Interactions Between Bovine Neutrophils and *Streptococcus uberis*

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Contents

Summary

Streptococcus uberis is a leading cause of mastitis worldwide with significant financial and welfare implications. It is uncontrolled by present disease control strategies and further knowledge of the interactions between host and pathogen are crucial to permit the development of novel control strategies.

Neutrophils (PMNs) migrate into the udder in large number and they are responsible for removing other udder pathogens. However previous work has shown that virulent *S. uberis* 0140J releases an unidentified soluble factor that inhibits the bactericidal activity of PMNs. This work set out to elucidate a better understanding of this host-pathogen interaction; firstly by investigation to determine whether avirulent strains of *S. uberis* were killed by PMNs, and secondly by assessing transcriptional changes between PMNs after exposure to milk and milk in which S. uberis has been cultured by microarray analysis.

Naturally occurring and mutant strains of *S. uberis* that show attenuation in the host were unaffected by the presence of functional PMNs, indicating that their attenuation in the host cannot be attributed to increased susceptibility to the bactericidal activities of PMNs.

PMNs incubated in milk in which *S. uberis* 0140J had been cultured (ST PMNs) had an altered transcriptome compared to PMNs incubated in milk. Genes involved in cell death and the inflammatory responses appear effected. A down-regulation of pro-apoptotic genes was observed, this finding was supported by a highly significant reduction in the presence of apoptotic cells, compared to cells incubated in milk. Transcription of pro-inflammatory cytokines including CXCL8, IL12β and CCL20 was up-regulated in ST PMNs and genes involved in cellular structure and metabolism had altered levels of expression. This data from this investigation has provided an initial insight into the mechanisms by which *S. uberis* may elicit changes in PMNs that may relate to inhibition the bactericidal function and/or other host pathogen interactions.

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1 Introduction

1.1 The Importance of Bovine Mastitis

Bovine mastitis, inflammation of udder, is a significant problem for the UK and world dairy industry. It has been estimated to be the most economically significant infectious disease of dairy cattle (Kossaibati and Esslemont, 1997), costing the dairy industry £200 billion worldwide and the UK around £200 million annually (Biggs, 2009). The disease also has major welfare implications; in 2009 the Farm Animal Welfare Council recommended that the incidence of mastitis should urgently be reduced (FAWC, 2009).

Mastitis is usually associated with the presence of an infectious agent. A large number of microbial agents come into contact with the udder and have the opportunity to enter the mammary gland via the teat canal; nevertheless most clinical mastitis cases are caused by only five causative bacteria: *Streptococcus uberis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Escherichia coli* (Bradley, 2002) giving some indication of the multiple host and pathogen factors required to establish infection.

Classical, infectious mastitis is defined by two parameters. Firstly by severity: in a subclinical case the cow shows no obvious outward signs of disease and the bacterial infection can become chronic. Clinical cases show adverse changes in the milk, the udder can be hot, swollen and painful, and as the severity of the case increases the cow becomes less productive, systemically unwell and in some cases can progress to septicaemia and death. Secondly, infections are classified based on whether the source of infection is either environmental or contagious. Although these definitions are quite arbitrary, they are still a useful starting point. Contagious pathogens, typically *S. aureus* and *S. agalactiae*, are found in the udder or on the udder or teat surface, and do not survive well in the environment. Infection is spread from one cow to another animal either directly or more usually via fomites during milking. On the other hand, pathogens that are considered environmental pathogens are those acquired from the cow's surroundings - from pasture, bedding and faecal material, where the bacteria persist well. Pathogens from the environment do not need to inhabit the bovine udder and cause disease for their survival. *S. uberis* and *E. coli* are typically classed as environmental pathogens.

The Five Point Plan outlining basic hygiene, husbandry, preventative measures and treatments for mastitis control has been universally implemented since the late 1960s (Bradley, 2002). This resulted in a dramatic decrease of mastitis due to contagious pathogens, which accounted for the vast majority of isolates (Wilson and Brookbanks, 1967). Intramammary infection by environmental pathogens was not controlled by these activities, and this has been reflected in the subsequent relative increase in their prevalence (Bradley et al., 2007, Bradley, 2002, Booth, 1997). Surveys suggest that environmental pathogens account for over 60% of cases (Bradley and Green, 2001, Bradley et al., 2007) and also indicate a possible increased incidence of mastitis in the past 10-20 years (Bradley et al 2007).

1.2 The Ubiquitous Nature of *S. uberis*

Many strains of *S. uberis* have been identified from the environment and from cases of clinical mastitis. The strain 0140J occurs within the sequence type 5 (ST-5) clonal complex, the sequence type most frequently associated with disease-causing strains in the UK (Coffey et al., 2006). *S. uberis* 0140J was first isolated from a clinical case of mastitis in the 1970s. This strain reproducibly infects and causes disease in the lactating gland (Leigh et al., 1990, Bannerman et al., 2004a), and has also been shown to cause disease in the non-lactating mammary gland (Hill, 1988). It has been used extensively worldwide to research the pathogenesis of *S. uberis*, and is the organism of choice for this project.

Mastitis caused by *S. uberis* is traditionally associated, in the UK, with housed cattle, especially those kept on straw. Straw has been shown to support higher *S. uberis* populations compared to wood-derived bedding (Hogan et al., 1989, Rendos, 1975). Although inorganic bedding (i.e. sand) has been reported to support lower bacterial numbers, the bacterial load increases with increasing organic contamination (Hogan et al., 1989). Pasture based systems are also associated with mastitis caused by *S. uberis* (Compton et al., 2007), with bacterial populations reaching levels comparative to that within housed farming systems (Harmon, 1992). *S. uberis* is found in the gastrointestinal tract of healthy cows and excreted in the faeces into the environment where it can survive for two weeks (Lopez-Benavides et al., 2007). This ability to survive outside the host, its presence in all farming systems and the ineffectiveness of the Five Point Plan in controlling it makes *S. uberis* a formidable pathogen, relevant to the dairy industry worldwide.

1.3 The Immune Response to Mastitis Pathogens

The bovine udder has an armoury of defences against invading pathogens; indeed it has been suggested that the mammalian mammary gland evolved as a secretory gland of the primitive immune system that produced a protective secretion, developing its nutritional function for supporting the neonate later (McClellan et al., 2008, Vorbach et al., 2006).

1.3.1 Physical Defences of the Mammary Gland

The streak canal, at the distal end of the teat, forms an initial barrier against invading pathogens. When the udder is not under a physiological state for milk let-down, the orifice is closed by constriction of sphincter muscles. It is only relaxed as part of the cascade of effects that oxytocin, released in response to stimulation by the calf or the routine of coming into the milking parlour, causes to result in milk-let-down. An additional crucial defence is the keratin plug which is formed from the constant turnover of the stratified squamous epithelial lining of the streak canal. The plug forms during the dry period and between milkings, and acts as a physical and bactericidal obstacle to invading pathogens. In some individuals the competency of these protective mechanisms can be low, increasing the likelihood of pathogens invading past the teat canal into the teat cistern. For example, closure of the end of the teat can be

inherently poor or damaged, due to over-milking or infection and in some individuals an effective keratin plug is not formed.

Where the streak canal meets the teat cistern, the lumen of the teat, is the anatomical formation known as the Rosette of Fürstenburg encircling the top of the streak canal. This non-muscular structure is formed by deep folds of the cuboidal epithelium that lines the teat cistern and is continuous with the rest of the epithelia of the mammary gland itself. It is thought that the folds of this structure produce a large surface area of the epithelium allowing leukocytes to rapidly access the area to defend against pathogens.

1.3.2 Cellular and Humoral Defences of the Mammary Gland

If microbes manage to penetrate past the streak canal, and into the mammary gland itself, then specific motifs associated with pathogens, so-called pathogen associated molecular patterns (PAMPs) are identified by pattern recognition receptors (PRRs) on mammary epithelial cells (MEC) and resident sentinel leukocytes. Toll-like receptors (TLRs) are a family of PPRs that recognise specific bacterial components, for example lipopolysacharide (LPS), peptidoglycan and lipoteichoic acid. Pathogen detection activates signalling cascades which result in the production of cytokines by the host cells (Lahouassa et al., 2007, Bannerman et al., 2004b), some are potent chemoattractants causing the influx of circulating leukocytes into the udder. Blood polymorph nuclear cells (PMNs) rapidly diapedese and enter into the udder in high numbers, this is seen clinically as a rise in somatic cell count (SCC). The key function of PMNs is to identify and remove pathogens by phagocytosis, this is a crucial step in defence of the mammary gland and highly effective against a variety of bacterial species (Hill et al., 1978, Schalm et al., 1976). Circulating monocytes are slower to enter into the udder; they mature into macrophages and also remove pathogens and importantly dead and dying PMNs from the milieu. If the infection persists despite this response T and B lymphocytes and monocytes increase. The role of lymphocytes in the response to mastitis is less well defined. T cell response populations in the non-lactating gland are primarily CD4+ (helper) dominated (Mehrzad et al., 2008, Asai et al., 1998). CD8+ cells become predominant during lactation (Asai et al., 1998) and in early *E. coli* infection (Mehrzad et al., 2008).

The udder also has a host of humoral protective mechanisms including adaptive antibody (Ab) and non-adaptive molecules including xanthine oxidase, lactoferrin, complement and lysosyme, all which may have an antibacterial role against the invading pathogen.

Opsonisation is an important step in early pathogen recognition and the importance of complement and different Ab are not fully defined. IgG_1, important for protection of the neonate, is selectively transferred from the bovine circulation into the udder and is the principal Ab found in normal milk, but has low opsonic activity. Levels of IgG_2 rapidly increase in the udder at the onset of infection, IgG_2 is a potent opsonin and rapidly becomes the

predominant Ab (reviewed Burton and Erskine, 2003). Activated PMNs in the udder have up-regulated expression of pathogen linked IgG_2 F_c receptors, leading to rapid pathogen clearance (Worku et al, 1994; Zang et al, 1995). Ab also binds to bacteria decreases the pathogens' ability to adhere to tissue.

The importance of IgM and complement are less defined. Although complement acts as an opsonin for mastitis pathogens, its actions are inhibited by milk. IgM requires complement activation to augment is opsonic capabilities but some work shows IgM to be a key opsonically active molecule in the udder (Williams and Hill, 1982). The IgM are thought to be primarily "natural antibodies", whose presence is as endogenous Ab and not the result of previous exposure to exogenous stimuli (Rainard and Riollet, 2006).

A multitude of pathogen and host factors determine the success of the immune response. This project aims to focus on the contribution of PMNs to the innate immune response of the bovine mammary gland during infection.

The pathology induced in a case of bacterial mastitis is as much due to the response of the immune system as it is to the pathogen itself. PMNs contain highly cytotoxic granules and via enzymatic degradation and the production of reactive oxygen species have been shown to induce tissue damage (Ledbetter et al., 2001, Zhao and Lacasse, 2008). Further understanding of how the interactions between pathogen and host influence the extent of pathology is required. Immunomodulatory therapies are a novel focus of research (Inumaru, 2010, Wedlock et al., 2008) aimed at limiting the negative effects of mastitis. Knowing which aspects of the immune response are appropriate or over-reactive would aid in targeting the development of potential immunomodulatory therapies which could enhance a specific response or dampen down an over exuberant one (Lauzon et al., 2005).

1.3.3 The Structure and Function of PMNs

PMNs are the principal cell type seen in the classical defined innate immune response, and the term is used interchangeably with neutrophils, although the PMN classification can also include eosinophils and basophils. In the bovine between 15-45 % of circulating leukocytes are PMNs (Kahn, 2005). In other species, they generally have a higher representation; for example in humans PMNs account for 35-80% of circulating leukocytes (Kumar, 1999).

Mature and circulating PMNs have been historically thought of as having very limited transcriptional potential and that their sole function was to phagocytose and kill foreign pathogens. This response was considered non-specific and a rather clumsy but effective first line defence. With the advancement of genome-wide and post-genomic studies this view is changing. The interaction of PMN and pathogen is complex and the response pathogen-specific. PMNs' ability to direct the course of both the innate and adaptive arms of the immune response is now a focus of research (Nathan, 2006, Whale and Griebel, 2009). Activated neutrophils undergo a burst of transcriptional activity of inflammatory related genes after migration to sites of inflammation, influencing other immune cells (Borregaard et al., 2007) and hence the progression of disease and tissue healing. Neutrophils, like specialized antigen presenting cells (APCs), are able to take up MHC class II from apoptotic and necrotic cells and can act as APCs (Whale et al., 2006a), capable of stimulating bovine T cells (Whale et al., 2006b) and dendritic cells in the murine model (Tateda et al., 2001). Clearly, PMNs, through interactions with other cells contribute dynamically to the overall progression and resolution of the inflammatory response. Further understanding of the PMN-pathogen interactions is important in elucidating the pathogenesis of infectious disease.

PMNs are produced in bone marrow in the myelopoietic tissue of the bone marrow. In a quiescent state, they continue to reside in the myeloid tissue for 7-10 days, and then enter the circulation where they are short lived (half-life 8.9 hours) (Carlson and Kaneko, 1975). Some sentinel PMNs enter tissue and, if unstimulated apoptose within 24-48 hours. Their high circulating density allows large numbers to influx into stimulated tissues, with their multi-lobed nuclei affords them the flexibility to move swiftly through endothelial tight junctions.

1.3.4 Mechanisms of Microbial Actions of PMNs

The role of PMNs and their mechanisms of pathogen elimination has been extensively reviewed (Segal, 2005, Paape et al., 2003, Hampton et al., 1998). These key cells identify pathogens via receptor-mediated recognition, engulf them to form a phagosome and then fuse with cytoplasmic granules, exposing the pathogen to their cytotoxic contents (Fig 1).

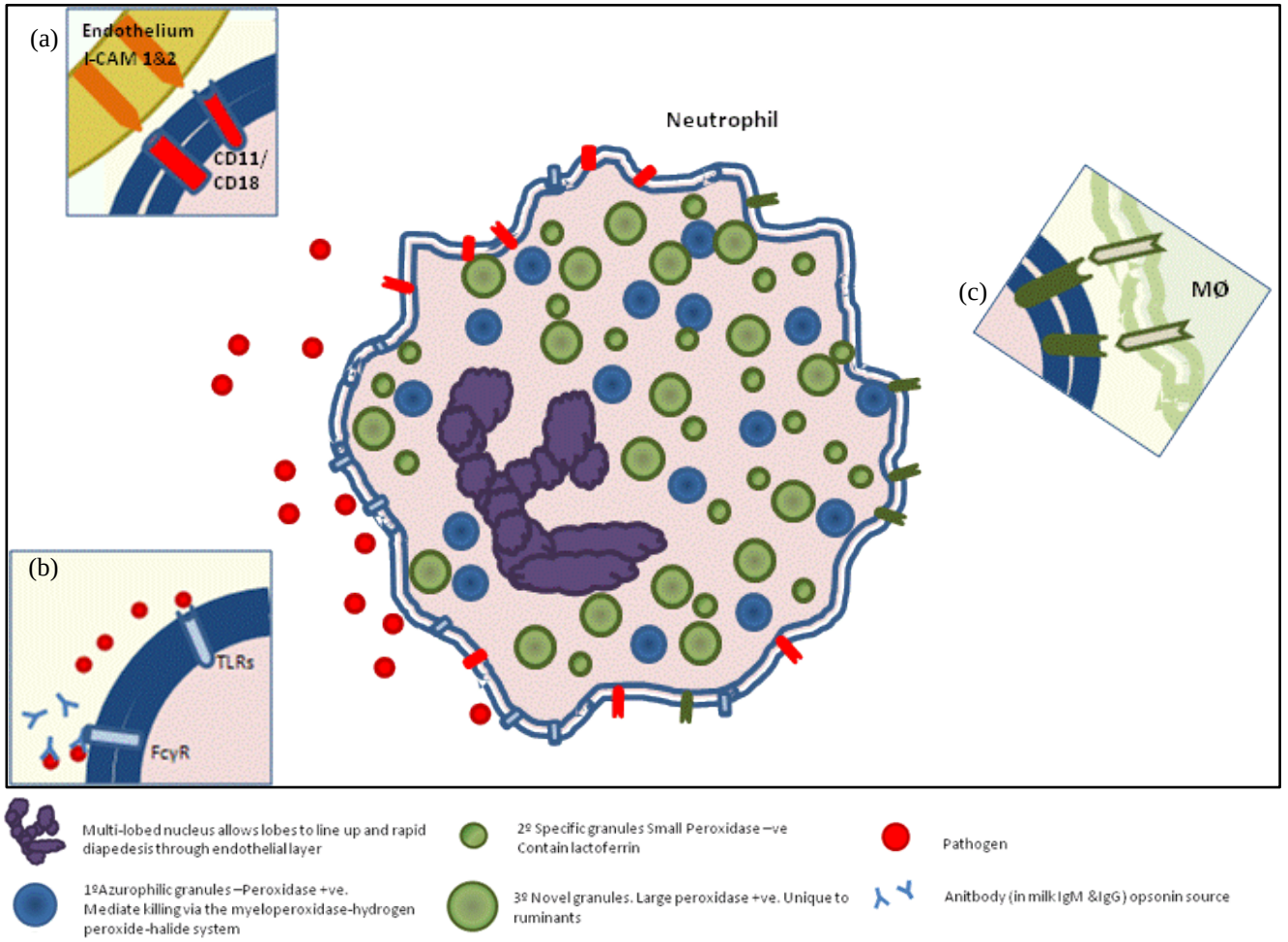
Bovine PMNs contain three types of cytoplasmic granules. Azurophilic granules mediate bacterial killing via the myleoperoxidase-hydrogen peroxide-halide system. Specific (secondary) granules contain lactoferrin and other antimicrobial compounds. Novel granules identified in domesticated ruminants (Gennaro et al., 1983), are the predominant granule seen

in mature bovine neutrophils. Novel granules appear unique to ruminants and are distinct from tertiary granules found in other species, i.e. humans (Dewald et al., 1982). They are the sole store for β defensins and bactenecins – highly cationic proteins; and, like other granules, they also contain lactoferrin.

