S. aureus*.

Finally, the dwindling numbers of therapeutic options for mastitis necessitates the development of novel antimicrobials. To this end, we used a bioinformatics approach to characterise the entire cathelicidin repertoire encoded in the bovine genome and profiled the expression of these AMPs in a range of tissues.

The specific aims of this thesis are to:

- Analysis the expression of candidate immune genes in liver tissue from both *S. aureus* challenged and control animals.
- Profile the expression of candidate immune genes in tissue from sterile unchallenged quarters of cows that had prior inoculation with *S. aureus* in the neighbouring ipsilateral quarters.
- Assay the relative levels of innate immune genes in tissue isolated from the alveolar, ductal, gland cistern and teat canal region of *S. aureus* infected and control quarters.
- Assay the relative levels of PRRs, pro- and anti-inflammatory cytokines, acute phase proteins and antimicrobial genes in primary mammary epithelial cells at 30 min, 1h, 3h and 6h post challenge with a low-virulence strain of *S. aureus*.
- Characterise the bovine cathelicidin AMP genes with respect to sequence, gene structure and genomic organisation.
- Assay the relative levels of cathelicidin gene in a panel of bovine tissue.

Chapter 2: Materials and Methods

2.1 Animal infection model and tissue collection

An experimental model, designed to mimic subclinical bovine mastitis, was established by our collaborators (Eckersall *et al.*, 2006) using a low-virulence strain of *S. aureus* (strain NCTC13047) originally isolated from a case of subclinical mastitis in Holstein- Friesian dairy cows (Young *et al.*, 2001). Intramammary infusion was carried out twice; for the first study period at day 0, and for a second study period 28 days later (Fig.2.1). All cows selected for the experiment had been free of clinical mastitis throughout the current and previous lactation and had individual quarter SCC below 0.1×10^6 cells/ml at monthly recordings during the current lactation. Before infusion, full bacteriological examination was carried out on milk samples from all 4 quarters of infused and control cows at several times, and shown to be negative for *S. aureus* and other major mastitis-causing pathogens. On day 0, both the right-fore (RF) and the right-hind (RH) quarters were infused with 5×10^4 cfu of *S. aureus* in 10 ml Ringer's 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 Ringer's solution (Fig. 2.1). 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) and from liver tissue, 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, Ayr, Scotland.

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 (Figure 2.1). In addition, liver tissue from *S. aureus* challenged (n=5) and control animals was used (n=3) (Table 2.1). These tissue samples, from both control and *S. aureus* challenged quarters and liver, were categorized as 48h 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.

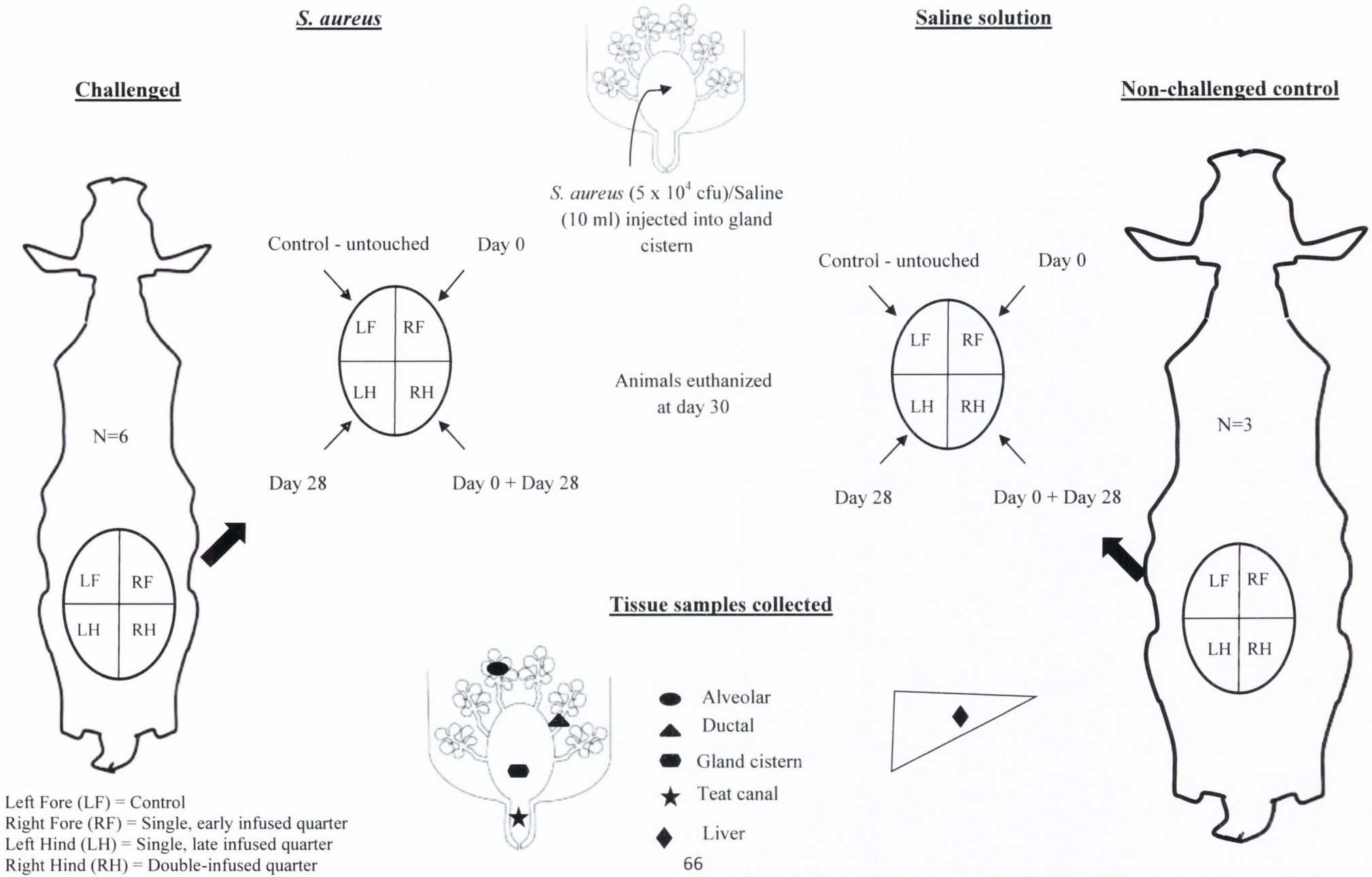


Figure 2.1 Experimental setup of intramammary infection with a low-virulence strain of *S. aureus* isolated from a natural case of subclinical mastitis.

2.2 Innate Immune gene expression profiling *in vivo*

2.2.1 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 thick) and placed in 2 ml microcentrifuge tubes. Total RNA was extracted from liver and mammary tissue using the RNeasy® lipid tissue mini kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions, with a DNase® (Qiagen Ltd., Crawley, UK) digestion step incorporated to remove any residual genomic DNA. RNA quality and yield was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Ireland Ltd., Cork, Ireland) and ND-1000 NanoDrop® spectrophotometer (Thermo Scientific, MA, USA), respectively.

The bioanalyzer is an automated bio-analytical device using microfluidics technology that provides eletrophoretic separations in an automated and reproducible manner (Mueller *et al.*, 2000). One microlitre of RNA are separated in the channels of micro-fabricated chips according to their molecular weight and subsequently detected via laser-induced fluorescence detection. The result is visualized as an electropherogram where the amount of measured fluorescence correlates with the amount of RNA of a given size (Fig. 2.2). RNA degradation is easily detected by a shift in the RNA size distribution towards smaller fragments and a decrease in fluorescence signal from the ribosomal peaks. The level of RNA degradation is reported as an RNA integrity number (RIN), with values ranging from 1 (low-quality degraded RNA) to 10 (high-quality intact RNA) (Fig. 2.2A-C).

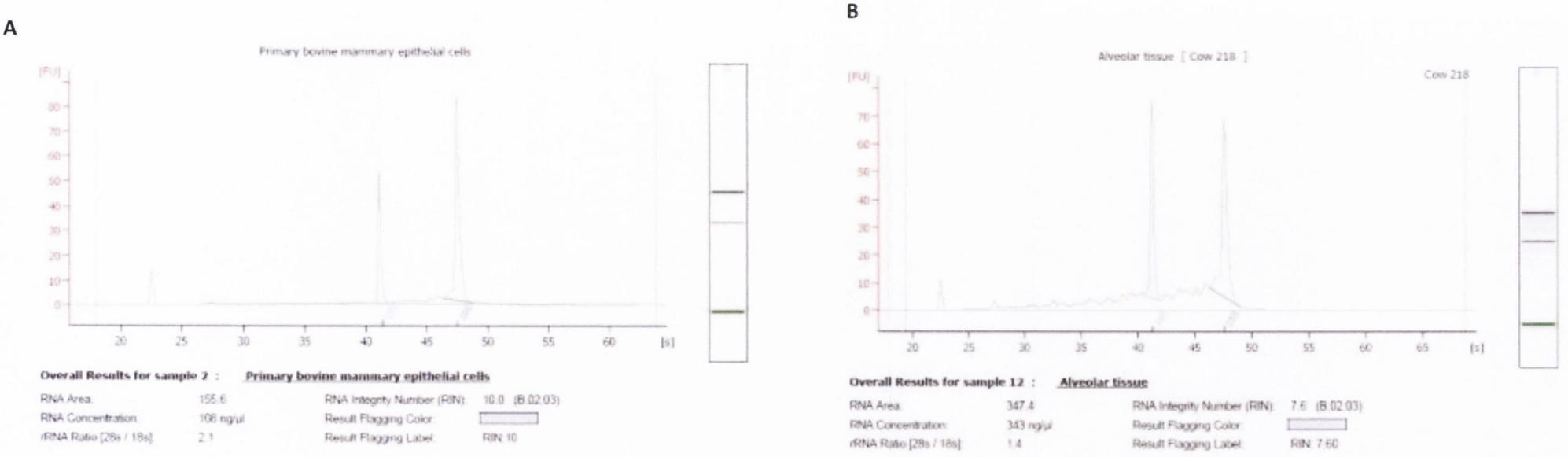


Figure 2.2 Selected RNA samples showing different degrees of degradation.

RNA extracted from primary bovine mammary epithelial cells (A), alveolar tissue (B) and testes tissue (C) was assessed using micro-capillary electrophoresis (Agilent Bioanalyzer). The reference ladder is visible as the first peak. RNA quantity is estimated as the area underneath the 28S and 18S ribosomal RNA band peaks. RNA quality is assessed by the degradation visible as smaller peaks between the reference ladder and the 18S band and is reported as an RNA integrity number (RIN). The level of RNA degradation is reported as an RNA integrity number (RIN), with values ranging from 1 (low-quality degraded RNA) to 10 (high-quality intact RNA). [FU] = Fluorescence units and [s] = seconds.

2.2.2 Quantitative real-time PCR (qPCR)

One microgram of total RNA from each sample was reverse transcribed into cDNA using OmniScript^{TM} 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 qPCR were designed using Primer Express 3.0 software (www.appliedbiosystems.com) (Table 2.1). Primers were designed to be located in the 3' end of the gene of interest in order to minimise potential 3' bias as a result of using oligo (dT) primers. In addition, where possible, they were designed to traverse introns in order to minimise the potential of amplifying genomic DNA. Primers were commercially synthesized (Invitrogen Ltd.). 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). Reaction conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30s, 60°C for 1 min and 72°C for 30s, and finally amplicon dissociation at 95°C for 1 min, 55°C for 30 s and 95°C for 30 s. Each reaction was carried out in duplicate in a total volume of 25µl with 2µl of cDNA, 12.5 µl of 2 × Sybr Green master mix (Stratagene Corp.) and 10.5µl primer/H_2O. 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 visualised on 1.5% agarose gels in 1X TAE buffer (50x stock: 121g Tris base, 28.5ml glacial acetic acid, 18.6g $Na_2EDTA.2H_2O$; adjusted to 500mls) with ethidium bromide (1:6000 dilution). Gels were run at 120V for 20-40 min and visualised under UV light.

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 (data not shown) were generated and efficiency determined with the formula $10^{(-1/slope)}$. Primer efficiency for *GAPDH* and *H3F3A* and all significantly differentially expressed genes was considered comparable and within the recommended range for using the $2^{-\Delta\Delta Cq}$ method (90–100% efficiency).

Table 2.1 Gene-specific oligonucleotide primers used for qPCR. NCBI Reference Sequence (RefSeq) numbers are provided along with amplicon size and optimum primer concentrations (continued on the next page).

Gene Symbol	Accession number	Forward primer (5'-'3)	Reverse primer (5'-'3)	Primer concentration (nM)	Amlicon size (bp)
ACTB	NM_173979	AGATGACCCAGATCATGTTCGA	GACCCCGTCACCGGAGTCCATCACGA	600	126
GAPDH	NM_001034034	CTCCCAACGTGTCTGTTGTG	TGAGCTTGACAAAGTGGTCG	600	222
PPIA	NM_178320	CCACCGTGTTCTTCGACAT	TCTGTGAAGCAGGAACCTTT	600	155
H3F3A	NM_001014389	CATGGCTCGTACAAAGCAGA	ACCAGGCCTGTAACGATGAG	300	136
RPS6	NM_001040584	CGCACGCTTCCCTATAAGAT	TTCACTTCCTGGGTCAGAGG	600	176
RPS9	NM_001101152	GATTACATCCTGGGCCTGAA	ATGAAGGACGGGATGTTCAC	600	161
RPS15	XM_585783	GCAGCTTATGAGCAAGGTCGT	GCTCATCAGCAGATAGCGCTT	900	151
TLR1	NM_001046504	ACTTGGAATTCCTTCTTCACGA	GGAAGACTGAACACATCATGGA	600	176
TLR2	NM_174197	GGTTTTAAGGCAGAATCGTTTG	AAGGCACTGGGTTAAACTGTGT	600	190
TLR3	NM_001008664	GATGTATCACCCTGCAAAGACA	TGCATATTCAAACTGCTCTGCT	600	195
TLR4	NM_174198	CTTGCGTACAGGTTGTTCCTAA	CTGGGAAGCTGGAGAAGTTATG	600	153
TLR5	NM_001040501	CCTCCTGCTCAGCTTCAACTAT	TATCTGACTTCCACCCAGGTCT	600	172
TLR6	NM_001001159	CCTTGTTTTTCACCCAAATAGC	TAAGGTTGGTCCTCCAGTGAGT	300	154
TLR7	NM_001033761	TCTTGAGGAAAGGGACTGGTTA	AAGGGGCTTCTCAAGGAATATC	600	205
TLR8	NM_001033937	TAACCTTCGGAATGTCTCCAGT	GTGGGAAATTCTGTTTCGACTC	900	232
TLR9	NM_183081	CTGACACCTTCAGTCACCTGAG	TGGTGGTCTTGGTGATGTAGTC	300	156
TLR10	NM_001076918	ATGGTGCCATTATGAACCCTAC	CACATGTCCCTCTGGTGTCTAA	600	248
NOD1	NM_001256563	TCTCGCTCTGGCTGTGAAGAA	TGAATGCAAGACTCAGGTTGGT	600	140
NOD2	NM_001002889	CGCCAAAGGACTTGCAAGA	CCTCGGAGCCAGACTTCCA	900	135
TNFA	NM_173966	CCATCAACAGCCCTCTGGTT	CTCACACCGTTGGCCATGA	900	150

Gene Symbol	Accession number	Forward primer (5'-'3)	Reverse primer (5'-'3)	Primer concentration (nM)	Amlicon size (bp)
IL6	NM_173923	TAAGCGCATGGTCGACAAAA	TTGAACCCAGATTGGAAGCAT	600	150
IL1B	NM_174093	CCCTGCAGCTGGAGGAAGTA	CTTCGATTTGAGAAGTGCTGATGT	600	150
IFNG	NM_174086	TTGAATGGCAGCTCTGAGAAAC	TCTCTTCCGCTTTCTGAGGTTAGA	600	150
IL8	NM_173925	AGAACTTCGATGCCAATGCAT	GGGTTTAGGCAGACCTCGTTT	600	150
TGFB1	NM_001166068	TGCTTCAGCTCCACAGAAAAGA	AGGCAGAAATTGGCGTGGT	600	116
IL10	NM_174088	AGAACCACGGGCCTGACA	ACCGCCTTGCTCTTGTTTTC	600	121
IL17	NM_001008412	TCGTTAACCGGAGCACAAACT	TGGCCTCCCAGATCACAGA	600	120
DEFB1	NM_175703	TCTTCCTGGTCCTGTCTGCT	TTACCTCCACCTGCAGCATT	300	164
DEFB4	NM_174775	ATCACCTGCTCCTCGCAGT	CCTACACGGCACAAGAACG	600	127
DEFB5	NM_001130761	ACCAGCATGAGGCTCCAT	TTGCCAGGGCACGAGAT	600	143
CATHL1	NM_174825	ATCACCTGTAATAATCACCAGAGCAT	CCCTTAGGACTCTGCTGGCTTA	600	150
CATHL2	NM_174826	GAGAATGGGCTGGTGAAACAG	GTTATCTGCCTATTGTTCACCGTCTA	600	141
CATHL3	NM_174001	TGACTTCAAGGAGAATGGGCTGGTG	GCCGGGGACGAATTCTCCTGACA	300	122
CATHL4	NM_174827	GGCACTCGAAAGCCTGTGA	GGCCATTTCCAGGGTAGGAT	600	200
CATHL5	NM_174510	AAGGAGAATGGGCTGCTGAAA	GCCATACTTCTTCCAAGCACGTA	600	150
CATHL6	NM_174832	GGAGGACGATGAGAACCCAAA	AGTAGCGGAATGACTGGAGAAAGT	600	270
CATHL7	NM_174831	CCCAGAGCAGTGTGACTTCAAG	AGCCCCGCACTCTGAATATTATTA	600	120
HAMP	NM_001114508	TTCTGCTCCTTGTCCTGCTC	AGATGCAGATGGGAAAGTGG	600	162
LEAP2	NM_174559	AAAGAGAAGGCCGAGGAGAATG	GAGCAGCGTCTTTTTCTGCAT	600	120
LAP	NM_203435	GACAGCATGAGGCTCCATC	CTCCTGCAGCATTTTACTTGGGCT	600	194
TAP	NM_174776	TCCTGGTCCTGTCTGCTTC	CTACAGCATTTTACTGCCCG	600	151
SAA3	NM_181016	CTCAAGGAAGCTGGTCAAGG	CTTCGAATCCTCCCGTACCT	600	240
HP	NM_001040470	TGGTCTCCCAGCATAACCTC	AGGGTGGAGAACCACCTTCT	600	185
LBP	NM_001038674	TCAGCTCTACGACCCCATTC	AAGTGCTGCACATCTGGAAA	600	151
AGP	NM_001040502	GTGCATAGGCATCCAGGAAT	CCGAGGGAACTGAGTTACAAA	600	192
CP	XM_592003	TTCATGCACATGGAATGACTT	TAAAGGCCCAATGAGTCCTG	600	236

2.2.3 Selection of a stably expressed reference gene

Gene expression quantification requires multi-step processes: RNA extraction, cDNA synthesis via reverse transcription and finally qPCR. During these processes, experimental errors are common place. RNA quality and quantity, reverse transcriptase enzyme efficiencies and qPCR reaction efficiencies can vary considerably between samples, and thus can result in inaccurate gene expression quantification between two experimental groups. To minimise this, each gene of interest is amplified in each sample in parallel with an internal reference gene. A reference gene is assumed to be stably expressed under any experimental condition, and thus acts as a 'normaliser'. Traditionally, either *GAPDH* or *ACTB* would have been used; however, it has been shown that certain experimental conditions can alter the expression of these genes (Dheda *et al.*, 2005). Therefore, for each experimental condition, it is critical to select a reference gene that is stably expressed.

A stable reference gene was determined using the geNorm software (version 3.4 Microsoft Excel add-in software package) (Vandesompele *et al.*, 2002). For each liver sample both *S. aureus*-induced IMI (n = 5) and from non-challenged controls (n = 3), seven putative reference genes [peptidylprolyl isomerise 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 were then analysed using the geNorm software to generate *M* stability. 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.

2.2.4 Data analysis

For differential expression analysis, qPCR data (Cq values) were converted to gene expression fold changes using the $2^{-\Delta\Delta Cq}$ (Cq represents the quantification cycle) method (Schmittgen and Livak, 2008), and recorded relative to control samples.

For baseline expression (expression in control samples) analysis, expression levels of the gene of interest (GOI) was determined as a ratio of the level of expression of the house keeping gene using the formula ($2^{-Cq(GOI)}/2^{-Cq(HK)}$).

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 (GraphPad Software, San Diego, CA). P values of <0.05 were considered statistically significant.

2.3 Innate immune gene expression profiling *in vitro*

2.3.1 Cultured primary mammary epithelial cells (PbMECs)

Primary bovine mammary epithelial cells (AvantiCell Science Ltd, Scotland, UK) isolated from animals (n=3) in their third trimester of pregnancy were cultured according to the manufacturer's instructions. Cells were thawed and cultured in 75cm^2 collagen-coated flasks (Cruinn Diagnostic Ltd) in a medium containing Medium 199/Ham's F12 (AnaLab supplies Ltd, Dublin, Ireland) (50:50) pH 7.4 supplemented with 8% horse serum, 2% fetal calf serum (Bio-Sciences Ltd, Dublin, Ireland), 5 µg/ml insulin, 1 µg/ml hydrocortisone, 3 µg/ml prolactin, 2 mM sodium acetate (Sigma-Aldrich Ireland Ltd, Dublin, Ireland), 10 mM Hepes and 1 U/ml penicillin/streptomycin (Bio-Sciences Ltd). Cells were grown to confluence, washed twice with Hank's Balanced Salt Solution (HBSS) (AnaLab supplies Ltd), then trypsinised with 0.25% trypsin/0.02% EDTA in HBSS solution (Sigma-Aldrich Ireland Ltd), and finally split into 6-well collagen-coated plates (Cruinn Diagnostic Ltd).

2.3.2 Cultured BME-UV cells

BME-UV cells (Bovine Mammary Epithelium - University of Vermont), originally described by Zavizion *et al.*, 1996, were cultured at 37°C with 5% CO_2 in 75 cm^2 cell culture flasks (Cruinn Diagnostic Ltd). Cultures were grown in bovine medium (pH 7.3) containing

21.25% Dulbecco's modified Eagle's medium, 21.25% Ham F-12 nutrient mixture, 25.5% RPMI 1640 (AnaLab supplies, Ireland), 17% NCTC 135, 10% fetal bovine serum, 3% newborn calf serum (Bio-Sciences Ltd.), 2% iron-supplemented newborn calf serum (Fisher Scientific, Dublin, Ireland), 1% penicillin/streptomycin (Bio-Sciences Ltd), 1% insulin–transferring–selenite, 1 mg/ml lactalbumin hydrosylate, 10 mg/ml L-ascorbic acid and 3 mM lactose (Sigma–Aldrich Ireland Ltd.). Cells were passaged with trypsin/EDTA (Bio-Sciences Ltd.) and seeded on 12-well plates or cell culture flasks (Cruinn Diagnostic Ltd) at a density of 2 x 10^4 cells/cm^2. All experiments were carried out on confluent monolayers to minimise cell proliferation in order to maintain reproducibility.

2.3.3 Bacterial strain preparation

Both *E.coli* 1303 and *S. aureus* strains 1027 were a kind gift from Dr Wolfram Petzl (Research Unit, Clinic for Ruminants, Ludwig Maximilians University, Munich, Germany). Bacteria were grown for 8 h in trypticase soy broth (TSB) (Cruinn Diagnostic Ltd, Dublin, Ireland) to the late logarithmic phase, the growth curves were determined through plating serial dilutions at various time points over 16 hr growth periods. Following incubation for 8 h, bacterial concentration was determined by plating serial dilutions. Bacteria were heat-killed for 1 hr (*E. coli* 60°C and *S. aureus* for 80°C), as verified through control plating. Cells were then centrifuged, washed twice in appropriate culture medium, then resuspended in culture medium at a density of 3 x 10^8 cfu/ml. Aliquots were stored at -20°C.

2.3.4 Dose response analysis

Concentrations of cfu used in experiments were derived from a dose-response analysis for BME-UV cells. Cells were challenged with *S. aureus* strain 1027 at various concentrations that corresponded to a multiplicity of infection (MOI) of 1, 10, 100 and 1000. Expression of the gene encoding the proinflammatory cytokine, IL-8, was quantified for each MOI.

2.3.5 Challenge with pathogens

Once the cells reached confluence, they were washed twice in PBS and the medium was replaced with, and without (controls), *S. aureus* 1027/*E. coli* 1303 at a final concentration of 8 x 10^8 cfu/ml which corresponded to MOI of approximately 100. After incubation for 30 min, 1 h, 3 h, 6 h supernatant was removed, cells were washed twice in PBS then lysed directly with RLT lysis buffer (Qiagen). Once lysed, cells where snap-frozen in liquid nitrogen and stored at -80°C for later processing.

2.3.6 RNA isolation and quality control

RNA was extracted from cultured PbMECs and the BME-UV cells as indicated in section 2.2.1. Ratios of 18S to 28S ribosomal RNA averaged >1.8 and the RNA Integrity Number (RIN) averaged > 9.5 for all cultured epithelial cells.

2.3.7 Quantitative real-time PCR (qPCR)

qPCR and qPCR data analysis were performed as indicated in section 2.2.2, section 2.2.3 and section 2.1.4, respectively.

2.3.8 Data analysis

qPCR data was analysed as described in section 2.2.4

Statistical analysis of qPCR results was carried out using the non-paired t-tests as implemented in version 5.01 of GraphPad Prism (GraphPad Software, San Diego, CA). P values of <0.05 were considered statistically significant

2.4 Characterisation of the cathelicidin gene repertoire and expression profile in cattle

2.4.1 General techniques used in bioinformatics

Bioinformatics is a broad term used to define the application of computers in biological research. Pattern recognition is one of the key tools of bioinformatics. Two of the principal methods used for this purpose are described below.

2.4.1.1 BLAST

Similarity searching is one of the central tools of bioinformatics and BLAST (Basic Local Alignment Search Tool) is the most widely used algorithm for this purpose. BLAST identifies homology by comparing a query sequence to all the sequences in a database in a pairwise manner. Many of the publicly available sequence databases contain millions of sequences composed of billions of bases or amino acids and as a consequence search methods using entire sequence lengths are not feasible due to processing speed and memory requirements. To overcome this, BLAST breaks the query and the database sequence into fragments known as "words" and then seeks matches between the fragments. Initial steps attempt to align the query words of length "W" to the target sequences. Matching word alignments which satisfy a threshold value, "T" is referred to as High-Scoring Segment Pairs (HSPs). HSPs alignments are then extended in either direction. Each alignment extension results in either an increased or decreased score depending on the quality of the newly added matches. When the alignment score drops below a second predefined threshold, "S", known as the Maximal-Scoring Segment Pair (MSP), the extension of the alignment stops (Altschul *et al.*, 1997).

Finally, the quality of each pairwise alignments are ranked based on the calculated bit-score and expectation value (E-value), which is a measure of the probability of any given hit occurring by chance. To calculate the E-value both the length of the query sequence and the size of the database being searched are considered. A low E-value indicates higher confidence in the query being a true homologue.

Similar BLAST programs use the method described above, though there are slight variations in the parameters employed by each one. BLAT, (BLAST-like Alignment Tool,

Kent, 2002), is similar in many ways to BLAST but is structured differently. For example, BLAT works by keeping an index of an entire genome in memory. Thus, the target database of BLAT is not a set of GenBank sequences, but instead an index derived from the assembly of the entire genome. BLAT has special code to handle introns in RNA/DNA alignments and can effectively "unsplice" mRNA onto the genome giving a single alignment that uses each base of the mRNA only once, and which correctly positions splice sites. As such, BLAT is commonly used to look up the location of a sequence in the genome or determine the exon structure of an mRNA.

2.4.1.2 Hidden Markov Models

Pairwise sequence comparison methods such as BLAST assume that all positions in the sequence are equally important. However, it does not take into account the relative importance of each residue in a protein as defined by its degree of conservation amongst closely related homologues (Eddy, 1998). Certain amino acid residues in a protein are more critical for correct structure and function and are thus highly conserved.

A Hidden Markov Model (HMM) is a statistical model which can exploit such position specific information derived from the alignment of multiple homologous protein sequences. The sensitivity of HMMs for detection of distant relatives based on the alignment of a set of known gene family members comes from the generation of a "fingerprint" which is unique to that set of sequences, and can be used to query a database of unknown proteins (Madera and Gough, 2002).

2.4.2 Bioinformatics

Publicly available protein sequences corresponding to the 7 known bovine cathelicidins were retrieved from Uniprot (http://www.uniprot.org/) (see Appendix). To carry out HMM (Eddy 1998) searches of the bovine genome for novel cathelicidin, the entire bovine genome was translated in all six reading frames using a purpose-written pearl script. To generate accurate HMM models representing the cathelicidin family, the protein sequences of the seven known cathelicidins were aligned using ClustralW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Fig. 2.3). The sequences were used in the construction of the HMM by the hmmbuild program in HMMER 2.1.1

(http://hmer.wustl.edu). The generated HMM profile was then searched against the translated genome to identify putative cathelicidin-like regions. Chromosomal location and strand orientation of the identified cathelicidins were determined using the BLAST-like Alignment Tool (BLAT) at the University of California—Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu). Genomic DNA corresponding to putative cathelicidins was retrieved using BLAT and used for prediction of intron/exon boundaries using GenScan (http://genes.mit.edu/GENSCAN) (Burge and Karlin, 1997). A qualitative estimation for the expression of cathelicidin genes was undertaken by a search of the bovine expressed sequence database (EST) usinq MegaBLAST (http://blast.ncbi.nlm.nih.gov/Blast.cg).

Figure 2.3 Multiple sequence alignment of bovine cathelicidins

Protein sequences corresponding to the 7 known bovine cathelicidins were retrieved from Uniprot. These sequences were aligned using CLUSTAL 2.1 multiple sequence alignment software. Dashes are inserted to optimise the alignment, and conserved residues are indicated by asterisk. *CATHL1*, Bactenecin-1 (Bac-1); *CATHL2* Bactenecin-5 (Bac-5); *CATHL3*, Bactenecin-7 (Bac-7); *CATHL4,* Indolicidin (Indol); *CATHL5*, Bovine myeloid antibacterial peptide 28 (BMAP28); *CATHL6,* Bovine myeloid antibacterial peptide 27 (BMAP27); *CATHL7*, Bovine myeloid antibacterial peptide 34 (BMAP34).

2.4.3 Bovine tissue panel

An extensive range of tissues were collected at a local abattoir from euthanized cattle and were immediately flash frozen in liquid nitrogen, these included tissue from the lung, rumen, small intestine, large intestine, testis, uterus, mammary gland, spleen, liver and lymph node.

Peripheral blood mononuclear cells were extracted from whole blood of uninfected cattle using a Percoll™ gradient (GE Healthcare UK, Buckinghamshire, UK) and previously described methods (Ulmer *et al.*, 1984). Briefly, blood was centrifuged in 50-ml sterile conical tubes at 1,800 rpm for 20 min at room temperature. The resulting buffy coats were pooled (3-4 tubes/new tube) for each animal, transferred to a new conical tube containing 34 ml of cold sterile phosphate-buffered saline (PBS), which were then under laid with 10 ml of Percoll™ (1.084 g/ml; Sigma-Aldrich Ireland Ltd: www.sigmaaldrich.com). Cells were then centrifuged at 1,380 rpm for 40 min at room temperature to separate erythrocytes and polymorphonuclear leukocytes from mononuclear cells. PBMC at the PBS-Percoll interface were pooled for each animal and transferred to a new 50 ml conical tube. The volume was brought up to 30 ml with PBS. After centrifugation at 1,800 rpm for 5 min at room temperature, all but 5 ml PBS was poured off and cell clumps were re-suspended in residual liquid.

2.4.4 Primary mammary epithelial cells (PbMECs) and *S.aureus* challenge

Primary bovine mammary epithelial cells (Avanticell Ltd.) were cultured as described in section 2.3.1 and challenged with *S.aureus* as described in section 2.3.5.

2.4.5 RNA isolation and quality control

RNA was extracted from bovine tissue and cultured PbMECs as described in section 2.2.1 and section 2.3.1, respectively.

2.4.6 Quantitative real-time PCR (qPCR)

cDNA synthesis, design of gene specific primers for qPCR (Table 2.1) and qPCR were performed as described in section 2.2.2 and section 2.2.3, respectively.