Human PMNs were discovered to produce Neutrophil Extracellular Traps (NETs), a potent bactericidal web-like structure thrown out by PMNs, after which the cell is dead. NETs consist of chromatin fibres adorned with histones and cytoplasmic granule contents, including elastase, cathepsin G, myleoperoxidase (1º granules) and lactoferrin (2º granules) (Brinkmann et al., 2004). NET formation has now been seen in several animal species including the mouse (Ermert et al., 2009) and cow (Grinberg et al., 2008). The importance of NETs is indicated by studies of human Chronic Granulomatous Disease. Affected individuals, with no, or very low levels, of NADPH oxidase activity (Heyworth et al., 2003) are unable to produce Reactive Oxygen Species (ROS), and work suggests that they are also unable to produce NETs. Supported by other evidence, this suggests that NETs are a sequel to ROS generation. However, in cattle, work has shown that whole milk inhibits ROS production and phagocytic function of PMNs compared to blood isolated cells but not the ability to form NETs (Lippolis et al., 2006).

Fig 1. PMN Structure

Structure of PMN and important receptors. Inset (a) CD11/CD18 cell surface receptors bind to ICAM receptors on the endothelium triggering adhesion and diapedesis. (b) Pathogen recognition mediated by cell surface receptors. Pathogens are subsequently taken up into a phagosome which fuses with cytoplasmic granules and the pathogen is exposed to their cytotoxic contents. (c) Fatigued PMNs express surface receptors identifying themselves for uptake by scavenging macrophages.



1.3.5 PMN Cell Death

The principal mechanisms of cell death are necrosis and apoptosis, although NET formation should probably also be classified as a recognised form of cell death which has been termed by some as "NETosis" (Brinkmann and Zychlinsky, 2007). During apoptosis (programmed cell death) the cell membrane remains intact. This contrasts with necrosis where cell death is mediated via cell lysis. Apoptotic neutrophils attract macrophages, and via the macrophage vitronectin receptor (Savill et al., 1990) are identified and phagocytosed. This promotes resolution of inflammation and tissue healing firstly by preventing the release of the neutrophils' cytotoxic contents, and secondly by leading to up-regulation of anti-inflammatory pathways in the macrophages (Fox et al., 2010). Modulation of PMN apoptosis by the human pathogen *Streptococcus pyogenes* to further its own survival has been recognised (Kobayashi et al., 2003). Human neutrophil functionality (motion, phagocytic ability and chemotaxis) decreases in apoptotic populations. (Whyte et al., 1993).

1.4 *Streptococcus uberis* Pathogenesis

1.4.1 Virulence Determinants of *S. uberis*

S. uberis 0140J, a virulent strain, was originally isolated from a clinical case of bovine mastitis in 1973. Multilocus sequence typing (MLST) has shown that 0140J falls within the ST-5 clonal complex, the major lineage associated with cases of mastitis in the UK (Coffey et al., 2006). Furthermore, it reliably produces clinical disease in both lactating (and non-lactating) animals, making experimental findings pertinent to on-farm disease.

In an established bovine intramammary infection model, despite a large influx of PMNs into the udder, no phagocytosis of *S. uberis* 0140J by PMNs was identified (Thomas et al., 1994) although the bacteria were taken up by macrophages (Grant R.G, 1996). *S. uberis* 0140J inhibited the phagocytic function of bovine PMNs and avoided being taken up (Field et al., 1997). The ability to evade the early host immune response and establish infections is characteristic of streptococcal disease in both humans and animals, and the mechanisms by which this is achieved are varied and not fully elucidated.

S. uberis EF20 is an avirulent field strain isolated from a cow in the late 1970s (J.A. Leigh, personal communication). The animal was reportedly clinically infected but all lactating animals challenged with the strain since reproducibly show only mild or no disease (Hill, 1988).

S. uberis 0145m, and 1095m are mutants generated from 0140J by random insertional mutagenesis and sub1154m is a deletion mutant generated by allelic exchange mutagenesis (Leigh et al 2010). These mutants have disrupted expression of surface proteins associated

with virulence and each shows an attenuated phenotype in lactating cattle, although the precise mechanisms underlying the attenuation in each case are unknown.

The cell surface of bacteria is covered with various protruding macromolecules, the attachment of which to the underlying peptidoglycan wall is facilitated by enzyme attachment. The transamidase SortaseA (SrtA) has been shown to be important in covalently attaching some proteins that may act as virulence determinants (Mazmanian et al., 1999, Lalioui et al., 2005, Comfort and Clubb, 2004) . Following experimental infection the insertional mutant of *S. uberis* 0140J that lacked the SrtA gene initially colonised the bovine udder to a similar bacterial load as the wild type, but was unable to continue to colonise to a greater extent. Infection with the mutant induced a similar SCC, but without clinical signs of mastitis. Ten surface proteins on *S. uberis* 0140J have been identified to be anchored by SrtA (Egan et al., 2010). Individual insertional / deletion mutants in which each of the genes encoding these proteins were disrupted were produced and used in a similar challenge study. Three of these lacking sub0145 – a lactoferrin binding protein, sub1095 - a collagen-like protein and sub1154 – serine protease with limited homology to C5a peptidase, were shown to be required for full expression of virulence (Leigh et al., 2010).

In addition, a mutant of *S. uberis* 0140J lacking a gene regulator protein known as virulence regulator uberis (Vru) has been assessed *in vivo* and shown to cause only very mild signs of disease (Leigh et al., unpublished). Whether the attenuation of any of these strains was due to enhanced clearance through the bactericidal action of PMNs was not investigated.

The human pathogen *Streptococcus pyogenes* (Group A Streptococci, GAS) produces a C5a peptidase which, via complement degradation, limits recruitment of complement activated PMNs, (Ji et al., 1996). It uses this, in addition to other mechanisms (Voyich et al., 2004), to circumvent the immune system and establish an infection. Knowledge of *S. uberis* virulence determinants and their role in pathogenesis may parallel and augment our understanding of other streptococcal diseases.

1.4.2 *S. uberis* in the Cow

Studies into how *S. uberis* isolates behave *in vivo* has focused on various aspects of the immune response, utilizing a diverse range of strains, animal factors and experimental design (Bannerman et al., 2004a, Hill, 1988, Moyes et al., 2009). However, the non-uniformity across studies makes it difficult to compare these and severely limits extrapolation from one study to another.

In experimental infection the strain UT888, reached peak bacterial numbers of 10^5 cfu ml^{-1} 72hr post infection (p.i) in second lactation heifers, 11-38 days in milk, with clinical signs of mastitis observed 12hr after this. In this experiment, concentrations of CXCL8, IL-1β and

TNFα in the whey were measured and shown to have increased significantly by 66 hours p.i. (Rambeaud et al., 2003). By comparison, strain 0140J in lactating heifers reproducibly reaches a peak of 10^7 cfu ml^{-1} at 24hrs p.i., bacterial numbers remain elevated and animals require treatment 3-4days p.i. (Leigh et al., 1990). CXCL8 was shown to peak at 36 and again at 72hr p.i., whilst IL-1 β and TNFα levels continued to increase from 30hr p.i. until the end of the experiment at 7days p.i. (Bannerman et al., 2004a). The variety of bacterial strains, experimental design and the parameters measured prohibits assuming the findings of one study are applicable under slightly different experimental conditions.

1.4.3 Hosts Effects on the Pathogenesis of Mastitis

Anecdotally, the breed of cow is often associated with differences in susceptibility to mastitis. A study by Rambeaud et al., in 2003 used a mixed population of Jersey and Holstein cows, and although it was not designed primarily to compare the breeds, no differences were observed. *In vivo* studies with *S. aureus* and *E. coli* looking for breed differences between Jerseys and Holsteins showed no significant differences in disease progression and cytokine profiles (Bannerman et al., 2008a, Bannerman et al., 2008b). However, in response to *S. aureus* Holsteins showed a higher circulating level of PMNs, though this did not translate into a higher SCC. Body weights are not mentioned, yet given the large body frame of most Holsteins it could be hypothesised that this reflects the greater amount of myelopoietic tissue and therefore larger reservoir of neutrophils which, when stimulated, enter the circulation. In Jerseys cytokine expression appeared slightly prolonged, and although this had no influence on disease it may be associated with longer suppression of milk yield following infection. Despite widely reported differences in breed susceptibility to mastitis (Youngerman et al., 2004, Berry et al., 2007) these data indicate that it may be due to factors other than the hosts' immune response. Together with the fact that the majority of the UK herd is Holstein based this implies that using the Holstein Friesian for future projects is a relevant model.

Within breeds, breeding for trait has also suggested a genetic basis to disease susceptibility. A recent study showed significant transcriptional differences in primary cell cultures, from cells isolated from German Holsteins predicted to be resistant or susceptible to mastitis based on the presence of a QTL associated with mammary health (Gunther et al., 2009).

Feeding dairy cows to meet their nutritional requirements and the metabolic demands of milk production is crucial to maintain their health. Mastitis cases peak in the first 11 weeks of lactation (Biggs, 2009), when animals are metabolically (and in some systems socially) most stressed. Negative energy balance (NEB) is caused by inadequate energy intake due to poor ration formulation, poor access to feed or concurrent disease limiting feeding activity. NEB has been shown to affect the transcriptional expression of TLR receptors and cytokines of neutrophils when challenged with intramammary *S. uberis* (Moyes et al., 2010a) although

clinical differences were not recorded. An unspecified feed additive is reported to also be associated with neutrophil apoptosis regulation (Wang et al., 2009) .

1.4.4 Long term and Systemic Effects of Mastitis

Acute cases of mastitis are often associated with a 5% decrease in milk yield for the whole lactation and in cases which are not cured rapidly, a prolonged infection and decrease in productivity (Seegers et al., 2003). Histological changes in mammary epithelium as a consequence of disease are seen (Zhao and Lacasse, 2008). Six days after inoculation with *S. uberis* 0140J changes consistent with early stage fibrosis were observed (Thomas et al., 1994). Work reviewed recently (Singh et al., 2010) indicates that *E. coli* and *S. uberis* intramammary infections can result in epigenetic, and even genomic, modification. Therefore, even epithelial tissue, although not histologically changed, may be less productive even after the infection has cleared.

The direct effect of the infection and also the often underestimated effect of pain on productivity (Huxley and Whay, 2006) have systemic consequences. Release of acute phase proteins is seen in response to mastitis-affected animals (Horadagoda et al., 1999), including cases due to *S. uberis* (Bannerman et al., 2004a) and sub-clinically infected animals (Gerardi et al., 2009). After intra-mammary challenge with *E. coli*, the liver's transcriptional profile alters (Jiang et al., 2008), supporting the need for a holistic approach when considering the consequences of an intramammary infection.

1.4.5 Microarray Analysis of *S. uberis* Mastitis Models

Multiple cell types are present and involved during infection of the bovine mammary gland. Studies highlighting the transcriptional response of the udder as a whole give an indication of inflammatory pathways involved (Moyes et al., 2009, Moyes et al., 2010a) but do not assess the contribution of specific cell populations. This knowledge is required to enable targeting of specific steps in the infection process to prevent progression of harmful inflammatory paths and encourage resolution of disease. Lactating cows in either a positive or negative energy balance were experimental challenged with *S. uberis* and the transcriptome of udder biopsies (Moyes et al., 2009, Moyes et al., 2010b) and circulating PMNs assessed (Moyes et al., 2010a). This work shows that within the mammary gland genes associated with immune function are differentially expressed in infection.

Comparing the transcriptome of the mammary gland and primary mammary epithelial cell cultures after challenge with *S. uberis* indicated that changes in immune gene expression in mammary epithelium is dependent on the presence of other cell types and their interactions (Swanson et al., 2009). Further insight into how individual cell populations may contribute to the immune response of the bovine mammary gland are required.

1.5 Hypothesis and Project Aims

Given the socio-economic and animal welfare implications of *S. uberis* mastitis within the dairy industry there is currently a major drive to understand the important host factors governing disease onset, disease outcome and resistance to infection. In tandem, work is currently underway to assess cell surface molecules of *S. uberis* as virulence determinants and to investigate subsequent knock-on effects during intra-mammary infection (reduced colonisation, decrease in clinical signs, altered innate immune response).

1.5.1 Hypothesis

The work presented herein seeks to address the hypothesis that the secretome of *S. uberis* strains of differing virulence will affect the survival, bactericidal actions and gene transcription of bovine PMNs.

1.5.2 Project aim

The project undertaken and reported here was focussed at the generation of information relating to changes, at the transcriptional level, in the bovine neutrophil following incubation in milk pre-conditioned by the growth of *S. uberis.* In so doing, I aim to test the hypothesis that factors released during the growth of *S. uberis* cause changes in gene transcription that correlate with the loss of neutrophil function.

2 Methods

2.1 – Preparation of Host Derived Samples

2.1.1 Isolation of Bovine Blood Derived Neutrophils (PMNs)

(See appendix for reagent preparation formulae).

Blood was collected by jugular venopuncture into a tube containing 1.5% EDTA.

Samples were centrifuged at 1,500 x *g* for 15min and the plasma, buffy coat and upper part of the red cell fraction removed. The remaining fraction was weighed and transferred to a conical flask. 10ml of Super-Q filtered water (SQW) was added for every 2.5g of the red cell fraction and agitated for 30sec. A half volume of 2.7% NaCl solution was added and agitated for a further 10sec. The solution was centrifuged at 125 x *g* for 10min and the supernatant discarded. The cell pellet was resuspended in PBS and centrifuged at 125 x *g* for 10min. Two further PBS washes were performed, and after which the cell pellet was resuspended in a small volume of PBS. 20µl of this suspension was mixed with 20µl of Trypan blue and a haemocytometer used to ascertain viability of the cells by exclusion of Trypan blue and their concentration. Cell concentration was adjusted to 1×10^8 cells ml^{-1}.

2.1.2 Assessing Purity of the Isolated Cell Population

The purity and viability of the PMN population isolated by the technique outlined in 2.1.1 was assessed on a Fluorescence Activated Cell Sorting (FACS) machine (FACSCalibur, Becton Dickinson), and the data analyzed using FCS Express Version3 software.

Cell viability was assessed by the exclusion of propidium iodide (PI) stain using the following method. Cells were suspended in PBS, centrifuged for 1min at 1200 x *g*, and the PBS removed. 25µl of 25ng $ml^{-1}$of PI was added to each well, the plate mechanically shaken for 1min, and then left for 10min. 100µl of PBS was added and the plate centrifuged again (1min, 1,200 x *g*) and the PBS removed. The cells were resuspended in 100µl PBS and added to 200 - 500µl PBS in a FACs tube.

2.1.3 Collection and Processing of Aseptic Skimmed Milk

Using current National Milk Record (NMR) data from the Mayfield Farm Dairy unit (IAH), four milking heifers between 21 and 100 days-in-milk, with no recent history of high SCC or disease, were selected for sample collection. Teats were aseptically prepared, the foremilk discarded and 50ml per quarter was collected. Samples were stored on ice during transport, prior to centrifugation at 2,000 x *g* for 10min at 4ºC, and the skimmed milk fraction transferred to new tubes. 500µl of milk from each quarter was plated out onto blood esculin agar, and incubated overnight at 37ºC to check for bacterial contamination. The skimmed milk was filtered twice using 0.45µm filters (Millipore, UK). Samples were combined, dispensed into aliquots and store at -20ºC. These skimmed milk aliquots were used to culture bacteria.

2.2 Preparation of Bacterial Strains

2.2.1 Bacterial Strains

All strains used were supplied by J.A. Leigh (Streptococcal Research Group, University of Nottingham) as 25% glycerol stocks, stored at -80°C. *S. uberis* 0140J and *S. uberis* EF20 are field strains isolated from cows in the 1970s; *S. uberis* 0145m, and 1095m are mutants generated by random insertional mutagenesis and sub1154m is a deletion mutant generated by allelic exchange mutagenesis (Leigh et al 2010).

The coliform *E. coli* P4, originally isolated form a clinical mastitis case and proven to induce mastitis (Bramley, 1976), is known to be susceptible to killing by PMNs. It was used in the bactericidal assays and to assess functionality of PMNs.

2.2.2 Bacterial Growth Conditions

2.2.2.1 Todd Hewitt Broth (THB) cultures

10ml of THB was inoculated from a stock culture of the required bacteria and left at 37ºC overnight. Growth curves of the *S. uberis* strains and *E. coli* P4 in THB were determined. THB was inoculated and cultured overnight as above. 1.5ml of each culture was centrifuged at 10,000 x *g* for 5min and pellet resuspended in 1ml of PBS. The optical density (OD) of each culture was measured in a spectrophotometer (UV-160A, Shimadzu, Shimadzu-Europa, UK) at 550nm and adjusted so that the OD of each was approximately equal to 1 at 550nm. These cultures were then diluted to approximately 100cfu ml^{-1} in THB and the OD measured for each sample in triplicate using the automated Bioscreen C **(**Oy Growth Curves Ab Ltd, Finland), measuring at OD 540nm at 37°C over 16hrs.

The viable population of bacteria in the THB cultures after overnight culture was confirmed, by serial dilution and spotting onto 1% sheep blood esculin agar. This was replicated six times.

2.2.2.2 Milk Cultures of *S. uberis* Strains

1ml of *S. uberis* THB culture was centrifuged (2,400 x *g*, 2min) and the cell pellet washed in PBS and re-centrifuged. 10µl of a 10^{-2} dilution was used to inoculate 10ml of aseptically collected skimmed milk (see 2.1.3) and left overnight at 37ºC. The resulting bacterial cultures are herewith termed "milk cultures".

The viable population of bacteria in the milk cultures after overnight culture was confirmed, by serial dilution and spotting onto 1% sheep blood esculin agar. This was replicated six times.

2.2.2.3 RPMI Culture of 0140J

10ml of RPMI-1640 (Sigma-Aldrich®, Switzerland) in a culture flask was inoculated with 500µl of THB culture and left overnight in a 5% CO_2 incubator at 37ºC.