2.4.7 Data analysis

qPCR data was analysed as described in section 2.2.4.

Chapter 3: Systemic Response at the Gene Transcription Level in Dairy Cows with *Staphylococcus aureus*-induced Subclinical Mastitis.

3.1 Introduction

The acute phase response (APR) is central to the action of the innate immune system in its response to trauma, inflammation, and infection. Following exposure to the damage associated molecular pattern (DAMP) molecules or pathogen-associated molecular pattern (PAMP) molecules, macrophages and epithelial cells produce cytokines such as IL-1β, IL-6, and TNF-α. As well as their important roles at the site of induction, IL-1β, IL-6, and TNF-α, upon entering circulation, ccoordinate the systemic response to infectious diseases and inflammation.

In the liver, these cytokines cause a profound effect with approximately 20% of all genes analysed up- or downregulated after intramammary exposure to *E.coli* lipopolysaccharide (LPS) (Jiang *et al.*, 2008). One of the most important hepatic adaptation to the APR is a shift in protein concentration (acute phase proteins (APP)) secreted by the liver into the blood. APPs play a variety of roles in the restoration of homeostasis (Moshage, 1997; Gabay and Kushner, 1999).

In any given species, particular APPs are classified as major, moderate or mild responders. For example, C-reactive protein (CRP) is a major APP, increasing 100 to 1000 fold in plasma concentration during the APR. In contrast, C-reactive protein is a constitutive plasma protein in cattle, little affected by the APR (Eckersall and Bell, 2010). Other important APPs are serum amyloid A (SAA), haptoglobin (HP), lipopolysaccharide-binding protein (LBP), α 1-acid glycoprotein (AGP) and ceruloplasmin (CP) all of which are important in the control of bacterial infections (reviewed in Chapter 1, section 1.7.1).

Another family of proteins that has a critical effect in innate immunity is the antimicrobial peptides (AMPs) (reviewed in Chapter 1, section 1.8). Liver-expressed antimicrobial peptide-2 (LEAP-2) and LEAP-1/hepicidin are blood-derived peptides that are predominantly expressed in the liver and highly conserved among mammals (Krause *et al.*, 2000, 2003).

A number of infectious diseases are known to stimulate the APR in cattle. Mastitis, a prevalent condition of lactating dairy cattle, is predominantly caused by bacterial infection of the mammary glands by a variety of gram-positive and gram-negative bacteria (Cullor and

Tyler, 1996). The pathogenesis of *Escherichia coli* mastitis is often characterized by an acute and severe inflammation both locally and systemically resulting in pathogen clearance.

In contrast, intramammary infections with *Staphylococcus 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).

In a recent investigation (Mitterhuemer *et al.*, 2010), it was shown that the systemic immune response induced as a result of intramammary *E. coli* infection had an influence on the transcriptome of neighbouring unchallenged quarters, which may impair the progression of subsequent infections of neighbouring quarters. Data on the systemic immune response to subclinical mastitis is limited, in particular, *S. aureus*-induced subclinical mastitis (Eckersall *et al.*, 2006; Yang *et al.*, 2008).

The use of within animal controls is a common and accepted practice because the udder quarters are generally considered to be separate, independent anatomical structures (Bannerman *et al.*, 2004; Lutzow *et al.*, 2008; Rinaldi *et al.*, 2010; Swanson., *et al.*, 2009) and reduces the between animal variation seen in outbred species such as cattle.

In this study, we investigate the systemic immune response to *S. aureus*-induced subclinical mastitis and determine whether the systemic response has an influence on neighbouring unchallenged quarters. The interplay of both local and systemic reactions may finally determine the pathogenesis of infection in individual animals.

3.2 Results

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

Increased somatic cell count (SCC) from pre-infusion to post-infusion of >2 × 10^5 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 quarters. The mean SCC in milk from *S. aureus* challenged 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 (Fig. 3.1). In mock inoculated and untreated neighbouring udder quarters of infected animals no significant increase in the number of somatic cells was observed (Fig. 3.1).

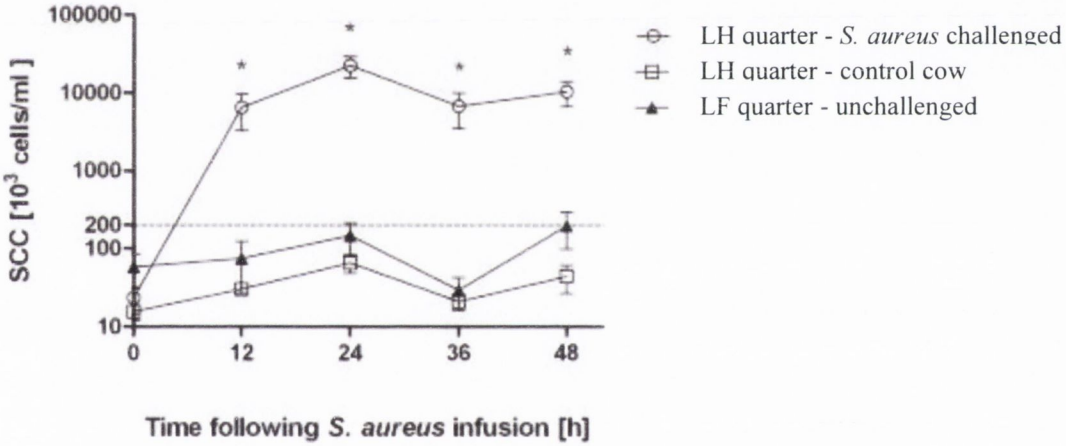


Figure 3.1 Elevated levels of somatic cells in milk in response *S. aureus* challenge
Analysis of milk somatic cell counts determined for milk isolated from *S. aureus* challenged and unchallenged quarters from the same animals (6 cows, 12 quarters), and non-challenged control quarters (3 cows, 3 quarters) for up to 48 h. Increased somatic cell count (SCC) from pre-infusion to post-infusion of >2 × 10^5 cells/ml (dashed line) 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 quarters. Mean values (bars denote standard error of the mean) are presented. Statistical significance is denoted as: $*P < 0.05$.

3.2.2 RNA quality

An essential requirement for a successful quantitative mRNA analysis using qPCR is the usage of intact RNA. Low-quality RNA may compromise the derived expression results. The assessment of RNA integrity is a critical first step in obtaining meaningful gene expression data (Bustin *et al.*, 2009). The extraction method used yielded RNA of high quality. For liver tissue, the RNA integrity number (RIN) averaged >6.8 (range, 6.2 to 7.7) and for alveolar tissue the RIN averaged >6.7 (range 6.2 to 8), all suitable for reverse transcription to cDNA and analysis using qPCR. Calculation and publication of an RIN facilitates reliable comparison of tissue RNA samples and facilitates accurate reproducibility of experiments. Figure 3.2A-C illustrates the high RNA quality isolated from liver tissue and mammary tissue.

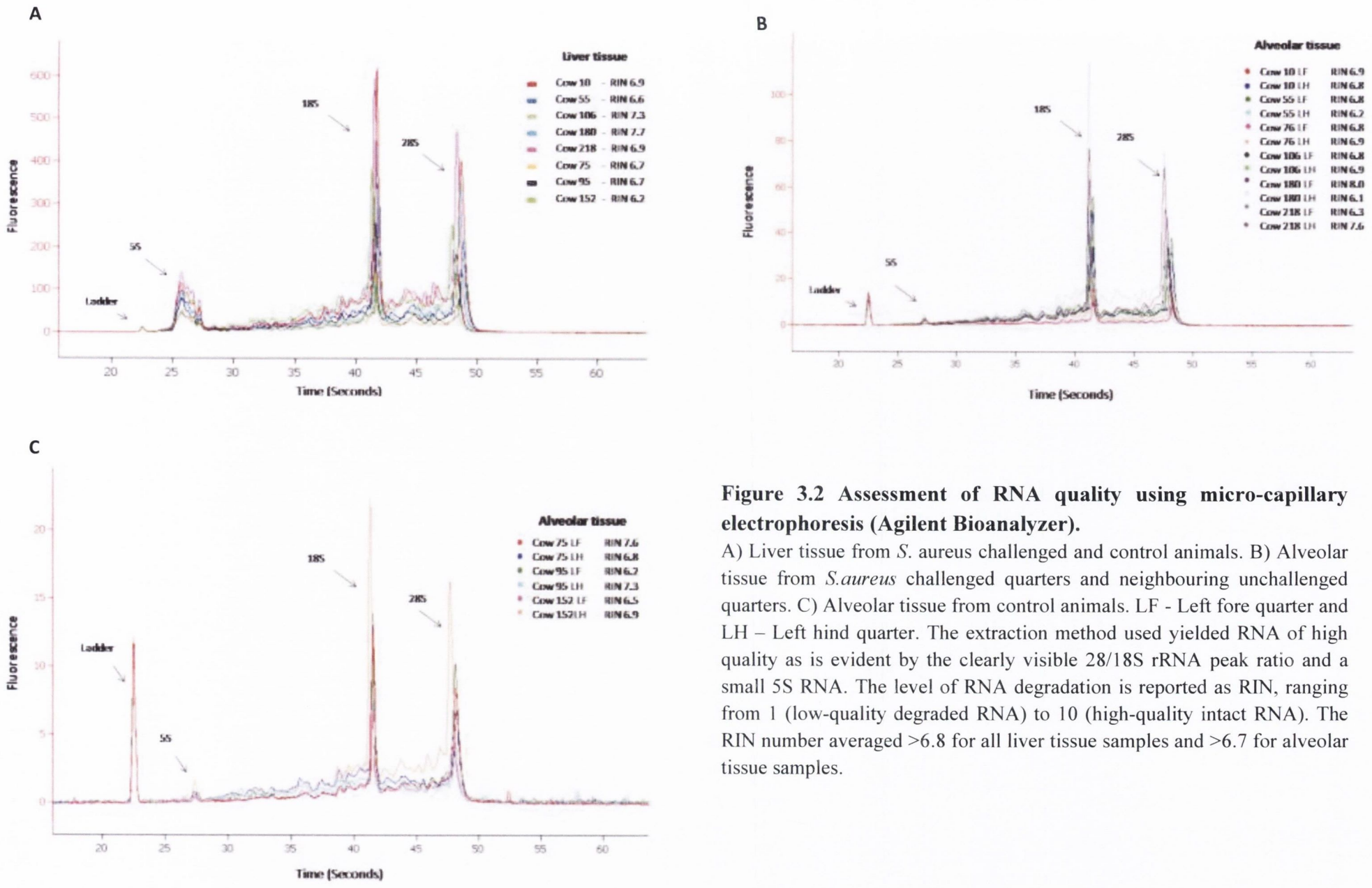


Figure 3.2 Assessment of RNA quality using micro-capillary electrophoresis (Agilent Bioanalyzer).

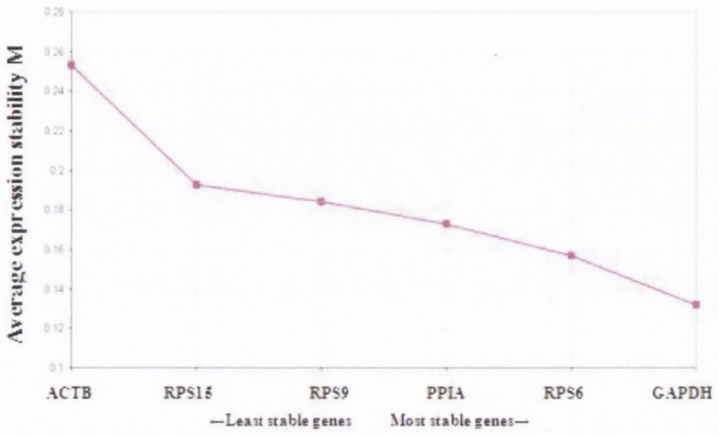
A) Liver tissue from *S.* aureus challenged and control animals. B) Alveolar tissue from *S.aureus* challenged quarters and neighbouring unchallenged quarters. C) Alveolar tissue from control animals. LF - Left fore quarter and LH – Left hind quarter. The extraction method used yielded RNA of high quality as is evident by the clearly visible 28/18S rRNA peak ratio and a small 5S RNA. The level of RNA degradation is reported as RIN, ranging from 1 (low-quality degraded RNA) to 10 (high-quality intact RNA). The RIN number averaged >6.8 for all liver tissue samples and >6.7 for alveolar tissue samples.

3.2.3 Selection of stable reference gene for qPCR

The expression of seven genes, *PPIA*, *GAPDH*, *ACTB*, *H3F3A*, *RPS6*, *RPS9* and *RPS15*, was examined in liver and mammary tissue. *GAPDH* demonstrated the greatest stability in liver tissue samples (Fig. 3.3A) and primer efficiency that was considered comparable and within the recommended range for using the $2^{-\Delta\Delta Cq}$ method (90–100% efficiency), and therefore selected as the most suitable genes for normalization of qPCR data.

H3F3A and *RPS9* demonstrated the greatest stability in mammary tissue (Fig. 3.3B), *H3F3A* was selected as the reference gene for mammary tissue based on its primer efficiency score (94%) which was greater than *RPS9* (89%).

A



B

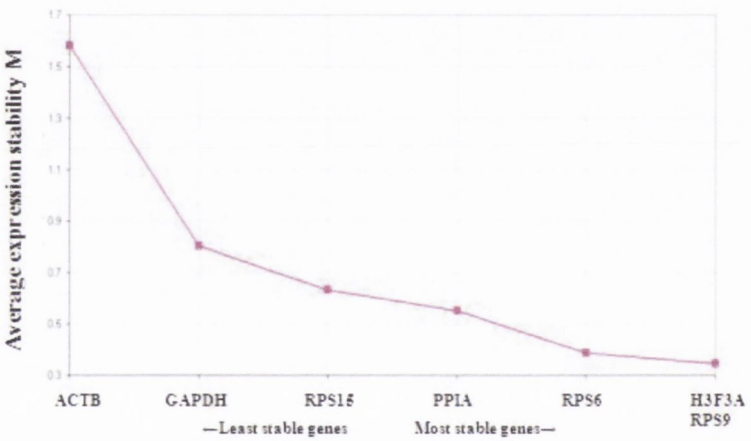


Figure 3.3 GeNorm analysis of reference genes

A) Stability of reference genes across liver tissue panel, animals with *S. aureus*-induced IMI and mock-challenged control cows. B) Stability of reference genes across alveolar tissue from both *S. aureus* challenged and non-challenged quarters (n = 6), and from the LH quarter of mock-challenged controls (n = 3) was determined. The recommended *M* value cut-off is 0.5 for homogeneous samples and can be higher for tissue samples.

3.2.4 Local and systemic immune responses to *S. aureus*

In order to determine if *S. aureus* inoculation had an effect on the transcriptome of neighbouring quarters, the expression of candidate immune genes (TLRs, cytokine, AMP and APP) was profiled in tissue from *S.aureus* challenged quarters and neighbouring unchallenged quarters and compared with the expression data from control cows at 48 h post challenge.

TLR1, TLR2, TLR4, and TLR6 are expressed on the cell surface and mainly detect bacterial molecules. Differential expression levels of each were profiled 48 h post challenge with *S. aureus* (Fig 3.4A-D). *TLR4* gene expression was significantly decreased in tissue from unchallenged neighbouring quarters relative to control cows (1.4 fold, $P < 0.05$; Fig. 3.4C). In contrast, *TLR4* showed no significant differential expression in *S. aureus*-challenged quarters relative to control cows (Fig. 3.4C). *TLR1*, *TLR2*, and *TLR6* showed no significant differential expression in *S.aureus* challenged and unchallenged quarters (Fig. 3.4A, B and D).

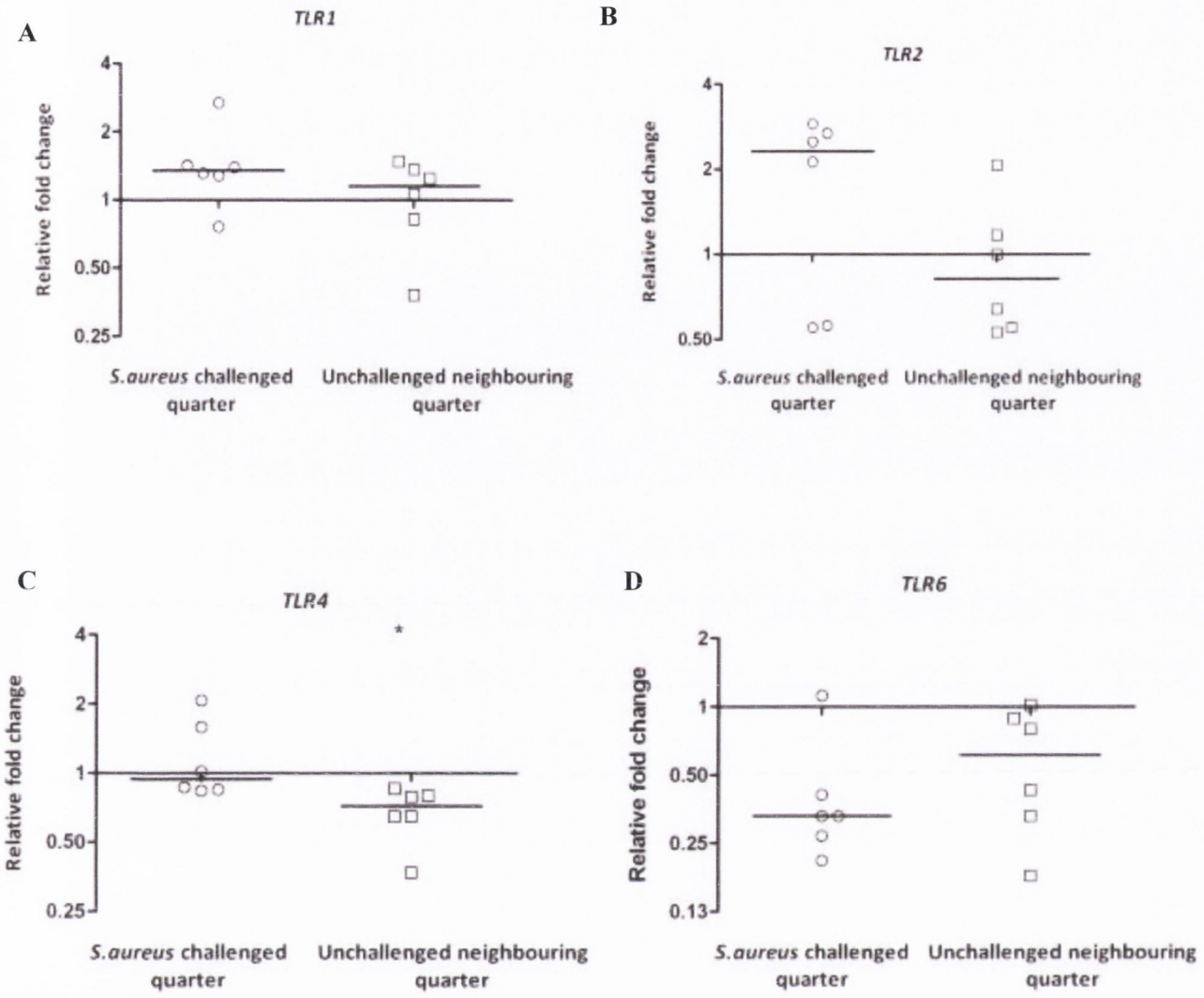


Figure 3.4 Toll-like receptor gene expression in mammary tissue isolated from the alveolar region of *S. aureus* inoculated and neighbouring unchallenged quarters.

Expression of A) *TLR1*, B) *TLR2*, C) *TLR4* and D) *TLR6* was analysed using qPCR. All samples were normalised to the reference gene *H3F3A*. Statistical analysis was carried out using the non-parametric Mann–Whitney U test. Median fold changes are shown for *S. aureus* challenged quarters and neighbouring unchallenged quarters (n=6) relative to mock-infected control animals (n=3) with statistical significance denoted as * = $P < 0.05$. Dashed line indicates statistical significance in gene expression between challenged quarters and neighbouring unchallenged quarters with * = $P < 0.05$.

Expression of the proinflammatory cytokine *IL6* gene was significantly increased in both *S.aureus* challenged and neighbouring unchallenged quarters when compared with control animals (5.1-, and 19.2-fold respectively, $P < 0.05$; Fig. 3.5C). There was also a significant difference in *IL6* expression between the *S.aureus* challenged and neighbouring unchallenged quarters ($P < 0.05$; Fig. 3.5C). In contrast, *IL1B* and *TNF* showed no significant differential expression in both quarters (Fig 3.5A and B). *IL8* showed a strong and significant increase in *S. aureus* challenged quarters (13-fold, $P < 0.05$; Fig. 3.5D) with no significant differential expression detected in neighbouring quarters (Fig. 3.5D). *TGFB* was significantly induced in unchallenged quarter (1.8-fold, $P < 0.05$; Fig. 3.5E).

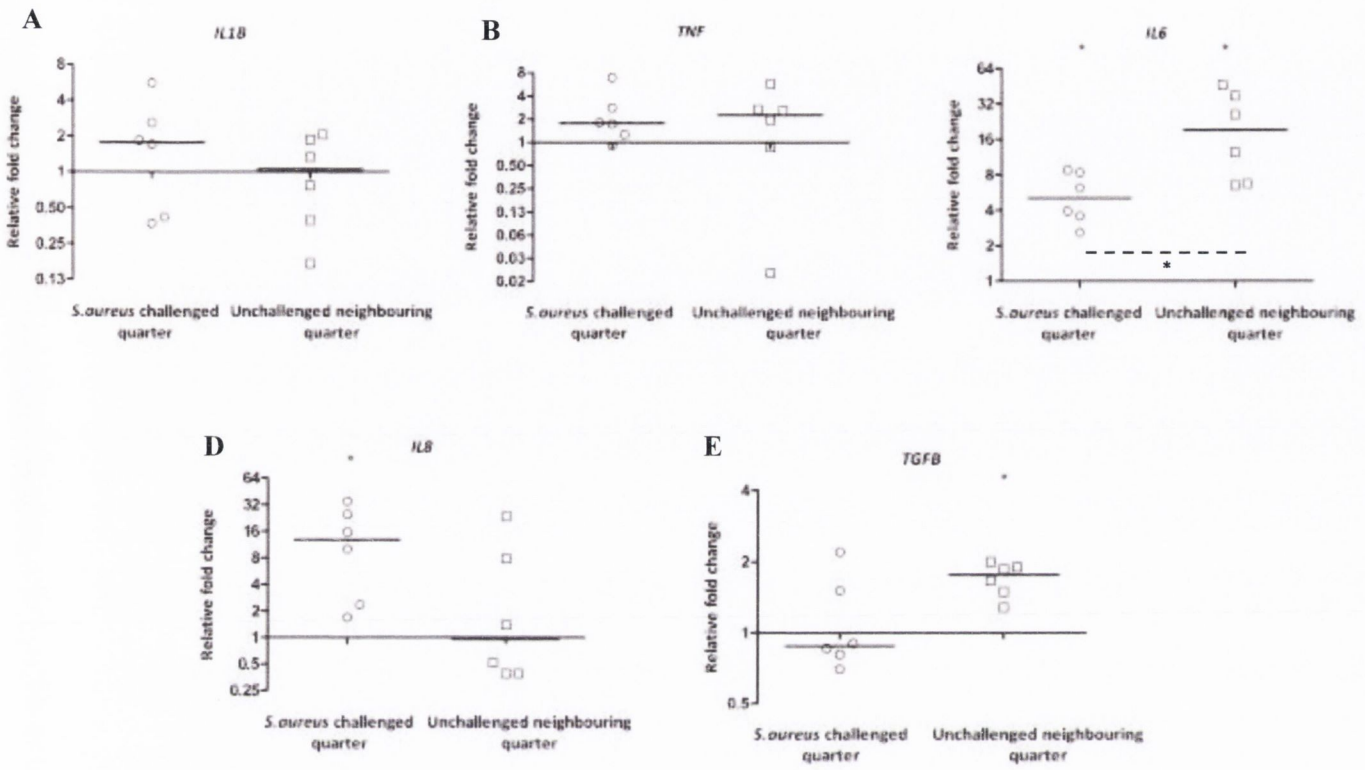


Figure 3.5 Cytokine gene expression in mammary tissue isolated from the alveolar region of *S. aureus* challenged and neighbouring unchallenged quarters

Expression of A) *IL1B*, B) *TNF*, C) *IL6* and D) *IL8* was analysed using qPCR. All samples were normalised to the reference gene *H3F3A*. Statistical analysis was carried out using the non-parametric Mann–Whitney U test. Median fold changes are shown for *S. aureus* challenged quarters and neighbouring unchallenged quarters (n=6) relative to mock-infected control animals (n=3) with statistical significance denoted as * = $P < 0.05$. Dashed line indicates statistical significance in gene expression between challenged quarters and neighbouring unchallenged quarters with * = $P < 0.05$.

Expression of the β-defensins, *DEFB4* and *DEFB5*, was strongly induced in *S. aureus* challenged quarters (9.4-and 27-fold respectively, $P < 0.05$; Fig. 3.6C and D). Tracheal antimicrobial peptide (*TAP*) showed a strong induction in unchallenged quarters compared to *S.aureus*-challenged quarters (38-fold, $P < 0.05$; Fig. 3.6B). Lingual antimicrobial peptide (*LAP*) was not significantly induced in any of the profiled quarters (Fig. 3.6A).

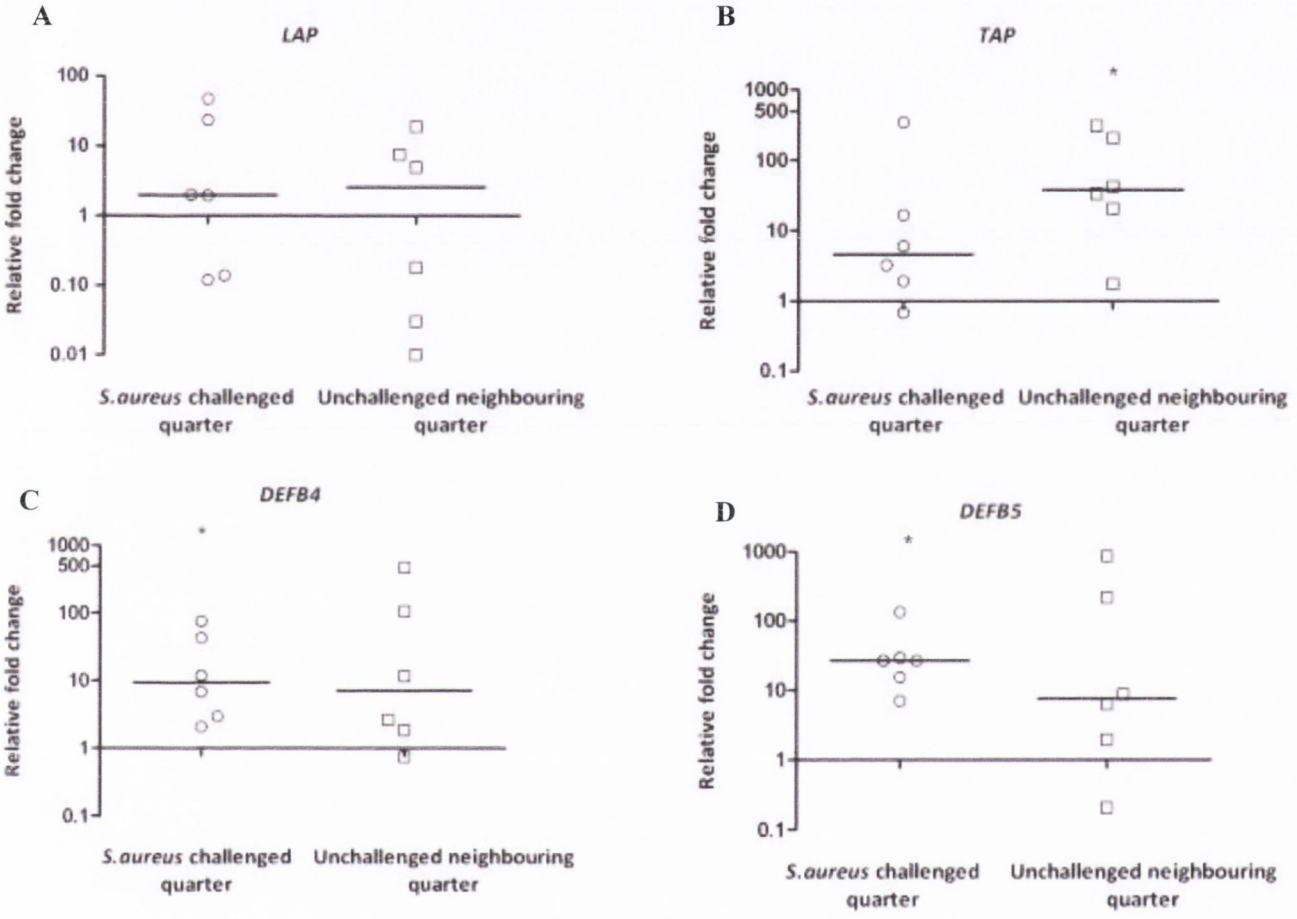


Figure 3.6 Antimicrobial peptide gene expression in mammary tissue isolated from the alveolar region of *S. aureus* challenged and neighbouring unchallenged quarters

Expression of A) *LAP*, B) *TAP*, C) *DEFB4* and D) *DEFB5* was analysed using qPCR. All samples were normalised to the reference gene *H3F3A*. Statistical analysis was carried out using the non-parametric Mann–Whitney U test. Median fold changes are shown for *S. aureus* challenged quarters and neighbouring unchallenged quarters (n=6) relative to mock-infected control animals (n=3) with statistical significance denoted as * = $P < 0.05$. Dashed line indicates statistical significance in gene expression between challenged quarters and neighbouring unchallenged quarters with * = $P < 0.05$.