2.3 Confirming the Identity of *S. uberis* Mutant Strains

In order to verify the identity of each of the *S. uberis* mutants used in these experiments, genomic DNA was extracted from each using a modification of a previously described method (Hill and Leigh, 1989). Polymerase chain reaction (PCR) was then performed comparing the PCR products obtained from the mutants to that from the field isolates, *S. uberis* 0140J and EF20.

2.3.1 Extraction of Genomic DNA from *S. uberis*

Genomic DNA was isolated using a modified, previously described method (Hill and Leigh, 1989). Briefly, bacterial cells were harvested by centrifugation (13,000 x *g*, 5min) from 1.5ml of overnight THB cultures of *S. uberis* strains 0140J, EF20 and *vru*, *srtA*, *sub0145*, *sub1095* and *sub1154* mutant strains. The cell pellets was resuspended in 0.5ml of TE Buffer (10mMTris-Cl, 5mM EDTA, pH7.8), centrifuged (13,000 x *g*, 5min) and the supernatant discarded. The cell pellet was resuspended in 375µl of cell wall disruption buffer (10mM Tris-Cl, 5mM EDTA, pH 7.8, containing 30units ml^{-1}mutanolysin (Sigma, UK) and 10mg ml^{-1} lysozyme (Sigma, UK)) and incubated at 37ºC. After 30min, 20µl of cell lysis buffer (SDS: 20%w/v in 50mM Tris, 20mM EDTA, pH7.8) and 3µl of proteinase K (Sigma, UK) were added and then the tube agitated and incubated at 37ºC for a further hour. Cell wall material was precipitated by the addition of 200µl of saturated salt solution and the tube vigorously agitated for 15sec then centrifuged (12,500 x *g*, 10min) to give a firm cell pellet.

Avoiding transfer of the precipitated protein, 400µl of the supernatant was transferred into a fresh tube and an equal volume of equilibrated phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma) was added. The tube was agitated and centrifuged (13,000 x *g*, 3min). Without disturbing any of the layer at the interface, 400µl of the upper aqueous phase was transferred to a fresh tube and 2volumes of ice-cold absolute ethanol was added to precipitate the DNA. DNA was recovered by centrifugation (13,000 x *g*, 5min) and air-dried for 30min before being resuspended in 40µl of TE buffer (10mM Tris, 1mM EDTA, pH8.0, containing RNAaseA (Sigma, UK) at 20µg ml^{-1}), incubated at 4ºC for 30min then further incubated at 37ºC for 20min.

2.3.2 Polymerase chain reaction (PCR) of Bacterial DNA

PCR amplification was carried out using gene primers that spanned sections of the coding sequence of interest. All reactions were carried out in 25µl aliquots as described below. Genomic DNA was diluted 1/10 in molecular grade water (Sigma, UK) with 1µl used in each reaction along with, 5µl 5×High Fidelity (HF) PCR buffer (New England Biolabs, UK), 2.5µl 2mM dNTP stock, 1.25µl 10mM forward primer, 1.25µl 10mM reverse primer and 13.5µl water. Reactions were placed on ice and 0.5µl Phusion polymerase (New England BioLabs, UK) added. The primers and amplification programs used are shown below in Table 2.1. 5µl of amplified DNA analysed by gel electrophoreses using a 0.8% agarose/TAE buffer gel, with Hyperladder™1 (Bioline, UK) was used as a marker to determine the PCR product size.

Table 2.1 Primers and Amplification Programs used for PCR of bacterial Genomic DNA

Mutant of Interest	Primers	Primer Sequence	Amplification Program
sub0145	Sense P635	262 bp upstream from sub0145 start codon 5'-TGATGTGATAGGGTACTTCAACTTTC-3′	98°C 30sec **x1** 98°C 10sec, 58°C 20 sec, 72°C 45 sec **x35** 72°C 2min30sec **x1**
sub0145	Antisense P636	1913 bp from sub0145 start codon, 365 downstream of stop codon 5'-TGATATGACCGATGAGATGGAC-3′	
sub1095	Sense P645	370 bp upstream of sub1095 start codon, 5'-ATCAAACTATTGTACCCTTTGGTGA-3′	As sub0145
sub1095	Antisense P646	1480 bp downstream of sub1095 start codon, 25bp downstream of stop codon, 5'-TGTAATGAATCTAGGAGGGGTAGGT-3′	
sub1154 - deletion mutant	Sense P615	162 bp upstream of sub1154 start codon, 5'-GAAATGATGATGAGAAATTGAGA-3'	98°C 30sec **x1** 98°C 10sec, 58°C 20 sec, 72°C 1min15sec x**35** 72°C 2min30sec **x1**
sub1154 - deletion mutant	Antisense P410	3557 bp from 1154 start codon, 5'-ATGTCAAAAGCCCGGTACCTTTACAG-3'	
SrtA (sub0881)	Sense P625	135 bp upstream from sub0881 start codon 5'-TGGAATTCTAATAGATACCAAGTGC-3'	98°C 30sec **x1** 98°C 10sec, 58°C 20 sec, 72°C 40sec x**35** 72°C 2min30sec **x1**
SrtA (sub0881)	Antisense P626	5'-CATTTAATTTCATATGGTACCCCTTAAA-3'	
vru (sub0144)	Sense P405	276 bp downstream from vru start codon, 5'-CATCGAAGGGGGTCTCATTTA-3'	98°C 30sec **x1** 98°C 10sec, 58°C 20 sec, 72°C 30sec x**35** 72°C 2min30sec **x1**
vru (sub0144)	Antisense P406	416 bp upstream from vru start codon, 5'-TTGAGAAAGCTGTCCCTGCT-3'	

2.4 Experimental Protocols to Investigate the Interactions of PMNs with *S. uberis* in Milk

2.4.1 Assessing the Susceptibility of *S. uberis* 0140J's to the Bactericidal Action of PMNs.

1 x10^7 of blood isolated PMNs were added to 3ml of 0140J milk culture, and placed on a Coulter Roller (Stuart, SRT9) at 37ºC. 100µl samples were taken at 0, 15, 30 60 and 120min. Serial dilutions performed for each time point, plated out onto blood esculin agar and incubated overnight at 37ºC. This was repeated using PMNs from 6 healthy animals.

2.4.2 RNA Processing to Assess the Effect of Exposure to *S. uberis* 0140J on PMN Transcription

At the time points listed in 2.4.1, 500µl samples were taken and centrifuged at 6,200 x *g* for 3min. The supernatant was discarded and the cell pellet resuspended in 1.5ml of Tris-EDTA buffer (Fluka® Analytical, Sigma Aldrich,UK). Samples were re-centrifuged and the pellet resuspended in 600µl of RLT buffer with 10µl of β-mercaptoethanol added per 1ml of RLT. The RNA was extracted using the RNeasy® Mini Kit (Qiagen, UK) and contaminating DNA removed (DNA-free, Ambion, UK).

2.4.3 Determining PMNs Ability to Kill Susceptible Bacteria

E coli P4 is known to be susceptible to the bactericidal actions of healthy PMNs. To confirm that the isolated PMNs from each animal were functional, a 3ml aliquot of milk, of the same stock as used to culture strains of *S. uberis* (as detailed in 2.4.1) was inoculated with 10^4 *E. coli* in 100µl of PBS. 1 x10^7 of blood-derived PMNs was then added, and placed on a Coulter Roller at 37ºC. 100µl samples were taken at 0, 15, 30 60 and 120min. Serial dilutions performed for each time point, plated out onto blood esculin agar and incubated overnight at 37ºC. .

2.4.4 Determining the Susceptibility of *S. uberis* EF20 and 0145m, 1095m, and 1154m to the Bactericidal Activity of PMNs

The procedures outlined in 2.4.1 was repeated using milk cultures of *S. uberis* strains EF20, 0145m, 1095m and 1154m.

2.5 Experimental Protocols to Investigate the Interactions of PMNs with the Supernatants from Cultures of *S. uberis* 0140J

The inhibitory effect of *S. uberis* 0140J on bovine PMNs also occurs if the cells are exposed to the supernatant of *S. uberis* 0140J milk cultures (Field et al., 1997), indicating that the effector molecule mediating the inhibition is released from the bacteria into the surrounding milieu and does not require the continued presence of the bacteria themselves.

2.5.1 Generating *S. uberis* Culture Supernatants

S. uberis milk cultures were prepared as described in 2.2.2.2. The milk was then passed twice through a 0.45µm filter (Millipore, UK) in aseptic conditions to remove the bacteria. A sample of the culture was serially diluted for assessment of bacterial load pre-filtering, and a post-filtering 500µl sample plated out onto blood esculin agar to confirm that the supernatant contained no bacteria. Supernatants were either used fresh or stored as aliquots (3ml) at -80ºC, until required. Aliquots of the same milk used to culture *S. uberis* were stored under identical conditions to ensure the equivalent control was used throughout further experiments.

S. uberis RPMI cultures were prepared as described in 2.2.2.3. RPMI supernatants were generated and stored as detailed above for the milk supernatants.

2.5.2 Assessing the Effect of Fresh and Frozen 0140J Culture Supernatants on the Bactericidal Activity of PMNs

The ability of 0140J culture supernatants to inhibit the bactericidal function of PMNs was assessed. In addition, the effect of freezing the supernatant was investigated. Freshly prepared supernatant was aliquoted into 900µl vials, and half of the samples frozen at -80ºC; and subsequently thawed at room temperature 30min later. Once all samples were at room temperature, 100µl of a 1 x 10^8 cells ml^{-1} PMN suspension was added to vials containing 900µl of milk, fresh milk supernatant (MS) or frozen milk supernatant (FMS) that had been thawed.

Samples were then placed on a Coulter Roller at 37ºC and incubated for 2hr. From each sample a 200µl aliquot was taken and used in a bactericidal assay. This was added to a vial containing 100µl 30%v/v complement-deactivated serum, as a source of opsonin, and 90µl of PBS. Finally, 30µl of $10^6 ml^{-1}$ *E. coli* P4 culture was added and the vials placed on a Coulter Roller at 37ºC for 90min. Bacterial serial dilution counts were performed using 100µl samples taken at 0 and 90min.

This was replicated using PMNs isolated from 6 different animals. The reduction in the *E.coli* populations in the presence of the differently treated PMNs was analysed by a one-way ANOVA with Tukey post test, using GraphPad Prism 4 software.

In parallel, the experiment was performed using RPMI, fresh RPMI supernatant and frozen RPMI supernatant.

2.5.3 Processing PMN-derived RNA

The remaining fraction containing $8x10^6$ PMNs from the incubation stage of 2.5.2 was collected in microfuge tubes and centrifuged at 6,200 x *g* for 1min at room temperature. The supernatant was removed and stored at -20ºC for further analysis (2.5.8). The cell pellet was resuspended in 1.5ml of TE buffer (10mM-Tris-HCl-1 EDTA pH 7.6), centrifuged at 6,200 x *g* for 1min and the supernatant discarded. Cells were then lysed by resuspending the pellet in 600µl buffer RLT (Qiagen RNeasy kit) with 10µl of β-mercaptoethanol added per every 1ml of buffer RLT. The RNA was isolated using the RNeasy® Mini Kit (Qiagen) and contaminating DNA removed (DNA-free, Ambion, UK).

2.5.4 Amplification of RNA for Microarray Analysis

Using the Amino Allyl MessageAmp™ II-96 Kit (Ambion®), 200ng of the RNA was amplified, according to the manufacturer's instructions, and including the optional second round amplification steps. The amplified RNA, 2.5µg of each sample, was labelled separately with Alexa Fluor® 555 or Alexa Fluor® 647 fluorescent probes. For each animal the labelled RNA from milk-treated PMNs was combined with oppositely labelled RNA from 0140J milk culture supernatant-treated PMNs. Samples were fragmented (RNA Fragmentation Reagents, Ambion, Texas, USA) and diluted in hybridization solution (SlideHyb™ Glass Array Hybridization Survey Kit and buffers, Ambion, Texas, USA).

2.5.5 RNA Hybridization for Microarray Analysis

BloPlus (Bovine Long Oligo Plus Microarray) (CAFG, Michigan State University, USA) contains approximately 8400 70mer oligos printed in duplicate, with numerous positive and negative control spots.

Pre-processing, hybridisation and washing of microarrays were performed in a Tecan HS400™ Pro Hybridization Station (Tecan UK Ltd, Reading, UK). Slides were pre-processed at 23°C with sodium dodecyl sulfate (SDS) 1% (SDS 10%, Gibco ultrapure, Invitrogen) for 2min, then with deionised water for 2min and dried at 30°C for 2min. The hybridization programme commenced with a wash step for the slides with medium stringency buffer at 45°C for 1min. Samples were then injected and automatically agitated. Hybridization at 45°C, with medium agitation and under high viscosity conditions was performed for 6hr. The hybridization step was repeated at 40°C then at 35°C. Slides were then washed with medium stringency buffer for a rinse step of 20sec and a soak time of 30sec for 5 cycles at 45°C, then with high stringency buffer for 5 cycles at 40°C, followed by 5 cycles at 40°C with the post wash buffer. Microarrays were dried for 2min at 30°C with nitrogen. See Appendix 1 for buffer recipes.

2.5.6 Microarray Analysis

The hybridised slides were scanned using an Axon GenePix 4000A microarray scanner (Molecular Devices Ltd., Wokingham, UK). Spots were manually checked using Axon GenePix Pro 6.0 software (Molecular Devices Ltd.). Limma (Smyth, 2004) within the R-project statistical package (Copyright, R-Foundation) was used to statistically analyse the data. All slides were normalized by Print-Tip Loess Normalization. Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis of all differentially expressed genes with a p-value of < 0.05 was performed in order to identify the genes of interest. Data were analyzed through the use of Ingenuity Pathways Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com).

As information regarding bovine genes is limited compared to human genes, human homologues to identified bovine genes were used as the input data set where possible. Pathways in the results section were generated by cross-referencing between IPA pathways and KEGG pathways (Kyoto Encyclopaedia of Genes and Genomes: *www.genome.jp/kegg/*).

2.5.7 Quantifying the Proportion of Apoptotic Cells in Isolated Cell Populations after Incubation in Milk or *S. uberis* 0140J Milk Supernatant

PMNs were incubated for 2hr in aliquots of milk and frozen ***S****. uberis* 0140J milk supernatant (see section 2.4.1),following the method outlined in 2.4.2, The cell pellet was isolated by centrifugation (4,000 x *g*, 2min) and washed twice in PBS. To block the Fc Receptor binding Annexin V, 1 $x10^6$ cells of each sample were resuspended in 100µl of mouse serum (Sigma) for 10min, centrifuged (1,000 x *g*, 2min) and the serum discarded. Cells were then stained with the Aposcreen™ Annexin V Apoptosis Kit (SouthernBiotech, USA), following manufacturer's protocol, and analysed by FACs.

2.5.8 Real Time Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (q-PCR) was used to assess levels of transcript in PMNs that had been exposed to milk or the supernatant of *S. uberis* 0140J milk culture as a means to indentify relevant time points from which microarray studies could be performed. In addition, q-PCR was used to validate up/down regulated genes identified from microarray studies.

2.5.8.1 Generation of PMN cDNA

In each case 100ng RNA was reverse transcribed using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, UK) according to the manufacturer's instructions. This was used as template for subsequent Q-PCR reactions.

2.5.8.2 qPCR Probes and Primers

Primers and probes for GAPDH (Glyceraldehyde 3-phosphate dehydrogenase.) (Collins et al., 1999), CXCL8 (Widdison et al., 2008), IL-6, IL-10 and IL-12p40 (Werling et al., 2002) have been previously reported. Additional primers and probes (Table 2.2), identified from microarray studies and similar projects carried out within the Bovine Genomics group, were designed using GenScript Real-time PCR (TaqMan) Primer Design tool (available at www.genscript.com). Where available primers and probes were chosen to cross intron:exon boundaries.

2.5.8.3 qPCR Protocol

Complementary DNA (100ng) was used as the starting material for qPCR with samples being assayed in triplicate. Primers and probes were mixed with the appropriate volume of TaqMan FAST Universal PCR Mastermix (Applied Biosystems, Warrington, UK) and applied to the wells before initiating the ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems). The amplification program used applied and initial denaturation step at 95°C for 20 seconds followed by 40 cycles at 95°C for 3 seconds and 60°C for 30 seconds. All results were quantified against plasmid standard curves. For each target gene, mRNA levels were normalised to the housekeeping genes (HK) according to the formula (Copy No. Target/Copy No.HK). Constitutive expression of GAPDH, β-actin and boRPL2 was assessed to validate their potential use as HK genes.

Table 2.2 qPCR Primer and Probe Sequences

ID	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5'FAM-3'TAMRA)
β-actin	GAGGCTCTCTTCCAGCCT	GAAGGTAGTTTCGTGAATGCC	TTCCTGGGCATGGAAT
RPLP0	GCACAATTGAAATCCTGAGTG	GGGTTGTAGATGCTGCCATT	AGCGAAGCCACGCTGCTGAA
RPLP2	TCAACAAGGTCATCAGTGAGC	CCGATACCCTGAGCAATGA	CGTCCTCGATGTTCTT
CCL20	TCAGAAGCAAGCACTTCGAC	GATGTCACAGGCTTCATTGG	CCAGCTGCTGTGTGAAGCCCA
COX-2	CTAACGACAAAGCTGACCCA	GAATTCGTAAAGAAGGAAGAGCA	CTGAACCATTCTGCCCGCCA
NFKB1A	TGCACTTAGCCATCATCCAT	GCGATTTCTGGCTGGTTAGT	CCTGCAGCAGACGCCACTCC

2.5.9 CXCL8 and IL12β Enzyme-Linked ImmunoSorbent Assay

Enzyme Linked ImmunoSorbent Assays (ELISAs) for CXCL-8 and IL12β ELISAs were performed on the supernatants of MT and ST PMNs (see 2.5.3) to reveal whether up-regulation of CXCL8 and IL12β at the transcript level equates to an increase in protein production.