Significant differential expression was detected for all four acute phase proteins (APP) genes profiled in both *S. aureus* challenged and unchallenged quarters relative to control quarters (Fig. 3.7A-D). Both *SAA3* and *HP* expression were strongly induced in *S.aureus* quarters (133- and 80-fold respectively, $P < 0.05$; Fig. 3.7A and B); with a moderate fold increases detected in unchallenged neighbouring quarters (9.4- and 9.7-fold respectively, $P < 0.05$; Fig. 3.7A and B). Lesser fold increases were detected for *LBP*, 3.1-fold in *S. aureus* treated quarters and 2.1 fold in neighbouring unchallenged quarters ($P < 0.05$; Fig. 3.7C). Expression of *AGP* was significantly decreased in *S. aureus* (54-fold, $P < 0.05$; Fig. 3.7A) and increased in neighbouring unchallenged quarters when compared to control animals (8.7-fold, $P < 0.05$; Fig. 3.7D). In additions, significant differential *AGP* expression was observed between the *S.aureus* challenged and neighbouring unchallenged quarters ($P < 0.05$; Fig. 3.7D)

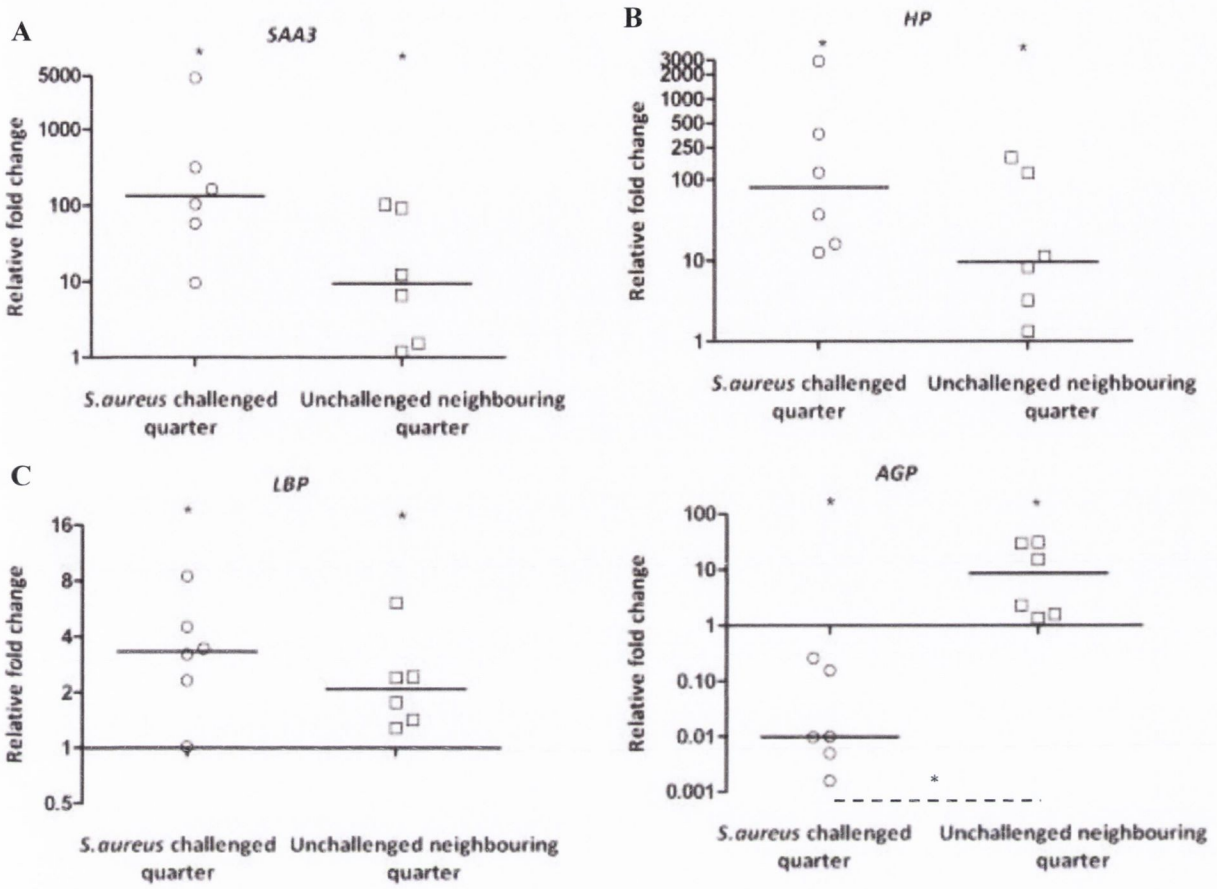


Figure 3.7 Acute phase protein gene expression in mammary tissue isolated from the alveolar region of *S. aureus* challenged and neighbouring unchallenged quarters

Expression of A) SAA3, B) HP, C) LBP and D) AGP was analysed using qPCR. All samples were normalised to the reference gene *H3F3A*. Statistical analysis was carried out using the non-parametric Mann–Whitney U test. Median fold changes are shown for *S. aureus* challenged quarters and neighbouring unchallenged quarters (n=6) relative to mock-infected control animals (n=3) with statistical significance denoted as * = $P < 0.05$. Dashed line indicates statistical significance in gene expression between challenged quarters and neighbouring unchallenged quarters with * = $P < 0.05$.

3.2.5 Effect of subclinical mastitis on hepatic gene expression of cytokines, antimicrobial peptides and acute phase proteins

To gain a better insight into the hepatic acute phase response in dairy cows with *S. aureus*-induced subclinical mastitis, we performed gene expression of selected innate immune genes using qPCR on liver tissue isolated 48 h post infection.

The proinflammatory cytokine *TNF* was significantly increased at 48 h post-challenge compared with control animals (Fig. 3.8). *IL1B* expression was not significantly altered at 48 h post-challenge (Fig. 3.8). *IL6* transcript was below the limit of detection in liver tissue from animals with mastitis and control animals.

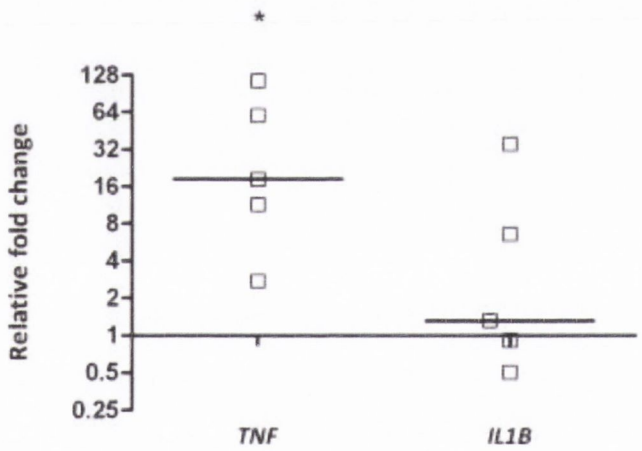


Figure 3.8 Cytokine gene expression in bovine liver tissue isolated from animals with *S. aureus* induced intramammary infection and mock infected control animals.
Differential expression of *TNF* and *IL1B* was analysed 48 h post challenge using qPCR. All samples were normalised to the reference gene *GAPDH*. Median fold changes are shown for *S. aureus* infected animals (n=5) relative to mock-infected control animals (n=3). Statistical significance is denoted as: * = $P < 0.05$.

The expression of the β-defensins, *TAP*, *HAMP* and *LEAP2* was not significantly induced at 48 h in liver tissue in response to subclinical mastitis (Fig. 3.9A). Analysis of baseline expression of these β-defensins in control animals showed *HAMP* to have the highest levels of expression followed by *LEAP2* then *TAP* (Fig. 3.9B). The expression of *LAP* was not detected in liver tissue.

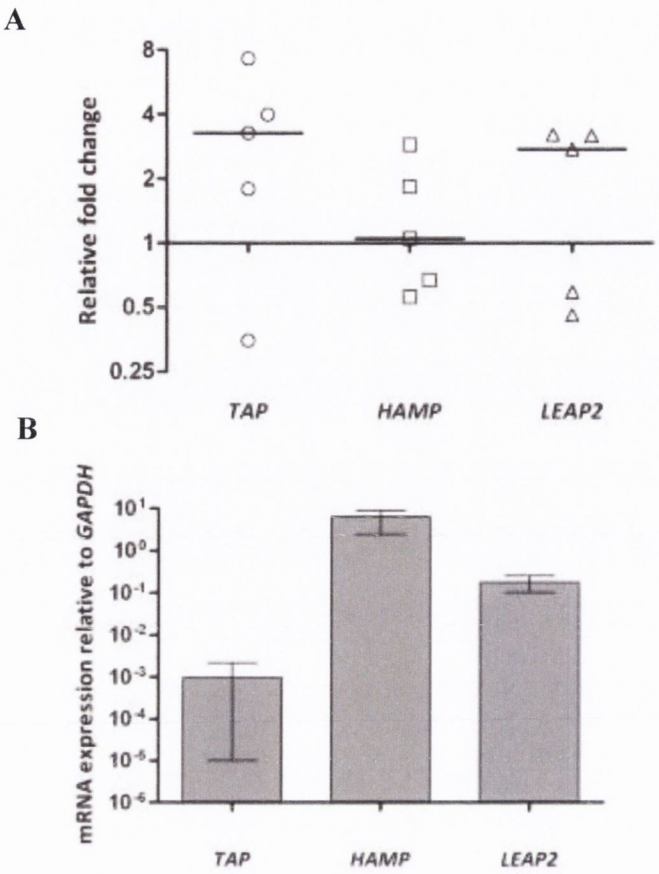


Figure 3.9 Antimicrobial peptide gene expression in bovine liver tissue isolated from animals with *S. aureus* induced intramammary infection and mock infected control animals.

A) Expression of TAP, HAMP and LEAP2 was analysed using qPCR. All samples were normalised to the reference gene *GAPDH*. Median fold changes are shown for *S. aureus*-infected animals [n=5, number of liver samples remaining following initial study (Eckersall *et al.*, 2006)] relative to mock-infected control animals (n=3). Statistical significance is denoted as: * = $P < 0.05$. B) Relative levels *TAP*, *HAMP* and *LEAP* expression in liver tissue from control animals.The expression of each gene in each sample is expressed relative to *GAPDH* expression using the formula $2^{-Cq(GOI)}/2^{-Cq(HK)}$. Data shown is the median (bars denote the upper and lower range) of n = 3 biological replicates. There were two technical replicates performed for each biological sample.

In agreement with previously reported data (Eckersall *et al.*, 2006), expression of the acute phase proteins, *SAA3* and *HP* was significantly elevated 48 h after challenge compared with control animals (13- and 14.8-fold respectively, $P < 0.05$; Fig. 3.10A). In contrast, the expression of *AGP* and *CP* was significantly decreased following *S. aureus* challenge (4.3- and 2.4-fold respectively, $P < 0.05$; Fig. 3.10A). *AGP* and *CP* were expressed at high levels in control animals compared to *SAA3*, *HP* and *LBP* (Fig. 3.10B).

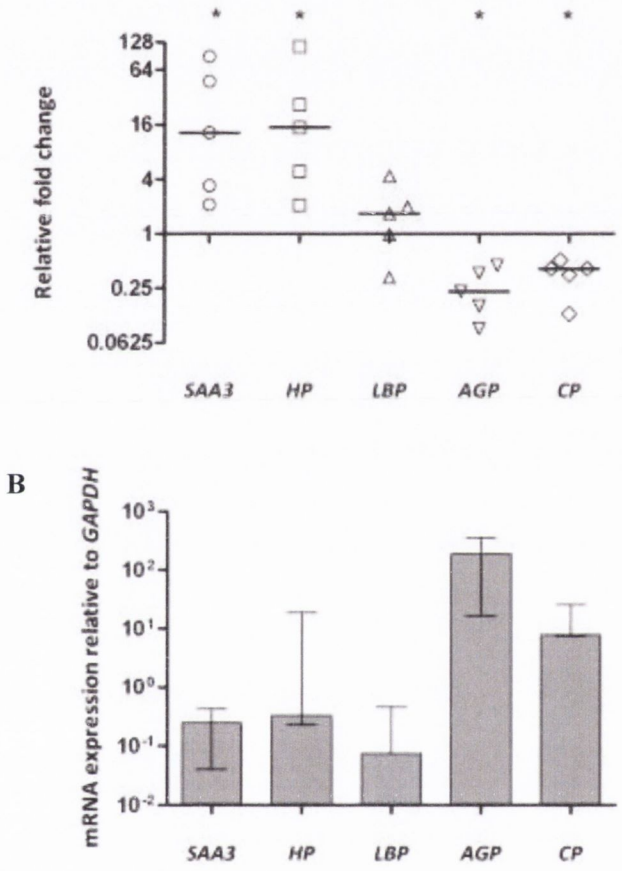


Figure 3.10 Acute phase protein gene expression in bovine liver tissue isolated from animals with *S. aureus* induced intramammary infection and mock infected control animals.

A) Expression of *SAA3*, *HP*, *LBP*, *AGP* and *CP* was analysed using qPCR. All samples were normalised to the reference gene *GAPDH*. Median fold changes are shown for *S. aureus* infected animals (n=5) relative to mock-infected control animals (n=3). Statistical significance is denoted as: * = $P < 0.05$. B) Relative levels of *SAA3*, *HP*, *LBP*, *AGP* and *CP* expression in liver tissue from control animals.The expression of each gene in each sample is expressed relative to *GAPDH* expression using the formula $2^{-Cq(GOI)}/2^{-Cq(HK)}$. Data shown is the median (bars denote the upper and lower range) of n=3 biological replicates. There were two technical replicates performed for each biological sample.

3.3 Discussion

Our results show that intramammary infection of the bovine mammary gland with *S. aureus* induces both a local and systemic immune response. Furthermore, the immune response induced in *S. aureus* inoculated quarters has an influence on the expression of innate immune genes in unchallenged quarters which we believe to be mediated systemically. Previous observations in cattle support this hypothesis of a systemic defence reaction which protects unaffected udder quarters (Merle *et al.*, 2007; Mitterhuemer *et al.*, 2010; Yang *et al.*, 2008).

In order to study this phenomenon, we profiled the expression of candidate innate immune genes in tissue from *S. aureus*-challenged quarters and neighbouring unchallenged quarters and compared these with the expression data from control cows at 48 h post challenge. In addition, we performed gene expression analysis in liver tissue isolated from the same animals in order to gain a greater insight into the hepatic acute phase response in dairy cows.

Increased somatic cell count (SCC) from pre-infusion to post-infusion of $>2 \times 10^5$ cells/ml in the absence of clinical signs was taken as an indication of subclinical mastitis, and was reported in milk from *S. aureus*-challenged LH quarters. In mock inoculated and untreated neighbouring udder quarters of infected animals no significant increase in the number of somatic cells was observed 48 h post challenge as shown in Figure 3.1.

Using the same model, our collaborators (Eckersall *et al.*, 2006) had shown that systemic immune response was initiated, as evidenced by the expression of the acute phase protein, *SAA3* and *HP* in liver tissue and their increase in protein concentration in serum. In this study, we investigated whether the systemic response influenced the local immune response in neighbouring unchallenged quarters.

Gene expression analysis of selected innate immune genes in tissue from the saline-infused left hind (LH) and untouched left forequarters (LF) showed no significant differences. The missing induction of proinflammatory molecules and the unchanged SCC in saline-inoculated udder quarters up to 48 h proves that the inoculation procedure neither provoked inflammation nor increased somatic cells in milk. This is in line with recently published data that showed saline treatment did not markedly alter the transcriptome of the control quarters (Mitterhuemer *et al.*, 2010). As a result, it was decided that the LH quarter of mock-challenged controls should be used for accurate quantification of relative gene expression changes in mammary tissue due to *S. aureus* challenge.

Cytokines are an important group of inflammatory mediators. Proinflammatory cytokines promote inflammation quickly after the perception of the pathogen, while anti-inflammatory cytokines suppress the activity of proinflammatory cytokines. IL-1β, TNF-α and IL-6 are major proinflammatory cytokines (reviewed in Chapter 1: Introduction, section 1.5). They are locally produced by many cell types and their expression is decisive for an effective inflammatory response, including pathogen clearance, wound healing, and return to the normal state. They are also key mediators of the acute-phase response in inflammation.

At 48 h after infection we observed a significant increase in *IL6* expression in *S.aureus* challenged and neighbouring unchallenged quarters. The increase in *IL6* expression was more pronounced in unchallenged quarters compared to *S. aureus* challenged quarters (Fig. 3.5), suggesting a key role for this cytokine in the defence against *S. aureus*. In contrast, *IL1B* and *TNF* showed no significant differential expression in any of the quarters profiled.

Similar results were observed in primary bovine mammary epithelial cells where *S. aureus* failed to induce *IL1A* and *TNF* expression while significantly inducing *IL6* expression, whereas all three genes were substantially induced in response to *E. coli* (Gunther at al. 2011). Reduced TNF-α and IL-1 secretion during mastitis caused by *S. aureus* and other Gram-positive bacteria has also been reported (Riollet *et al.*, 2000; Rainard *et al.*, 2008). The lack of a significant increase of *IL1B* and *TNF* 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.*, 2008b; Sutra and Poutrel, 1994), whereas, *E. coli* infection usually resolves within a short period of time (Blum *et al.*, 2000b; Hoeben *et al.*, 2000). IL-6 is one of the key mediators of the acute phase response (Heinrich *et al.*, 2003) and may be a central cytokine involved in activation of the systemic response in our model.

The expression of these proinflammatory cytokines was also profiled in liver tissue. *TNF* was significantly increased at 48 h post-challenge compared with control animals (Fig. 3.8), whereas *IL1B* and *IL6* were altered at 48 h post-challenge. Similar results were reported for liver tissue in response to intramammary infection (IMI) with *E. coli*, however TNF-α was not detected in serum after 24 h, although hepatic gene expression of *TNF* was still increased at 48 h (Vels *et al.*, 2009). Understanding the contribution of the liver to the circulating levels of cytokines requires further work.

The chemokine *IL8* was induced in *S. aureus* challenged quarters (13-fold, $P < 0.05$; Fig. 3.5D) with no significant differential expression detected in neighbouring quarters (Fig. 3.5D). Chemokines such as IL8 are chemotactic for neutrophils, which play a key role in destroying invading pathogens. The observed enhanced expression of *IL8* in infected quarters may explain the influx of somatic cells observed in these quarters only (Fig. 3.1).

Expression of *TGFB* was also profiled. Significantly increased expression was detected in the control quarters of *S. aureus* challenged animals. This is in line with previous data which found that uninfected control quarters of *S. aureus*-infected cows had increased expression of *TGFB* (Yang *et al.*, 2008). TGF-β has been shown to block TLR signalling through ubiquitination and subsequent proteasomal degradation of MyD88 (Naiki *et al.*, 2005). Increased expression may be a mechanism by which *S. aureus* can evade or delay the host's immune response.

β-Defensins have also been shown to be bactericidal against mastitis-causing pathogens (Diamond *et al.*, 1991; Isobe *et al.*, 2009). Expression of the β-defensins, *DEFB4* and *DEFB5*, was strongly induced in *S. aureus* challenged quarters and tracheal antimicrobial peptide (*TAP)* showed a strong induction in unchallenged quarters compared to *S.aureus* challenged quarters, suggesting a key role for these peptides in the defence against *S. aureus*. Although not significantly expressed, the baseline expression of hepcidin antimicrobial peptide (HAMP) and liver expressed antimicrobial peptide (LEAP-2) was very high in liver tissue, suggesting an import role for these peptides in maintaining a constitutive defence barrier. Understanding the contribution of the liver to the circulating levels of antimicrobial peptides requires further work.

In addition to hepatic production following infection or injury, APPs are also induced at the site of infection where they augment the local immune response (Chapwanya *et al.*, 2009; Cheng *et al.*, 2008; Eckersall *et al.*, 2006). Significant differential expression was detected for *SAA3, HP, LBP* and *AGP* in both *S. aureus* challenged and unchallenged neighbouring quarters (Fig. 3.7A-D). This systemic effect on APP expression was also observed in response to *E. coli*-induced mastitis at 24 h – *E. coli* challenged quarters, *SAA3*, 60-fold and *LBP*, 20-fold, as well as in neighbouring uninfected quarters, *SAA3*, 3-fold and LBP, 4-fold (Mitterhuemer *et al.*, 2010).

SAA3 and *HP* were significantly induced in liver tissue (Fig. 3.10A). This is in agreement with our collaborator's findings (Eckersall *et al.*, 2006). In contrast, the expression of *AGP* and *CP* was significantly decreased following *S. aureus* challenge (4.3- and 2.4-fold respectively, $P <$

0.05; Fig. 3.10A). Although not significantly induced, *AGP* was expressed at high levels in control animals compared to *SAA3*, *HP*, *CP* and *LBP* (Fig. 3.10B). Alpha 1-Acid glycoprotein (AGP) has been shown to be present at high concentrations in the blood of healthy animals compared with SAA and Hp (Vels *et al.*, 2009). AGP might be important in the early phase of the acute phase response (APR). Interestingly, *AGP* was significantly decreased in *S. aureus* challenged quarters and increased in the neighbouring unchallenged quarter compared to the *S. aureus* challenged quarter. Whether this is a result of *S. aureus* modulating the host immune response in order to enhance survival requires a greater understanding of the immunological function of AGP. Taken together, these findings suggest a key role for APPs in the establishment and maintenance of both local and systemic inflammation.

The mechanism by which the infection of neighbouring quarters affects the transcriptome of uninfected quarters is unknown. Intramammary infection with *S. aureus* induces a systemic effect inducing transcriptional changes in the liver which leads to the production of acute phase proteins which are released into the blood (Eckersall *et al.*, 2006). This suggests that the systemic response has an influence on the expression of innate immune genes in unchallenged quarters. This has also been postulated to explain the transcriptional profile in healthy quarters of animals infected with *E. coli* (Mitterhuemer *et al.*, 2010). However, in a similar infection model, analysis of TLR and β-defensin expression in the local and peripheral lymph nodes of animals with *S. aureus* or *E. coli* intramammary infection showed that the systemic effect is quite limited (Jensen *et al.*, 2013). Local cross-talk between the udder quarters could also play a role in priming the neighbouring udder quarters. Indeed, lymphocytes have been shown to be capable of migrating between mammary gland quarters (Kimura *et al.*, 2005) and therefore the presence of a more localized interaction between quarters is probable and requires further investigation.

3.4 Conclusion

It has previously been reported that during *E. coli* infection the transcriptome of neighbouring, sterile quarters is modified (Mitterhuemer *et al.*, 2010). Our results show that a similar phenomenon occurs during *S. aureus* infection of the bovine mammary gland, suggesting that the systemic response primes the uninfected quarters to prevent or limit the spread of infection, and is therefore worthy of further investigation considering the frequency of mastitic infections on dairy farms. Furthermore, these results have important implications for the design

of appropriate models to study bovine mastitis. To date, most experimental designs used in mastitis research lack external controls and are thus not able to differentiate between local and systemic responses to the pathogen. As a result of these finding, a comprehensive quantification of innate immune genes was carried out on mammary tissue from *S. aureus* challenged quarters and compared with gene expression levels in mammary tissue from external control animals (Chapter 2: Materials and Methods, section 2.1).

Chapter 4: Innate Immune Gene Expression Profiling in Bovine Mammary Gland in Response to *S. aureus-induced* Subclinical Mastitis

4.1 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 *Staphylococcus aureus* predominantly causes subclinical mastitis resulting in chronic infection that can persist for the life of the animal (Schukken *et al.*, 2011). 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 (Elazar *et al.*, 2010). Toll-like receptor 2 (TLR2) 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, has been reported (Goldammer *et al.*, 2004; Günther *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 and chemokines, 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; Lutzow *et al.*,

2008) 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 also results in expression of effector molecules of the innate immune response, including antimicrobial peptides (AMPs) and acute phase proteins (APPs). AMPs 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. β-defensins are an important family of AMP in cattle (Lai and Gallo, 2009). 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). Several β-defensins, including *LAP*, *TAP* and *DEFB5* have been found to be expressed in mammary tissue in both a constitutive and inducible manner in response to bacterial challenge (Goldammer *et al.*, 2004; Petzl *et al.*, 2008; Swanson *et al.*, 2004; Tetens *et al.*, 2010).

Induction of the acute phase response (APR) is also prominent during intramammary 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 (Uhlar *et al.*, 1994), SAA3 has been shown to be the main SAA protein present in bovine colostrums (McDonald *et al.*, 2001).

Several studies have quantified levels of cytokine, APP and AMP expression in bovine milk following experimentally induced intramammary infection with *E. coli* (Bannerman *et al.*, 2004; Chockalingam *et al.*, 2005; Shuster *et al.*, 1997). Furthermore, recent studies have described transcriptome wide changes in gene expression in 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*.

Following on from our initial study (Chapter 3) which demonstrated both a local and systemic immune response to *S.aureus*-induced subclinical mastitis, a comprehensive quantification of innate immune genes was carried out on mammary tissue from the same

infection model (Chapter 2: Materials and Methods, section 2.1). 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 were profiled. For simplicity and in order to facilitate a comparison between the tissue regions, qPCR data from Chapter 3 was incorporated into this study.

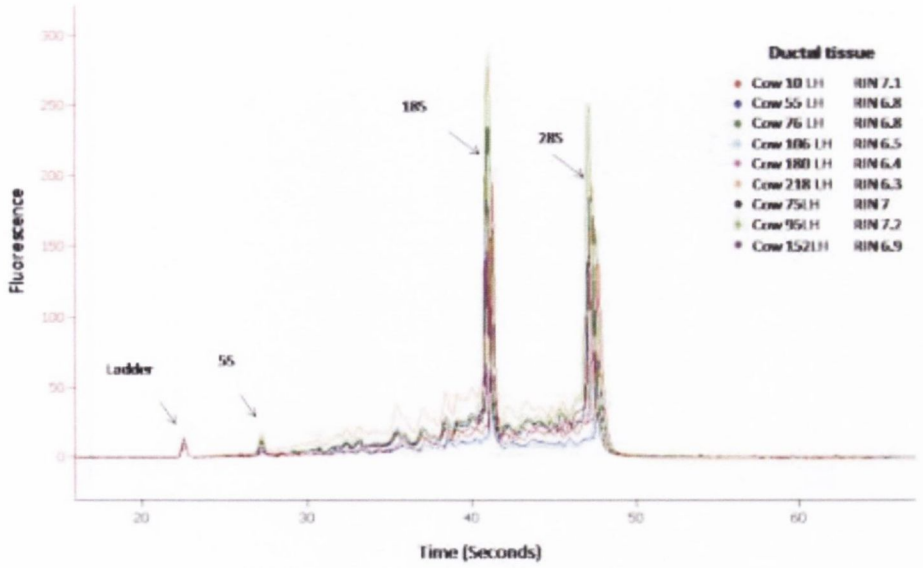
4.2 Results

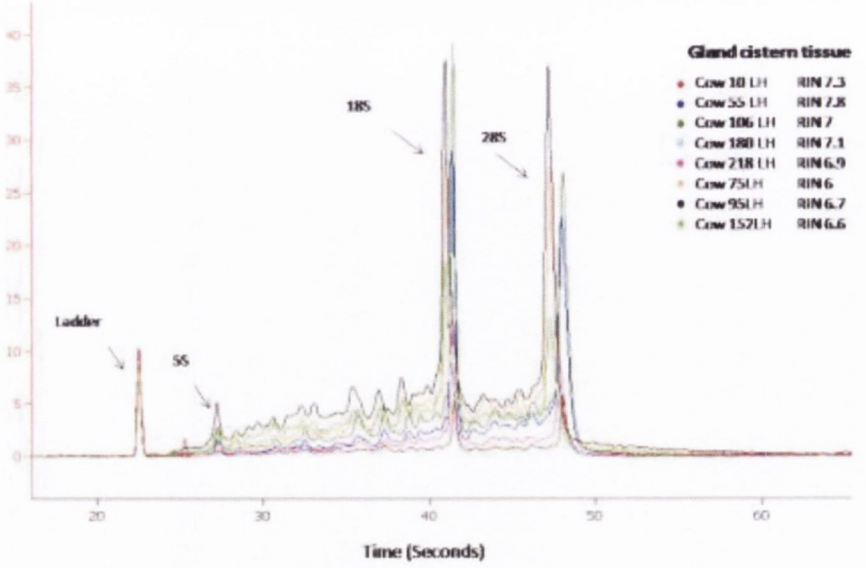
4.2.1 Elevated levels of somatic cells in milk in response *S. aureus* challenge

The same animals that were used in our initial study (Chapter 3; Results, Fig 2.2B and C) were used for this study. The mean somatic cell count in milk from *S. aureus* challenged left hind (LH) quarters remained elevated over the 48 h period. The corresponding LH quarters of the controls animals did not exceed 2×10^5 cells/ml over the 48 h period (Chapter 3: Results, Fig. 2.1).

4.2.2 RNA quality

For each quarter, tissue was taken aseptically from four different tissue regions (alveolar, ductal, gland cistern and teat canal). Total RNA was extracted and its quality assessed using the Agilent 2100 Bioanalyzer (Chapter 2: Materials and methods, section 2.2.1). The extraction method used yielded RNA of high quality. The RNA integrity number (RIN) for RNA extracted from alveolar tissue averaged >6.7 (Chapter 3: Results, Fig. 2.2B and C); for tissue isolated from the lactiferous ductal region, the RIN averaged >6.7, gland cistern region >6.8 and teat canal region >6.7 (Fig. 4.1A-C).





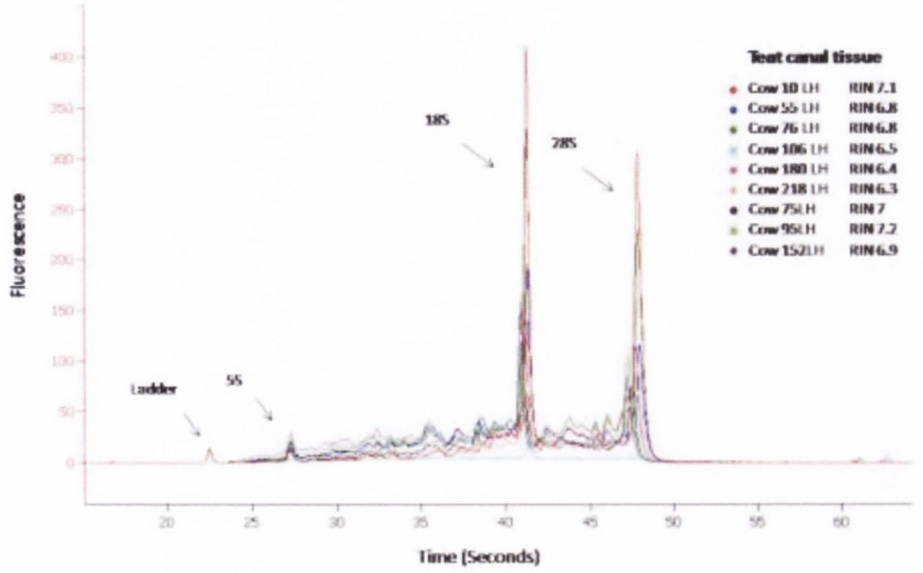


Figure 4.1 Assessment of RNA quality using micro-capillary electrophoresis (Agilent Bioanalyzer).

RNA quality analysis of mammary tissue from *S.* aureus-challenged and control animals. A) Tissue isolated from lactiferous ducts. B) Tissue isolated from teat sinus. C) Tissue isolated teat sphincter. The extraction method used yielded RNA of high quality as is evident by the clearly visible 28/18S rRNA peak ratio and a small 5S RNA. The level of RNA degradation is reported as RIN (ranging from 1 (low-quality degraded RNA) to 10 (high-quality intact RNA). The RIN number averaged >6.7 for ductal tissue, >6.8 gland cistern tissue and >6.7 for teat canal tissue.

4.2.3 Selection of stable reference gene for qPCR

The selection of a stable reference gene for qPCR was performed as described in section 3.2.3.

4.2.4 Selection of control animals for qPCR

As a result of our initial study (Chapter 3), where we showed that immune response induced in *S. aureus* challenged quarters had an influence on the expression of innate immune genes in neighbouring unchallenged quarters (Chapter 3: Results, Fig. 2.8, Fig 2.9 and Fig. 2.10) the left hind (LH) quarter of mock challenged animals was used for accurate quantification of relative gene expression changes due to *S. aureus* challenge.

4.2.5 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. 4.3), with *TLR8* having the least expression in comparison to the other PRRs profiled (Fig. 4.4A). Differential expression levels of each PRR were further profiled in the four tissue regions 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. 4.5B, C and D). *TLR3* showed a moderate fold increase in teat canal tissue from challenged quarters (2.5-fold, $P < 0.05$; Fig. 4.5D). 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. 4.5C). *TLR5* and *TLR7* were also significantly increased in alveolar tissue from challenged animals (1.8– and 2.3-fold respectively, $P < 0.05$; Fig. 4.5A). 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. 4.5D), *TLR6* in ductal tissue (1.1-fold, $P < 0.05$; Fig. 4.5B) and *TLR8* in gland cistern tissue (1.9-fold, $P < 0.05$; Fig. 4.5C). *TLR2*, *TLR9*, and *TLR10* showed no significant differential expression across the four tissue regions (see Appendix).

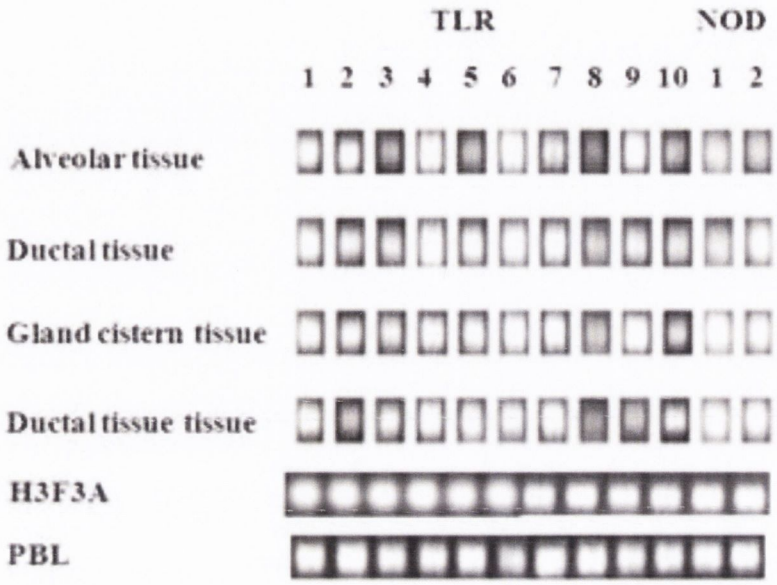


Figure 4.2 Tissue expression profile of bovine pattern recognition receptors genes in mammary tissue 48 h post-challenged with *S. aureus*.

PCR (40 cycles) was used to profile the expression of TLRs 1-10 and NOD 1-2 in tissue isolated for the alveolar, ductal and teat canal regions of both *S. aureus* challenged and control animals. Amplicons were visualised on 1.5% agarose gels. A representative result of n = 6 is shown. H3F3A = housekeeping gene. PBL (peripheral blood leucocytes) = positive control. No amplicons were observed in the negative controls (no template controls) (data not shown).

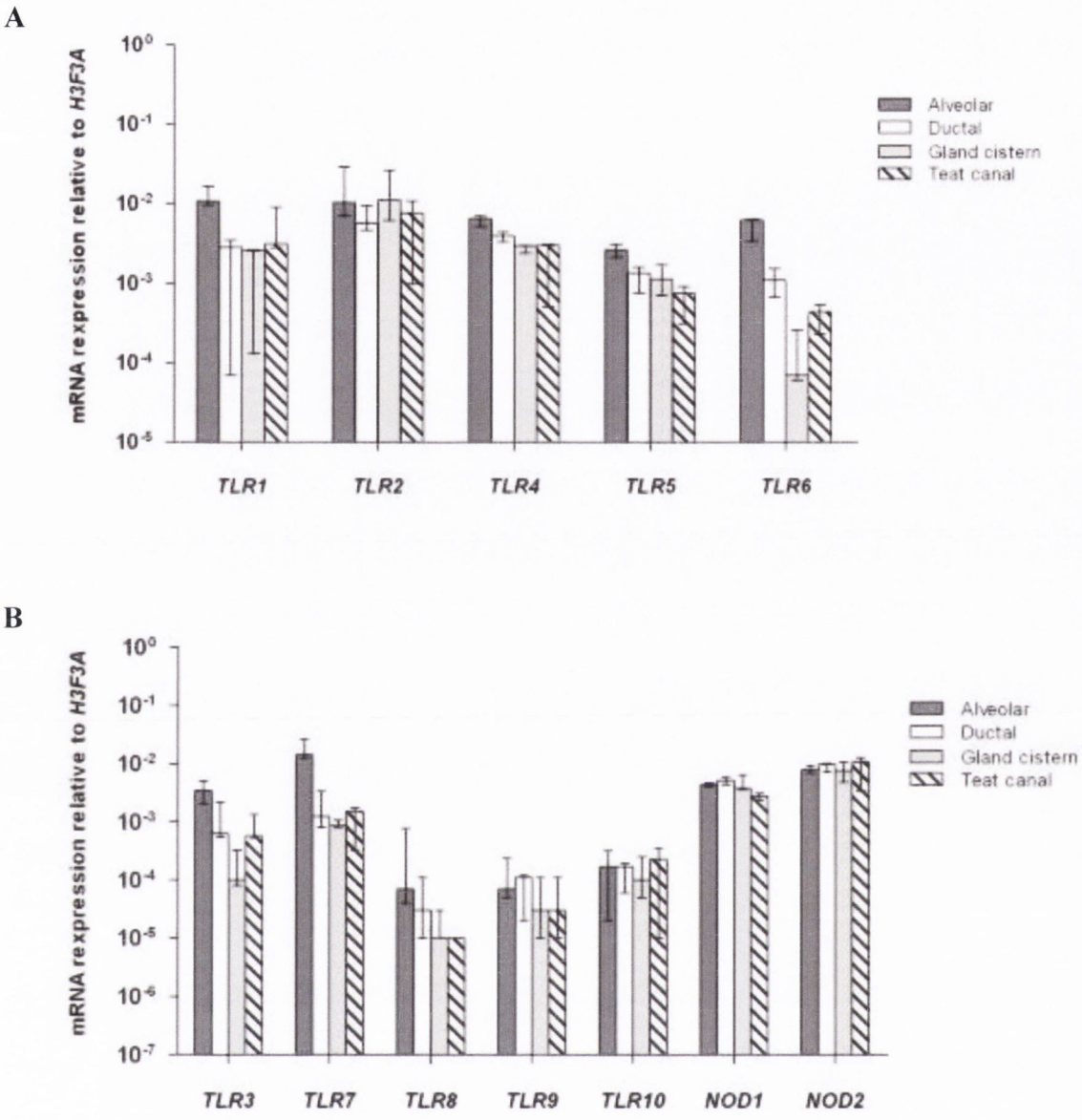


Figure 4.3 Relative gene expression levels of bovine pattern recognition receptors in control animals

A) Cell surface receptors. B) Intracellular receptors. Relative levels of PRR expression in alveolar, ductal, gland cistern and teat canal tissue from control animals. The expression of each gene in each sample is expressed relative to *H3F3A* (10^0) expression using the formula $2^{-Cq(GOI)}/2^{-Cq(HK)}$. Data shown is the median (bars denote the upper and lower range) of n = 3 biological replicates. There were two technical replicates performed for each biological sample.

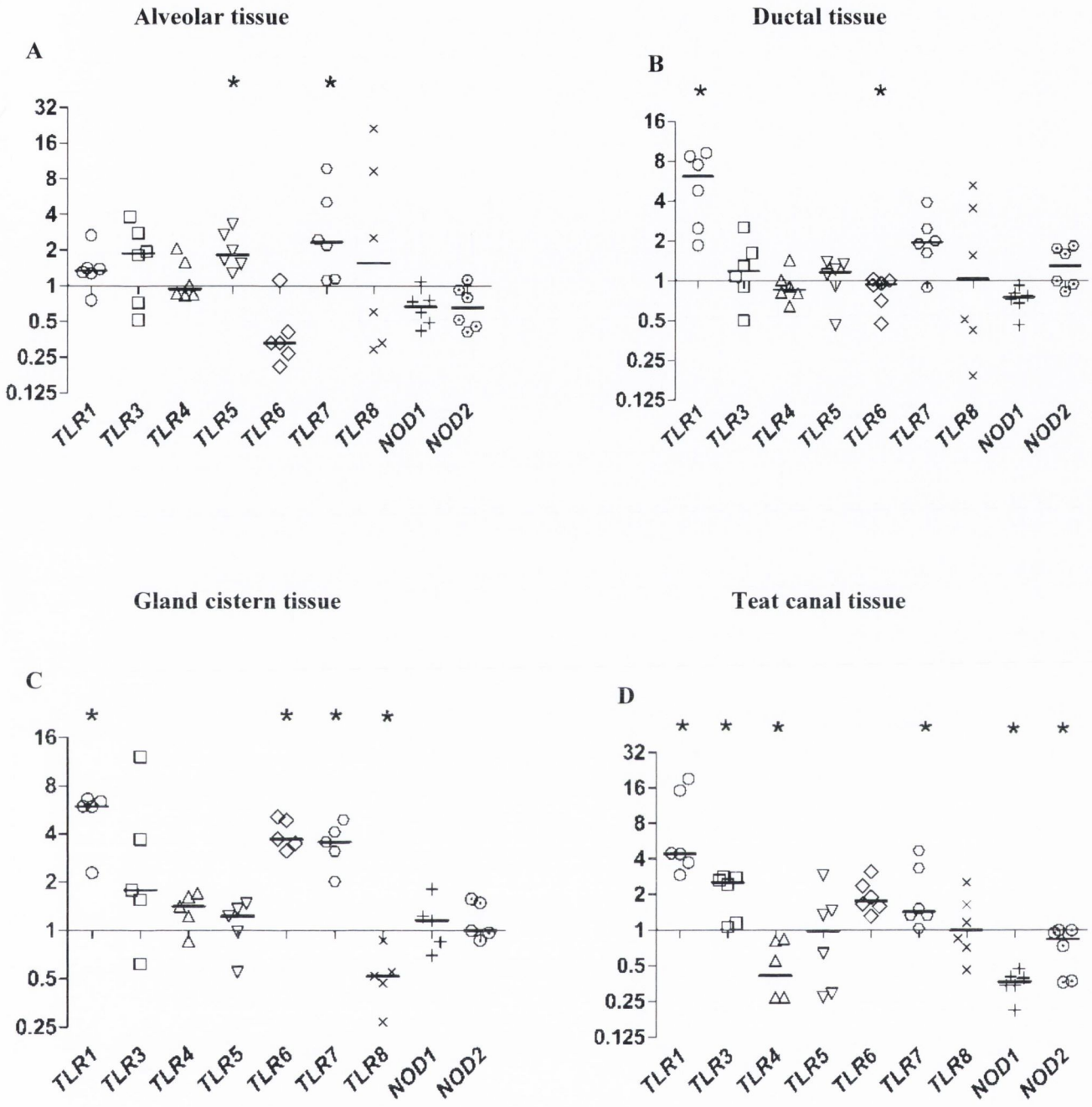


Figure 4.4 Pattern recognition receptor (PRR) gene expression in mammary tissue isolated from *S. aureus*-challenged and control quarters

(A)-(D) PRR expression in bovine mammary tissue isolated from alveolar, ductal, gland cistern and teat canal regions of infected and control quarters with *S. aureus*. Median fold changes are shown for *S. aureus* infected animals (n=6) relative to non-infected control animals (n=3). Statistical significance is denoted as: * = $P < 0.05$.

4.2.6 Regional variation in cytokine gene expression

Expression of the proinflammatory cytokine *IL6* was 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. 4.6A, 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. 4.6B). *TNF* showed no significant differential expression across the four tissue regions (see Appendix). *IL8* showed a strong and significant increase in alveolar tissue (13-fold, $P < 0.05$; Fig. 4.6A) and a moderate fold increase in gland cistern tissue (2.1-fold, $P < 0.05$; Fig. 4.6C). A similar fold increase in teat canal tissue was observed for the gene encoding *IFNG* (11-fold, $P < 0.05$; Fig. 4.6D). 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. 4.6A–D). Gene expression of the proinflammatory antagonists *TGFB* and *IL10* was also profiled. *TGFB* expression was moderately increased in ductal tissue and a moderate decrease was evident in teat canal tissue (1.4- and1.8- fold respectively, $P < 0.05$; Fig. 4.6B and D). *IL10* showed a strong increase in both ductal and teat canal tissue (8.4- and 3.2-fold respectively, $P < 0.05$; Fig. 4.6B and D).

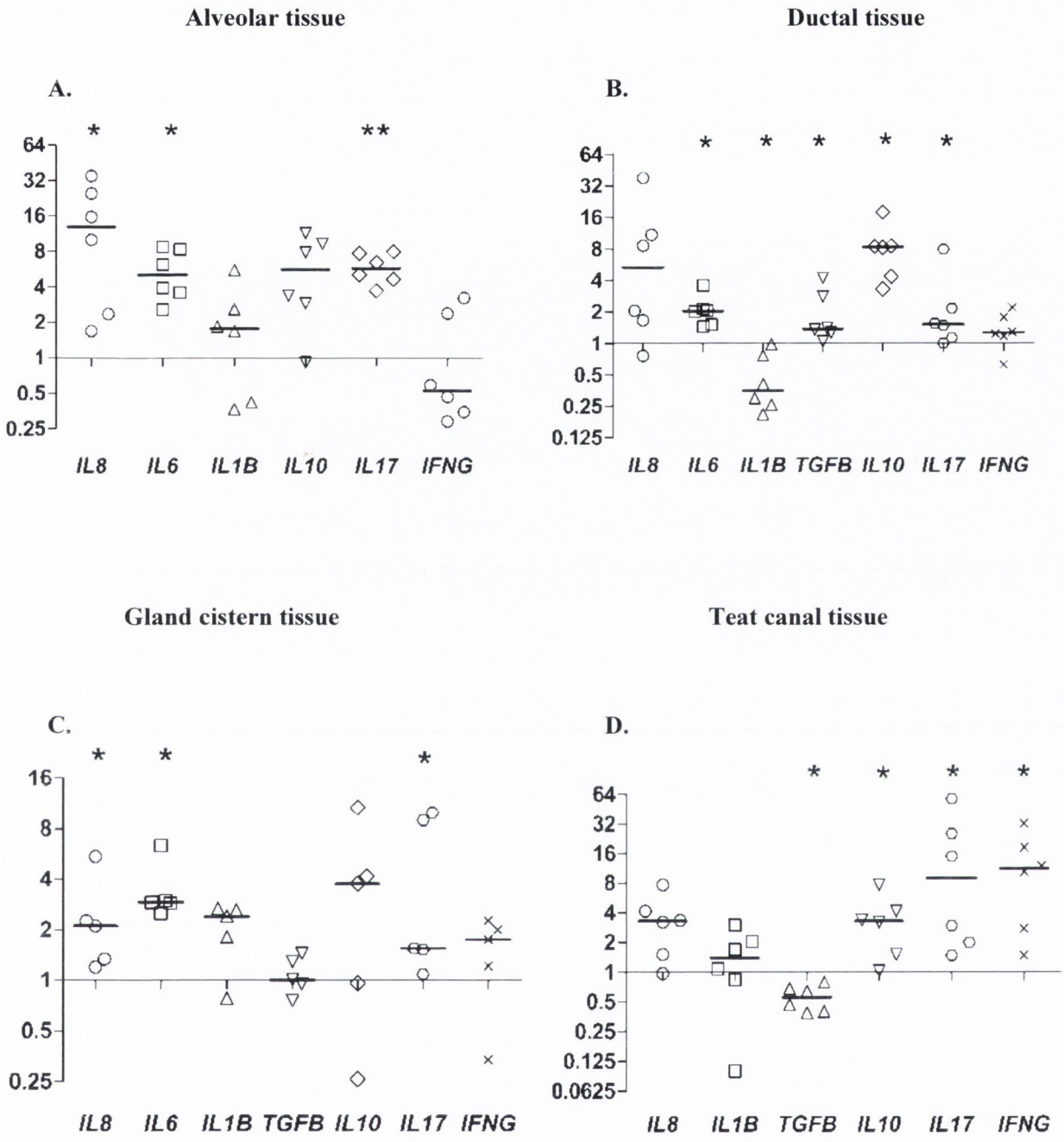


Figure 4.5 Cytokine gene expression in mammary tissue isolated from *S. aureus*-challenged and control quarters

(A)-(D) Cytokine expression in bovine mammary tissue isolated from alveolar, ductal, gland cistern and teat canal regions of infected and control quarters with *S. aureus*. Median fold changes are shown for *S. aureus* infected animals (n=6) relative to non-infected control animals (n=3). Statistical significance is denoted as: * = $P < 0.05$ and ** $P < 0.05$.

4.2.7 Acute phase protein gene expression in mammary tissue

Baseline expression levels of *SAA3*, *HP*, *LBP*, *AGP* and *CP* are shown in Figure 4.7. *SAA3* had the greater levels of expression compared to *HP*, *LBP*, *AGP* and *CP* with expression levels highest in gland cistern tissue (Fig. 4.7) Of the 5 APP 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. 4.8A and B), with a moderate fold increase detected in gland cistern tissue (4.1-fold, $P < 0.05$; Fig. 4.8C). Similarly *HP* expression was strongly induced in both alveolar and ductal tissue (80- and 30-fold respectively, $P < 0.05$; Fig. 4.8A and B), with a moderate fold increase detected in gland cistern tissue (5.6- fold, $P < 0.05$; Fig. 4.8C). Lesser fold increases were detected for *LBP* (alveolar, ductal and gland cistern, 3.3-, 3.3- and 1.7-fold respectively; $P < 0.05$ – Fig. 4.8A, B and C) and *CP* (gland cistern and teat canal, 3.2- and 1.9-fold respectively; $P < 0.05$– Fig. 4.8C and D). Expression of *AGP* was significantly decreased in alveolar tissue (54-fold, $P < 0.05$; Fig. 4.8A) and increased in ductal and gland cistern tissue (5.1- and 7.2- fold respectively, $P < 0.05$; Fig. 4.8B and C).

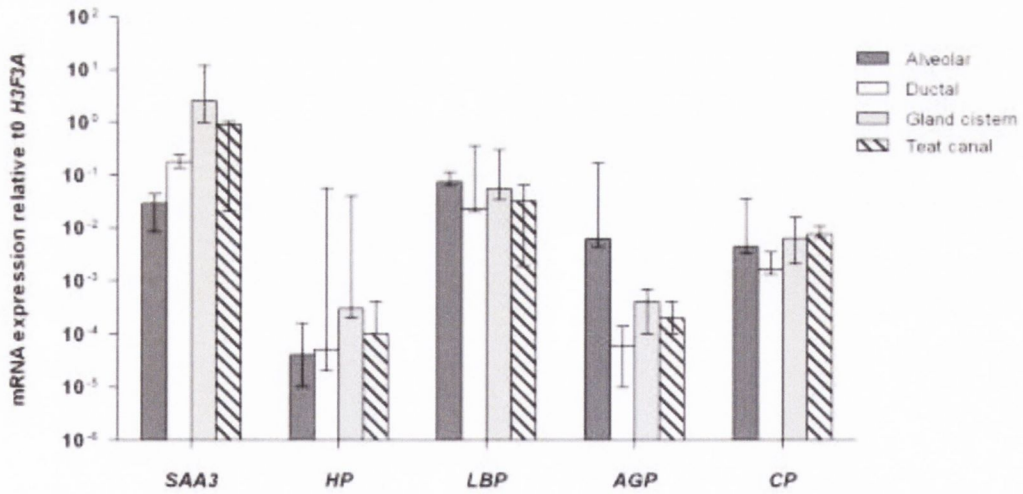


Figure 4.6 Relative gene expression levels of bovine acute phase proteins in control animals. Baseline levels of acute phase protein expression in alveolar, ductal, gland cistern and teat canal tissue from control animals. The expression of each gene in each sample is expressed relative to *H3F3A* (10^0) expression using the formula $2^{-Cq(GOI)}/2^{-Cq(HK)}$. Data shown is the median (bars denote the upper and lower range) of n = 3 biological replicates. There were two technical replicates performed for each biological sample.

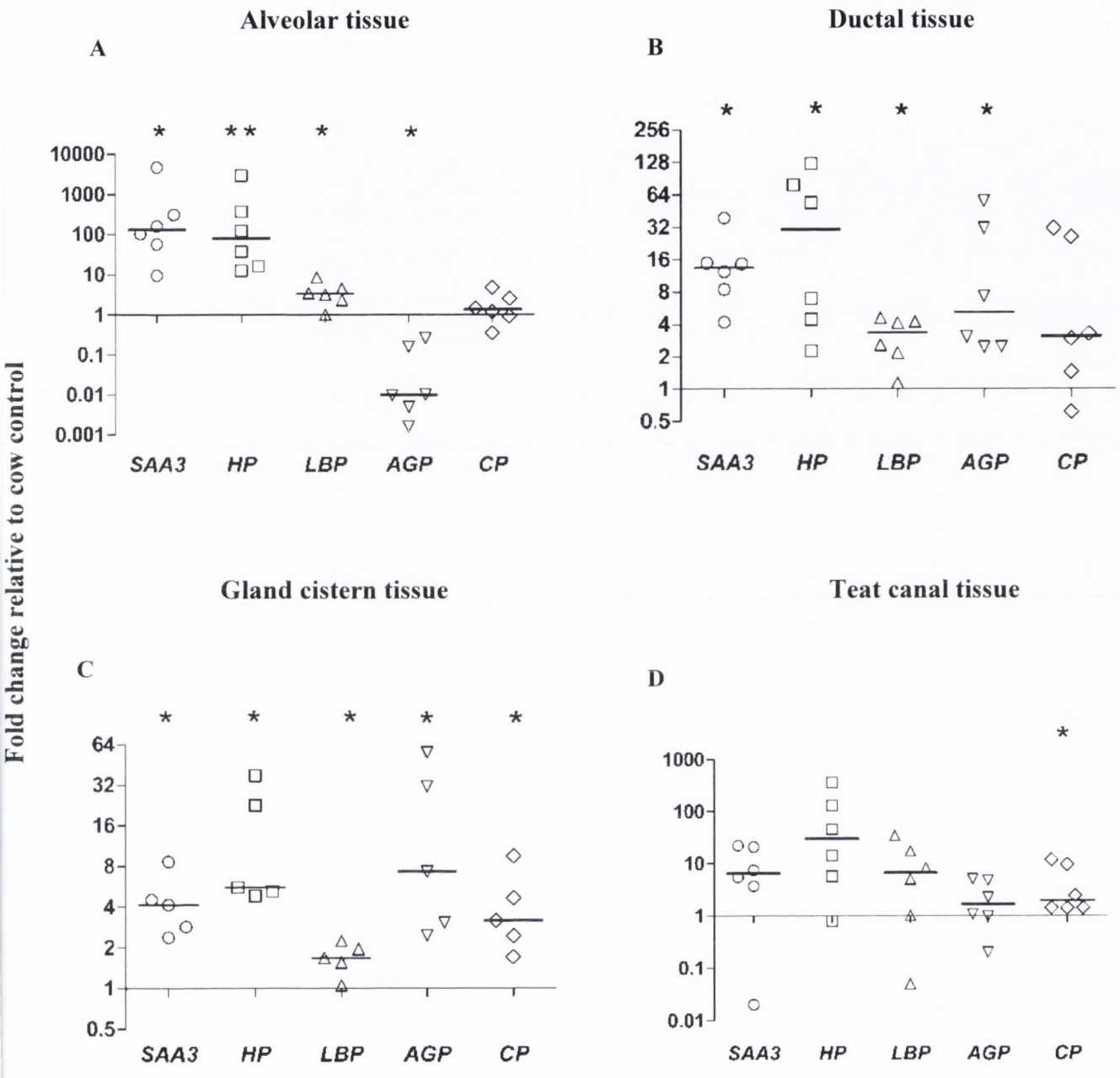


Figure 4.7 Acute phase protein (APP) gene expression in mammary tissue isolated from *S. aureus*-challenged and control quarters

(A)-(D) APP expression in bovine mammary tissue isolated from alveolar, ductal, gland cistern and teat canal regions of infected and control quarters with *S. aureus*. Median fold changes are shown for *S. aureus* infected animals (n=6) relative to non-infected control animals (n=3). Statistical significance is denoted as: * = $P < 0.05$ and ** $P < 0.05$.

4.2.8 β-Defensin gene expression

The baseline expression of *LAP* in alveolar, ductal gland cistern and teat canal tissue was orders of magnitude higher than that for *DEFB*4, *DEFB5*, *DEFB1* and *TAP* (Fig. 4.9). Expression of the β-defensins, *DEFB4* and *DEFB5*, was strongly induced in both alveolar (9.4-and 27-fold respectively, $P < 0.05$; Fig. 4.10A) and gland cistern tissue (10- and 34-fold respectively; $P < 0.05$; Fig. 4.10C) from challenged quarters. *DEFB5* showed similar strong induction in ductal tissue (22-fold, $P < 0.05$; Fig. 4.10B). *DEFB1* was moderately induced in the alveolar region (4.9-fold, $P < 0.05$; Fig. 4.10A) with no significant induction detected in the remaining three regions. Similarly, expression of the β-defensins *LAP* and *TAP* was not significantly induced in any of the regions profiled (Fig. 4.10A–D).

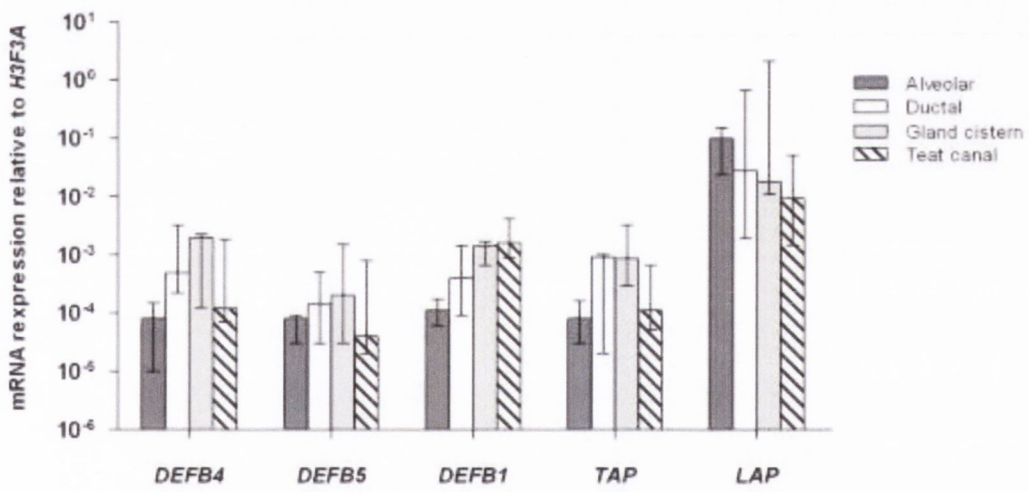


Figure 4.8 Relative gene expression levels of bovine β-defensins in tissue control animals.
Baseline levels of acute phase protein expression in alveolar, ductal, gland cistern and teat canal tissue from control animals. The expression of each gene in each sample is expressed relative to *H3F3A* (10^0) expression using the formula $2^{-Cq(GOI)}/2^{-Cq(HK)}$. Data shown is the median (bars denote the upper and lower range) of n = 3 biological replicates. There were two technical replicates performed for each biological sample.

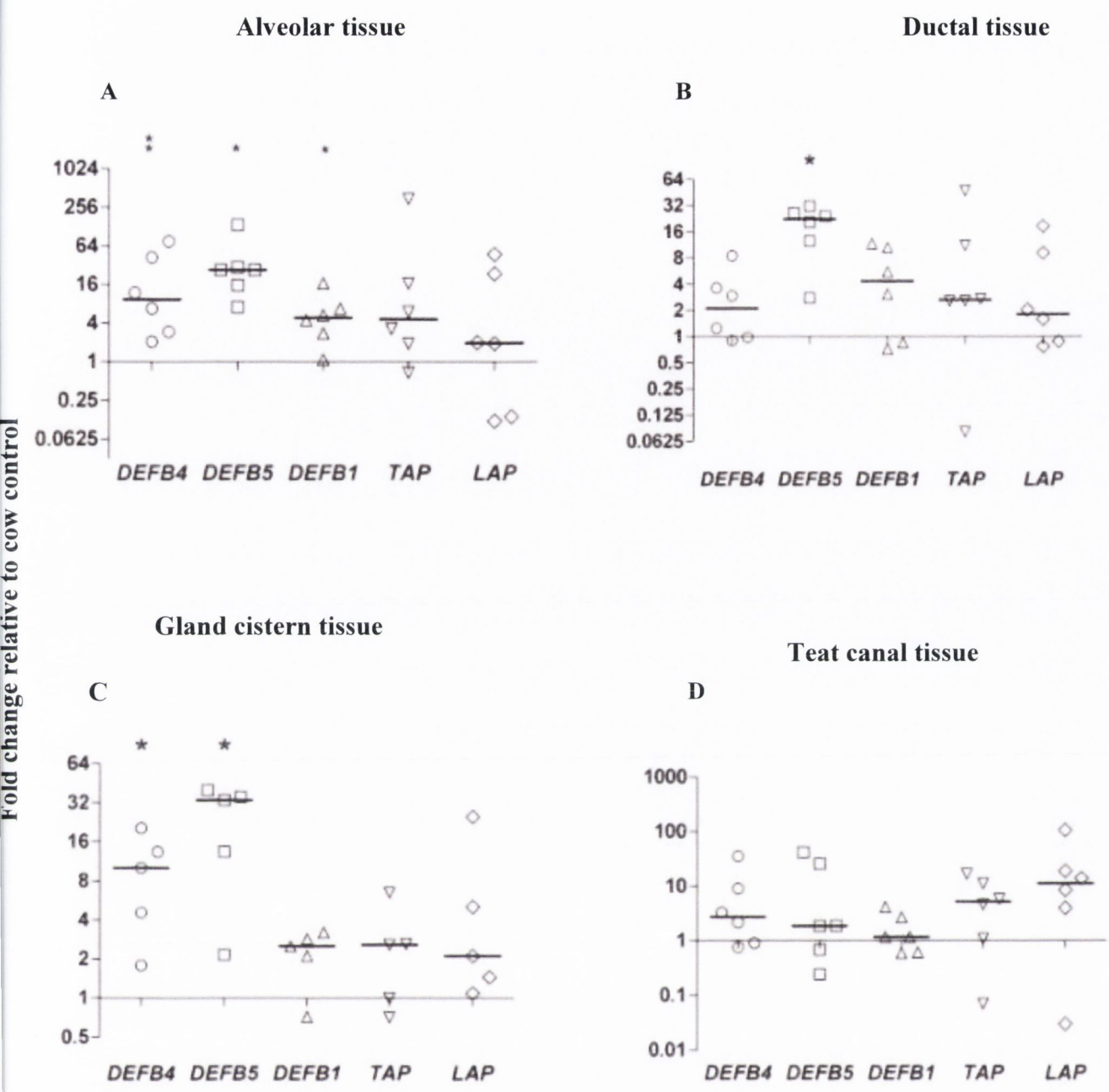


Figure 4.9 β-defensin gene expression in mammary tissue isolated from *S. aureus*-challenged and control quarters

(A)-(D) β-defensin expression in bovine mammary tissue isolated from alveolar, ductal, gland cistern and teat canal regions of infected and control quarters with *S. aureus*. Median fold changes are shown for *S. aureus* infected animals (n=6) relative to non-infected control animals (n=3). Statistical significance is denoted as: * = $P < 0.05$ and ** $P < 0.01$.

4.3 Discussion

A greater 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. An experimental model of *S. aureus*-induced subclinical mastitis had previously been established by our collaborators (Eckersall *et al.*, 2006), who profiled the expression of the acute phase proteins, serum amyloid A (SAA) and haptoglobin (HP), in milk and serum. In this study, a comprehensive quantification of innate immune genes was 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 reference gene *H3F3A* (Fig. 4.4A and B), 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 intracellular 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. The combination of bovine TLR2 and TLR1 can recognise gram-positive cell wall component (Werling *et al.*, 2009). Interestingly, significant increases in the expression of TLRs that recognise viral ligands were also observed (*TLR3* and *TLR7*). Human monocytes also upregulate TLR3 and TLR7 in response to both *S. aureus* and IL6 treatment (Zarember and Godowski, 2002). *TLR8* showed a low level of expression in the four regions profiled. *TLR8* has been shown to be 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 which could explain the low levels of *TLR8* expression in mammary tissue. Similar low levels of *TLR8* expression was observed in bovine endometrial tissue, with no transcript detected in purified stromal or 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 a high level of constitutive expression in all four tissue regions (Fig. 4.4A). 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-challenge (Chapter 3: Results, Fig. 2.1), and through the induction of proinflammatory cytokines (Fig. 4.6A-D); 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* was not significantly increased across the mammary gland. However, the *IL6* gene, the protein product of which regulates the APR through the synthesis of APPs (Jensen and Whitehead, 1998), was significantly induced in three of the four tissue regions profiled. Our observations are consistent with recently published data which demonstrate that *S.aureus* can induce *IL6* expression in mammary epithelial cells but fails to induce *IL1A* and *TNF* (Mitterhuemer *et al.*, 2010). Reduced TNF-α and IL-1 secretion during mastitis caused by *S.* aureus and other Gram-positive bacteria has also been reported (Riollet *et al.*, 2000; Rainard *et al.*, 2008).

A similar proinflammatory cytokine response has been observed *in vivo* 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 expression of *TNF* increased in 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 within a short period (Blum *et al.*, 2000; Hoeben *et al.*, 2000).

IFNG, *IL17A*, and *IL8* were also significantly increased. The gene encoding *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-challenge. At 48 h post-challenge there was an increase in cell infiltration into the mammary gland (Fig. 3.1) Chemokines such as IL8 are chemotactic for neutrophils. The observed enhanced expression of *IL8* in infected quarters may explain the influx of somatic cells observed in these quarters only. Interestingly, in humans, *IL17A* has also been shown to be involved in the recruitment

of neutrophils through regulating IL8 expression (Laan *et al.*, 1999). Increased expression of a number of genes encoding cytokines, chemokines and antimicrobial peptides has been demonstrated in primary bovine mammary epithelial cells (PbMECs) in response to recombinamt bovine IL-17A (Bougran *et al.*, 2011). In addition, the expression of *IL8* was augmented by the combination of IL-17A with staphylococcal lipoteichoic acid or muramyl dipeptide, suggesting that IL-17A has the potential to modulate the mammary gland immune response to mastitis-causing pathogens (Bougran *et al.*, 2011).

Expression of molecules involved in the resolution of infection was also profiled. While the expression of *TGFB1* was similar to that of control animals, *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.