Capture mAb, MAB208 (R&D Systems, UK) and CC301 (IAH,UK), were diluted in 50mM carbonate/bicarbonate buffer pH 9.5, applied to the wells of 96-well Maxisorp plates (Thermo Scientific, UK) and incubated overnight at room temperature (RT). Following five washes with PBS Tween20 (0.05% v/v), plates were blocked by the addition of 300µl/well of blocking buffer (1mg/ml casein in PBS) and the plate incubated at RT for 1hr. After washing, 90µl/well of supernatant from the MT and ST incubations was added to wells, in duplicate, as well as 90µl of standards (100 $ngml^{-1}$, four further 1:3 dilutions in blocking buffer and a negative control).

Samples were incubated at RT for 1hr, washed and 100µl/ well of biotinylated detection antibody diluted in blocking buffer applied to each well (IL-8; BAF-208, IL-12; CC326B). Samples were incubated at RT for 1hr and the plate washed as previously described. Biotinylated mAb was detected by the application of 100µl/well Streptavidin-HRP solution (Amersham-Pharmacia, UK) (in PBS, Tween 20 0.05% v/v). Following a final incubation of 45 min and subsequent washing, bound streptavidin-HRP was detected using Super Signal® ELISA Femto Solution (Thermo Scientific, UK) and the plate was read immediately using a flurometer (FLUOstar Optima, BMG Labtech, UK). Following construction of a standard curve, concentrations of chemokines in supernatants was evaluated.

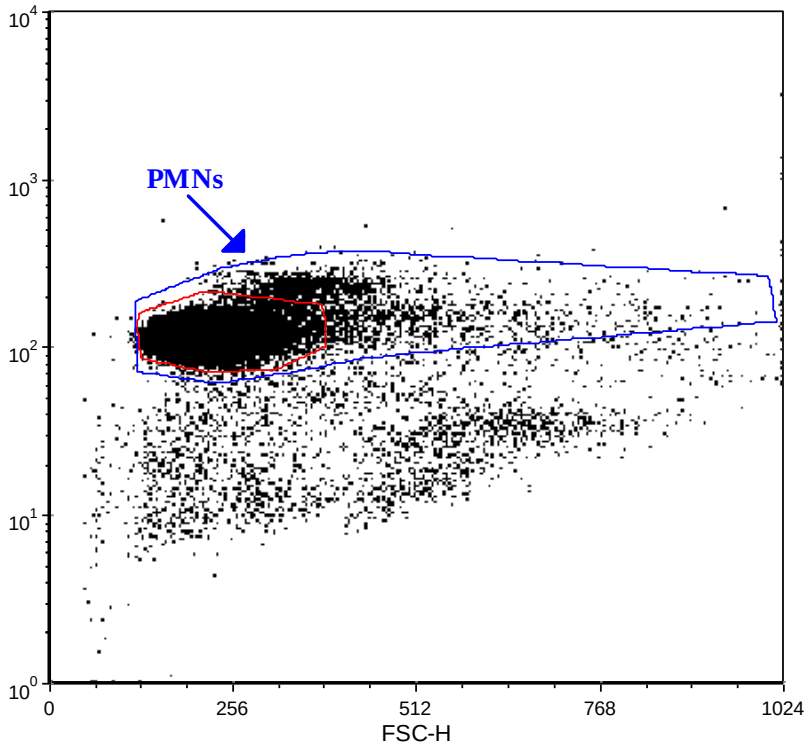
3 Results

3.1 PMN Population Purity

The hypotheses this project tests are based on data derived from experiments in which PMNs have been isolated as described in section 2.1.1.. The population of cells isolated from four animals that also provided the cells for RNA extraction and functional assays were assessed for composition and viability by Fluorescence Activated Cell Sorting (FACS) analysis. Of the cells in the population, 94.8% (SD 0.83) were PMNs; of this population 99.3% of the cells were propidium iodide –ve (P.I.-ve)(n=4; SD=0.74). Hereafter, the isolated population will be known as PMNs throughout this work.

Fig 3.1 FACs Plot of Cell Population Isolated from Whole Blood

Using the Methods described in section 2.1.1 a blood-derived PMN were isolated from 4 animals. FACs analysis revealed a purity of 94.8% (SD 0.83). Within the PMN population (blue arrow and outline) 96.7% (SD 0.43) were within one subset (red outlined area). Small populations (1.8% (SD 0.15) and 1.5% (SD 0.43)) appeared either, slightly larger and more granular - higher side scatter levels (y axis) or larger - higher forward scatter levels (x axis).



3.2 Bacterial Growth

To adequately compare different strains of *S. uberis* 0140J it is essential to know if they behave similarly in growth media, and attain similar population levels in the stationary phase.

3.2.1 Growth of Different Strains of *S. uberis* and *E. coli* P4 in THB

Growth of the different bacterial strains over 16hrs in THB was assessed using the Bioscreen C automated system. *S. uberis* strains grew to a similar optical density, although strain EF20, and the mutants Vru and SrtA grew to slightly higher optical densities than the wild type 0140J and the sub0145, sub1095 and sub1154 mutants. *S. uberis* strains 0140J, EF20, and the mutant Vru attained viable counts of approximately 5 x 10^7 cfu ml^{-1} after overnight incubation of THB cultures at 37°C in glass universal vials. Mutant strains SrtA , sub0145 and sub1095 grew to a slightly lower level with the latter only attaining 6 x 10^6 cfu ml^{-1}. The mutant sub1154 appeared to grow slightly better than the others tested reaching 8 x 10^7 cfu ml^{-1}. The Bioscreen revealed that the mutant with a lesion in sub1095 showed a greater lag and grew at a slightly slower rate than the other strains. *E.coli* P4 grew to a higher magnitude, to a viable count of approximately 1 x 10^9 cfu ml^{-1}. (Fig 3.2 (a) and (b)).

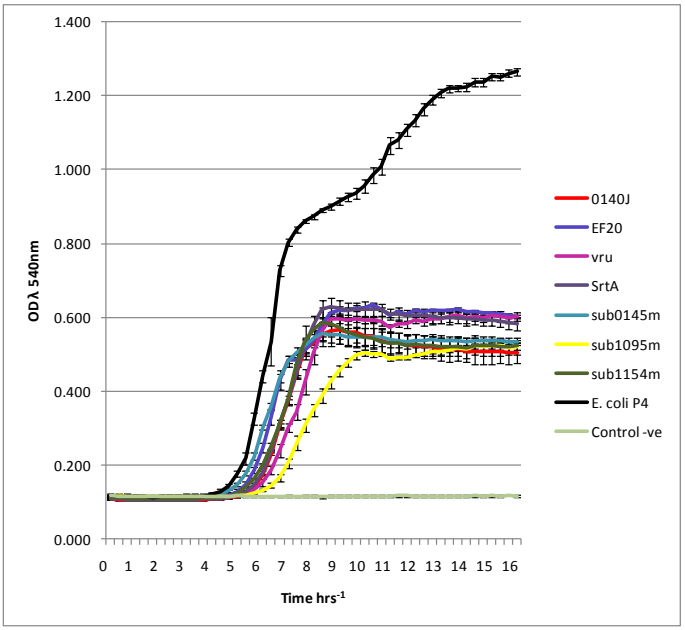
3.2.2 Growth of *S. uberis* Strains in Overnight Milk Cultures

As described in section 2.2.2.2 *S. uberis* strains were cultured in milk. *S. uberis* strains 0140J, EF20 and mutants Vru and SrtA attained viable counts of 1x10^8 cfu ml^{-1} after overnight incubation in milk at 37°C in glass universal vials. Mutant strains sub0145, sub1095 and sub1154 reached 1 -3 x 10^7 cfu ml^{-1}. (Fig 3.3).

Fig 3.2 Growth Curve of Different Strains of *S. uberis* and *E. coli* P4 in THB

Growth of strains of *S. uberis and E. coli* P4 in THB at 37ºC was assessed in triplicate by both **(a)** optical density; a 16hr growth curve determined using the automated Bioscreen C and **(b)** viability; counts performed by serial dilution and spotting onto 1% sheep blood esculin agar plates after growth in glass vials overnight. Plots show mean values, error bars represent standard deviation (SD).

(a)



(b)

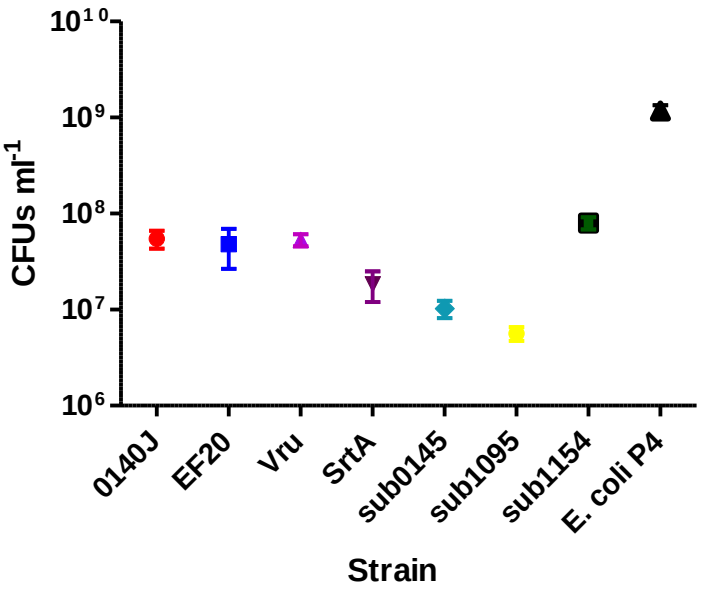
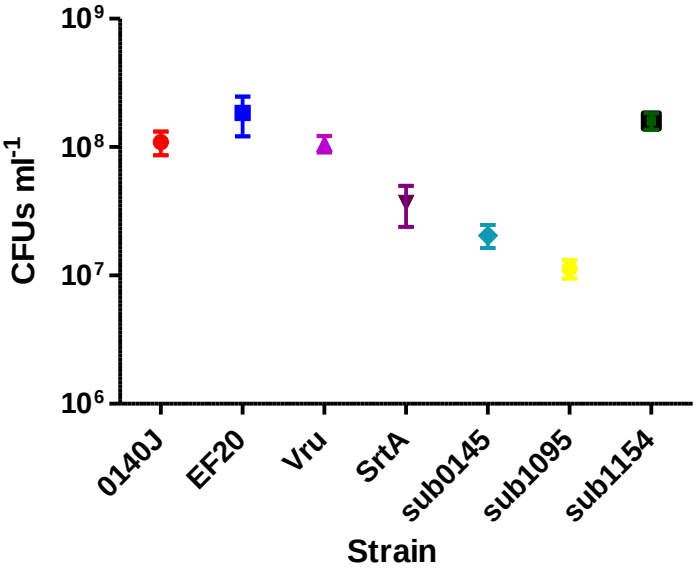


Fig 3.3 Growth of *S. uberis* Strains in Overnight Milk Cultures

Growth of strains of *S. uberis and E. coli* P4 in milk at 37ºC was assessed by viability counts performed by serial dilution and spotting onto 1% sheep blood esculin agar plates after inoculation of 10ml of milk with approx 10^4 bacteria in triplicate and incubation in glass vials overnight. One-way ANOVA revealed no significant differencs between growth rates of strains. Plot shows mean values, error bars represent SD.



3.2.3 Confirmation of the identity of Mutants of *S. uberis* 0140J by PCR Amplification

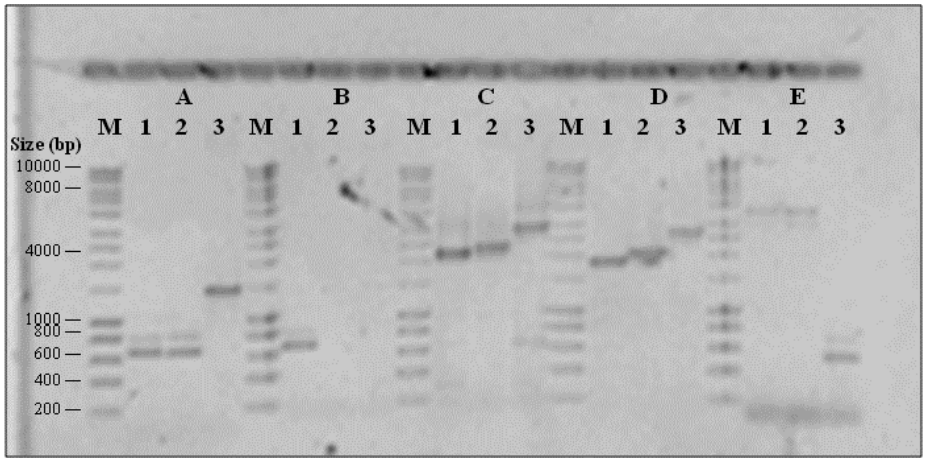
To confirm to identity of the mutant strains, genomic DNA was isolated and polymerase chain reaction (PCR) carried out (See Fig 3.4).

The mutant strains of Vru (lane A,3),SrtA (lane B,3 – not easily visibly), sub0145 (lane C,3) and sub1095 (lane D,3) each have an 800bp insertion within the gene of interest compared to wild type 0140J (lanes 1), confirming disruption of the gene via plasmid insertion (Vru (Ward et al., 2003); SrtA, sub0145, sub1095 (Leigh et al., 2010)). The mutation in the *sub1154* gene (lane E,3) was produced by allelic exchange, removing 3700bp of the gene (Leigh et al., 2010). This is confirmed by the production of an amplicon of approximately 500bp.

Comparison of the avirulent wild type *S. uberis* EF20 (lanes 2) to the virulent 0140J, shows gene products of identical size for the *vru* (A) and *sub1154* genes (E). The gene products of *S. uberis* EF20 for Sub0145(C), sub1095 (D) are slightly larger compared to 0140J. The genome sequence of *S. uberis* EF20 to date is unpublished and small differences in PCR product sizes most likely reflect coding but not necessarily functional differences in these genes (P.N. Ward, unpublished data). For SrtA (B,2) no band is seen for *S. uberis* EF20. This may reflect the mismatch of 5 bp compared to the antisense primer, which under these annealing conditions, could be sufficient to prevent amplification of the DNA (P.N. Ward, unpublished data).

Fig 3.4 Confirmation of Individual *S.uberis* 0140J Mutants by Polymerase Chain Reaction.

PCR products from primer sequences for the following genes are shown with *S. uberis* 0140J and *S. uberis* EF20 strain products in lanes 1 and 2 respectively and the product form each individual mutant in lane 3. Amplification of specific genes was as follows; **A**: vru, **B**: SrtA, **C**: sub1045 **D**: sub1095 and **E**: sub1154. Lane M indicates the DNA marker Hyperladder, with all sizes represented in base pairs (bp).



3.3 Interaction of PMNs with Different Strains of *S. uberis* in Milk Culture

To explore the development of an *in vitro* model to investigate the early interactions between PMN and pathogen different strains of *S. uberis* were cultured in milk overnight and blood-derived PMNs added.

3.3.1 Evaluation of the Susceptibility of Different Strains of *S. uberis* to the Bactericidal Actions of PMNs

The field isolated *S. uberis* EF20 and the mutants Vru, SrtA, sub0145, sub1095 and sub1154 all show reduced virulence in the host compared to *S. uberis* 0140J. To ascertain if this attenuation is due to susceptibility to the bactericidal actions of PMNs, blood-derived PMNs were added to milk cultures of *S. uberis* strains. Bacterial counts were performed over 2hrs (Fig 3.5 (a)). SrtA was not tested. In parallel, an aliquot of the same skimmed-milk was inoculated with *E. coli* P4 to achieve an equivalent population as for *S. uberis* cultures, PMNs were then added. No significant changes in the populations of the different strains of *S. uberis* were seen. The *E. coli* P4 population was reduced to less than 1% of the original level (Fig 3.5 (b); a level significantly different from the populations of *S. uberis* 0140J, EF20, Vru, and sub 1154 ($p<0.01$**).

Fig 3.5 (a) Bacterial Counts of Milk Cultures, in the Presence of PMNs

PMNs were added to overnight milk cultures of different *S. uberis* strains and milk into which an equivalent number of *E. coli* P4 had been added. Bacterial viability counts performed by serial dilution and spotting 1% sheep blood esculin agar plates were carried out at 0, 30, 60 and 120min.
n=6, graph show mean values, error bars represent SD

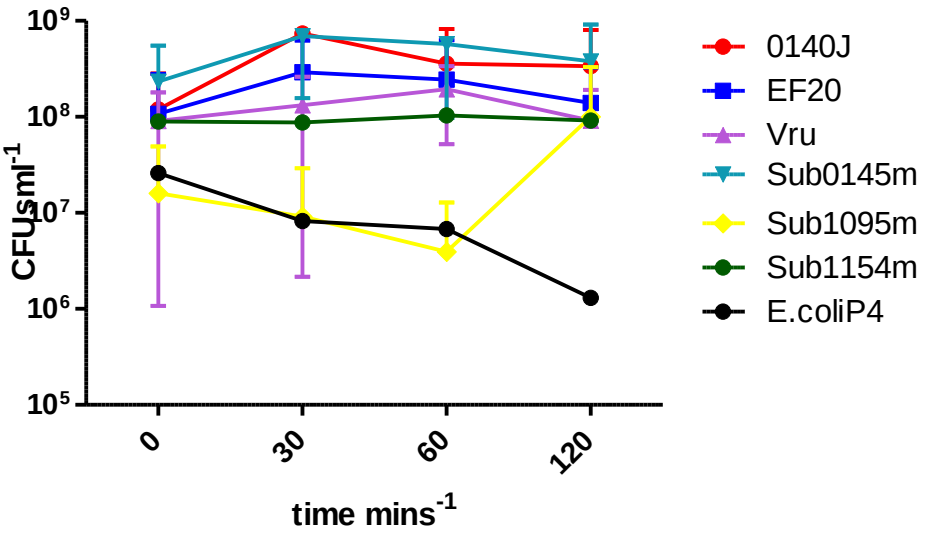
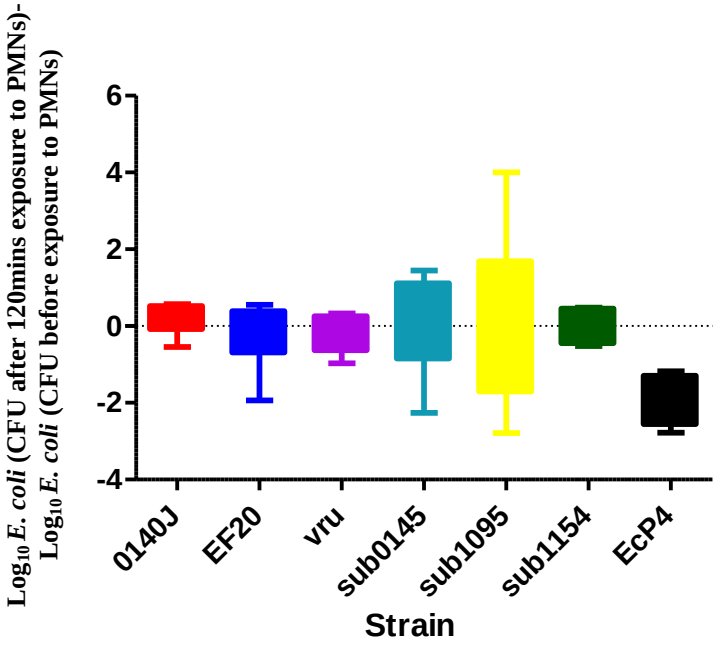


Fig 3.5 (b) Log_{10} Survival of Bacterial Strains in Milk in the Presence of PMNs

One-way ANOVA analysis of bacterial survival over the 120min incubation period with PMN, revealed *E.coli* P4 population was significantly different $p<0.01$ to those of *S. uberis* strains 0140J, EF20, Vru, sub1154. *S. uberis* population were not significantly changed by the presence of PMNs.