The increased cytokine gene expression, in conjunction with the APP expression supports a proinflammatory host response to *S. aureus* infection. *IL6* expression has also been demonstrated in milk somatic cells from cows infected with *S. aureus* (Lee *et al.*, 2006). It has been suggested that APPs detected in the mammary gland were a result of leakage from the serum in times of stress or infection (Salonen *et al.*, 1996). In this study, we confirmed the expression of acute phase protiens, SAA3 and HP at the mRNA level, reaffirming Eckersall's findings of the local production of these two major APPs throughout the mammary gland in response to *S. aureus* infection (Eckersall *et al.*, 2006). *SAA3* and *HP* genes were particularly highly induced in alveolar tissue 48 h post-challenge (Fig. 3.8 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 against gastrointestinal infections by inducing mucin production in the intestine resulting in reduced pathogen adherence (Gardiner *et al.*, 2009; Larson *et al.*, 2003). The high levels of expression of *SAA3* in control animals (Fig. 4.7) would also add weight to this argument. 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. HP may limit the availability of iron (Schaer *et al.*, 2002) that is necessary for *S. aureus* to proliferate (Skaar *et al.*, 2004). Alternatively, it may reduce 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). 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 (LBP), ceruloplasmin (CP) and α-1-acid glycoprotein (AGP) throughout the mammary gland. Induction of the gene encoding LBP was observed in all four tissues (Fig. 4.8A-D). Increased concentration of LBP 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, LBP has been shown to bind to cell wall components of gram-positive bacteria, namely lipoteichoic acid (Schröder *et al.*, 2003). CP gene expression was significantly increased in gland cistern and teat canal tissue (Fig. 4.8 C and D). Increased concentration of CP has been reported in the milk of cows with subclinical mastitis (Tabrizi *et al.*, 2008). Interestingly, AGP gene expression was significantly decreased in the alveolar region alone (54-fold), whereas expression was induced in both ductal and gland cistern tissue (Fig. 4.8 A, B and C). AGP has been identified in bovine colostrum and milk and expression of mRNA localised to mammary tissue (Ceciliani *et al.*, 2005). Immunomodulatory functions of AGP have also been demonstrated. In particular, it has been shown to inhibit bovine neutrophil respiratory burst activity while enhancing IL8 production. However, this inhibitory effect did not affect the ability of neutrophils to phagocytose 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 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 differentially expressed across the mammary gland. *DEFB5* was strongly induction in the alveolar (27-fold), ductal (22-fold) and gland cistern tissue (34-fold) (Fig. 4.10A, B and C). Similarly high differential expression was detected for *DEFB4* in alveolar and gland cistern tissue (Fig. 3.10A and C). A previous study showed that following intramammary infection with *S. aureus*, expression of *DEFB5* was not detected until 84 h post-challenge (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. 2.1 Material and Methods) must be considered when interpreting these results, since the expression of innate immune genes were significantly increased in the neighbouring unchallenged quarters compared to control animals (Chapter 3: Results, Fig. 3.5, Fig. 3.6 and Fig. 3.7). 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 (Chapter 2: Materials and Methods, Fig. 2.1).

Having shown their inducible (Fig. 4.9) and constitutive (4.10) expression in mammary tissue 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, 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 observed in response to *E. coli* infection (Rinaldi *et al.*, 2010). It was suggested that regional differences are a result of the time frame 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, maybe a result of differences in resident cell populations throughout the mammary gland (Nickerson and Pankey, 1983; Nickerson *et al.*, 1984) or differences in the expression of PRRs (Fig 4.4A). The contribution of each cell population to the challenge-related changes in gene expression remains to be elucidated.

4.4 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.

Chapter 5: Innate immune gene expression profiling in bovine mammary epithelial cells in response to a low-virulence strain of *S.aureus*

5.1 Introduction

The udder is a complex organ composed of several cell types all likely to contribute differentially to the immune competence of the udder (reviewed in Chapter 1: Introduction, Section 1.9). The immune capacity of milk against mastitis has been well characterised (Burvenich *et al.*, 2003; Dosogne *et al.*, 2002; Paape *et al.*, 2002). However, the immune relevance of mammary epithelial cells has only recently been recognised (Gunther *et al.*, 2010; Juliane *et al.*, 2009; Griesbeck-Zilch *et al.*, 2008; Pareek *et al.*, 2005) These cells are the dominant cell type in healthy, uninfected mammary quarters and, therefore, are the first cells most likely to encounter pathogens after entering the udder.

In contrast to other epithelia, such as the upper respiratory and intestinal epithelia which are constantly exposed to pathogens, the mammary gland is normally sterile, and as such, and any bacterium that enters can be considered an intruder. The immune make-up of the mammary gland, including the repertoire and distribution of pathogen receptors and the expression of bactericidal molecules in mammary epithelial cells, is likely to be conditioned by the aseptic nature of the mammary gland. In this respect, the mammary gland closely resembles more the urinary system.

Although recent studies have demonstrated the enhanced expression of a variety of immune genes in response to mastitis-causing stimuli, little information is available describing the early immune response in mammary epithelium after pathogen challenge (Gunther *et al.*, 2010), in particular, the early expression of bactericidal molecules such as antimicrobial peptides and acute phase proteins, in response to *S.aureus* challenge.

To address this, we used primary isolates of bovine mammary epithelial cells (PbMECs) (Chapter 2: Materials and Methods, Section 2.3.1) to investigate the early immune response to pathogen stimulation. We were particularly interested in the early immune response to *S. aureus*, which upon intramammary infection causes predominately subclinical mastitis which can lead to chronic infection. The early host response induced in mammary epithelial cells during infection may be crucial determinants for the pathogenesis after infection with *S.aureus.* Proper recognition of the pathogen by the host and the rapid

expression of bactericidal effector molecules likely represents a key event in the pathogenesis of infectious diseases.

Our results from chapter 4 demonstrated the expression of *TLRs 1-10* and *NODs 1-2* throughout the mammary quarter as well as the spatial and inducible expression of a panel of acute phase protein and antimicrobial peptide genes in response to *S. aureus*-induced subclinical mastitis. Histopathological analysis reported minimal inflammation. Therefore, we hypothesised that the mammary epithelial cell was a primary contributor to immune activity in response to *S. aureus* infection. In order to test this, qPCR was used to measure the expression of genes encoding pattern recognition receptors (PRR), pro- and anti-inflammatory cytokines, acute phase proteins (APP) and antimicrobial peptides (AMP) at early time points in primary bovine mammary epithelial cells (PbMEC) following stimulation with *S. aureus*. We were unable to obtain the same strain of *S. aureus* (strain NCTC13047) that was used in our *in vivo* study (Chapter 4). In order to facilitate a comparison between our *in vivo* and *in vitro* infection models, we used a strain of *S. aureus* (*S. aureus 1027*) that had previously been isolated from a case of subclinical mastitis (Petzl *et al.*, 2008).

5.2 Results

5.2.1 **Characterisation of the purity of PbMECs** (Work carried out by AvantiCell Science Ltd)

Primary bovine mammary epithelial cells (PbMECs) were derived from mammary parenchyma. The animals were in their third trimester of pregnancy and recorded as BSE-free. Post-mortem analysis was negative for bovine viral diarrhoea. Cells were isolated by enzymic digestion (collagenase, hyaluronidase) then enriched for luminal epithelial cells by differential density gradient centrifugation. The resulting cells were cytokeratin 18 positive, cytokeratin 19 negative and vimentin negative, indicating the presence of epithelial cells.

5.2.2 RNA quality

The extraction method used yielded RNA of excellent quality (Fig. 5.1). The RNA integrity number (RIN) averaged >9.7 (range, 6.2 to 7.7) and for all primary bovine mammary epithelial cell samples the RIN averaged >6.7 (range 9.5 to 10), all suitable for reverse transcription to cDNA and analysis using qPCR.

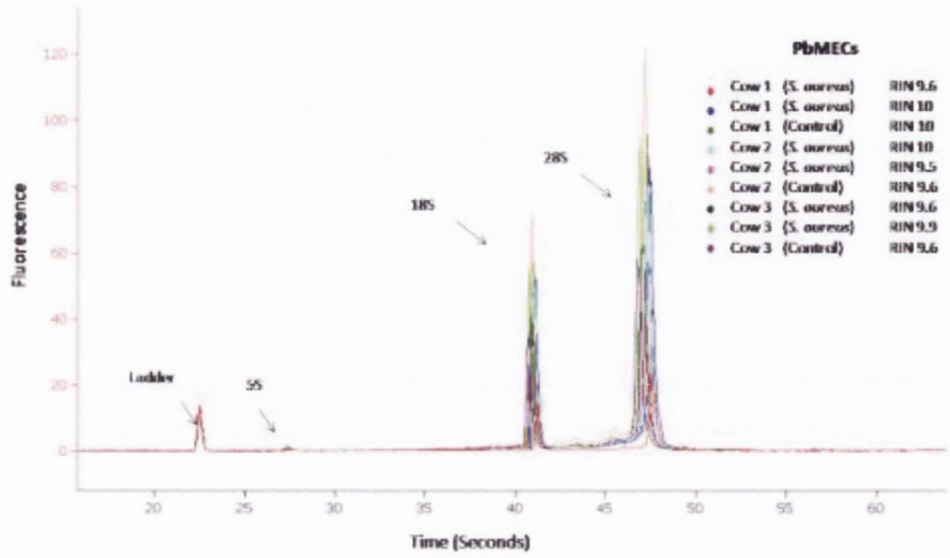


Figure 5.1 Assessment of RNA quality using micro-capillary electrophoresis (Agilent Bioanalyzer)

Assessment of RNA quality from primary bovine mammary epithelial cells (PbMECs) stimulated with *S. aureus* and unstimulated control cells. A representative result is shown for primary bovine mammary epithelial cells isolated from 3 individual cows. The extraction method used yielded RNA of excellent quality as is evident by the clearly visible 28/18S rRNA peak ratio and a small 5S RNA.The level of RNA degradation is reported as an RIN (ranging from 1 (low-quality degraded RNA) to 10 (high-quality intact RNA). The RIN number averaged >9.7 for all PbMEC samples.

5.2.3 Selection of stable normaliser gene for quantitative real-time PCR (qPCR)

The expression of seven genes, *PPIA*, *GAPDH*, *ACTB*, *H3F3A*, *RPS6*, *RPS9* and *RPS15*, was examined in cDNA from each time point of both *S. aureus* stimulated and unstimulated control cells. *RPS9* demonstrated the greatest stability across all time points from both stimulated and unstimulated cells (Fig. 5.2) and was therefore selected as the most suitable gene for normalisation of qPCR data. Primer efficiency for *RPS9* and all significantly differentially expressed genes were considered comparable and within the recommended range for using the $2^{-\Delta\Delta CT}$ method (90 to 100% efficiency).

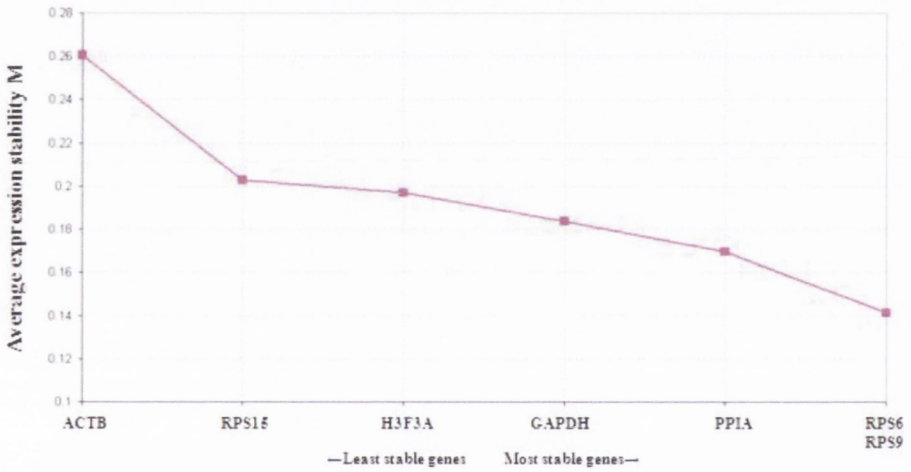


Figure 5.2 The stability of reference genes in PbMECs in response to *S. aureus* challenge.

A stable reference gene was determined using the geNorm software (version 3.4 Microsoft Excel add-in software package) (Vandesompele *et al.*, 2002). Seven putative reference genes [peptidylprolyl isomerise 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 were then analysed using the geNorm software to generate *M* stability. 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. RPS9 demonstrated the greatest stability across all time points from both *S. aureus* stimulated and unstimulated cells.

5.2.4 Multiplicity of infection

Preliminary experiments to decide on final treatment parameters were carried out on a bovine mammary epithelial cell line in order to minimise costs. We chose to use a cell line called BME-UV (Bovine Mammary Epithelium – University of Vermont) which was established by Zavizion and colleagues in 1996 (Zavizion *et al.*, 1996). This cell line is derived from mammary tissue of a lactating pregnant Holstein cow.

Cells were challenged with *S. aureus* 1027 at various concentrations that corresponded to a MOI of 1, 10, 100 and 1000. Gene expression of the proinflammatory cytokine, IL8, was quantified for each MOI. *IL8* expression reached a plateau at a MOI of 100 (data not shown) and as a result this concentration of heat inactivated *S. aureus* 1027 was used for subsequent experiments.

5.2.5 TLR 1-10 and NOD 1-2 expression in mammary epithelial cells

Relative expression levels of TLRs 1-10 and NOD 1-2 were compared in control (unstimulated) primary mammary epithelial cells (PbMECs). *NOD1* and *NOD2* were highly expressed, *TLR1*, *TLR2*, *TLR3*, *TLR4*, *TLR6* and *TLR7* were moderately expressed and there was little expression of *TLR5* and *TLR9* detected (Fig. 5.3). No expression of *TLR8* and *TLR10* was detected in control cells (Fig. 5.3 and 5.4). The expression of *TLRs 1-10* and *NOD 1-2* were detected after treatment with heat-inactivated *S.aureus* 1027 for 3 hours (Fig. 5.4).

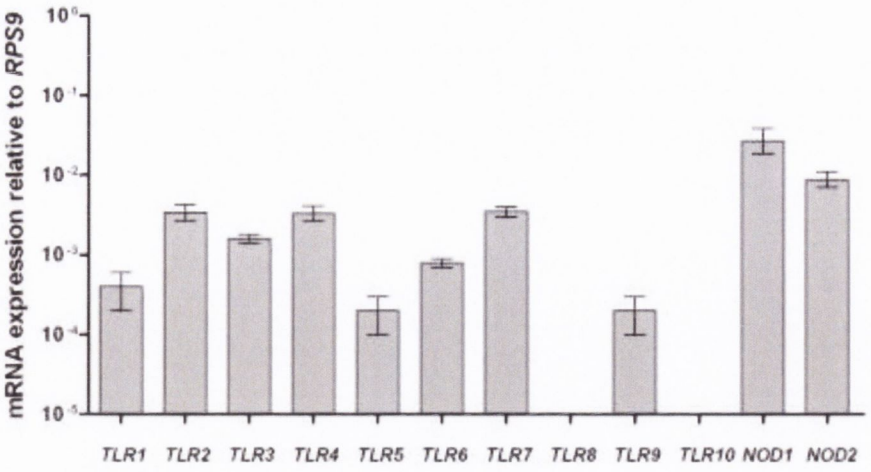


Figure 5.3 Relative gene expression levels of bovine pattern recognition receptors in primary bovine mammary epithelial cells (PbMECs)

Relative levels of PRR expression in unstimulated PbMECs (baseline expression). The expression of each gene in each sample is expressed relative to *RPS9* (10^0) expression using the formula $2^{-Cq(GOI)}/2^{-Cq(HK)}$. Data shown is the median (bars denote the upper and lower range) of n = 3 biological replicates. There were two technical replicates performed for each biological sample.

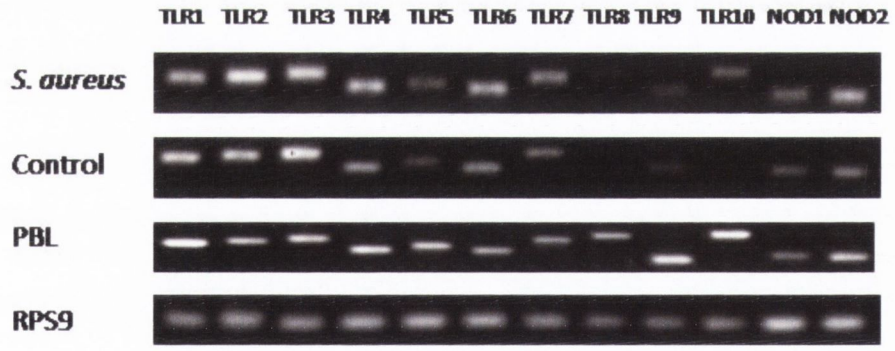


Figure 5.4 Expression profile of TLRs 1-10 and NOD 1-2 in mammary epithelial cell

qPCR (40 cycles) was used to profile the expression of TLRs 1-10 and NOD 1-2 in cells that were challenged with *S. aureus* 1027 for 3h. The expression of *TLRs 1-10* and *NOD 1-2* were profiled and compared to the expression in non-challenged controls A representative result is shown (n=3). PBL = peripheral blood leucocytes cDNA. Rps9 = housekeeping gene. No amplicons were observed in the negative controls (no template controls) (data not shown).

Expression of a panel of TLR genes was further profiled in PbMECs following stimulation with heat-inactivated *S. aureus* 1027. Expression levels of all 4 TLRs profiled (*TLR1, TLR2, TLR4* and *TLR6*) remained relatively unchanged over 6 hours, with the exception of *TLR2* at 30 min which was significantly increased (1.4-fold, $P < 0.05$; Fig. 5.5A) *TLR6* was also significant increased at 6 h post stimulation (5.3-fold, $P < 0.05$; Fig. 5.5D). The expression of *TLR1* was slightly decreased at 30 min post stimulation (1.9-fold, $P < 0.05$; Fig. 5.5A). Similarly, the expression of *TLR4* and *TLR6* was decreased at 1 h post stimulation (2- and 2.2-fold, respectively; $P < 0.05$, Fig. 5.5B).

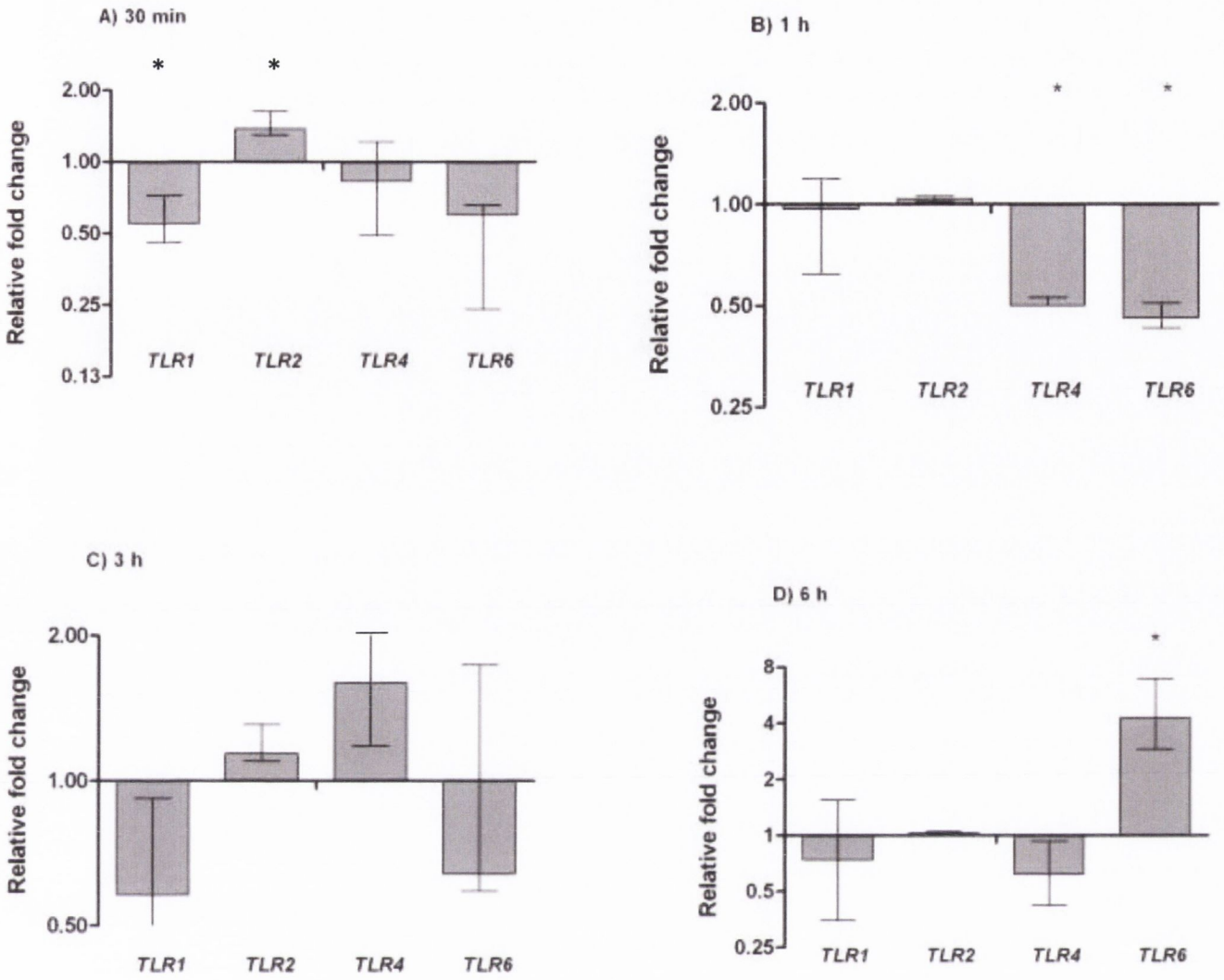


Figure 5.5 Toll-like receptor gene expression in primary bovine mammary epithelial cells (PbMECs) stimulated with heat-inactivated *S. aureus*.

(A)-(D) qPCR analysis of challenged related changes in the expression of TLRs in PbMECs at 30 minutes,1 h, 3 h and 6 h post stimulation with heat-inactivated *S. aureus* 1027. Median (bars denote the upper and lower range) fold changes are shown for *S. aureus* challenged cells (n=3) relative to control cells (n=3). Two technical replicates were performed for each biological sample. Statistical analysis was performed using unpaired t-tests (Significance is denoted as: * = $P < 0.05$).

5.2.6 Strong induction of TNF and IL1B in epithelial cells

In order to gain a better understanding of pathogen-specific immune responses, PbMECs were also stimulated with a stain of *Escherichia coli* (*E. coli* 1303) (Chapter 2: Materials and Methods, section 2.3.5) that was previously shown to cause acute mastitis *in vivo* (Petzl *et al.*, 2008). A strong induction of the proinflammatory cytokine genes *TNF* and *IL1B* was observed in cells stimulated with heat-inactivated *E coli 1303* compared to *S aureus* stimulated cells (Fig. 5.6A and B). *TNF* was induced significantly in cells incubated with heat-killed *E. coli* for 1 h (28-fold, $P< 0.05$), with expression levels >500-fold at 3 h and >70-fold at 6 h ($P< 0.05$; Fig. 5.6A) In comparison, *TNF* expression was moderately increased at 30 min, 1 h and 3 h post stimulation with *S. aureus* (2.3-, -4 and 10-fold respectively, $P < 0.05$; Fig. 5.6A). *IL1B* expression was significantly increased after 30 min and remained significantly elevated over the course of the experiment (3.6-, 3.4-, 77- and 30-fold respectively, $P < 0.05$; Fig. 5.5B). Expression of *IL1B* was significantly increased at 3 h (4.8-fold, $P < 0.05$; Fig. 5.6B), with a further increase at 6 h (13.8-fold, $P < 0.05$; Fig. 5.6B).

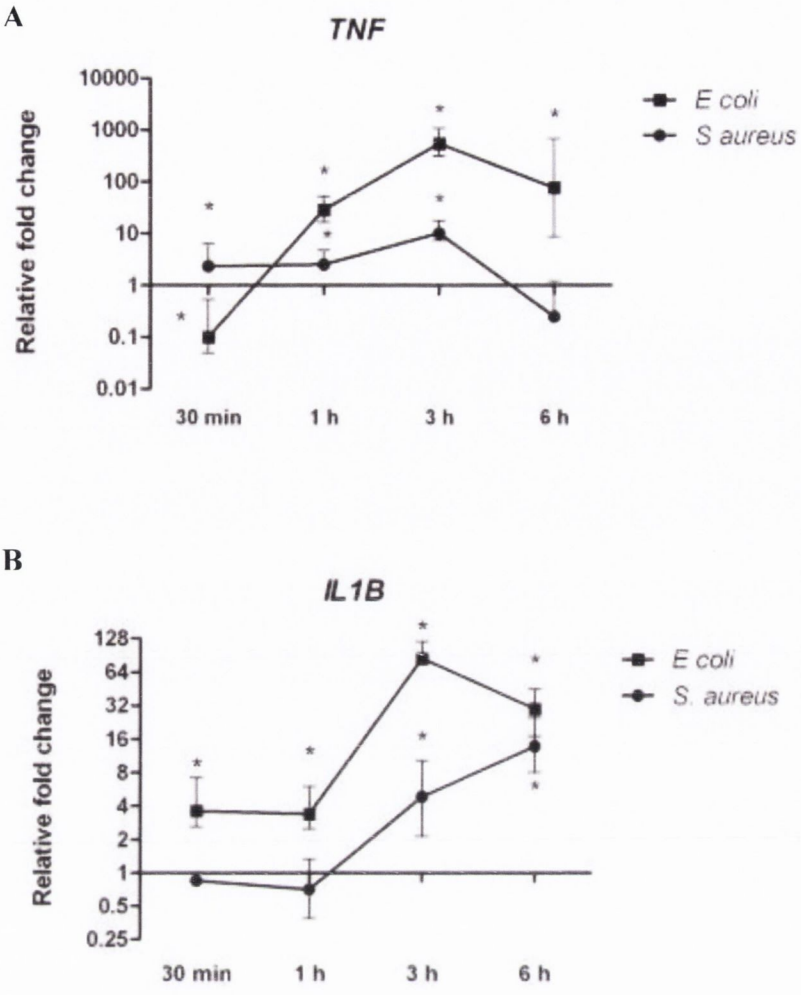


Figure 5.6 Cytokine gene expression in primary bovine mammary epithelial cells (PbMECs) stimulated with heat-inactivated *S. aureus* and heat-inactivated *E.coli.*

qPCR analysis of challenged related changes in the expression of A) *TNFA* and B) *IL1B* in PbMECs at 30 minutes,1 h, 3 h and 6 h post stimulation with heat-inactivated *S. aureus* 1027 and heat-inactivated *E.coli* 1303. Median (bars denote the upper and lower range) fold changes are shown for pathogen stimulated cells (n=3) relative to control cells (n=3). Two technical replicates were performed for each biological sample. Statistical analysis was performed using unpaired t-tests (Significance is denoted as: * = $P < 0.05$).

A significant decrease in *IL6* expression was detected at 1 h post stimulation (2.5-fold, $P < 0.05$; Fig. 5.7A). The expression of the chemokine *IL8* was moderately decreased at 1 h and increased at 6 h (2.3- and 4-fold respectively, $P < 0.05$; Fig. 5.7B). *TGFB* showed no significant differential expression across the time course. Expression of *IL10*, *IFNG* and *IL17* was not detected in control or *S. aureus* stimulated cells.

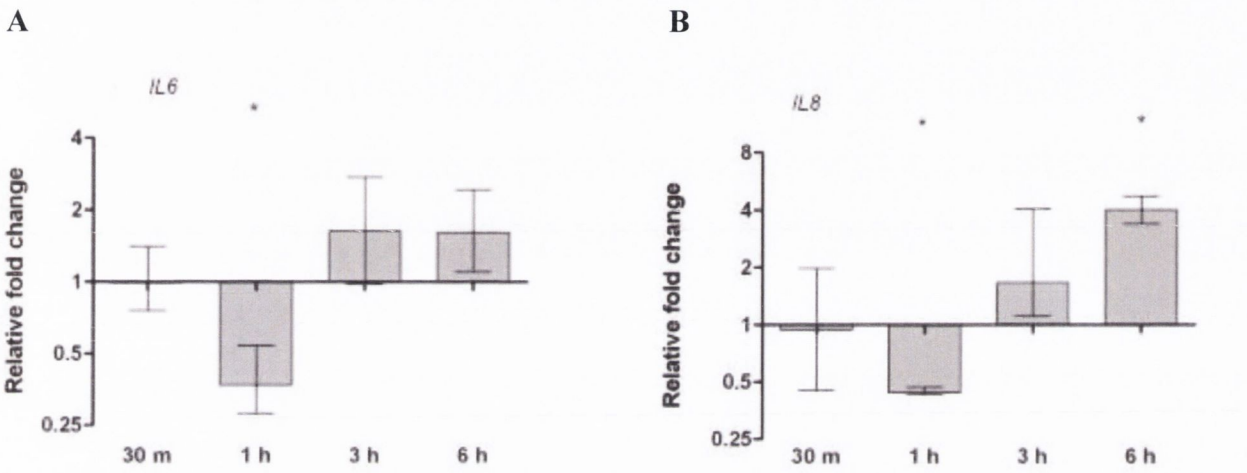


Figure 5.7 Cytokine and chemokine gene expression in primary bovine mammary epithelial cells (PbMECs) stimulated with heat-inactivated *S. aureus* and heat-inactivated *E.coli.*

qPCR analysis of challenged related changes in the expression of A) *IL6* and B) *IL8* in PbMECs at 30 minutes,1 h,3h and 6 h post stimulation with heat-inactivated *S. aureus* 1027. Median (bars denote the upper and lower range) fold changes are shown for pathogen stimulated cells (n=3) relative to control cells (n=3). Two technical replicates were performed for each biological sample. Statistical analysis was performed using unpaired t-tests (Significance is denoted as: * = $P < 0.05$).

5.2.7 Expression of acute phase protein genes

Transcript from all 5 acute phase genes profiled was detected in epithelial cells. Expression of *SAA3* was significantly increased after 3 h (5.8-fold, $P < 0.05$) with levels of expression >40 ($P < 0.05$) fold at 6 h post challenge (Fig. 5.8A). *HP* expression was moderately increased at 6 h post challenge (2.7-fold, $P < 0.05$; Fig. 5.8B). Expression of *AGP* and *LBP* was significantly decreased at 1 h (2.4- and 3-fold respectively, $P < 0.05$; Fig. 5.8C and D) *CP* expression was significantly decreased at 1 h and significantly increased at 3h post stimulation (2.3-and 41-fold respectively, $P < 0.05$; Fig. 5.8D).

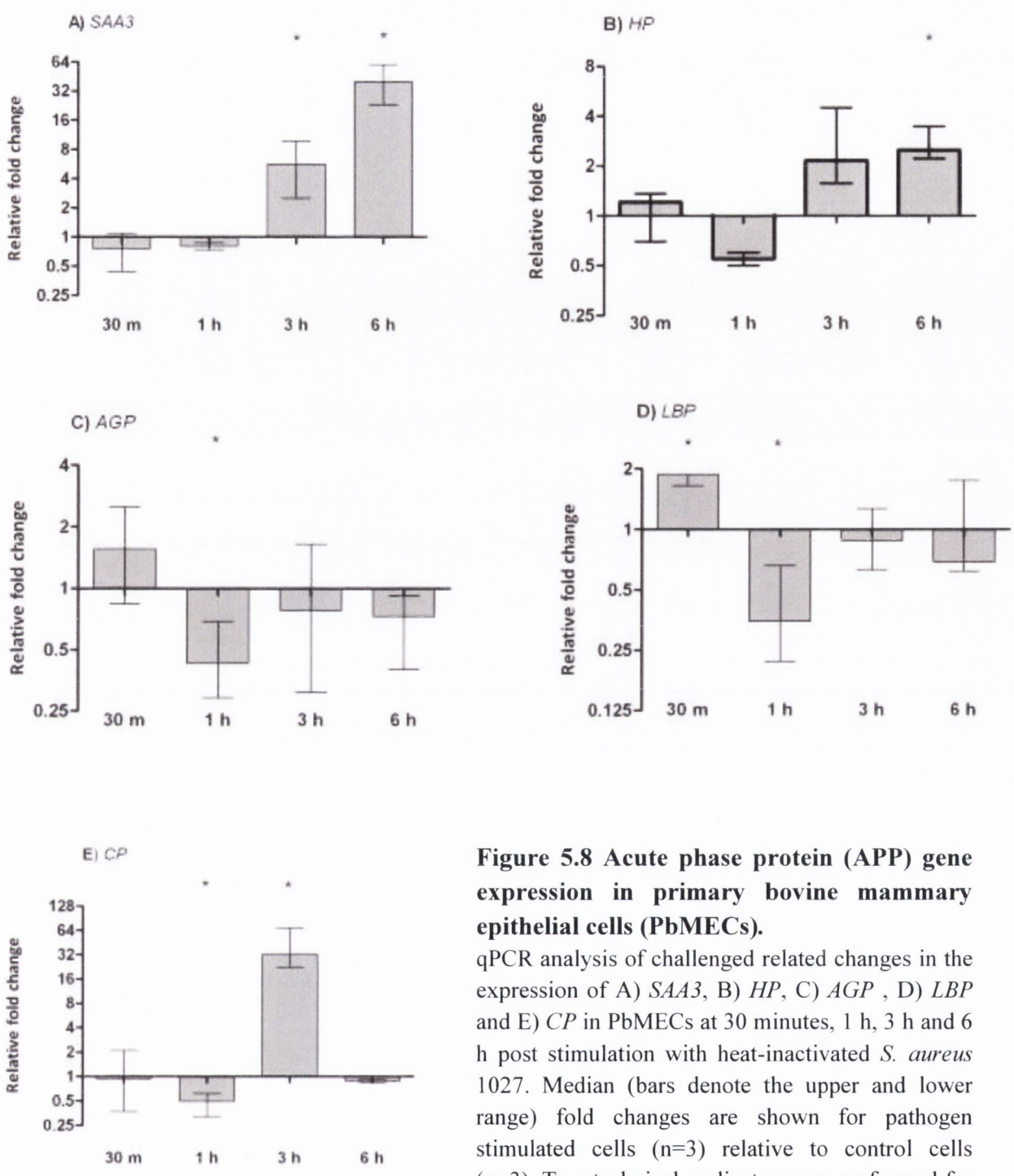


Figure 5.8 Acute phase protein (APP) gene expression in primary bovine mammary epithelial cells (PbMECs).

qPCR analysis of challenged related changes in the expression of A) *SAA3*, B) *HP*, C) *AGP* , D) *LBP* and E) *CP* in PbMECs at 30 minutes, 1 h, 3 h and 6 h post stimulation with heat-inactivated *S. aureus* 1027. Median (bars denote the upper and lower range) fold changes are shown for pathogen stimulated cells (n=3) relative to control cells (n=3). Two technical replicates were performed for each biological sample. Statistical analysis was performed using unpaired t-tests (Significance is denoted as: * = $P < 0.05$).

5.2.8 β-defensin gene expression in epithelial cells early post challenge with S. aureus

Expression of the beta-defensins *LAP*, *TAP*, *DEFB5*, *BNBD4* and *DEFB1* was also profiled. Both *TAP* and *LAP* were moderately expressed (Fig. 5.9) and there was little expression of *DEFB4* and *DEFB5* in both control and *S. aureus* stimulated cells (Fig. 5.10A). *LAP* expression was significantly increased at 6 h post challenge (2.5-fold, $P < 0.05$; Fig. 5.10B). In contrast, *TAP* expression was slightly decreased at 1h and 6h, with an increase in expression detected at 3 h post challenge (1.7- and 1.4-fold respectively, $P < 0.05$; Fig. 5.10A). *DEFB1* was not significantly differentially expressed at any of the time points analysed. *DEFB5* and *BNBD4* transcripts amplified very late in the qPCR (> 38 cycles) with no consistency in the expression between samples. Therefore, the challenge-related changes in their expression could not be quantified accurately.

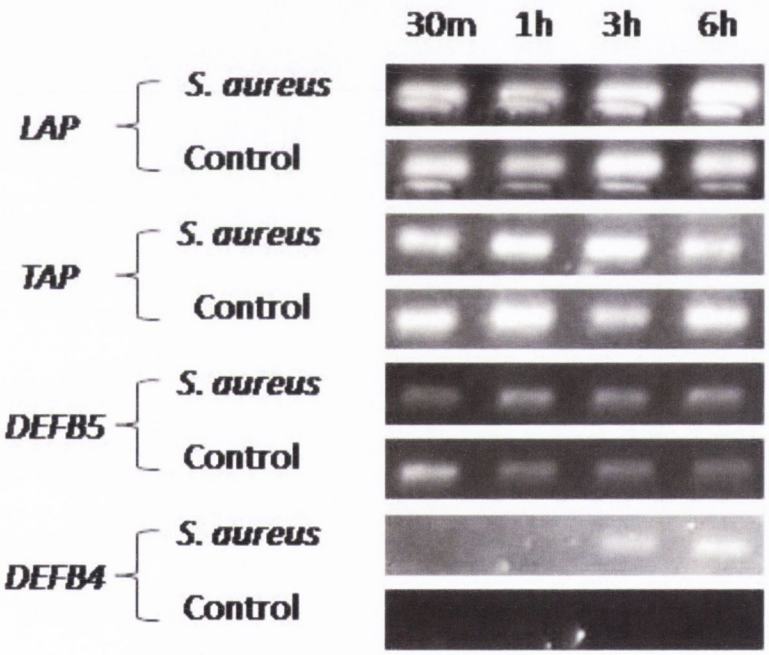


Figure 5.9 Expression profile of β-defensins in primary mammary epithelial cell
qPCR (40 cycles) was used to profile the expression of *LAP*, *TAP*, *DEFB5* and *DEFB4* in cells that were challenged with *S. aureus* 1027 for 30 m, 1 h, 3 h and 6 h and unchallenged controls. A representative result is shown (n=3). No amplicons were observed in the negative controls (no template controls) (data not shown).

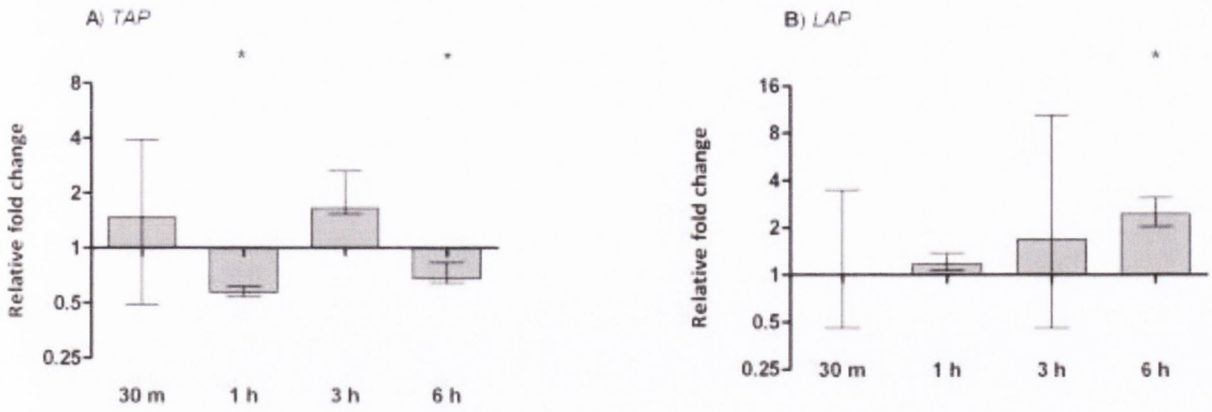


Figure 5.10 Antimicrobial peptide (AMP) gene expression in primary bovine mammary epithelial cells (PbMECs).

qPCR analysis of challenged related changes in the expression of A) *TAP* and B) *LAP* in PbMECs at 30 minutes, 1 h, 3 h and 6 h post stimulation with heat-inactivated *S. aureus* 1027. Median (bars denote the upper and lower range) fold changes are shown for pathogen stimulated cells (n=3) relative to control cells (n=3). Two technical replicates were performed for each biological sample. Statistical analysis was performed using unpaired t-tests (Significance is denoted as: * = $P < 0.05$).

5.3 Discussion

Primary bovine mammary epithelial cells (PbMECs) are well established model analysing the immune activity in the udder (Gunther *et al.*, 2011, 2010; Yang *et al.*, 2008; Strandberg *et al.*, 2005). Mammary epithelial cells (MEC) line the alveoli, ducts, gland cistern and teat canal of the mammary gland (Tanhuanpaa, 1995) and are highly relevant sentinel as well as effector cells of udder immunity (Juliane *et al.*, 2009; Lahouassa *et al.*, 2007; Petzl *et al.*, 2008; Strandberg *et al.*, 2005). They are well suited to exert these immunological functions due to their exposed position as epithelial cells and their abundance in the healthy mammary gland, and as a result are most likely the first cells to engage the pathogen.

Subsequent to stimulation with *S. aureus*, PbMECs were analysed for the expression of genes involved in pathogen recognition, mediation of the immune response as well as for molecules involved in the effector arm of the innate immune response. While attempting to keep the stimuli as similar as possible to a natural physiological infection, we challenged the PbMECs with heat inactivated *S. aureus* in order to provide standardised and reproducible conditions. Although no pathogen derived virulence factors were present, *S. aureus* cell wall PAMPs, lipoteichoic acid (LTA) and muramyl dipeptide, have been shown to increase the expression of a number of genes encoding cytokines, chemokines and antimicrobial peptides in PbMECs as a result of activation cell membrane TLRs (Bougarn *et al.*, 2007; Strandberg *et al.*, 2005). Furthermore, muramyl dipeptide activates the intracellular NOD2 receptor (McDonald *et al.*, 2005) and therefore the response to *S. aureus* particles is similar to the response encountered by the host during intramammary infection with live *S. aureus* (Bannerman *et al.*, 2004; Petzl *et al.*, 2008).

qPCR analysis confirmed the expression of *TLRs 1-10* and *NOD 1-2* by PbMECs in response to stimulation with heat-inactivated *S. aureus*. With the exception of *TLR8* and *TLR10*, transcripts for remaining TLRs and *NOD1* and *NOD2* were detected in unstimulated cells. Expression profiles of PRRs is indicative of a heightened ability to respond to appropriate PAMPs, for example intestinal epithelial cells have low levels of *TLR4* gene expression and as a result are unresponsive to LPS (Abreu *et al.*, 2001). It seems likely then that mammary epithelial cells are very sensitive to bacterial ligands as a result of the high level of baseline expression of *TLR1*, *TLR2*, *TLR4*, *TLR5*, *TLR6*, *TLR9* and *NOD1* and *NOD2* which are involved in the sensing of bacterial ligands both on the cell surface and intracellularly (Takeuchi and Akira, 2010).

TLR1, *TLR2*, *TLR4* and *TLR6* were further profiled using qPCR. With the exception of *TLR6* whose expression was increased by 4-fold at 6 h, expression levels were largely uninfected over the time course in response to *S. aureus* stimulation suggesting that activation of the innate immune response is initiated through baseline expressed TLRs or other receptors. Similar results were observed for *TLR2* and *TLR4* expression in mammary epithelial cells at 1 h and 6 h post stimulation with *S. aureus* and *E. coli* (Griesbeck-Zilch *et al.*, 2008). Activation of TLRs by the appropriate ligands culminates in the expression of multiple proinflammatory genes, such as cytokines (Takeuchi and Akira, 2010).

Cytokines promote inflammation quickly after detection of a pathogen. IL-1 and TNF-α are major proinflammatory cytokines that trigger an inflammatory cascade promoting the clearing of infection. They are locally produced by many cell types including mammary epithelia (Gunther *et al.*, 2011). Our data (Chapter 4) demonstrated that the expression of *IL1B* and *TNF* was not significantly increased across the mammary gland. A similar proinflammatory cytokine response has been observed *in vivo* with *S. aureus* strains that caused subclinical infections (Bannerman *et al.*, 2004; Lee *et al.*, 2006; Petzl *et al.*, 2008). This is in contrast to results observed following intramammary infection with *E. coli* where expression of *TNF* increased in 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 within a short period (Blum *et al.*, 2000; Hoeben *et al.*, 2000).

In order to determine if pathogen-specific immune response was evident, PbMECs were also stimulated with a stain of *Escherichia coli* (*E. coli* 1303) that was previously shown to cause acute mastitis *in vivo* (Petzl *et al.*, 2008). A strong induction of the proinflammatory cytokine genes *TNF* and *IL1B* was observed in cells stimulated with heat-inactivated *E coli 1303* compared to *S aureus* stimulated cells (Fig. 5.6A and B). Similar results were observed in primary bovine mammary epithelial cells using the same strain as we used in our study (*S. aureus* 1027 and *E. coli* 1303) (Gunther *et al.* 2011). The researchers showed that *S. aureus* failed to induce *IL1A* and *TNF* expression while significantly inducing *IL6* expression, whereas all three genes were substantially induced in response to *E. coli,* highlighting the importance of TNF-α, IL-α and IL1-β in the pathogenesis of subclinical mastitis.

Expression of molecules involved in the resolution of infection was also profiled. Although we found *IL10* to be significantly increased in our *in vivo* study (Fig 4.6), we could not detect *IL10* mRNA in our PbMECs cultures. Hence, controls dampening inflammation

may be missing in PbMEC models. The absence of *IL10* expression also highlights the complexity of the udder which is composed of multiple cell types (Nickerson and Pankey, 1983; Nickerson *et al.*, 1984), all of which are likely to contribute differentially to mastitis infection.

Transcripts of all 5 APPs profiled were detected in cultured mammary epithelial cells. SAA was significantly increased 3 h post challenge with *S. aureus* in mammary epithelial cells with levels of induction > 30 fold at 6h post challenge. However *S. aureus* had limited impact on the expression of LBP in epithelial cells. Marked induction of *CP* was observed in mammary epithelial cells at 3 h post challenge with *S.aureus.* Decreased expression of AGP was also detected in mammary epithelial cells at 1h and 6h post challenge.

Expression levels of the beta-defensins profiled remained relatively unchanged over the course of the challenge with the exception of *LAP* whose expression was significantly induced (2.5-fold) at 6 h post stimulation with *S. aureus.* Similar results were observed in primary mammary epithelial cells in response to *E.coli* stimulation where the extent and the time course of induced expression of *TNF* was dissimilar to induced *LAP* and *SAA3* expression (Seyfert, 2009). Induction of β-defensin often involves signaling mediated by proinflammatory cytokines. For example, in keratinocytes, human β-defensin 2 (hBD2), hBD3 and hBD4 are induced by stimulation with TNF-α and IL1-β (Harder *et al.*, 2001; Harder *et al.*, 2004). Similarly, IL-1β markedly induces hBD-2 expression by tracheal epithelial cells (Tsutsumi-Ishii and Nagaoka, 2003). Conceivably, transcription factors other than those activated directly by heat-inactivated *S. aureus* are eventually turned on by previously synthesised cytokines in mammary epithelial cells.

Similar to our findings, previous research with *S. aureus* 1027 and *E. coli* 1303 showed that both pathogens induce *IL8* and *TNFA* gene expression in primary mammary epithelial cells (Yang *et al.*, 2008). The magnitude of expression was much higher in response to *E. coli*. This muted proinflammatory response to *S. aureus* was paralleled by a complete lack of NF-kB activation by *S. aureus* in contrast to *E. coli*. To rule out differences in the ability to engage both TLRs, they showed that heat inactivated bacteria from both pathogens activate bovine TLR2 and TLR4 receptors equally to induce NF-kB activation. They speculated that *S. aureus* 1027, which upon intra-mammary infection induces subclinical mastitis, (Petzl *et al.*, 2008), impairs NF-kB activation in mammary epithelial cells by an unknown mechanism thus resulting in a weakened immune response in the udder. They concluded that the muted immune response in mammary epithelial cells was the result of the activation of a cell type dependent blocking of TLR-dependent NF-kB activation, mediated by

the heat-inactivated *S. aureus* pathogen or even just the lipoteichoic acid (LTA) isolated from this strain. Recently, the same group profiled the kinetics and extents of global changes in the transcriptome of primary bovine mammary epithelial cells (MEC) after challenging them with heat-inactivated preparations of *E. coli* 1303 or *S. aureus* 1027 pathogens (Gunther *et al.*, 2010). They demonstrated that the *S. aureus* driven immune response was IL-6 mediated, which is in agreement with our observation in Chapter 3 and Chapter 4 where we showed that *S. aureus* induced IL6 both locally and systemically. In addition, their data shows that heat-inactivated S. aureus 1027 induces an early inhibitor of NF-κB activation (TNFAIP3) as well as NFKBIZ. These factors are crucial for induced IL-6 expression but also block TNF-α synthesis (Matsuo *et al.*, 2007 Yamamoto, *et al.* 2004).

5.4 Conclusion

Collectively, our data demonstrate that mammary epithelial cells are fully equipped to counteract *S. aureus* infection through the expression of PRRs and through the induction of AMPs and APPs. However, our results also comply with previous findings that a strong induction of the proinflammatory cytokine genes *TNF* and *IL1B* was observed in cells stimulated with heat-inactivated *E coli 1303* in comparison to *S aureus* stimulated cells. These results further support the hypothesis that TNF-α and IL-1β play a key role in dictating the pathogenesis of infection caused by these strains of bacteria.

Chapter 6: Characterisation of the Cathelicidin Gene Repertoire and Expression Profile in Cattle

6.1 Introduction

A rapid and effective response to challenge by pathogens is essential for the survival of all living organisms. Among the several different defence mechanisms which have evolved to meet this requirement is the production of a large variety of antimicrobial peptides (AMPs). These peptides play a major role in the immune defence of invertebrates, while in vertebrates they act as a first line of defence against invasion by pathogens and in the control of the natural flora.

Cathelicidin genes code for a family of AMPs that have been found in invertebrate [hagfish (Uzzell *et al.*, 2003)] and vertebrate species including amphibians (Hao *et al.*, 2011), fish (Chang *et al.*, 2005), birds (Lynn *et al.*, 2004), snakes (Zhao *et al.*, 2008) and mammals (Zanetti, 2005). Initially categorised by their ability to act as endogenous antibiotics as a result of microbial cell membrane disruption (Brogden, 2005), it is now becoming clear that their biological activity is multifunctional and extends beyond microbial killing (Lai and Gallo, 2009; Wuerth and Hancock, 2011). For example, the human cathelicidin peptide LL-37 (37 amino acids in length beginning with two leucines), is chemotactic for neutrophils, monocytes and T cells (De *et al.*, 2000).

Cathelicidins are so named based on a highly conserved N-terminal-coding region of the precursor protein known as the cathelin domain, followed by a highly variable antimicrobial domain (Fig. 6.1)(Zanetti *et al.*, 1995). The cathelin domain contains two disulfide bonds between cysteine residues C85-C96 and C107-C124 (Fig. 6.1) and was given its name based on it sequence identity to a protein called cathelin, a member of the cystatin superfamily of cysteine proteinase inhibitors (cathe-l-in is an acronym for cathepsin L inhibitor) (Ritonja *et al.*, 1989).

Although found ubiquitously in mammals, the number of cathelicidin genes in any single species varies greatly. For instance, the majority of the Euarchontoglires, which includes humans and mice, have a single cathelicidin gene; in contrast, in Laurasiatheria, which includes cattle and sheep, multiple cathelicidin genes have been reported (Tomasinsig and Zanetti, 2005; Zaiou and Gallo, 2002; Zanetti, 2004, 2005).

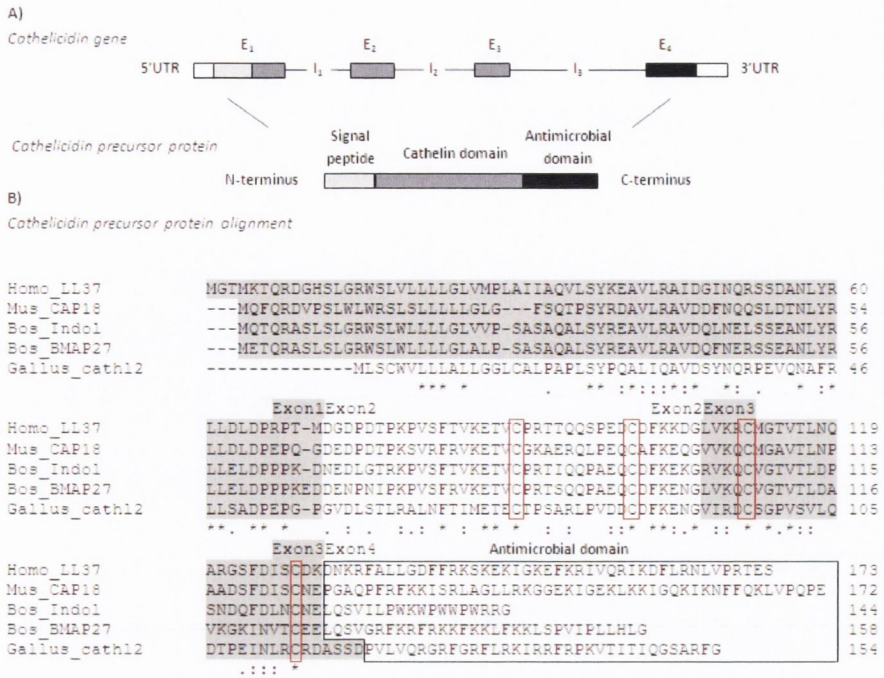


Figure 6.1 Cathelicidin structure

A) Cathelicidin genes are approximately 2 kilobase in size with a conserved four exon – three intron arrangement. **B)** Alignment of cathelicidin precursor proteins from human (*Homo sapian*, LL37), mouse (*Mus musculus*, CAP18), cow (*Bos taurus*, indolicidin and BMAP27) and chicken (*Gallus gallus*, cathl2). Conserved residues are indicated by the asterisk. Exons 1-3 are highly conserved between species with a considerable variable sequence within the exon 4 region found both within and between species. These proteins contain two disulfide bonds between cysteine residues C85-C96 and C107-C124

The bovine lineage was one of the first in which cathelicidins were discovered during studies of the antimicrobial activity of bovine neutrophil lysates (Ritonja *et al.*, 1989). Subsequent studies resulted in the identification of seven cathelicidins (see Appendix) using molecular cloning strategies (Gennaro *et al.*, 1989; Storici *et al.*, 1992; Zanetti *et al.*, 1993). When tested for *in vitro* antimicrobial activity, cathelicidins rapidly killed a wide range of microorganisms (reviewed in (Zanetti, 2004, 2005). However, the contribution of these molecules in protecting the host from pathogens *in vivo* is not fully understood. The importance of these peptides *in vivo* can be inferred from their specific localisation at sites which are exposed to microbial invasion as well as in the professional immune cells. To date, the expression of these peptides has been detected in phagocytic cells (Zanetti *et al.*, 1995) with limited information on cathelicidin expression in non-myeloid cells (Ibeagha-Awemu *et al.*, 2010; Tomasinsig *et al.*, 2010).

LL-37 has been detected in neutrophils, mast cells, monocytes and macrophages (Agerberth *et al.*, 1995; Cowland *et al.*, 1995; Di Nardo *et al.*, 2003; Koon *et al.*, 2011) Epithelial cells in skin, gut, lung, epididymis and mammary gland are other important sources of LL-37 (Bals *et al.*, 1998b; Hase *et al.*, 2002; Malm *et al.*, 2000; Murakami *et al.*, 2005b; Murakami *et al.*, 2004), which is therefore thought to have an important role in host defence at sites that are in contact with the external environment (Zasloff, 2006).

The aim of this study was to re-examine, using new bioinformatic technologies, the number of cathelicidin genes in the bovine genome and to identify sites of expression. Firstly, a bioinformatics strategy was adopted which uses the homology-based search methods of the BLAST family of programs (Altschul *et al.*, 1997) and the more sensitive Hidden Markov Models approach (Eddy, 1998) (reviewed in Chapter 2: Materials and Methods, section 2.4.1). Use of HMM profiling searches allows us to avail of position-specific information unique to the cathelicidin gene family and improve the sensitivity of gene search efforts compared to the use of BLAST alone which assumes all positions in a protein to be equally important. Secondly, we used qPCR to profile the expression of these cathelicidins, in a wide range of bovine tissues, including the respiratory tract (lung), digestive tract (rumen, small intestine and large intestine), reproductive system (uterus, mammary gland, mammary and testis) and representative organs and cells of primary immune function (spleen, liver, lymph node and PBMCs). In addition, we profiled cathelicidin expression in mammary epithelial cells stimulated with *Staphylococcus aureus* a causative agent of mastitis. Finally, we measured cathelicidin expression in milk leukocytes from naturally occurring mastitis infections.

6.2 Results

6.2.1 Characterisation of the bovine cathelicidin locus

The BosTau7 assembly of the bovine genome provided us with the opportunity to reconstruct the full cathelicidin region for this species. A HMM profile was constructed based on the alignment of protein sequences corresponding to the seven known bovine cathelicidins (Chapter 2; Materials and Methods, Fig 2.3). This was then used to search the sequenced bovine genome which had been translated in all six reading frames. This search led to the cathelin containing motifs additional to the seven already annotated. Chromosomal location and strand orientation of the identified cathelicidins was determined using the BLAST like Alignment Tool (BLAT) at the University of California—Santa Cruz genome browser (Fig. 6.2). The predicted novel cathelicidin genes were named *CATHLnew*, *CATHLpsuedo* and *CATHLnovum* (Fig. 6.2).

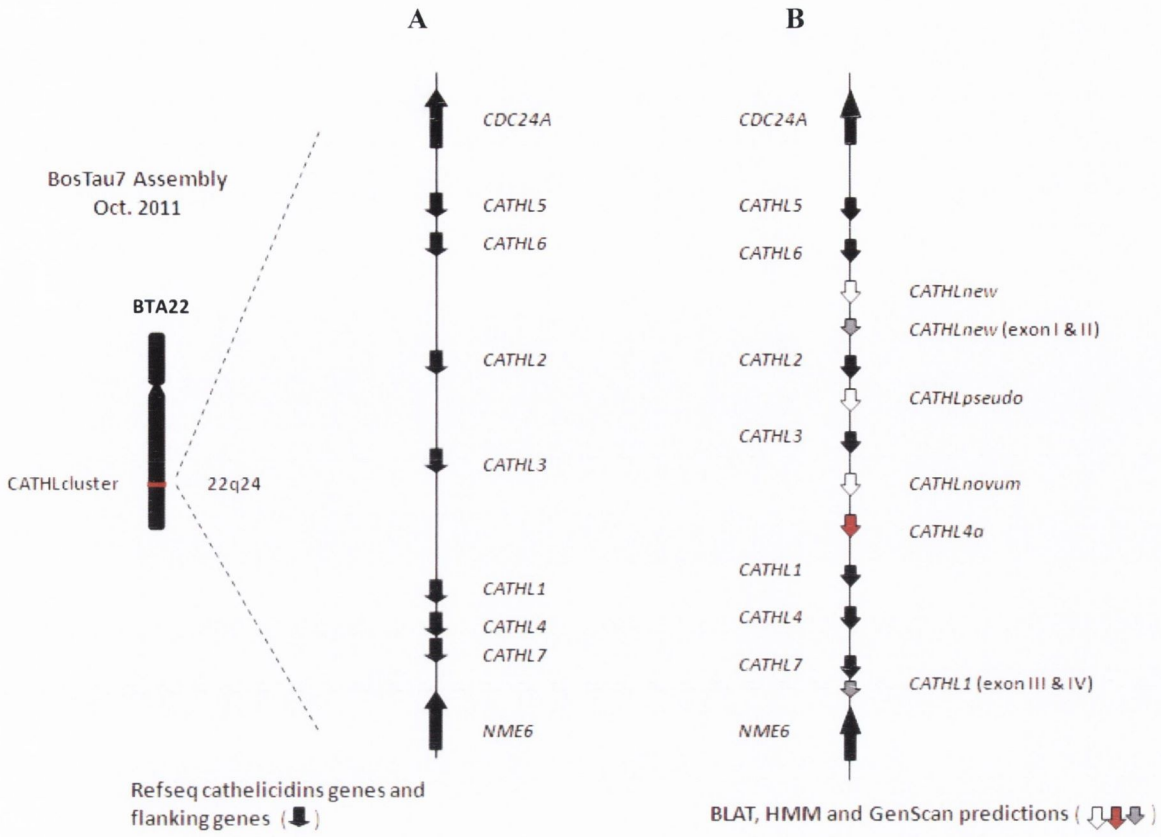
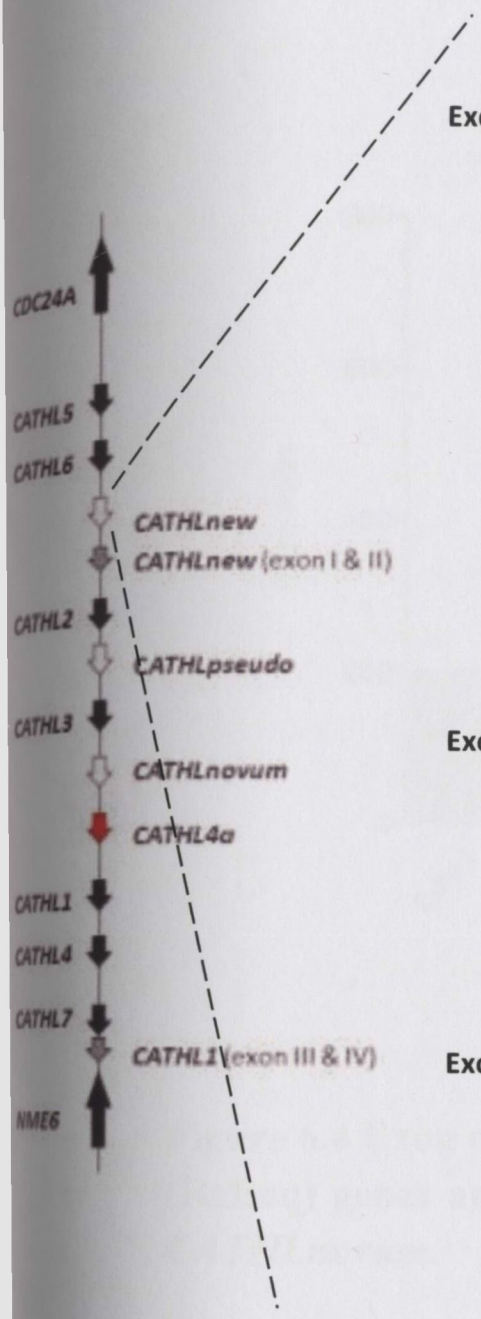


Figure 6.2 Genomic organisation of the bovine cathelicidin gene cluster on bovine chromosome 22 (BTA22)

A) Genomic organisation of the seven known bovine cathelicidins prior to our investigation. B) Genomic organisation following our homology-based search methods using the BLAST family of programs (Altschul *et al.*, 1997) and the more sensitive Hidden Markov Models (Eddy, 1998). Genes are shown as arrows, and indicate direction of transcription. Black arrows indicate refseq cathelicidins. White arrows indicate novel cathelicidin and grey arrows indicate partial genes. The red arrow indicates a duplicate copy of *CATHL4*

Genomic DNA corresponding to putative cathelicidins was retrieved using BLAT then used for prediction of intron/exon boundaries with GenScan software (Fig. 6.3). Translations of the predicted genes revealed stop codons in the first exon of *CATHLpsuedo* and *CATHLnovum* (see Appendix). In contrast, *CATHLnew* was intact and displayed all the characteristics of a functional cathelicidin including 2 kilobase size, a conserved four exon – three intron arrangement (Fig.6.3 and Fig. 6.4). Alternative 5' splice junctions were predicted, changing the 3' boundary of exon 2 and exon 3 when compared to cathelicidins 1-7 (Fig. 6.5A and B).




```
Exon I      52633015  aggcttgaggtaagaccagcccacaccctgggaggagggcagggatggggcgggtcagga
            52632955  agactcctggtagagcttttgcatcagggctcagactgggcataaaagaagggtcccttg
            52632895  ggctgggaggaggcagactcgggaccATGGAGACCCAGAGGGCCAGCCTCTCCCTGGGGC
                                                  M··E··T··Q··R··A··S··L··S··L··G··
            52632835  GCTGGTCGCTGTGGCTGCTGCTGCTGGGACTAGCGCTGCCCTCGGCCAGCGCCCAGGCCC
                      R··W··S··L··W··L··L··L··L··G··L··A··L··P··S··A··S··A··Q··A··
            52632775  TCAGCTACAGGGAGGCCGTGCTTCGTGCTGTGGATCAGCTCAATGAGAGGTCCTCAGAAG
                      L··S··Y··R··E··A··V··L··R··A··V··D··Q··L··N··E··R··S··S··E··
            52632715  CTAATCTCTACCGCCTCCTGGAGCCATCTCCTCCCAGCCTTGGCCACACTgtcgcccttt
                      A··N··L··Y··R··L··L··E··P··S··P··P··S··L··G··H··T··········
            52632655  cgctcaggctggtcctcctgtcaggaaggcacttttccctctaggtgggttcccacctct
            52632595  tccaggaaaccttcccagacctgggtcatctcccagcaccaggcttcctgtcttagcatc
            52632535  tctgctgtgggaacaggcgccctgcacacctggctcaggttccctggacttctgggagct
            52632475  ccagggatggaggggtcacaggctctgtgaggtgacttccctcctaatatcctctctgca
            52632415  cctcggtgtctctctgccaggaggagctctgtcagcctggaggctccagtgacaagggct
            52632355  ctccctgcaggcggccctgacctccctcagcccctctgaggggaggcgctgccatcagcg
            52632295  ctgctgtgagggccactcctgctctctgtgtgcccgtgaggccaggcacgggctctgtcc
            52632235  cctcccctgtgctgccagcaccaagcccagggctggacacacaggggggctggagaggctg
            52632175  cggtccgggttgggggcagggagacagatcagagaaggaaacatgagcctgagcccagtc
Exon II     52632115  cccccactttgatccttgaccagGTGGAGGACCCGGGAGCTCGAAAGGCTGTGAGCTTCA
                                                  V··E··D··P··G··A··R··K··A··V··S··F··
            52632055  GGGTGAAGGAGACTGTGTGCCCCAGGCCGAGCCTGCAGCCCCCAGAGCAGTGTGACTTCA
                      R··V··K··E··T··V··C··P··R··P··S··L··Q··P··P··E··Q··C··D··F··
            52631995  AGGAGAATGGGCTGACCTTGGGGGCTGAGACTGAGGGCTGGGATCAATGCTTCTCAGCGC
                      K··E··N··G··L··T··L··G··A··E··T··E··G··W··D··Q··C··F··S··A··
            52631935  GAGCTGAACAGGGAACTTCAGGGAATgtttccagcccctggcaggtgaggtaagctgagc
                      R··A··E··Q··G··T··S··G··N···································
Exon III    52631875  ctgggagattatggcccggggtttccagtttgaccttgagctccccttccagCTGGTGAA
                                                                        L··V··K
            52631815  ACAGTGTTTGGGGACAGTCAGCCTGGACCGGTCCGATGACCAGTTTGACATAAACTGTAA
                      ··Q··C··L··G··T··V··S··L··D··R··S··D··D··Q··F··D··I··N··C··N
            52631755  TGAGGCGAGTGGCCCCTTCTGTgttgggcatatgctaacagggtgggttgagaaacatcc
                      ··E··A··S··G··P··F··C···································
            52631695  tttggaccaatgacctgctgctccatctagggtagagaaaaggccctcctatctgggccc
            52631635  accctccccaatccctaggtctccagccctggctctgcatcctttagagaagtggctgtc
            52631575  taacggggtccccacccaggaactcacaggaaggcagattctcagccccaccgagactcc
            52631515  tgaatcagactttggggtgggccccggcatttgcgctttcacaaggcctccaggggttc
            52631455  tgacagtgctgaaattgtggcagcctgacctgggcggtggtctcgaggccatgctccagt
            52631395  ctacctttgcccggatgggcttgtgacccttggaagccccttgtcatctctgggatcagt
            52631335  ttccccatatgttgtgggtttaggtattcaatcacatgctccaaagatcactgccagagg
            52631275  atgatctggggccaaagttcctttggtggctcagtttgggggttgttcatgtggggagag
            52631215  agtggtcttctcttgacccttgcccagtctcacaagtaatctcttccattgtggttcaca
Exon IV     52631155  gcttcagagtgtcagggctaactgacctcagcctcccaaacctcggcctcctcacagGCC
                                                                        A·
            52631095  AAGGCCAAGGCCACGGCCATGGTTCCCACCAAGATTCCCTGGAAAACGGTGAAGGACTGG
                      ·K··A··K··A··T··A··M··V··P··T··K··I··P··W··K··T··V··K··D··W·
            52631035  CTATCATACCTATTAATGGCTTTTGGTGAATTCCGAGCCtgagggaagcattttaaagat
                      ·L··S··Y··L··L··M··A··F··G··E··F··R··A··x···················
            52630975  atgatttgttctggatcagaattctggacggtgaaaaataaatcttgtgaaaacaacttc
            52630915  ctccaggcttcaatttctattatttccctttttccagcaatggcaccccactccagtact
```

Figure 6.3 Predicted structure of novel cathelicidin gene.