3.4 The Effect of Supernatants from Cultures of *S. uberis* 0140J on PMNs

To investigate the effects of the soluble factors released by *S. uberis* on PMN function experiments exposing PMNs to the supernatants of bacterial cultures were performed. This enables the distinction of effects mediated by the products of *S. uberis* rather than its presence.

3.4.1 Inhibition of the Bactericidal Activity of PMNs by Incubation in the Supernatant of *S. uberis* 0140J Milk Cultures. Freezing at -80°C does not Negate its Inhibitory Effects

To standardise experimental conditions over time, the use of the same bacterial-derived inhibitory media on different days would be advantageous. Capsular extract from *S. uberis* cultures has previously been shown to inhibit PMNs phagocytic actions. To corroborate these findings and investigate if storing the culture supernatants at -80°C affects their inhibitory effect, PMNs were incubated for 2hr in milk ("milk treated" (MT), fresh *S. uberis* 0140J milk culture supernatant ("supernatant treated" (ST)) and *S. uberis* 0140J milk culture supernatant that had been frozen at -80°C and thawed before use ("Frozen supernatant treated" (FST)). The PMNs were then isolated and their effect over 2hr on a population of *E. coli* P4 measured (Fig 3.6). MT PMNs reduced the *E. coli* P4 population by 3.5 $\log_{10}$. This reduction was significantly ($p<0.01$**) greater than that detected in ST and FST PMNs. Freezing the milk supernatant prior to use had no effect on its inhibitory activity towards bovine PMN.

3.4.2 Bactericidal Activity of PMNs is not Significantly Affected by Incubation in the Supernatant of *S. uberis* 0140J Grown in RPMI

It is not known whether *S. uberis* 0140J cultured in media other than milk produces the soluble factor(s) that cause the inhibition of the PMNs bactericidal actions. Milk-derived media will inherently vary in composition continuously. A more robust model using a standardised, commercially available media would be preferential; RPMI is widely used worldwide to culture mammalian cells including PMNs. Although PMNs incubated in RPMI showed greater bactericidal action than PMNs incubated in either the fresh or frozen *S. uberis* 0140J RPMI culture supernatant, this difference was not significant (Fig 3.7).

Fig 3.6 *E. coli* P4 survival in the presence of PMNs incubated in milk & in *S. uberis* 0140J milk culture supernatant

The ability of PMNs to kill *E. coli* P4 after incubation for 2hr in milk (MT), supernatant from *S. uberis* 0140J milk culture (ST) and supernatant from *S. uberis* 0140J milk culture that had been stored at -80ºC then thawed (FST) was assessed. Box and whisker plots are shown, box represents interquartile range; error bars indicate maximum and minimum values. n=6. The *E. coli* population in the presence of MT PMNs was reduced significantly different level compared to *E. coli* P4 in the presence of ST and FST PMNs. There was no statistical difference between the reduction of the *E. coli* populations in the presence of ST and FST PMNs.

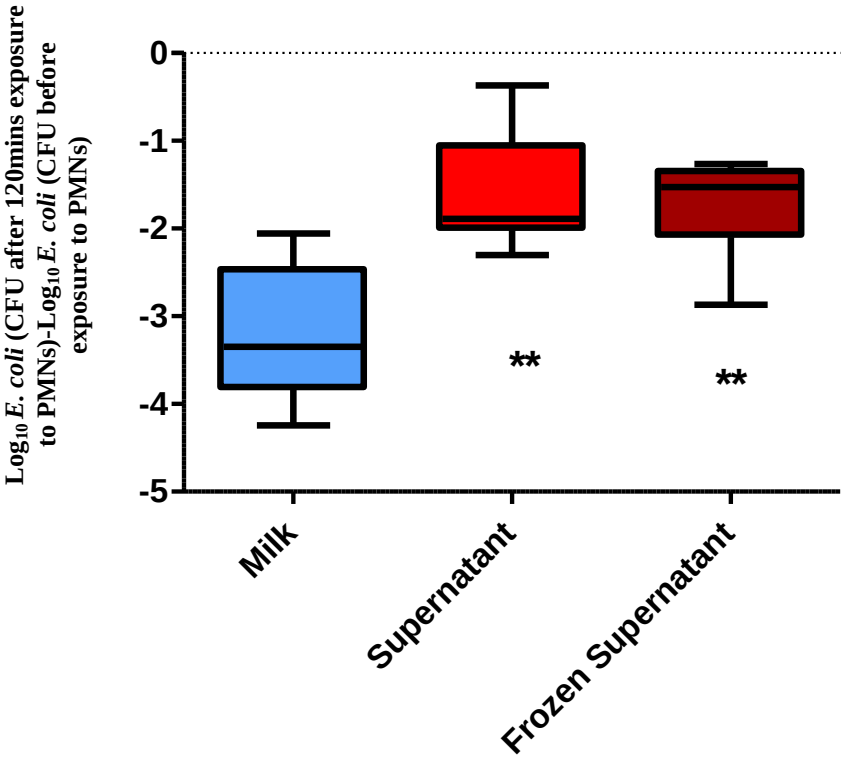
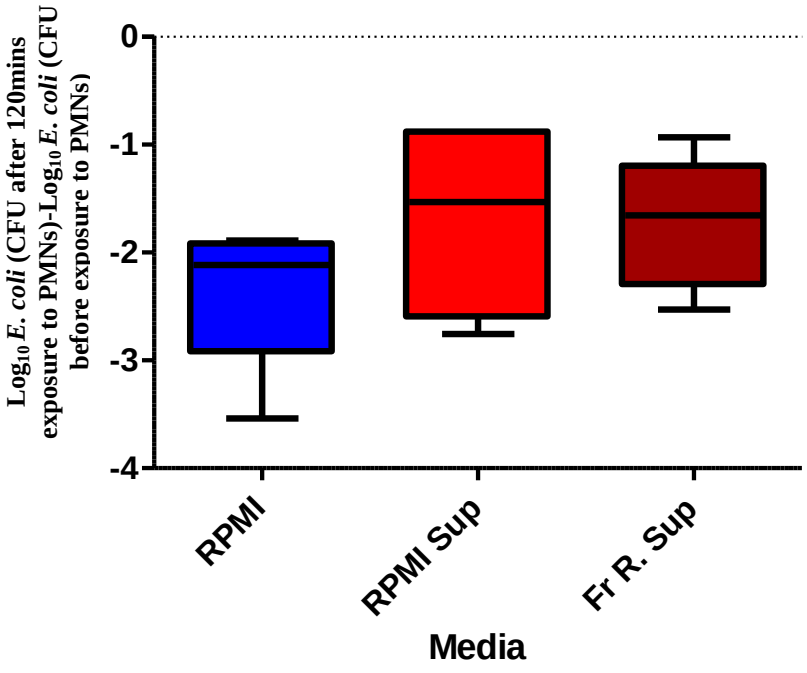


Fig 3.7 *E.coli* P4 Survival in the Presence of PMNs Incubated in RPMI or *S. uberis* 0140J RPMI Culture Supernatant

The ability of PMNs to kill *E. coli* P4 after incubation for 2hr in RPMI, supernatant from *S. uberis* 0140J RPMI culture and supernatant from *S. uberis* 0140J RPMI culture that had been stored at -80ºC then thawed was assessed. Box and whisker plots, box represents interquartile range; error bars indicate maximum and minimum values. n=6. No statistical differences in the reduction of the *E. coli* populations were seen.



3.4.3 Differences in the Transcriptome of PMNs Incubated in *S. uberis* 0140J Milk Culture Supernatant from that of PMNs Incubated in Milk and the Functions of the Differentially Expressed Genes

RNA from MT and ST PMNs was amplified and used to perform a dye-swap microarray experiment. The data was captured using an Axon GenePix Scanner with Gene Pix Pro 6.0.

The results were then analyzed using R which identified that of the 8400 genes represented on the BloPlus Microarray, 328 were differentially expressed in the ST PMNs compared to MT PMNs, having a p-value of < 0.05. These were termed the differentially expressed genes (DEG). For each DEG the human homologues was identified and the online IPA Ingenuity software used to identify associations between them. When the adjusted p-values were calculated (0.99 for all DEG) these indicated that with the present data set no results can be classed as statistically significant.

Tables 3.2 and 3.3 shows the top DEGs in ST PMNs relative to the expression levels in MT PMNs. Table 3.2 shows the top 15 up-regulated DEGs and Table 3.3 the top 15 down-regulated DEGs. Of these up-regulated genes, 14 are known to be involved in inflammatory pathways or intracellular function, the function of the remaining one is unknown. Of the down-regulated genes, three are known to occur in apoptotic pathways, while others relate to cell membrane and cytoplasmic structure and sequestration of metal ions.

IPA Networks are molecular maps representing proven molecular relationships allowing *in silico* combination of established pathways. IPA identified networks in which the DEG are represented, this is a basis of directing further analysis at the metabolic area of relevance; Associated Network Functions (Table 3.4 (a)), Diseases and Disorders (Table 3.4(b)) and Molecular and Cellular Functions (Table 3.4(c)). Although more applicable to a whole tissue model, this data is a useful way of analysing the results obtained, and has given an indication of the networks and pathways affected in the ST PMNs.

Inflammatory and apoptotic pathways were identified by the IPA analysis as key areas of interest. Using the software, lists of DEGs associated with these pathways were generated (Tables 3.5 and 3.6.) and a speculative pathway designed. These are represented graphically in Fig 3.8 and 3.9.

Table 3.2 Top 15 Differentially Up-regulated Genes in PMNs Incubated in *S. uberis* 0140J Supernatant Compared to PMNs Incubated in Milk and their known Functions.

Fold Change and Log Ratio shown.

Molecule	Log Ratio	Fold Change	Also Known As	Function
SUPT3H	2.05	4.15	Suppressor of Ty 3 homolog/ SPT3 homolog	Predicted: Transcription initiation protein
PYROXD2	1.80	3.47	Pyridine nucleotide-disulphide oxidoreductase domain 2	A FAD-dependent pyridine nucleotide-disulphide oxidoreductase, most likely involved in Respiratory Burst Pathways
PTGS2	1.73	3.31	COX-2	Inducible catalyst of the rate-limiting step of prostaglandin production. Major mediator of inflammation
ATP2B1	1.60	3.03	plasma membrane Ca ATPase	Critical role in intracellular calcium homeostasis, Mg dependent
IL12B	1.57	2.96	NK cell stimulatory factor chain 22	Notably expressed by activated macrophages/monocytes, Tcells and mast cells. Broad biological activities
SLC7A5	1.39	2.62	MPE16/LAT1	Involved in cellular amino acid uptake
BAT2	1.33	2.51	HLA-B associated transcript 2	Gene co-localised with TNF alpha and TNF beta genes. In the human, all within the MHC class III region.
CCL20	1.30	2.46	MIP3A	Chemotactic factor that attracts lymphocytes and, slightly, neutrophils, but not monocytes
CPT1A	1.28	2.42	-	Key enzyme in mitochondrial oxidation of long-chain fatty acids
SPIB	1.23	2.34	-	PU-box specific transcriptional activator, expressed in hematopoietic cells including neutrophils
IL8	1.16	2.23	CXCL8	Released in response to inflammatory stimulus. Chemotactic for neutrophils, basophils and T-cells. Involved in neutrophil activation
MAPKAPK2	1.15	2.22	MK2	Regulated by p38 MAP kinase. Involved in stress and inflammatory responses, nuclear export, and gene expression regulation
STARD7	1.13	2.19	StAR-related lipid transfer (START) domain containing 7	Unknown function. Gene contains a region similar to the START domain, which is often present in proteins involved in the cell signaling mediated by lipid binding
MKNK1	1.12	2.17	MAP kinase interacting serine/threonine kinase 1	Appears to regulate transcriptionand play a role in the response to environmental stress and cytokines
BCL2A1	1.11	2.15	BCL2-related protein A1	Its encoded protein decreases caspase activation and CytochromeC release (pro-apoptotic) from mitochondria . Its transcript, via NF-kappa B, is up-regulated by several extracellular signals, including GM-CSFand inflammatory cytokines TNF and IL-1.

Table 3.3 Top 15 Differentially Down-regulated Genes in PMNs Incubated in *S. uberis* 0140J Supernatant Compared to PMNs Incubated in Milk and their known Functions

Fold Change and Log Ratio shown.

Molecule	Log Ratio	Fold Change	Also Known As	Function
DFF40	-1.14	2.20	CAD/DFFB	DFFA is the substrate for caspase-3 and triggers DNA fragmentation during apoptosis
RNF145	-1.10	2.15	ring finger protein 145	Unknown
MAPK9	-1.10	2.14	JNK2/SAPK	Targets specific transcription factors,. Mediates immediate-early gene expression in response to various cell stimuli
GUSB	-1.09	2.13	beta-glucuronidase2	Hydrolase that degrades glycosaminoglycans
MICAL1	-1.07	2.10	NICAL	A microtubule associated monoxygenase thought to be a cytoplasmic scaffolding protein
CYB561D1	-1.04	2.06	Cytochrome b-561 domain containing 1	Secretory vesicle-specific electron transport protein. It is an integral membrane protein.
AGPAT3	-1.04	2.06	1-AGP acyltransferase3	May be an integral membrane protein. Key enzyme in de novo phospholipid biosynthetic pathway
DCUN1D5	-1.03	2.03	DCN1	scaffold-like E3 ligase for cullin neddylation
FOS	-1.00	2.00	FBJ murine osteosarcoma viral oncogene/AP-1	Forms the transcription factor AP-1 (with JUN) . Implicated as regulator of cell proliferation and and also in apoptotic cell death
UCP2	-1.00	1.99	uncoupling protein 2	Mitochondrial uncoupling protein, separates oxidative phosphorylation from ATP synthesis with energy dissipated as heat
SLC39A1	-0.99	1.99	ZIP1	Mediates zinc uptake. May function as a major endogenous zinc uptake transporter in many cells of the body
Unknown	-0.99	1.99	?	?
FGL2	-0.98	1.97	fibrinogen-like 2 /T49	Serine protease activity. Immune coagulant
LTB	-0.95	1.94	lymphotoxin beta / Tumor necrosis factor C	Member of the TNF superfamily. Forms part of the primary ligand for the lymphotoxin-beta receptor. An inducer of the inflammatory response system.
SLC40A1	-0.93	1.91	Solute carrier family 40 (iron-regulated transporter), member 1-like	Identified in Macrophages and endothelial cells. Important in iron-sequestering

Table 3.4 (a) IPA Analysis, List of Top Networks

Tables show the Top Bio Functions identified by IPA, including both direct and indirect relationships between DEG. p-values shown reflect the range of significance of the differential expressed genes within the identified pathway/function

Associated Network Functions	Score
Cellular Function and Maintenance, Hair and Skin Development and Function, Molecular Transport	39
Antigen Presentation, Cellular Movement, Genetic Disorder	38
Organ Morphology, Reproductive System Development and Function, Cancer	32
Cancer, Tumor Morphology, DNA Replication, Recombination, and Repair	27
Lipid Metabolism, Nucleic Acid Metabolism, Small Molecule Biochemistry	24

Table3.4 (b) IPA Analysis, List of Top Diseases and Disorders

Diseases and Disorders	p-value	# Molecules
Dermatological Diseases and Conditions	1.21E-07 - 1.73E-03	35
Cancer	4.10E-07 - 2.34E-03	88
Organismal Injury and Abnormalities	7.67E-07 - 2.47E-03	24
Infection Mechanism	1.64E-06 - 2.27E-03	19
Inflammatory Disease	1.72E-06 - 2.06E-03	74

Table3.4 (c) IPA Analysis, List of Top Molecular and Cellular Functions

Molecular and Cellular Functions	p-value	# Molecules
Cell Death	5.84E-10 - 2.38E-03	77
Cellular Movement	9.97E-08 - 2.50E-03	56
Cellular Growth and Proliferation	1.98E-07 - 2.34E-03	87
Cellular Development	2.95E-07 - 2.44E-03	71
Cell Morphology	1.36E-06 - 2.44E-03	28

Table 3.5 Table of Up-regulated and Down-regulated genes associated with cell death pathways

IPA Analysis of DEG found in ST PMNs compared to MT PMNs that have been shown to be associated with cell death pathways.