The coding sequence is in uppercase letters, noncoding sequence are in lowercase letters. The line numbers represent the base pair position on BTA22 according to the 2011 assembly (BosTau7). The deduced amino acid sequence of the open reading frame is indicated in single letter code and the stop codon is indicated by an x. The TATA-box signal is underlined and the polyadenylation signal is double underlined.

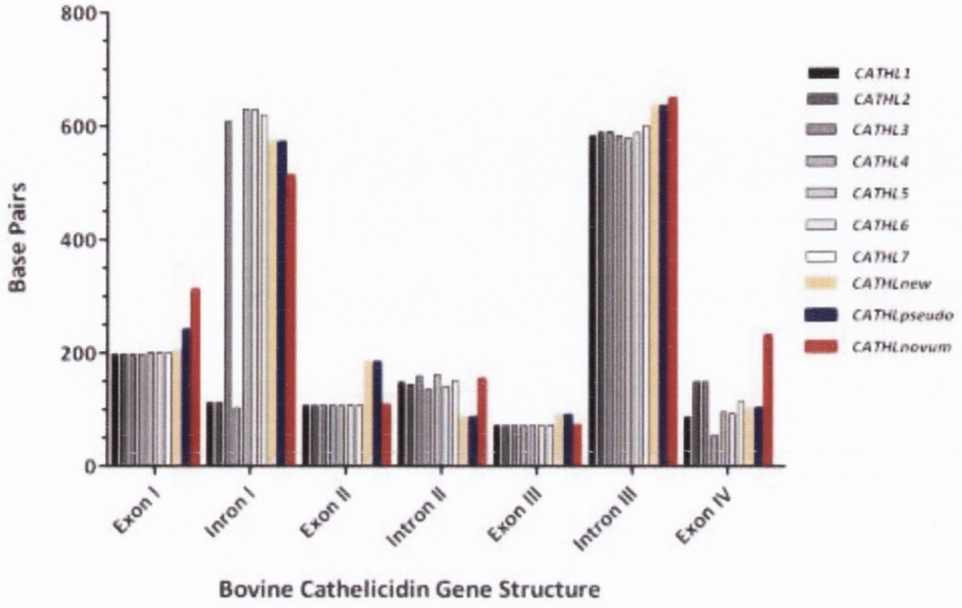


Figure 6.4 Exon and intron length (base pairs) of cathelicidin NCBI Reference Sequence (Refseq) genes and predicted novel cathelicidin genes, *CATHLnew*, *CATHLpseudo* and *CATHLnovum*.

A)

```
CATHL7_Exn2         GTGGAGCACCCGGGAGCTCGAAAGCCTGTGAGCTTCACAGTGAAGGAGACCGTGTGCCCC 60
CATHLnew_Exn2       GTGGAGGACCCGGGAGCTCGAAAGGCTGTGAGCTTCAGGGTGAAGGAGACTGTGTGCCCC 60
CATHL3_Exn2         GTAGAGGACCGGGGAGCTCGAAAGCCTACAAGCTTCACGGTGAAGGAGACTGTGTGCCCC 60
CATHL4_Exn2         AATGAAGATCTGGGCACTCGAAAGCCTGTGAGCTTCACGGTGAAGGAGACTGTGTGCCCC 60
CATHL5_Exn2         GATGAGAACCCAAACATCCCGAAGCCTGTGAGCTTCAGGGTGAAGGAGACCGTGTGCCCC 60
CATHL6_Exn2         GATGAGAACCCAAACATCCCGAAGCCTGTGAGCTTCAGGGTGAAGGAGACCGTGTGCCCC 60
CATHL1_Exn2         GATGAAGACCCAGACAGCCCGAAGCGGGTGAGCTTCAGGGTGAAGGAGACCGTGTGCTCC 60
CATHL2_Exn2         GACTTGGACCCAGGCACCAGAAAGCCTGTGAGCTTCAGGGTGAAGGAGACCGATTGCCCC 60
                    .:  :. * * .....  . .***   . .******* .********** *: *** **

CATHL7_Exn2         AGGACAACCCCGCAGCCCCCAGAGCAGTGTGACTTCAAGGAGAATGGGGT---------- 110
CATHLnew_Exn2       AGGCCGAGCCTGCAGCCCCCAGAGCAGTGTGACTTCAAGGAGAATGGGCTGACCTTGGGG 120
CATHL3_Exn2         AGGACGAGCCCGCAGCCCCCGGAGCAGTGTGACTTCAAGGAGAATGGGGT---------- 110
CATHL4_Exn2         AGGACGATTCAGCAGCCCGCGGAGCAGTGTGACTTCAAGGAGAAAGGGGT---------- 110
CATHL5_Exn2         AGGACGAGCCAGCAGTCCCCGGAGCAGTGTGACTTCAAGGAGAATGGGGT---------- 110
CATHL6_Exn2         AGGACGAGCCAGCAGCCCGCAGAGCAGTGTGACTTCAAGGAAAATGGGGT---------- 110
CATHL1_Exn2         AGGACCACCCAGCAGCCCCCCGAGCAGTGTGACTTCAAGGAGAATGGGGT---------- 110
CATHL2_Exn2         AGGACAAGCCAGCAGCCCCTGGAGCAGTGTGACTTCAAGGAGAATGGGGT---------- 110
                    ***.* *  * **** **   ********************.**:*** *

CATHL7_Exn2         ------------------------------------------------------------
CATHLnew_Exn2       GCTGAGACTGAGGGCTGGGATCAATGCTTCTCAGCGCGAGCTGAACAGGGAACTTCAGGG 180
CATHL3_Exn2         ------------------------------------------------------------
CATHL4_Exn2         ------------------------------------------------------------
CATHL5_Exn2         ------------------------------------------------------------
CATHL6_Exn2         ------------------------------------------------------------
CATHL1_Exn2         ------------------------------------------------------------
CATHL2_Exn2         ------------------------------------------------------------

CATHL7_Exn2         -----
CATHLnew_Exn2       AATGT 185
CATHL3_Exn2         -----
CATHL4_Exn2         -----
CATHL5_Exn2         -----
CATHL6_Exn2         -----
CATHL1_Exn2         -----
CATHL2_Exn2         -----
```

B)

```
CATHL1_Exn3         CTGCTGAAACGCTGTGAGGGGACAGTCACCCTGGACCAGGTCAGGGGTAACTTCGACATC 60
CATHL5_Exn3         CTGCTGAAAGAGTGTGTGGGGACAGTCACCCTGGACCAGGTCGGGAGTAACTTCGACATC 60
CATHL7_Exn3         CTGGTGAAACAGTGTGTGGGGACAGTCACCCGGTACTGGATCAGGGGTGATTTCGACATC 60
CATHL3_Exn3         CTGGTGAAACAGTGTGTGGGGACAATCACCCTGGACCAGTCCGATGACCTATTTGACTTA 60
CATHLnew_Exn3       CTGGTGAAACAGTGTTTGGGGACAGTCAGCCTGGACCGGTCCGATGACCAGTTTGACATA 60
CATHL2_Exn3         CTGGTGAAACAGTGTGTGGGGACAGTCACCCTGGACCCATCAAATGACCAATTTGACATA 60
CATHL4_Exn3         CGGGTGAAACAGTGTGTGGGGACAGTCACCCTGGACCCATCCAATGACCAGTTTGACCTA 60
CATHL6_Exn3         CTGGTGAAGCAATGTGTAGGGACAGTCACTCTGGATGCGGTGAAAGGCAAAATTAACGTC 60
                    * * ****. . *** :.******.***  * * *   .   .. ..  : :* .** *.

CATHL1_Exn3         ACCTGTAATAATGT------------------ 74
CATHL5_Exn3         ACCTGTGCTGTGGT------------------ 74
CATHL7_Exn3         ACTTGTAATAATGT------------------ 74
CATHL3_Exn3         AACTGTAATGAGGT------------------ 74
CATHLnew_Exn3       AACTGTAATGAGGCGAGTGGCCCCTTCTGTGT| 92
CATHL2_Exn3         AACTGTAATGAGGT------------------ 74
CATHL4_Exn3         AACTGTAATGAGGT------------------ 74
CATHL6_Exn3         ACCTGCGAGGAGGT------------------ 74
                    *. ** .. .: *
```

Figure 6.5 Alignment of cathelicidins 1-7 and *CATHLnew* amino acid sequences.
A) Exon 2 alignment with donor sites and alternative donor sites highlighted in grey. **B)** Exon 3 alignment with donor sites and alternative donor sites highlighted in grey. These sequences were aligned using CLUSTAL 2.1 multiple sequence alignment software. Conserved residues are indicated by asterisk.

Alignment of *CATHLpseudo*, *CATHLnovum* and *CATHLnew* sequences with the seven known bovine cathelicidins revealed that these novel genes had the highest nucleotide identity to *CATHL3*. To test the authenticity of *CATHLpsuedo, CATHLnovum* and *CATHLnew*, bovine EST databases were searched. In addition, primers were designed to co-amplify all three predicted transcripts (Fig. 6.6A). EST analysis revealed that neither exon 2 nor exon 3 were in the bovine EST database. In addition, PCR could not detect the novel transcripts in RNA from spleen or milk somatic cells isolated from a naturally occurring mastitis infection (Fig. 6.6B).

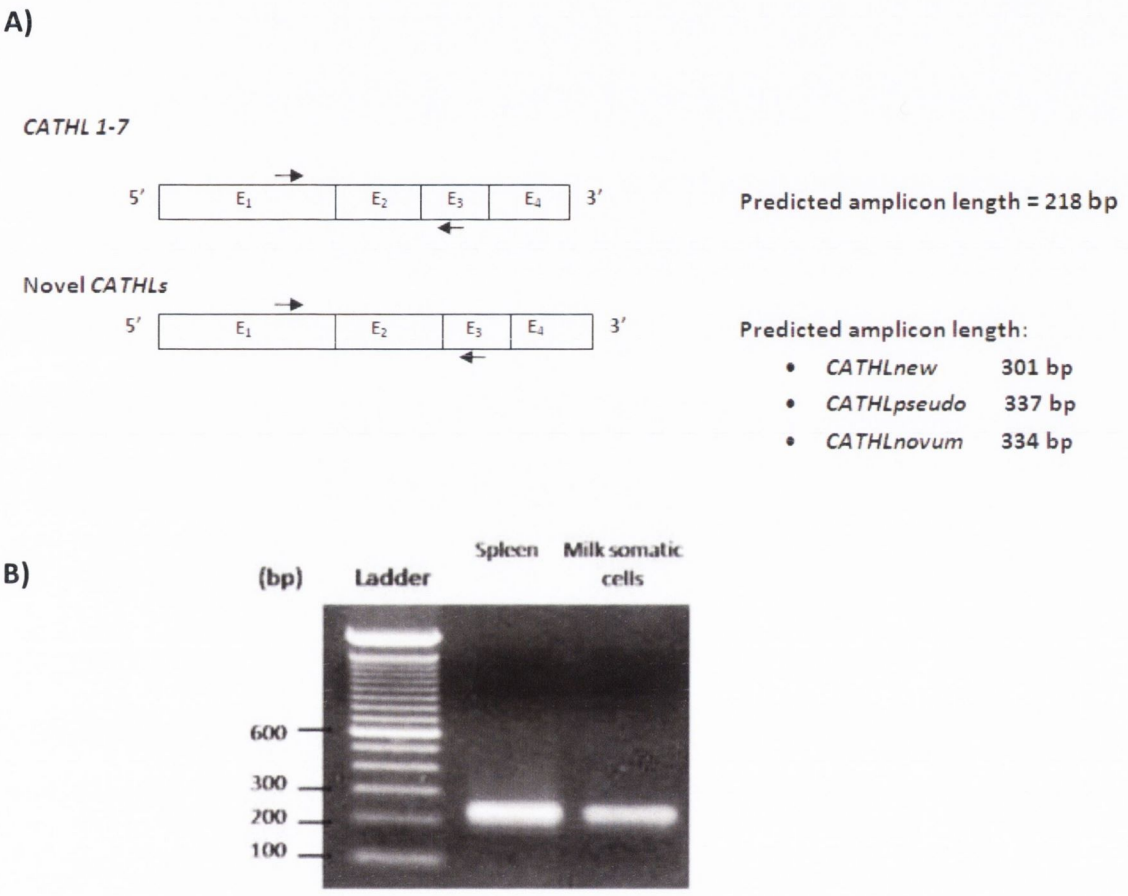


Figure 6.6 Untranscribed pseudogene.

A) A PCR assay was performed using primers spanning exons 1-3 of cathelicidins 1-7 (*CATHLs* 1-7) and *CATHLnew*, *CATHLpseudo* and *CATHLnovum*. B) PCR amplification of cathelicidins in spleen and milk somatic cell cDNA from a naturally occurring mastitis infection in a Holstein-Friesian. Band size corresponds to predicted amplicon length for *CATHL1-7*.

6.2.2 Tissue expression pattern of cathelicidins 1-7

We next examined the tissue expression patterns of cathelicidins 1-7 in bovine tissues as a first step toward understanding their *in vivo* biological functions. Distribution of cathelicidin mRNA was assayed by quantitative real-time PCR which provides sensitive detection and quantitative enumeration of transcript. Multiple sequence alignments were used to select primers for each member of the cathelicidin family. qPCR primers were designed to traverse introns, where possible, to ensure that only cDNA made from mRNA extracted was amplified and not genomic DNA. Single PCR bands corresponding to our *in silico* amplicon lengths were observed on agarose gels (Fig. 6.7). PCR products were then sequenced to validate primer specificity.

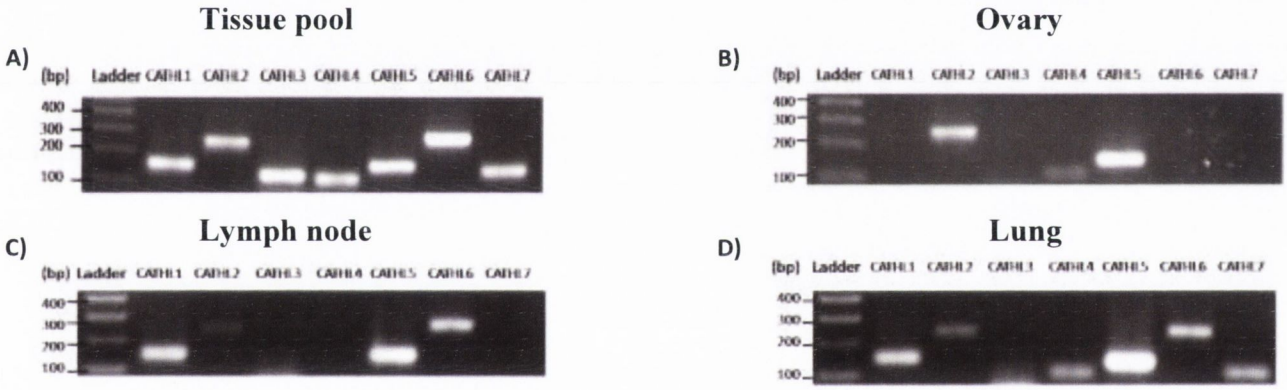


Figure 6.7 Primer validations

A) A tissue pool representing RNA pooled from a range of tissues including the liver, mammary gland, lymph node, pituitary gland, hypothalamus, brain, heart, spleen, foetal tissue, ovary and conA-stimulated leucocytes, was used as a positive control for cathelicidin expression. **B)** Expression profile in Ovary. **C)** Expression profile in lymph node. **D)** Expression profile in Lung

To facilitate comparison between tissues, all samples were normalized to the expression of the house keeping gene *GAPDH*. Most tissues tested expressed at least one cathelicidin (Fig. 6.8A-Ns) Tissues involved in immune function, including spleen, lymph node and milk leukocytes, displayed the most diverse repertoire of cathelicidin expression, as did those constantly exposed to microbial products such as the lungs and liver (Fig. 6.8I, J, N, L and H respectively). Similarly, testis expressed multiple cathelicidins (*CATHL1*, *CATHL2*, *CATHL4*, *CATHL5* and *CATHL7*; Fig. 6.8C). Tissue from fallopian tube, uterus and PBMCs expressed a single cathelicidin gene, *CATHL5*, *CATHL5* and *CATHL6*, respectively (Fig 6.8B, D and K, respectively). *CATHL5* expression was detected in all tissues profiled except the small intestine (Fig. 6.8F). In contrast, *CATHL6* was detected in two out of the 11 tissues profiled (Fig. 6.8J and L). Restricted expression of *CATHL6* compared to others was also evident in the bovine EST database.

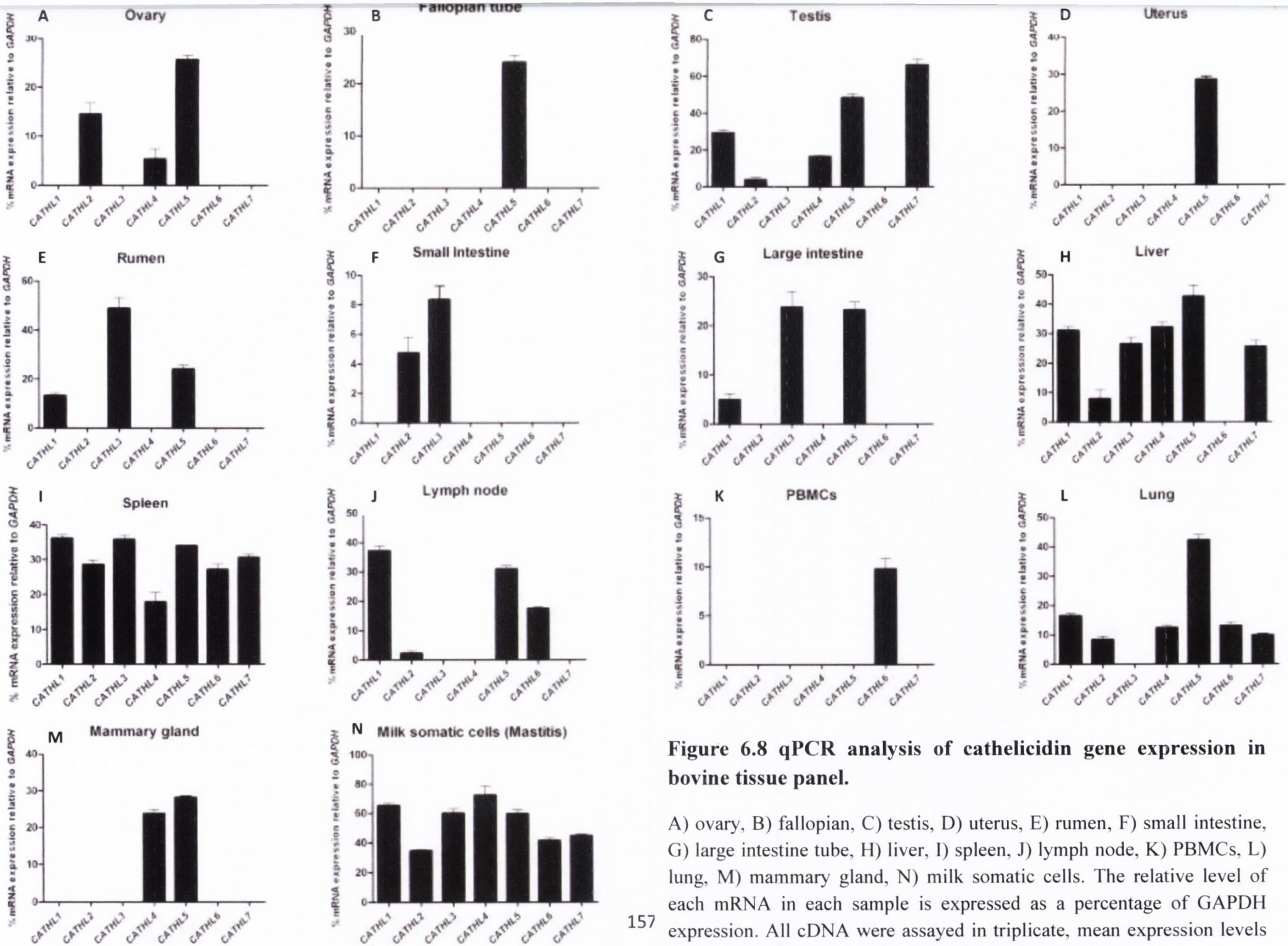


Figure 6.8 qPCR analysis of cathelicidin gene expression in bovine tissue panel.

A) ovary, B) fallopian, C) testis, D) uterus, E) rumen, F) small intestine, G) large intestine tube, H) liver, I) spleen, J) lymph node, K) PBMCs, L) lung, M) mammary gland, N) milk somatic cells. The relative level of each mRNA in each sample is expressed as a percentage of GAPDH expression. All cDNA were assayed in triplicate, mean expression levels are shown (error bars denote standard deviation of the mean).

6.2.3 Rapid induction of cathelicidin gene expression in mammary epithelial cells

Because the mammary gland is constructed of different cell types, we further analysed the expression of *CATHL4* and *CATHL5* in mammary epithelial cells, which are the predominant cell type in the mammary gland. We profiled their expression at early time points post stimulation with heat-inactivated *Staphylococcus aureus*. *CATHL4* and *CATHL5* were detected in both control and *S. aureus* stimulated cells at 30 min, 1 h, 3 h and 6 h (Fig. 6.9A). CA*THL5* expression was not significantly inducted across the time course. *CATHL4* was significantly increased at 30 min post stimulation with a further increase detected at 1 h post stimulation with *S. aureus* (5- and 60-fold respectively, $P < 0.05$; Fig. 6.9B) and maintained up to 6 h post stimulation. These genes were further profiled across the mammary gland in tissue from our animal model of *S. aureus*-induced subclinical mastitis. Expression of *CATHL4* and *CATHL5* remained unchanged.

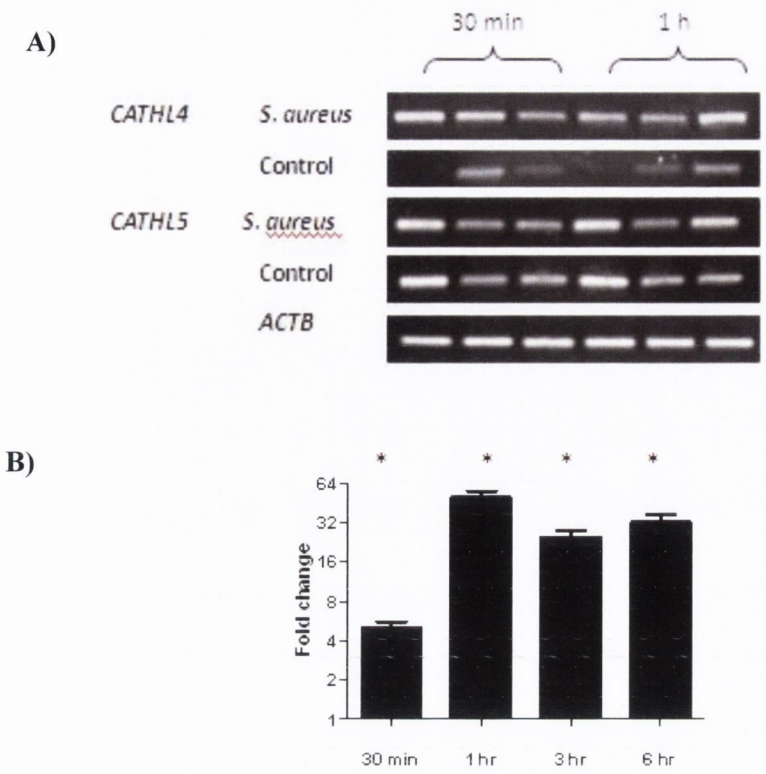


Figure 6.9 Cathelicidin gene expression in mammary epithelial cells.

A). PCR (40 cycles) analysis of *CATHL4* and *CATHL5* expression in primary mammary epithelial cells. A representative result is shown (n=3). No amplicons were observed in the negative controls (no template controls) (data not shown). **B)** qPCR analysis of challenge related changes in gene expression of *CATHL4* at 30 min, 1h, 3h and 6 h post-challenge with heat-killed *S. aureus*. Mean (bars denote the SEM) fold changes are shown for *S. aureus* challenged cells (n=3) relative to control cells (n=3). Statistical significance is denoted as: * = $P < 0.05$.

6.3 Discussion

In this study, we used the recently released BosTau7 assembly of the bovine genome to identify known and novel cathelicidins. This search identified seven Refseq genes cathelicidin1-7 and three novel genes clustered on chromosome 22 (Fig. 6.2).

Cathelicidin genes have been identified in mammals, chickens, fish and hagfish (Chang *et al.*, 2005; Goitsuka *et al.*, 2007; Uzzell *et al.*, 2003) indicating that the origin of this class of immune molecule evolved before the emergence of vertebrates. Humans, have one cathelicidin gene, it is widely expressed in both myeloid and epithelial cells and therefore thought to contribute substantially to host defence at sites that are in contact with the external environment (Lai and Gallo, 2009).

Cathelicidins play a central role in protecting the host from microbial infection. This can be inferred from, for example, the correlation between cathelicidin knockout mice and susceptibility to Group A Streptococcus (Lee *et al.*, 2005; Nizet *et al.*, 2001), herpes simplex virus (Howell *et al.*, 2006), Escherichia coli (Chromek et al., 2006) and vaccinia virus (Howell *et al.*, 2004).

The recent discovery of multiple cathelicidin in both the wallaby genome and the platypus genome suggests that common ancestors of placental and marsupial mammals most likely expressed a broad repertoire of cathelicidin peptides to protect their young. With the evolution of longer gestation periods resulting in the birth of young with greater immune competence, cathelicidin genes may have been lost from the eutherian lineage which is evident in humans and mice having only one cathelicidin gene. Subsequent gene duplications in sheep, cows and pigs have led to the expanded cathelicidin gene family in the Cetartiodactyla lineage. The duplication and subsequent diversification of these genes in the Cetartiodactyla lineage could be indicative of some species-specific or clade-specific pathogenic challenge against herd animals, which is not a factor for the human lineage.

In cattle at least seven distinct protein-coding cathelicidin genes exist in the bovine genome and are known to cluster in chromosome 22 (Tomasinsig and Zanetti, 2005). Cathelicidin expression has been detected in myeloid-derived cells (Zanetti *et al.*, 1995) but limited evidence is available on their expression in nonmyeloid cells, although cathelicidin expression has been reported in the bovine mammary gland (Ibeagha-Awemu *et al.*, 2010; Tomasinsig *et al.*, 2010).

In all other species investigated the first three exons code for the cathelin domain whilst the fourth exon codes for the mature antimicrobial domain (Fig. 6.1). Although the function of the

cathelin domain is uncertain, the widespread distribution of this highly conserved domain in mammals suggests it has considerable value. Likewise, all of the predicted full-length cathelicidins genes identified in our investigation contain the cathelin domain coded for by exon 1, 2 and 3. Translations of the predicted genes revealed stop codons in the first exon of *CATHLpsuedo* and *CATHLnovum* (see Appendix). *CATHLnew* was intact and displayed all the characteristics of a functional cathelicidin gene including, approximately 2 kilobase in size with a conserved four exon – three intron arrangement with, a TATA-box just upstream from the transcription start site and a polyadenylation signal located 54 bp from the stop codon (Fig. 6.3). In addition, several potential recognition sites for transcription factors involved in the transcription of immune-related gene are found in the 5' flanking region of *CATHLnew* (see Appendix). However, we observed that the predicted sequences and their intron-exon boundaries were not consistent with cathelicidins 1-7, and as a result, the cathelicidins did not display the canonical cysteine spacing that is invariably conserved among cathelicidin family members (Zanetti, 2005). This may have disrupted the two disulfide bonds between cysteine residues C85-C96 and C107-C124 leading to loss of function and its transcription lost over time. Alternatively, *CATHLnew* could be expressed in tissues other than the ones that we examined or their intron-exon boundaries are consistent with cathelicidins 1-7 and have the same amplicon length as predicted for cathelicidins 1-7 (Fig 6.6B). It should also be considered that the observed extension of exon II and exon III in the *CATHLnew* gene (Fig 6.5) may be specific to the Hereford breed from which the original whole genome sequence was obtained. Further work is required to determine if these novel cathelicidins are transcribed.

Incomplete cathelicidins were identified (exon I and exon II of *CATHLnew* and exon III and exon IV of *CATHL1*). In the case of *CATHL1*, (Scocchi *et al.*, 1997) previously reported two copies of this gene in the bovine genome that were picked up by southern blotting with a 3' specific probe. This probe was designed to hybridize to exon IV of the *CATHL1* gene; as a result, the authors concluded that two copies of *CATHL1* exist in the bovine genome. Scocchi's results support our evidence that the *CATHL1* fragment (exon III and exon IV only) identified (Fig. 6.2B) is in fact a pseudogenised gene and not errors resulting from the assembly process. Similarly, the bovine genome also codes for two *CATHL4* genes (*CATHL4* and *CATHL4a*) (Fig. 6.2). Two adjacent indolicidin-coding genes in cattle genome have also been reported (Scocchi *et al.*, 1997). Furthermore, a recent study profiling gene copy number variation in different bovine

breeds (Bickhart *et al.*, 2012) reported 4 copies of *CATHL4* in a Holstein and 6 in Hereford, which could potentially underlie inherited differences in innate immunity and disease resistance.

In line with our data, both the human (LL37) and mouse (CRAMP) cathelicidins are constitutively expressed in several tissues such as bone marrow, thymus, liver, spleen, testis, stomach, and intestine (Bals *et al.*, 1998a; Gallo *et al.*, 1997). Constitutive expression of cathelicidins in a variety of tissues suggests that these tissues may have an innate ability to resist microbial infection without stimulating an inflammatory cell influx, or these peptides possess additional biologic functions to maintain homoeostasis (Lai and Gallo, 2009). Further work to evaluate cell type-specific expression and biological function of cathelicidin *in vivo* is needed to address these issues directly.

Greater numbers, and higher levels of expression was detected in organs and cells of primary immune function such as spleen (Fig. 5.9I) and milk leukocytes (Fig. 5.9N) from a case of naturally occurring mastitis. At the cellular level, cathelicidins are most abundant in granules of neutrophils (Zanetti, 2005), which are the predominant cells in mastitic milk. They are also expressed by other cell types either constitutively or in an inducible manner, for example, LL-37 is expressed by monocytes, NK cells, B cells, and γ δT cells (Agerberth *et al.*, 2000), which may account for the expression levels observed in our spleen tissue.

Of the cathelicidin genes profiled, *CATHL6* expression was most restricted. The bovine EST database also demonstrated restricted expression and there were few hits compared to other cathelicidins. Our findings of higher levels of expression in milk somatic cells (Fig. 6.8N), indicates that *CATHL6* is inducible only in certain conditions. Interestingly, only *CATHL6* was detected in PBMCs from our healthy animal, whereas expression of *CATHL*1, 2, 4, 5, 6 and 7 (*CATHL3* was not tested) had been detected in PBMCs from cattle with *Trypanosoma congolense* infection (data not shown), possibly indicating an important role for cathelicidins in blood borne infections and their potential use as biomarkers of infection. Increased cathelicidin expression in blood or milk may therefore be a useful biomarker of infection.

In vitro, most AMPs are pleiotropic and can act against many different types of microbes including gram-negative and gram-positive bacteria, protozoa, fungi as well as some viruses (Lai and Gallo, 2009). This is particularly true for the mammalian AMPs that have maximal effectiveness against specific groups of organisms relevant to the tissue where the AMP is expressed. For instance, β-defensins exhibit activity against *Staphylococcus aureus* and

Pseudomonas aeruginosa (Huang *et al.*, 2007), which are relevant to skin infections, whereas α-defensins expressed in the gut are able kill important enteric pathogens such as Salmonella (Salzman *et al.*, 2003). Similarly, selective antimicrobial killing was observed for bovine cathelicidins *in vitro* (Tomasinsig *et al.*, 2010). Determining the location of endogenously expressed cathelicidins is an important first step in identifying which AMPs could be more effective in killing specific pathogens. For example, six out of the seven cathelicidins profiled were expressed in lung tissue (Fig. 6.8L). Both the human (LL37) and mouse (CRAMP) cathelicidins are important in the mucosal immune response in the lung to *Klebsiella pneumonia* and *P. aeruginosa* infections (Byfield *et al.*, 2011; Kovach *et al.*, 2012). Sheep cathelicidin (SMAP29) had greater bactericidal activity than LL37 and CRAMP against clinical isolates of *P. aeruginosa* (Saiman *et al.*, 2001). The bovine cathelicidins expressed in the lung could represent a novel class of antimicrobial agents. Their antimicrobial activity may be enhanced when multiple cathelicidins are present.

As an initial step to evaluate the role of the bovine cathelicidins in mammary gland infection, the expression of cathelicidins 1-7 was analysed by qPCR using RNA extracted from bovine mammary gland tissue, from primary mammary epithelial cells, and from milk somatic cells. Cathelicidin proteins are a major component of the neutrophil secondary granule and increased levels of these proteins are observed in a range of inflammatory conditions (Zanetti, 2005). 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) and this could account for the high expression of cathelicidin 1-7 in milk cells from a naturally occurring case of mastitis. This is in line with prior data which showed expression of cathelicidin protein in bovine milk (Smolenski *et al.*, 2007; Smolenski *et al.*, 2011) and the expression of a panel of cathelicidin genes in milk cells. This is the first report of the expression of all seven protein-coding cathelicidins in mastitis milk.

Expression of *CATHL4* and *CATHL5* was detected in our mammary tissue (Fig 6.8M). Cathelicidin transcription was previously demonstrated in healthy mammary gland tissue (Tomasinig *et al.*, 2010). *CATHL4* was found to be up-regulated in heat inactivated *S. aureus* 1027 treated mammary epithelial cells (Fig. 5.10). Similar upregulation of *CATHL4* expression was observed in a bovine mammary epithelial cell line in the presence of *S. aureus* (Ibeagha-Awemu *et al.*, 2010). Our results from chapter 5 would suggest that upregulation of *CATHL4*

expression is NF-κB independent. Previous research has shown that *S. aureus* 1027 induces early inhibitor of NF-κB activation (TNFAIP3) as well as NFKBIZ (Gunther *et al.*, 2010). These factors are crucial for induced IL-6 expression but also block TNF-α synthesis (Matsuo *et al.*, 2007 Yamamoto, *et al.*, 2004). These molecules may also play an important role in the expression of *CATHL4* which codes for the peptide indolicidin which has been shown to kill bacterial isolates obtained from cases of mastitis *in vitro* (Tomasinsig *et al.*, 2010).

6.4 Conclusion

In this study, we used the recently released BosTau7 assembly of the bovine genome to reconstruct the full cathelicidin repertoire for this species. In addition to known (cathelicidins 1-7) we identified three novel cathelin-coding domains clustered on bovine chromosome (BTA22). Our initial investigations would suggest these are pseudogenes that are not transcribed, however further work is required to support this supposition.

We also identified two *CATHL4* genes adjacent to each other on BTA22. A recent study profiling gene copy number variation in different bovine breeds (Bickhart *et al.*, 2012) reported 4 copies of *CATHL4* in a Holstein and 6 in Hereford. In the same study, 6, 7 and 11 *CATHL4* genes were reported in 3 Angus individuals. This observed variation in *CATHL4* copy number between individual animals could form the basis for selective breeding towards resistance against mastitis.

This is the first study to characterise the expression of bovine cathelicidins in a wide range of bovine tissues. It also the first report of the expression of all seven protein-coding cathelicidins in mastitis milk. The simultaneous presence of all the peptides as a result of infection may produce a far more robust response than that of a single peptide. Their antimicrobial activity may be enhanced when multiple cathelicidins are present. Their distinct and complementary functions may contribute to a thorough and sustained response to infection and point to a protective role of cathelicidins in bovine mastitis. In addition, our results also suggest that they may be useful biomarkers of infection.

7. Final discussion

Among the ailments that affect dairy ruminants, infection and inflammation of the mammary gland (mastitis) plays a prominent part. Mastitis causes major economic losses through reduction in milk yield and milk unfit for consumption and is a major cause of premature culling. In addition to the agricultural cost, continuous use of antibiotics is contributing to the emergence of antibiotic resistant strains of micro-organisms which can infect many species including humans. Milk and dairy products are an essential source of food for the majority of the world population. To meet the growing global demand and to keep the dairy farming profitable at the same time, prevention and treatment of mastitis are primary concerns of the dairy industry. In spite of the efforts made to control it, the rate of mastitis incidence has been quite stable for years and the highest of all the cattle diseases. Furthermore, as a result of the long-lasting feature of subclinical mastitis, the most common form of the mastitis, its prevalence in dairy herds is a major concern on an international scale. In this thesis, novel components of the bovine innate immune system are identified and characterised.

7.1 The systemic immune response

Our results show that intramammary infection of the bovine mammary gland with *S. aureus* induces both a local and systemic immune response. Furthermore, the immune response induced in *S. aureus* inoculated quarters has an influence on the expression of innate immune genes in unchallenged quarters which could be mediated systemically. It has previously been reported that during *E. coli* infection the transcriptome of neighbouring, sterile quarters is modified (Mitterhuemer *et al.*, 2010). Our results are the first to show that a similar phenomenon occurs during *S. aureus* infection of the bovine mammary gland.

The mechanism by which the infection of neighbouring quarters affects the transcriptome of uninfected quarters is unknown. Intramammary infection with *S. aureus* induces a systemic effect inducing transcriptional changes in the liver which leads to the production of acute phase proteins which are released into the blood (Eckersall *et al.*, 2006). This suggests that the systemic response has an influence on the expression of innate immune genes in unchallenged quarters. This has also been postulated to explain the transcriptional profile in healthy quarters of animals infected with *E. coli* (Mitterhuemer *et al.*, 2010). However, in a similar infection model, analysis

of TLR and β-defensin expression in the local and peripheral lymph nodes of animals with *S. aureus* or *E. coli* intramammary infection showed that the systemic effect is quite limited (Jensen *et al.*, 2013). Local cross-talk between the udder quarters could also play a role in priming the neighbouring udder quarters. Indeed, lymphocytes have been shown to be capable of migrating between mammary gland quarters (Kimura *et al.*, 2005) and therefore the presence of a more localized interaction between quarters is probable and requires further investigation.

Furthermore, these results have important implications for the design of appropriate models to study bovine mastitis. To date, most experimental designs used in mastitis research lack external controls and are thus not able to differentiate between local and systemic responses to the pathogen.

These results introduce a new concept to mastitis research and may yield new targets for prevention and therapy of subclinical mastitis. Moreover, the observed variation in the systemic response between individual animals could form the basis for selective breeding towards resistance against *S. aureus* induced mastitis.

7.2 Region-specific changes in innate immune gene expression

Following on from our initial study, a comprehensive quantification of innate immune genes was carried out on mammary tissue from the same infection model. Expression of genes encoding PRRs, pro- and anti-inflammatory cytokines, APPs and AMPs in tissue from the alveolar, ductal, gland cistern and teat canal regions of control and *S. aureus* challenged mammary quarters were profiled.

Of the regions profiled, 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 observed in response to *E. coli* infection (Rinaldi *et al.*, 2010). The fact that the alveolar region appears more sensitive to pathogens in both infection models regardless of the point of entry of the pathogen, maybe a result of differences in resident cell populations throughout the mammary gland (Nickerson and Pankey, 1983; Nickerson *et al.*, 1984) or differences in the expression of PRRs (Fig 4.4A). The contribution of each cell population to the challenge-related changes in gene expression remains to be elucidated. Furthermore, in order to develop efficient vaccines against mastitis a greater understanding of the

resident cell populations in the bovine mammary gland is important and requires further investigation.

7.3 Cathelicidins

The dwindling numbers of therapeutic options for bacterial 'superbugs' necessitates the development of novel antimicrobials. The development of microbial resistance against naturally occurring antimicrobials peptides is rare. To this end, we used a bioinformatics approach to characterise the entire repertoire of cathelicidin, important antimicrobial peptides, encoded in the bovine genome.

This is the first detailed study of cathelicidins in a ruminant species. The work describes the first comprehensive annotation of the cathelicidin locus in *Bos taurus* using a bioinformatics approach. In addition to known cathelicidins, we detected three novel cathelicidin genes, and found that two of these novel genes are pseudogenes while one is a potentially functional cathelicidin gene. In addition, we have characterised expression of bovine cathelicidin genes in a wide range of bovine tissues, highlighting their importance at sites which are exposed to microbial invasion as well as in professional immune cells. It is also the first report of expression of all seven protein-coding cathelicidin genes in mastitic milk cells, suggesting that they may provide a useful biomarker of infection. This is backed up by the finding that cathelicidin levels do increase in milk following intramammary infection (Smolenski *et al.*, 2011).

In addition, we found early expression of cathelicidin genes in primary mammary epithelial cells after *S. aureus* stimulation. *CATHL4* and *CATHL5* was significantly increased as early as 30 min in mammary epithelial cells stimulated with S. aureus (>60-fold). The bovine cathelicidin gene, *CATHL4*, codes for the peptide indolicidin. Indolicidin is one of the shortest cathelicidin peptides (13 amino acids), that exhibits potent and wide antimicrobial activity against Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria (Selsted *et al.*, 1992) as well as fungi (Lee *et al.*, 2003). Additionally, modified synthetic versions of indolicidin have improved antibacterial and antifungal activity in vitro and also have reduced cytotoxicity compared to the native peptide (Falla and Hancock, 1997; Ryge *et al.*, 2004). However, direct killing of microbes may not be the primary role that cathelicidins perform in the innate immune response as these functions do not occur at physiological salt and peptide concentrations (Bowdish *et al.*, 2005a). The role of bovine cathelicidins as immunomodulatory molecules has received some recent

attention (Bowdish *et al.*, 2005c; Mookherjee *et al.*, 2006b). These studies demonstrated that indolicidin, like LL-37, inhibited LPS-induced TNF-α secretion and that they do this by immunomodulatory mechanisms, such as suppressing translocation of NF-kB subunits, in addition to direct binding to LPS. It is tempting to speculate that the induction of *CATHL4* by *S aureus* 1027 may be a mechanism by which *S. aureus* can evade the innate immune response. Interesting, the expression of *CATHL4* in response to *E. coli* 1303 was downregulated at 1 h post-stimulation (preliminary data - not shown). This hypothesis is further backed by previous research with *S. aureus* 1027 and *E.coli* 1303 (Yang *et al.*, 2008). They showed that both pathogens induce *IL8* and *TNFA* gene expression in primary mammary epithelial cells. However, the magnitude of expression was much greater in response to *E. coli*. This muted proinflammatory response to *S. aureus* was paralleled by a complete lack of NF-kB activation by *S. aureus* in contrast to *E. coli*. To rule out differences in the ability to engage both TLRs, they showed that heat inactivated bacteria from both pathogens activate bovine TLR2 and TLR4 receptors equally to induce NF-kB activation. They speculated that *S. aureus* 1027, which upon intra-mammary infection induces subclinical mastitis, (Petzl *et al.*, 2008), impairs NF-kB activation in mammary epithelial cells by an unknown mechanism thus resulting in a weakened immune response in the udder. They concluded that the muted immune response in mammary epithelial cells was the result of the activation of a cell type dependent blocking of TLR-dependent NF-kB activation, mediated by the heat-inactivated *S. aureus* pathogen or even just the lipoteichoic acid (LTA) isolated from this strain. Recent data from the same group showed that heat-inactivated *S. aureus* 1027 induces an early inhibitor of NF-κB activation (TNFAIP3) as well as NFKBIZ. These factors are crucial for induced IL-6 expression but also block TNF-α synthesis (Matsuo *et al.*, 2007 Yamamoto, *et al.* 2004). Similar to my experiments, they used heat-inactivated *S. aureus* 1027 and as such blocking of NF-kB activation is mediated through cell membrane PAMPs interacting with epithelial cell PRRs and not secreted virulence factors. Interestingly, LTA from *S. aureus 1027* strongly induce *TNF* and *IL8* expression in human neutrophils (Hattar *et al.*, 2006). The fact that indolicidin is bovine specific could explain this. Further work is required to determine the immunoregulatory properties in mammary epithelial cells.

Interestingly, the bovine genome also codes for two *CATHL4* genes (*CATHL4* and *CATHL4a*) (Fig. 6.2). Two adjacent indolicidin-coding genes in cattle genome have also been

reported (Scocchi *et al.*, 1997). Furthermore, a recent study profiling gene copy number variation in different bovine breeds (Bickhart *et al.*, 2012) reported 4 copies of *CATHL4* in a Holstein and 6 in Hereford. In the same study, 6, 7 and 11 *CATHL4* genes were reported in 3 Angus individuals. This observed variation in *CATHL4* copy number between individual animals could form the basis for selective breeding towards resistance against mastitis. Our results revealed that high level of expression of all seven protein-coding cathelicidins were detected in milk somatic cells. Taken together, cathelicidin expression levels could potentially underlie inherited differences in innate immunity and disease resistance.

8. References

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9. Appendix

Chapter 4: Relative median fold changes of a range of innate immune genes profiled using qPCR.

Table 1Mean fold changes are shown for *S. aureus* infected animals (n=6) relative to non-infected control animals (n=3). Statistical significance is denoted as *P <0.05 and **P <0.01. n.d = transcript not detected.

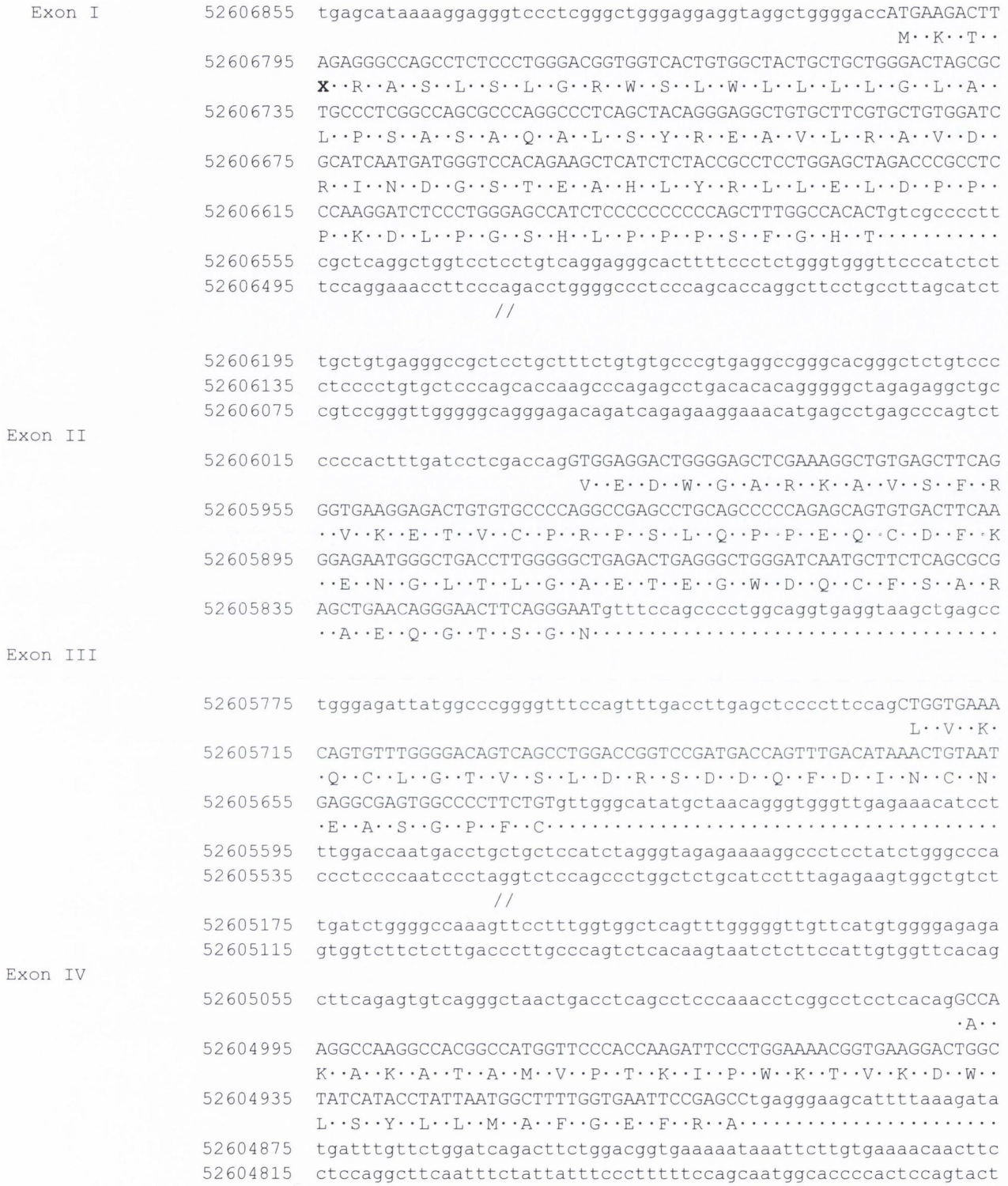
	Gene	Regional Mammary Tissue			
Gene Class	**symbol**	Alveolar	Duct	Cistern	Teat
PRRs	*TLR 1*	1.4	6.1*	6*	4.2*
	TLR 2	2.3	1.4	1.5	-0.5
	TLR 3	1.9	1.2	1.8	2.5*
	TLR 4	-0.1	-1.2	1.4	-2.8*
	TLR 5	1.8*	1.2	1.2	-0.1
	TLR 6	-3	-1.1*	3.7*	1.8
	TLR 7	2.3*	1.9	3.6*	1.4*
	TLR 8	1.6	-0.2	-1.9*	0.1
	TLR 9	1.3	1.1	2.3	1.9
	TLR 10	5.3	1.8	1.1	2.0
	NOD1	-1.5	-1.4	1.2	-2.7*
	NOD2	-1.6	0.3	1	-1.2*
Mediators	*TNFA*	1.8	0.3	1.3	2.8
	IL1B	1.8	-2.9*	2.4	1.4
	IL6	5.1*	2.1*	2.9*	n.d
	IFNG	-2	1.3	2.3	11.2*
	Il17	5.7**	1.5*	1.5*	9*
	IL8	13*	5.4	2.1*	3.4
	TGFB	-1.2	1.4*	1	-1.8*
	IL10	5.6	8.4*	3.7	3.2*
APPs	*SAA3*	133*	14*	4.1*	6.4
	HP	80**	30*	5.6*	30
	LBP	3.3*	3.3*	1.7*	6.6
	A1AG	-54*	5.1*	7.2*	1.6
	CP	1.3	3.1	3.2*	1.9*
AMPs	*DEFB1*	4.9*	4.3	2.5	1.2
	DEFB4	9.4**	2.1	10*	2.7
	DEFB5	27*	22*	34*	1.9
	TAP	4.6	2.6	2.6	5.2
	LAP	1.45	1.8	2.1	11

Chapter 6: Cathelicidin Uniprot Accession numbers

Protein Name	Accession Number	Gene Name
Bactenecin 1	P22226	Cathelicidin 1
Bactenecin 5	P19660	Cathelicidin 2
Bactenecin 7	P19661	Cathelicidin 3
Indolicidin	P33046	Cathelicidin 4
Myeloid antibacterial peptide 28	P54229	Cathelicidin 5
Myeloid antibacterial peptide 27	P54228	Cathelicidin 6
Myeloid antibacterial peptide 34	P56425	Cathelicidin 7

Chapter 6: Gene-specific oligonucleotide primers used for qPCR

Gene Symbol	Gene Name	Forward primer (5'-'3)	Reverse primer (5'-'3)	Amlicon size (bp)	Accession Number
CATHL1	Cathelicidin 1	ATCACCTGTAATAATCACCAGAGCAT	CCCTTAGGACTCTGCTGGCTTA	150	NM_174825
CATHL2	Cathelicidin 2	GAGAATGGGCTGGTGAAACAG	GTTATCTGCCTATTGTTCACCGTCTA	141	NM_174826
CATHL3	Cathelicidin 3	TGACTTCAAGGAGAATGGGCTGGTG	GCCGGGGACGAATTCTCCTGACA	122	NM_174001
CATHL4	Cathelicidin 4	GGCACTCGAAAGCCTGTGA	GGCCATTTCCAGGGTAGGAT	200	NM_174827
CATHL5	Cathelicidin 5	AAGGAGAATGGGCTGCTGAAA	GCCATACTTCTTCCAAGCACGTA	150	NM_174510
CATHL6	Cathelicidin 6	GGAGGACGATGAGAACCCAAA	AGTAGCGGAATGACTGGAGAAAGT	270	NM_174832
CATHL7	Cathelicidin 7	CCCAGAGCAGTGTGACTTCAAG	AGCCCCGCACTCTGAATATTATTA	120	NM_174831



Chapter 6 Predicted structure of *CATHLpseudo*

The coding sequence is in uppercase letters, noncoding sequence are in lowercase letters. The deduced amino acid sequence of the open reading frame is indicated in single letter code and the stop codon is indicated by an X.

```
Exon I          52579495  ctggggaccATGGAGACCCAGAGGGCCAGCCTCTCCCTGGGGCGCTGGTCGCTGTGGCTG
                                    M··E··T··Q··R··A··S··L··S··L··G··R··W··S··L··W··L·
                52579435  CTGCTGCTGGGACTAGTGCTGCCCTCGGCCAGCGCCCAGGCCCTCAGCTACAGGGAGGCC
                           L··L··L··G··L··V··L··P··S··A··S··A··Q··A··L··S··Y··R··E··A·
                52579375  ATGCTTCGTGCTGTGGATCAGCTCAATGAGCGGTCCTCAGAAGCTCATCTCTACCGCCTC
                          ·M··L··R··A··V··D··Q··L··N··E··R··S··S··E··A··H··L··Y··R··L·
                52579315  CTGGAGCTAGACCCGCCTCCCTAGGACATGAGCTGGGGAGGGGACCGGGGACGAGCCTTT
                          ·L··E··L··D··P··P··P··X·····································
                52579255  ctcctgcctgctttggccacactgttgccccttcactctggctgtacctcctgtcaggaa
                52579195  ggcacttttccctctaggtgggttcccacctcttccaggaaaccttcccagacctgggtc
                52579135  atctcccagcaccaggcttcctgtcttagcatctctgctgtgggaataggcaccctgcac
                                              //
                52578895  cagcccctgtgaggggaggcgctgccatcagcgctgctgtgagggccgctcctgctctct
                52578835  gtgtgcccgtgaggccgggcacgggctctgtcccctcccctgtgctcccagcaccaagcc
                52578775  cagggccggacacacagtgggctggagaggctgccgtctgggttgggggcagggagacag
Exon II
                52578715  atcagagaaggaaacATGAGTGAACCTAGTTTCCCCACTTTGACTGTTGACCAGGTGGAG
                                                                              V··E·
                52578655  GACCTGGGAGCTCGAAAGCCTGTGAACTTCAGGGTGAAGGAGACCGTGTGCCCCAGGTCG
                          ·D··L··G··A··R··K··P··V··N··F··R··V··K··E··T··V··C··P··R··S·
                52578595  AACCTGCAGCCCCCAGAGCAGTGTGACTTCAAGGAAAATGGGgtgagcctggggactgag
                          ·N··L··Q··P··P··E··Q··C··D··F··K··E··N··G···················
                52578535  actgagggctgggaataatgcttctcagtgcgagctgaacagggaaatcgggaaggtttc
                52578475  cagcatctagagggtgaggtgagcctgggagattatggcccgggggttccagtttgacct
Exon III
                52578415  tgagctacccttccagCTGGTGAAACAGTGTGTGGGGACACTCAGCCTGTACCGGTCTGA
                                        L··V··K··Q··C··V··G··T··L··S··L··Y··R··S··D
                52578355  TGACCCATTCGGTCTAAACTGTAATGAGgtgagtggccctttctgtgttatgcagatgct
                          ··D··P··F··G··L··N··C··N··E·····························
                52578295  aacaaggtgggttgtggaacatgcttaggaccgatgacccgctgccccatccagggcaga
                52578235  gaaaggccctcctacccgggcccctccctccccgagccccaggtctccagccctggctc
                52578175  tgcatcccttagagcagtgtttctgtaatgcagtccccaccccggaactgacatgagaca
                                              //
                52577875  aatcatatgcttcaaagataacagccagagggtgaacagggcccaacactcgtggtgtcc
                52577815  cagttagagggctgttcaggtgtgaagtgagggatcttgtcttgacccttgcccagtccc
                52577755  acaacaaatctgttttgtcatggtttacagcttcagagtgtcaggagacttcatccccag
Exon IV
                52577695  catcaacgtttcccaagGGAAAGGCCATGGCCAAAGCCATTGTCATTACCACTGCCAAGG
                                          G··K··A··M··A··K··A··I··V··I··T··T··A··K··
                52577635  CCAGGGCCAAGGCCATGGCCAAAACCCCTGTGATAATCATTGTGAAGGCCAAGGTCAAGT
                          A··R··A··K··A··M··A··K··T··P··V··I··I··I··V··K··A··K··V··K··
                52577575  TTAGCGCTGTGGCCAAGACCAATGCCAAGTCCACGTCCATTCCTGTTGACGTCTCTCATT
                          F··S··A··V··A··K··T··N··A··K··S··T··S··I··P··V··D··V··S··H··
                52577515  CCAAGGTGTCCTTGAAAATGGTGAAGGATTGGCTGCCATCACACCCATTAAAGAATTTTG
                          S··K··V··S··L··K··M··V··K··D··W··L··P··S··H··P··L··K··N··F··
                52577455  GTGAATCCTGAgtccacagaagagtcctaatgttctgatttgttttggcttagactgctg
                          G··E··S·····································
                52577395  gatggtgaaaaataaattcttgtgaaaacaacttcctccaggcttcaatttctactttct
                52577335  cacctcttcccacttattgagactgagtccttaactctcagagggctactctgtgtgtgt
```

Chapter 6: Predicted structure of *CATHLnovum*

The coding sequence is in uppercase letters, noncoding sequence are in lowercase letters. The deduced amino acid sequence of the open reading frame is indicated in single letter code and the stop codon is indicated by an X.

NF-κB

GCTAGAGGAGAACAAAGCAACCCACTCCAGTATTCTTGCCTGGAGAATCCCATGGATAGT

GAGCCTGGCAGGCCACAGTCCGTAGGGTCTCAAAGAGTCTCAAAGAGTCAGACACGACTG

C/EBPα

AAGCGACTGAGCACACATGCATGCACACATAGCTGATTAACAAATCATGTTATGATAGCT

TCAGGTGAAGAGCAAAGGGACTCAGCCATCTGTATATATGTATCCATTCTCTTCCAACCT

AP-1 GATA

GGAGGCGAACTTCCTAACTGAGAAACAGTGGAGATGAATCCAGGAGAGAGGACAGATGGA

GCCAAGCTCCTGACAGGCTGACGGCTGGTCCCAAAGACAGGATGGTGTAGGGTTGATGGC

TGCCTAGGGACAGGCTGGGTGGGTAAGGCTCAGAAGGGGTTGGTCCTGTCATCCACTCAT

CAAATGTTAAATGAGTGCATACTGTGTGCCAGGCAGTGCCTAGAGCTGGAGGTTGAGTGC

TGAATATACTCCTTTACCCTGAATCCCGAGGAGCCCATGGTGTGGAGGCAGAGGGGATGC

C/EBP

AGACAGTGCCTGGTCCCTCCCTCTCCTGGAGGACACAACCCTCTTGAGCAAGCCTGCCGG

TGACACTCCCCATGGGATCTAGGAGAGGCCAAGAAGTTGTCAGGAGATGGAGAAACCATT

Sp1 NF-κB

TCTTCACCTTGCAAACGGGCCTCCCTGCCCACTGCCCAGAAATCCCATGAGGCAACAGCA

AGGCTTGAGGTAAGACCAGCCCACACCCTGGGAGGAGGGCAGGGATGGGGCGGGTCAGGA

AGACTCCTGGTAGAGCTTTTGCATCAGGGCTCAGACTGGGC*ATAAAA*GAAGGGTCCCTTG

GGCTGGGAGGAGGCAGACTCGGGACC **ATG** GAG ACC CAG AGG GCC AGC CTC TCC

Chapter 6: DNA sequence of the *CATHLnew* promoter region.
Translation start codon ATG is in bold and TATA-box is in italics. Predicted transcription binding sites were identified using alibaba2 and TFSEARCH programs and highlighted.