Up-regulated Apoptosis Associated Genes		Down-regulated Apoptosis Associated Genes	
PTGS2	PCSK6	DFF40	HCK
ATP2B1	COL18A1	MAPK9	APBB3
IL12B	CSF1	FOS	JNK
CXCL8	SELS	LTB	STIP1
FBXW7	TLR2	CAD	DDB2
NFKB1A	SFRP1	UCP2	BAX
BCL2A1	PLAUR	PGL2	GAPDH
CCL3	PTPRE	ARRB2	GCLC
SFRS5	EPHA2	A2M	S100A8
CDKN1A	KSR1	TXNDC17	CCNL2
KAT5	EIF2AK3	EGLN2	CDK5R1
RBL1	TNIP	EGR1	DDX5
NSMCE1	MYLK	MLL	CD47
ATM	HDAC3	CEBPD	HMGB2
ATR	LMNB1	GADD45A	ATF4
TMEM102	NDEL1	DUSP1	YY1
TXNRD1	PTPRCAP	RARA	ITPRP
NDUFV2	CDH5	NFKB1	MAPK10
IL1RN	MSN	HSPA1A	CGT

Table 3.6 Table of Up-regulated and Down-regulated genes associated with inflammatory response pathways

IPA Analysis of DEG found in ST PMNs compared to MT PMNs that have been shown to be associated with inflammatory response pathways.

Up-regulated Immune Associated Genes	Down-regulated Immune Associated Genes
PTGS2	FOS
IL12B	JNK2
CCL20	SLC39A1
CXCL8	LTB
CCL3	HCK
NFKB1A	CEBPD
CXCL5	RARRES2
MHCII	RARA
PSMD3	NFKB1
IL1RN	AKNA
TLR2	IFI30
SELS	TOB1
HLA-DQB1	IFNGR2
PLAUR	S100A8
SLC11A1	CFP
	ARRB2
	MAPK10
	MHC1
	NMI
	HSPA1A

Fig 3.8 Genes Associated with Apoptosis

IPA Analysis of DEG found in ST PMNs compared to MT PMNs that have been shown to be associated with cell death pathways. Up-regulated genes are shown in red, down-regulated genes are shown in green. Depth of colour indicates level of relative expression, stronger colours = greater differential expression. DEG arranged based on where they are known to occur: from top of graphic: extracellularly, within the cell membrane (blue bi-layer), within the cytoplasm and associated to mitochondria (central pink coloured organelle) and within the nucleus (brown oval).

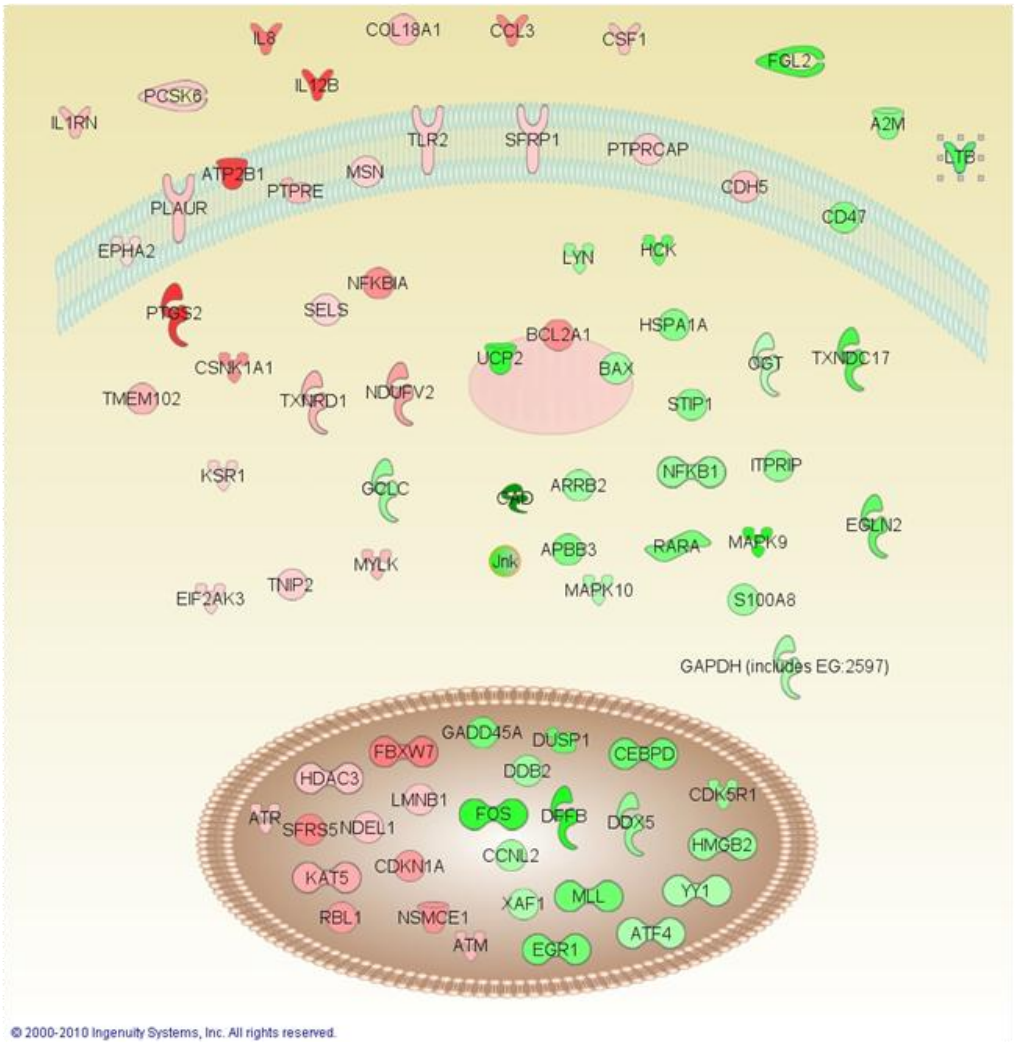
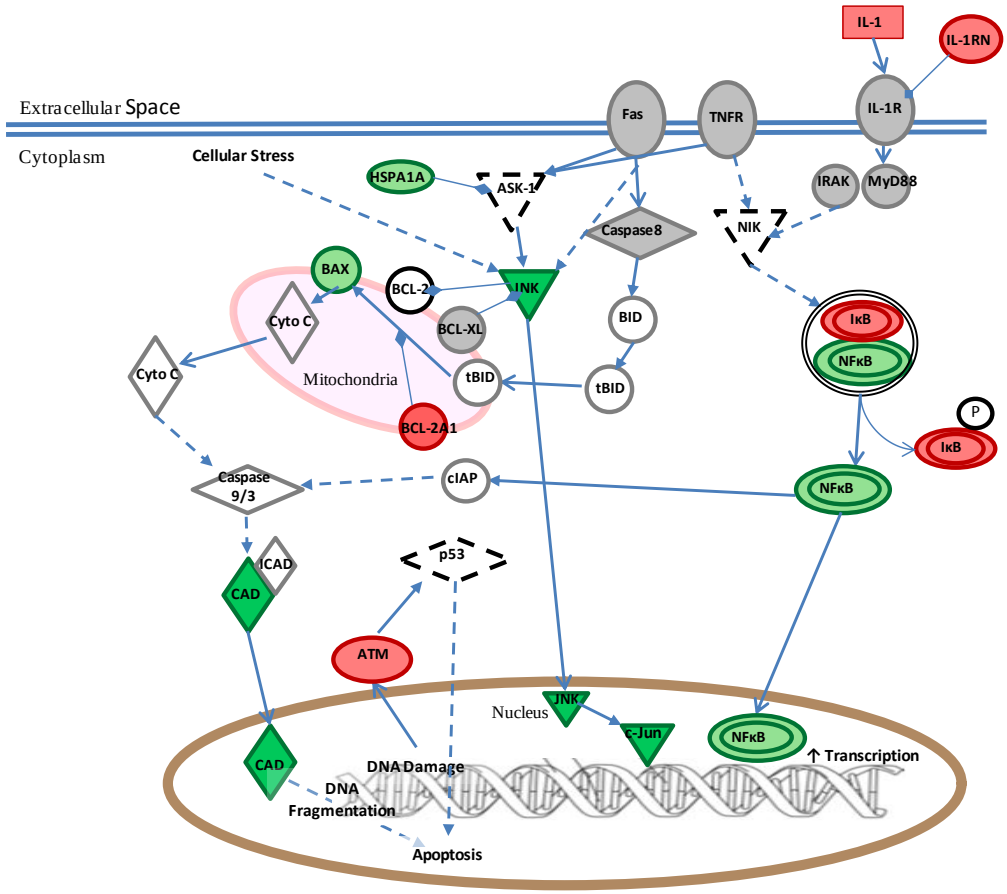


Fig 3.9 Potential Apoptotic Pathway Affected by Exposure to the Supernatant of *S. uberis* 0140J milk-culture

Speculative pathway of DEG involved in apoptotic pathway based on cross-referencing between IPA pathways and KEGG pathways. Up-regulated genes are shown in red, down-regulated genes are shown in green. Depth of colour indicates level of relative expression, stronger colours = greater differential expression. Grey outlined (no fill) are present in the bovine, grey filled in shapes are present in the bovine & on the CAFG BloPlus microarray slide. Black dashed outlines signify products known in other species (principally human) but not seen/yet discovered in the bovine.



3.4.4 Proportion of Apoptotic and Dead Cells in the PMN Population Following Incubation in *S. uberis* 0140J Milk Culture Supernatant Compared to Incubation in Milk

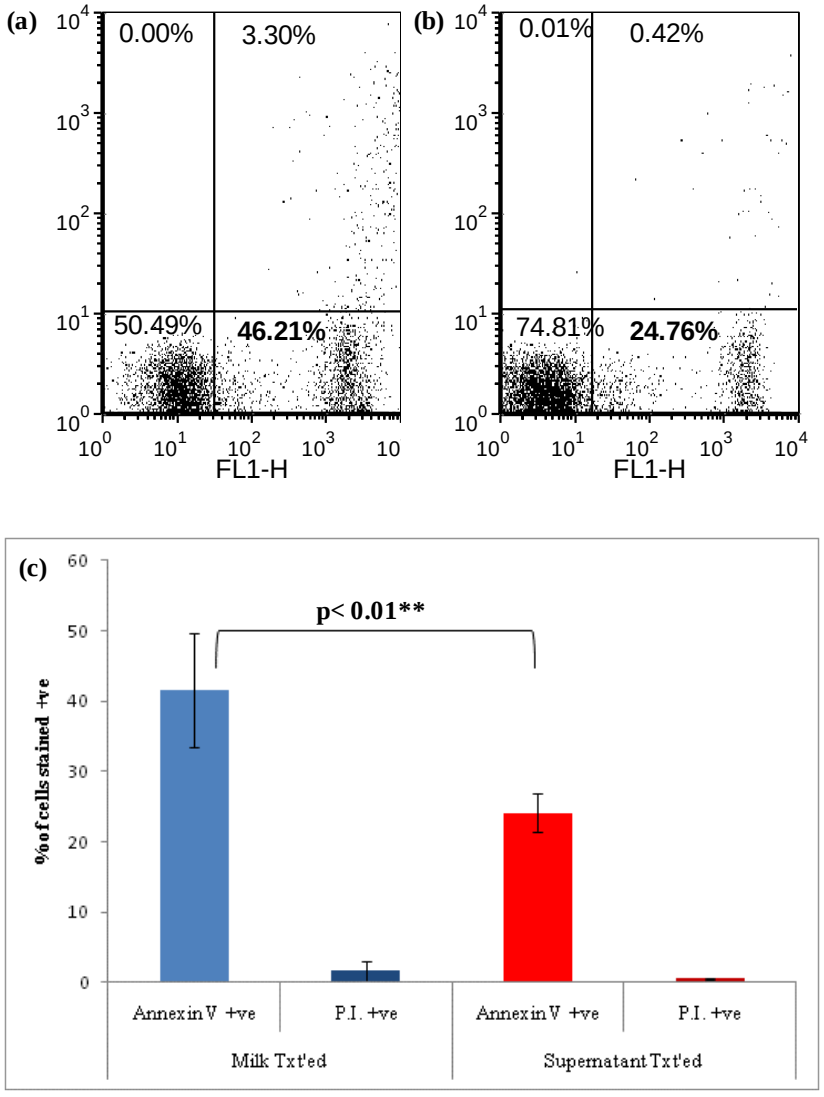
Analysis of the DEG identified by the microarray analysis indicated that genes involved in apoptotic pathways were differential expressed between the two groups.

During apoptosis, phosphatidylserine (PS) translocates from the inner to the outer leaf of the phospholipid bilayer. Annexin-V binds to phospholipids containing PS with high affinity. As the Annexin-V is conjugated with the fluorochrome Fluorescein isothiocyanate (FITC), Annexin-V labelled cells are detected in the FL1-H channel by FACs. As cells die, P.I. is able to enter the cell and bind DNA. P.I. fluoresces in the FL2-H channel.

PMNs isolated, as previously, from 3 animals were incubated in either milk (MT) or *S. uberis* 0140J milk culture supernatant (ST) for 2 hrs and then labelled with Annexin V-FITC and P.I (Fig3.10 (a) and (b)). The population of ST PMNs had significantly less apoptotic cells compared to the MT PMN population (24% (SD 2.7) Vs 42% (SD 8.1). The proportion of dead cells was also lower in the ST PMN population (0.57% (SD 0.13) Vs 1.64% (SD 1.46)) (Fig 3.10(c)).

Fig 3.10 FACs Results for PMNs Incubated in Milk or *S. uberis* 0140J Milk Culture Supernatant.

(a) and **(b)** show FACs plots of PMNs incubated in milk or culture supernatant respectively. The percentage of cells represent in each quadrant is noted. Cells positive for Annexin V-FITC, are appear in the lower right quadrant. Cells positive for both Annexin V-FITC and P.I. appear in the upper right quadrant. **(c)** shows the combined data (n=3). Live, non-apoptotic cells are shown in the lower left quadrant of the FACs plots, apoptotic cells are in the lower right quadrant and dead cells in the upper right quadrant. ST PMNs have a lower proportion of apoptotic cells than MT PMNs ($p<0.01$).

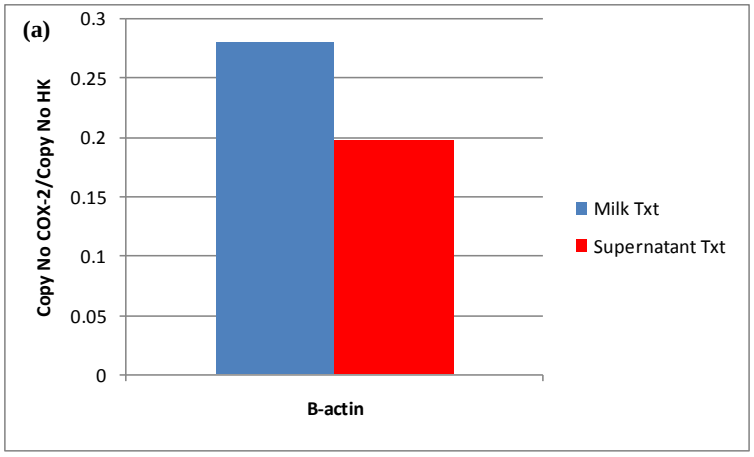


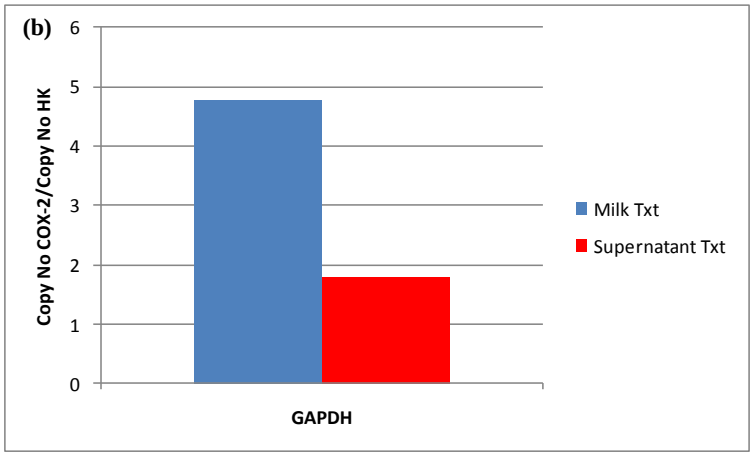
3.4.5 Validation of Microarray data by qPCR

To validate the microarray findings q-PCR was performed on six of the identified DEG, using three potential housekeeping gens as reference. Results for COX-2 are shown here.

Fig 3.12 Transcript levels of COX-2 relative to 3 Potential Housekeeping genes in MT and ST PMNs

qPCR was used to validate the microarray findings. The mean transcript levels of COX-2 from 3 animals' PMNs were assessed using (a) β-actin, (b) GAPDH and (c) boRPL2 as the housekeeping (HK) gene.





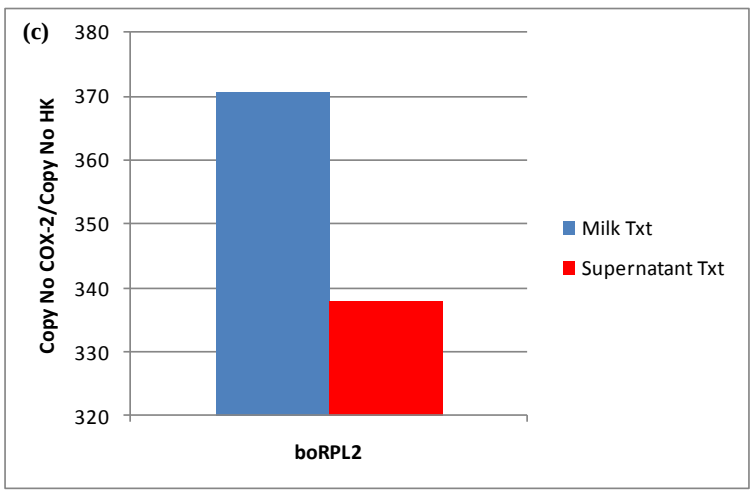
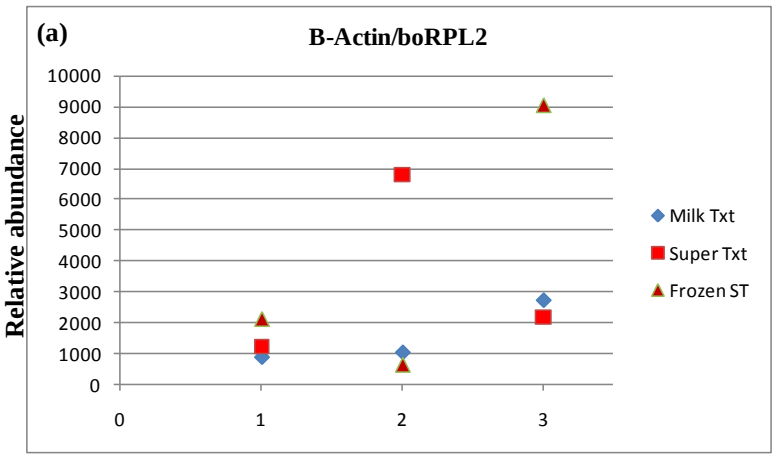
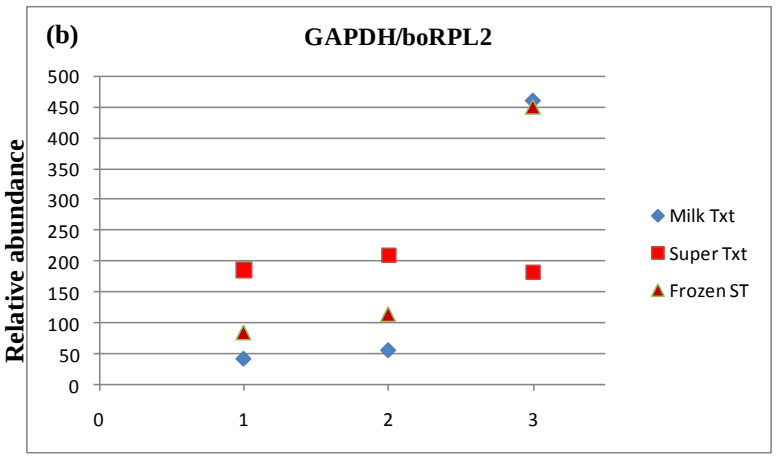


Fig 3.11 Relative abundance of known constitutively expressed endogenous genes identified in previous studies to assess their ability to act as housekeeping genes for q-PCR.

The RNA expression of PMNs from 3 animals that were exposed to milk (Milk Txt), the supernatant of 0140J milk cultures (Super Txt), and the 0140J milk culture supernatant that had been previously frozen (frozen ST) are shown. (a) shows abundance of β-actin relative to boRPL2, (b) GAPDH relative to boRPL2 and (c) β-actin relative to GAPDH. If the two compared genes are constitutively expressed, then for each animal PMNs the relative abundance should be constant in all the test conditions.





4 Discussion

Streptococcus uberis is a leading cause of mastitis worldwide impacting on all dairy husbandry practice (Leigh, 1999). This economically important disease appears uncontrolled by current hygiene methods (Bradley, 2002), and worryingly its incidence may be increasing (Bradley et al., 2007).

With this mounting disease challenge and public concern over the routine use of antibiotic treatments in the farming industry (NMCRC, 2004), novel methods are sought to control this pathogen; these include vaccination and immune-modulatory therapies (Denis et al., 2009, Wedlock et al., 2008). To permit the development of these, further knowledge of the interactions between host and pathogen is crucial.

After the initial penetration of a pathogen past the physical barriers of the streak canal, its presence is recognised by sentinel leukocytes and the mammary epithelial cells. These release cytokines to initiate a leukocyte influx from the blood led, and dominated by, neutrophils. The primary function of neutrophils is to phagocytose invading pathogens but it is increasingly recognised that they are crucial in signalling to other cells, directing the progression and resolution of the immune response (Whale and Griebel, 2009, Nathan, 2006).

The interactions of virulent *S. uberis* 0140J and bovine PMNs has been a focus of much research. Previous work has shown that during experimental challenge despite their large numbers, no PMNs appear to internalize *S. uberis* (Thomas et al., 1994). *In vitro* studies with *S. uberis* have shown the pathogen releases a soluble factor that inhibits the phagocytic function of blood-derived PMNs; this inhibition is associated with altered PMN cytoplasmic and membrane structure (Field et al., 1997). However, the inhibitory substance remains unidentified and the mechanisms by which it alters PMN function and structure are unknown. This work set out to elucidate a better understanding of this host-pathogen interaction; firstly by assessing if avirulent strains of *S. uberis* were killed by PMNs, and secondly by measuring changes in the transcriptome of PMNs after exposure to this unknown inhibitory factor.

4.1 Excluding the Mutant Lacking sub1095, the Growth of *S. uberis* 0140J, EF20and Mutant Strains Lacking Vru, SrtA and sub1045, sub1154 are Similar in THB and Milk Cultures

All strains of *S. uberis* tested grew to similar levels in THB, although the mutant lacking sub1095 had a slower rate of growth. In milk, all reached a similar level except for the mutant lacking sub1095, which grew less well. In the host this mutant, which lacks a collagen-like protein, initially colonizes the udder like the virulent 0140J, but after 24hr its numbers decline (Leigh et al., 2010), whether its reduced growth *in vitro* is related to its decreased pathogenicity is not yet determined.

4.2 The Attenuation of *S. uberis* EF20, and Mutants Lacking Vru, sub0145, sub1095 and sub11454 is Not Due to Susceptibility to Killing by PMNs

The wild type *S. uberis* strain EF20 and mutants of *S. uberis* 0140J lacking: Vru, sub0145, sub1095 or sub1154 failed to establish the severe clinical infection seen following infection with the virulent strain *S. uberis* 0140J (Leigh et al., 2010, Leigh et al., 1990). Whether attenuation of any of these mutants was due to decreased resistance to killing by PMNs was unknown. In this study, the populations of these strains after culture in milk were not decreased by the presence of PMNs. The absence of susceptibility to the bactericidal action of PMNs implies that in no instance was attenuation likely to be due to removal by PMNs.

To confirm that the lack of susceptibility to the bactericidal actions of PMNs is due to a similar inhibition of the phagocytic function as occurs with 0140J and not due to, for example bacterial avoidance of opsonisation, the phagocytic assay of *E. coli* after incubating PMNs in milk pre-conditioned by the growth of these attenuated strains should be conducted.

4.3 The Inhibitory Effect of Supernatant of *S. uberis* 0140J Milk Cultures on PMNs is Not Affected by Storage at -80°C

The identity of the soluble factor which mediates inhibition of phagocytosis by PMNs is unknown. Previously however, it has been shown that this effect is present in milk culture supernatant after removal of *S. uberis* (Field et al., 1997). Given the inherent variability of the composition of milk between cows and over time, creating a standardised experimental model is challenging. To permit a more consistent experimental design, the effect of freezing (and thus storage of) the supernatant of *S. uberis* milk cultures was investigated. PMNs from six animals were incubated in both fresh supernatant from *S. uberis* 0140J milk culture (milk supernatant: "MS") and the same supernatant that had been frozen and then thawed (frozen milk supernatant: "FMS"). Both MS and FMS were shown to significantly inhibit the bactericidal actions of PMNs ($p<0.01$**). Although it appeared that MS was slightly more effective at inhibiting PMNs than FMS, there was no statistically significant difference. This confirms that freezing does not affect the inhibitory action of the supernatant from *S. uberis* milk cultures, allowing a stock of MS to be made, stored frozen and used for various experiments over time.

4.4 The Supernatant of *S. uberis* 0140J Culture Grown in RPMI is Not a Useful Model of the Inhibition of Bactericidal Actions of PMNs

If *S. uberis* produced the soluble factor that causes the inhibitory effect on PMNs in a standardised commercial available media it would be globally useful, enabling the experimental design of such studies to be consistent between laboratories. To assess the

possibility of this, the inhibitory effects of the supernatant of *S. uberis* grown in RPMI tissue media culture were assessed.

In RPMI, *S. uberis* grew to a final cell density of 2-8 x 10^8cfuml^{-1}, ten-fold greater than its growth in milk. Although the bactericidal action of PMN incubated in fresh or frozen supernatant of *S. uberis* RPMI cultures ("RS" and "FRS" respectively) was less than in RPMI, this was not statistically significant. This suggests that, despite a *S. uberis* population ten times greater than that in milk, the inhibitory substance is produced at a much lower level, if at all. The possibility also remains that the inhibitory activity was derived from milk itself through the action of *S. uberis*; this was not investigated in the present study.

4.5 Transcriptional Changes of PMNs Exposed to the Supernatant of *S. uberis* 0140J Milk Culture

4.5.1 Experimental Design

Microarray analysis is a useful tool to assess changes across the transcriptome (Allison et al., 2006). In the bovine, it has been used to address a wide range of scientific questions including investigations into mammary gland function and host response to mastitis (Connor et al., 2008, Moyes et al., 2009, Swanson et al., 2009).

The Centre for Animal Functional Genomics (CAFG) at Michigan State University (MSU) (http://www.cafg.msu.edu/) have developed the BloPlus Oligo microarray which encompasses genes known to be associated with the immune response, and has been used to investigate transcriptional changes in leukocytes associated with infectious disease (Verschoor et al., 2010).

The microarray slide contains 8400 70-mer oligonucleotides, printed in duplicate, each of which represents a single gene. With an oligonucleotide probe length of 70 base-pairs (bp), the BloPlus array has superior specificity compared with the widely used Affymetrix GeneChip Bovine Genome Array which uses a probe length of 25bp (http://www.affymetrix.com). Although the GeneChip array is more comprehensive with probes for 23,000 bovine transcripts the BloPlus microarray includes probes for more recently discovered gene products, including highly relevant TLR transcripts. The BloPlus array was a more appropriate tool to use than a genome wide chip given the incomplete status of the current bovine genome assembly, the greater probe specificity, more updated probe design and the focus on genes relevant to the immune systems. Although the genome-wide chip may have produced more data, without annotation the conclusions achievable would be limited.

This experiment assessed the differences in transcription between PMNs incubated in milk ("milk –treated" (MT PMNs)) and supernatant from *S. uberis* 0140J milk cultures ("supernatant-treated" (ST PMNs) from healthy lactating heifers.

4.5.2 The Implications of the Differentially Expressed Genes Identified

Although the transcriptional changes detected were small, the observation that only 328 (4% of the oligos expressed on the BloPlus Array these data suggest that only specific genes were affected by exposure to the supernatant of *S. uberis* milk culture. Further analysis using IPA Ingenuity analysis revealed several pathways and processes in which multiple DEG occur, indicating that these may be functionally relevant during the initial interaction with *S. uberis* and possibly the early stages of intramammary infection.

4.6 Cellular Functions Affected

4.6.1 Down-Regulation of Genes Involved in Apoptosis

Individual PMNs undergo apoptosis to control their own death and identify themselves for uptake by macrophages. This leads to their removal from the milieu without discharging their cytotoxic contents and internalised pathogens, and thus preventing further tissue damage. Apoptosis plays a crucial role in resolving the inflammatory response. As well as bringing about their own removal, apoptotic cells modify macrophage transcription to produce the anti-inflammatory cytokines IL-10 and TGF-β (Voll et al., 1997). Progression of apoptosis directly correlates to loss of PMN function (Whyte et al., 1993), and is accompanied by characteristic morphological changes including fragmentation of chromatin, condensation of cytoplasmic contents and blebbing of the cell membrane (Reed, 2000).

Nearly a quarter of all DEG identified by IPA analysis were associated with cell death. DNA Fragmentation Factor 40 (DFF40), also known as Caspase-activated DNase (CAD), is an endonuclease activated by Caspase 9. It is specific for double stranded DNA and brings about the fragmentation of DNA (Widlak, 2000). DFF40 was the most down-regulated gene in ST cells, potentially indicating a strong anti-apoptotic response after exposure to the supernatant of *S. uberis* milk culture. Other down-regulated genes with crucial roles in the apoptotic pathway are transcription initiators, FOS and MAPK8 and 9 (also known as JNK and JNK2/SAPK respectively).

The effect on apoptotic pathways was verified functionally by the Annexin-V FITC Apoptosis assay. This assay showed a highly statistically significant difference in the ST PMNs which had approximately half the number of apoptotic cells compared to the MT PMNs ($p<0.01$), and after the 2hr incubation period the number of ST cells staining positive for propidium iodide, and therefore classed as dead, was only $1/8^{th}$ of that of MT cells.

4.6.1.1 Does Down-Regulation of Apoptosis Benefit the Host or the Pathogen?

This observed effect corroborates *in vivo* work demonstrating that mastitis pathogens stimulate a decrease in the proportion of apoptotic lymphocytes (Slama et al., 2009, Sladek and Rysanek, 2001). Human neutrophils also showed decreased apoptosis and prolonged half life following stimulation with *Streptococcus haemoliticus* (Colotta et al., 1992).

Whether the apoptotic state observed favours the host or the pathogen is dependent on the nature and stage of the complex interactions between host and pathogen. Later in the progression of an infection, it would be beneficial for a PMN which has ingested several pathogens to rapidly apoptose to ensure the removal of itself and hence the contained bacteria from the local environment, this has been shown to occur in human PMNs after uptake of bacterial pathogens (Kobayashi et al., 2003). Other pathogens are able to manipulate apoptosis to promote their own survival and replication cycle; for example virulent *Mycobacterium tuberculosis* which is able to survive within macrophages disrupts the normal progression of apoptotic cell death in favour of necrosis which aids its spread within tissues (Gan et al., 2008).

The human pathogen *S. pyogenes* shows resistance to phagocytosis (Voyich et al., 2004) and the ability to survive intracellularly (Medina et al., 2003). *S. pyogenes* modulates apoptosis in human neutrophils differently to other human pathogens, by down-regulating genes associated with the IFN response and by up-regulating pro-apoptotic genes (Kobayashi et al., 2003). Although some common features exist between the findings of Kobayashi et al. and those from this study, PMN exposed to *S. uberis* culture supernatants showed a down- (rather than up-) regulation of apoptosis. IPA analysis identified 77 differentially expressed genes associated with cell death. However, molecules in pathways were not concomitantly up- and down-regulated in association with their neighbours; this may be a reflection of either the complexity and subtlety of the system or of responses of contaminating cells disrupting the evidence of pathways in neutrophils.

4.6.2 Inflammatory Pathways Affected

Several genes involved with inflammatory pathways were differential expressed. Although coherent pathways could not be identified the modulation of transcription of several key players in the immune response increases the likelihood that these DEG are relevant to the interaction of *S. uberis* with host cells, but further work is required to confidently define their roles.

4.6.2.1 Pro-inflammatory cytokine expression is up-regulated

Increases in cytokine expression are associated with mastitis infections, with some pathogen-specific patterns (reviewed (Bannerman, 2009)). In the present study expression of the

cytokines CXCL8, IL-12β, CCL20 CXCL5 and CCL3 were all up-regulated in ST PMNs. Inflammatory cytokines are produced by leukocytes, including neutrophils; a principal role of some cytokines is as chemokines. They attract other leukocytes to the site of inflammation and their induced expression directs the resulting immune response.

CXCL-8 (IL-8) acts as an attractant for neutrophils and monocytes and is also able to activate neutrophils; it is produced by many cells, including neutrophils, and is a key pro-inflammatory cytokine. It has previously been shown to be up-regulated in experimental infections with *S. uberis* 0140J (Bannerman et al., 2004a) and it was the top up-regulated DEG in mammary tissue following infection with *S. uberis* (Moyes et al., 2009).

IL-12β is the p40 subunit that heterodimerises with the p38 subunit to form the active dimer IL-12 and is also part of the IL-23 heterodimer. Cells of the monocyte lineage are the main producers of IL-12β, however PMNs have been shown to produce IL-12 (reviewed by (Scapini et al., 2000)). The overwhelming presence of PMNs during mastitis suggests they could significant contribute to the ongoing immune response. IL-12 triggers an IFNγ response in immune cells and is important in the defence against intracellular pathogens, mediating a Th1 response (Scapini et al., 2000). Bannerman and colleagues found that experimental infection with *S. uberis* 0140J increases levels of IL-12 which correlates with IFNγ production (Bannerman et al., 2004a).

IL-23, initially shown to be produced by activated dendritic cells (Oppmann et al., 2000), activates a neutrophil-mediated response via IL-17 leading to IL-1, IL-6 and TNFα release (Langrish et al., 2004). Whether the IL-12β transcription detected by microarray is directed via IL-12 or IL-23 cannot be determined, in fact it is likely that both are relevant as in any inflammatory process the resulting immune response is due to the balance between many pro and anti-inflammatory molecules. In experimental challenge, IL-12β levels increases in response to *S. uberis* strain 0140J, but not in response to strain EF20 (Coffey et al personal communication). However, the increase in response to 0140J is much less than with the attenuated mutant strains lacking SrtA and Vru (Coffey et al personal communication). Even during infection *in vivo*, where numerous cytokines were measured, it is hard to definitively link the levels of IL-12β seen with biological function. As a general trend, increases in IL-12β production correlate with TNFα, IL-8 and initially bacterial numbers in the udder, but IL-12β negatively correlates with IL-6, perhaps indicating an overall anti-inflammatory bias in its effect. Though some parallels may be drawn between the data generated from *in vivo* and *in vitro* studies, with regards to Il-12β production, the exact contribution of IL-12 is clouded by the fact that the IL-12β subunit is common to both IL-12 and IL-23. The contribution of IL-23 to the immune response to infection must therefore be investigated to assess the role of IL-23 during experimental challenge.

CCL20, also termed macrophage inflammatory protein 3α (MIP-3α), is a chemoattractant for neutrophils and lymphocytes but not monocytes. CCL20 mRNA and protein has been detected in human neutrophils as have transcripts for CCR6, the CCL20 receptor (reviewed by (Schutyser et al., 2003)). Human CCL20 has been shown to have a direct antimicrobial action against several pathogens including streptococci (Yang et al., 2003), although the activity of bovine CCL20 against *S. uberis* is unknown, it is an interesting finding to explore further.

4.6.2.2 Increased Expression of Toll-Like Receptor 2 (TLR2)

TLR2 gene expression was up-regulated. Bovine TLR2 is a PRR important in binding the peptidoglycan of Gram-positive bacteria (Werling et al., 2006). Following mastitis infection due to various pathogens, TLR2 transcription has been shown to be up-regulated in infected quarters (Goldammer et al., 2004); in NEB (compared to PEB) cows TLR2 transcription also increases in circulating PMNs following intramammary infection with *S. uberis* (Moyes et al., 2010a). Although TLR2 activation can promote transcription of the cytokines that were also up-regulated (described previously, Section 4.6.2.1), key intermediates (i.e. MyD88) were not. It is therefore likely that increased transcription of TLR2 was in response to the stimulation of the PMNs. However due to the early time point investigated this did not yet equate to increased expression of the receptor, and thus the TLR2 pathway had not yet been activated. This illustrates the caution required when trying to relate transcriptional expression with the resulting pathway activation and protein and functional changes.

4.6.2.3 Altered Transcription of MHC in ST PMNs

Transcription of the *Major Histocompatibility Group II (MHCII)* transcription was increased whilst *MHCI* transcription decreased. MHCII is responsible for antigen presentation in professional APCs. Some recent work suggests that PMNs may be able to take up MHCII from apoptotic and necrotic cells though no evidence was found to support constitutive MHCII expression by PMNs (Whale et al., 2006a) which has been suggested by some work with human PMNs (Gosselin et al., 1993).

4.6.3 Bivalent Cations may be Important in Interactions Between *S. uberis* and the Host

Expression of *SLC40a1*, also known as ferroportin, was down-regulated in the ST cells. Iron is an essential trace element for both microbes and vertebrates, Various host defences, including lactoferrin and lipocalin and ferroportin exist that sequester iron, rendering it unavailable for microbial metabolism (Ganz, 2009). Ferroportin is a membrane protein identified in macrophages and epithelial cells which exports iron out of the cell. It is also a receptor for the hormone hepicidin; the activity of which leads to internalization and breakdown of ferroportin. This results in the retention of iron in the cytoplasm as ferritin, thus is decreasing iron availability for extracellular pathogens. A TLR-4-dependent down-regulation of ferroportin was detected in macrophages (but not in non-myeloid cells) in

response to bacterial pathogens in a murine model (Peyssonnaux et al., 2006), and was postulated to favour successful defence against extracellular pathogens.

If the differential expression of ferroportin in the present study was due to contaminating macrophages within the cellular population isolated from blood, then the mechanisms underlying its down-regulation may be more complicated. It is known that macrophages, unlike neutrophils, are capable of phagocytosing *S. uberis* (Grant R.G, 1996). Under these conditions, higher intracellular iron availability is beneficial to the pathogen, providing it is accessible to the phagosome. *Neisseria meningitidis* is capable of initiating ferritin breakdown in infected epithelial cells (Larson et al., 2004). Importantly ferroportin transcription in murine macrophages with mycobacterial-infected phagosomes was shown to be directed by the presence of the pathogen (Van Zandt et al., 2008). Various streptococci have been reported to manipulate the host cell after phagocytosis to benefit their own survival and replication (Quach et al., 2009, Phelps and Neely, 2007). The iron transporter within *S. suis* has been shown to be crucial to intracellular survival and pathogenesis (Aranda et al., 2009).

The zinc transporter *SLC39A1* (*ZIP*) was also down-regulated in the present study. Knowledge of the importance of zinc during infection is limited; however its acquisition has been shown to be essential for virulence of *S. pyogenes* (Weston et al., 2009). Crystallization of the *S. pyogenes* laminin-binding protein reveals it targets zinc, and it is thought that it mediates invasion via adhering to zinc-bound laminin (Linke et al., 2009).

The microarray results obtained in this study highlight the requirement for further investigation into the mechanisms of bivalent cation metabolism in both the success of pathogen and of host defences with regards to infection with *S. uberis*.

Although there is no clear answer to the underlying basis of the reduction in the bactericidal function of PMN, the microarray analysis has revealed evidence that *S. uberis* manipulates the host response and has provided an initial insight into the possible early stages of the host pathogen interactions.

4.6.4 Influence of Incubation Period

Following experimental challenge it has been reported that bacterial numbers typically increase to around 10^7 cfu ml^{-1} from time of challenge to 24hr post infection. The SCC typically remained at the pre-infection level until the second milking after infection (at 24hr). This reflects the influx of PMNs from the blood by that time point. Both bacterial and PMN levels remained elevated over the subsequent milkings and remained elevated until the onset of clinical signs. PMN functionality has been shown to be rapidly compromised after exposure to *S. uberis* 0140J (J.A. Leigh, unpublished data).

The aim of this project was to look at the earliest time points in the interaction between PMNs and pathogen. The experiment was designed to mimic the 24hr time point of *in vivo S. uberis* 0140J challenge.

This project showed that strains of *S. uberis* grow to around 10^7 cfu ml^{-1} in overnight milk cultures. These cultures were then filtered to generate the supernatants. Incubating the PMNs in the supernatants for 120min aimed to reproduce the level of bacteria-derived soluble factors that these cells would be exposed to *in vivo*, and also to expose them to it for a length of time known to result in loss of PMN bactericidal activity. The microarray analysis investigated the transcriptional changes that may contribute to this loss of functionality.

In this study, 120min was chosen as the time point at which to assess transcriptional changes in ST exposed PMNs based on preliminary phagocytic assays in milk cultures showing that bacteria susceptible to phagocytosis were reduced to 10% of their original population by 90min and 0.1% by 120min. As this study aimed to understand the early interactions between products of *S. uberis* and PMNs, 120min was a justified time point to investigate by microarray.

In phagocytic assays with human PMNs and various human bacterial pathogens, only *S. pyogenes* showed resistance to the phagocytic actions of PMNs (Kobayashi et al., 2003). 90min post challenge, 65% of *S.* pyogenes were viable whilst other pathogens' survival was <20%. Interestingly this coincided with *S. pyogenes*- challenged PMNs having two and half times more DEG compared to those challenged by all other pathogens, and a very different transcriptional profile. *S. pyogenes*-challenged PMNs had differential expression of genes associated with apoptotic pathways; many of which were also differentially expressed in this study. However, the *S. pyogenes*-challenged PMNs had an accelerated rate of apoptosis compared to PMNs challenged with other pathogens (Kobayashi et al., 2003). This would suggest that although *S. pyogenes* and *S. uberis* are resistant to the phagocytic actions of PMNs, the process of events following the interaction in the PMNs is very different. Nevertheless the evidence from Kobayashi *et al* and the results of this study show that interactions at the time point chosen for this study are relevant to unravelling the early interactions between streptococcal pathogens and PMNs.

4.6.5 Validation of Microarray Data

To investigate if the DEG identified by the microarray hybridisation technique are corroborated by other recognised and routinely used techniques, qPCR, ELISA as well as the apoptotic cell assay previously discussed, were used.

Real-time polymerase chain reaction (qPCR) is a sensitive and specific technique that is often used for the validation of microarray data (Rajeevan et al., 2001). In this study, qPCR was performed on those genes which were seen to be amongst the ones most differentially expressed and where assays for detection were already available. As a result five DEG were further analysed, including COX-2, CXCL-8, IL-12β and CCL20, which all occur in the top 10 up- regulated genes in ST PMNs.

qPCR results for *COX-2*, *CXCL-8*, *IL-12β*, *CCL20* and *NFKβ*, normalised to *β-actin*, *boRPL2* and *GAPDH*, showed no correlation with the findings of the microarray data. Results for COX-2 are shown, this was the third highest up-regulated DEG identified by microarray in ST PMNs a finding not supported by qPCR. Several factors may be responsible for this finding, RNA degradation lead to an incomplete data set for all transcripts for all of the 6 animals used for qPCR. In addition, wide biological variation and inevitable variation if the amount and type of contaminating cells within the isolated PMN population (see below) lead to mixed results. In addition, levels of IL-12β and CCL20 were too low to be accurately quantified by the qPCR, suggesting at this early time point (2hr) transcriptional changes are below the level of detection of the available assays.

Also the use of *β-actin*, *boRPL2* and *GAPDH* as housekeeping genes is questionable. Indeed *β-actin* and *GAPDH* were found as DEG on the microarray. *boRPL2* was identified as a HK from *in-vivo* microarray analysis of 0140J mammary gland infection, however none of these three appeared consistently endogenously expressed in this work.

ELISAs were used to investigate if the transcriptional up-regulation of *CXCL8* and *IL-12β* identified by microarray was reflected at the protein level. However, cytokine levels were below the detection range of the technique (results not shown).

The Annexin-V apoptotic assay showed a significantly lower percentage of apoptotic cells in the ST PMN population compared to the MT PMNs; excitingly, this correlates well with the down-regulation of genes associated with the apoptotic pathways, giving confidence in the validity of the DEG found relating to this pathway.

Future optimisation of various aspects of the experiment (discussed below) should lead to increased sensitivity of the techniques and reliability of results, improving the likelihood of successful validation of the microarray findings.

4.7 Factors Affecting the Lack of Statistical Significance

Microarrays are a fairly new technique and no flawless statistical algorithm to handle the large amount of data for all experimental designs has yet been developed. A p-value of <0.05 (which was the value used to identify DEG in this study) assumes each test was carried out separately and indicates a 5% chance of that result being a false positive. However, the microarray technique seeks to test many hypotheses, and in this case explores the differential transcription of 8,400 features simultaneously. The effect of multiplicity increases the chances of those DEG being deemed false positive. In an attempt to overcome this effect, a False Discovery Rate (fdr) adjustment is routinely made. This generates an "adjusted p-value" for the DEG which takes into account the data, number of hypotheses being tested and the number of experimental replicates (Pawitan et al., 2005). An fdr of 5%, as was used here, attributes equivalent significance to a result of $p<0.05$ if only a single hypothesis was tested. For the DEG found here the adjusted p–values were 0.99, indicating no statistical significance. Several reasons may underlie this observed lack of statistical relevance and these are discussed further (below).

4.7.1 The Number of Genes Differentially Expressed is Low

Three hundred and twenty-eight differentially Expressed Genes (DEG) were identified with a p-value of <0.05, this represented 4% of the oligos expressed on the BloPlus Array. As only 4% of all oligos (328 out of 8400) were differentially detected, with an fdr of 5% it is impossible to differentiate true positive from false positive results, and hence in such a situation the resulting adjusted p-value would always appear non-significant. The inability of fdr adjustments to identify DEG when only a small proportion of tested gene products are differential expressed is an on-going drawback (Rajeevan et al., 2001). In these cases, DEG can still be significant and biological knowledge of what would be expected to be affected, over representation in specific pathways and validation by other tests can aid in identifying true positive results. Furthermore, although not a good indication of true significance in systems with "background noise" (*www-stat.stanford.edu/~tibs/ftp/FCTComparison.pdf*), fold changes are a useful guide and DEG with a high fold change value are more likely to be biologically significant. In this study only 36 out of the 328 DEG had a fold change greater than two.

4.7.2 Suboptimal Number of Biological Replicates

It is widely suggested that a minimum of five biological replicates are optimal when designing microarray studies (Allison et al., 2006), and the experiment was designed to sample seven animals. Animals were selected for use based on NMR records to ensure no present or recent history of disease, as the transcriptome of circulating PMNs is altered in disease states and

NEB (Moyes et al., 2010a). Unfortunately issues with acute mastitis and other concurrent diseases on the day of blood sampling, time and licence limitations (on the number animals to be used) and degradation of RNA samples reduced the number of suitable animals to four. In an attempt to overcome the issue with limited biological replicates, the microarray design incorporated "dyeswaps" whereby the same sample was used for two separate arrays, with the sample labelled with each of the two microarray dyes.

4.7.3 Potential Effects of Contaminating Cell Types

Historically PMNs were considered to have limited transcriptional potential, though those working with PMNs would debate this (Nathan, 2006). These cells are reported to have $1/10^{th}$ to $1/20^{th}$ of the total RNA content of other cells (Cassatella, 1995). Thus, the contribution of contaminating cells to the overall RNA levels is potentially more of an issue for studies of PMNs than for other cell population. The purity of the isolated cell population was 94.8% (n=4, SD 0.83); if PMNs have a RNA content $1/10^{th}$ of other cells then PMN-derived RNA could account for 65% of the total RNA. If the PMN RNA content is $1/20^{th}$ compared to other cells then PMN-derived RNA would only account for 48% of the total RNA.

The definition "PMN" can be unclear. It is used interchangeably with the term "neutrophil" but is also an umbrella term equivalent to "granulocyte". With respect to this study, the actual "PMN" population was a granulocyte population as the isolation technique did not differentiate between neutrophils, eosinophils and basophils. However, the aim of the project was to investigate the interactions of *S. uberis* and neutrophils; in functional studies the presence of a low level of contaminating eosinophils and basophils would not impact on the results, however with studies into gene expression the composition of the population should be acknowledged.

The contribution of eosinophils to the PMN population is highly variable between individuals (see Table 4.1) and thus will influence the relative quantity of RNA from each cell type between animals. The presence of eosinophils in the PMN population was indicated during FACs by high autofluorescence of some cells (Shapiro, 2003).

Table 4.1 Reference intervals for clinically healthy lactating dairy cows

Table adapted from Brun-Hansen et al., 2006; values calculated from samples from 75 animals.

Granulocyte	cells/L
Neutrophils	$1.3\text{-}5.3 \times 10^9$
Eosinophils	$0.1\text{-}1.7 \times 10^9$
Basophils	$0\text{-}0.1 \times 10^9$

Several methods for isolating neutrophils from whole blood exist and debate surrounds the most appropriate method to derive the cells without influencing functionality and limiting the effects of contaminating cells. The major contaminating cells are thought to be monocytes and lymphocytes (Soltys et al., 1999), and the presence of cells other than neutrophils potentially impacts on transcriptional studies.

In this study it is unknown what, if any, changes the isolation technique may have induced in the PMNs, but taken in context it is not a major concern. The effect of contaminating cell types however should be considered in future experimental design; it is inevitable that in this study the RNA would not have been entirely derived from neutrophils. High expression of *SLC40A1* (ferroportin) by macrophages and enterocytes cells is well documented (Anderson and Vulpe, 2009), though no evidence has been presented for its production by PMNs (Peyssonnaux et al., 2006). Its occurrence in the top fifteen down-regulated genes in ST cells is possible evidence that contaminating cells are contributing to the transcripts being analysed. Thus, the differentially expressed genes identified may reflect changes in cell types other than neutrophils.

The use of different gradient techniques to isolate human neutrophils (Percoll, Ficoll-Hypaque/Dextran sedimentation, Mono-Poly Resolving Medium) (Venaille et al., 1994) all yielded a population of equivalent purity (93-94%) and viability (98%) as the technique employed in this study. It is clear that groups have varying success with implementing established isolation techniques. The continual Percoll density gradient technique yielded bovine neutrophil purities of 81% +/- 3% (Soltys et al., 1999), >87% (Van Oostveldt et al., 2002), 94% (Wang et al., 2009) and >95% (Rinaldi et al., 2007).

Methods implementing erythrocyte lysis achieved PMN populations of 90% (Van Oostveldt et al., 2002) and 94.8 % in this study. Hypotonic shock, using ammonium-chloride or water is a standard technique to lysis erythrocytes; however this has been suggested to affect functionality by altered transcription of cell surface receptors (Macey et al., 1995, Youssef et al., 1995).

Clearly, established techniques for PMN isolation although adequate for functional studies have limitations when used to isolate cells for microarray analysis. Future studies would benefit from investigating novel PMN isolation techniques, and three methods which merit further investigation are suggested below.

Following whole blood lysis, the use of anti-bovine neutrophil specific monoclonal antibody labelled biomagnetic beads resulted in a neutrophil population of 95.5% +/- 1.8% purity and a marked decrease in the level of contaminating leukocytes and monocytes with no detrimental

effects on neutrophil functionality (Soltys et al., 1999), although effects on the transcriptome were not assessed.

Neutrophils have a very high expression of the FcγRIIIB (CD16b) surface receptor, which binds pathogen-bound immunoglobulin. Combining a discontinuous Percoll gradient and FACs sorting of cells positive for $CD16^{bright}$ a 99.9% pure human neutrophil population with a viability of 90% was achieved (Lichtenberger et al., 1999), the authors suggest that this method enables RNA studies to confidently assess transcriptional changes in neutrophils. Whether this method could be adapted for successful bovine neutrophil isolation is unknown.

Suitable reagents are not always available for bovine studies, as they are for human or murine studies, and this can have implications for studies. In addition, markers suitable for identifying neutrophils in the human system do not necessarily hold true for isolating the equivalent cell population in other systems, such as the bovine. Using FACsAria technology, populations of cells can be isolated based on the forward and side scatter patterns without the need for cell markers. Forward scatter correlates to increasing cell diameter and side scatter correlates with the granularity of a cell. PMNs are large granular cells, which produce a scatter profile different to other leukocytes, allowing their differentiation. Using this, it may be possible to remove the contaminating cells from the granulocyte population.

Clearly, isolating a pure population of neutrophils is a technical hurdle that affects human and animal research worldwide; however now it is acknowledged and improved isolation techniques are being developed.

4.8 Concluding Remarks

PMNs lack bactericidal efficacy against avirulent strains of *S. uberis*, as has previously been shown with virulent *S. uberis* 0140J. This suggests that the virulence determining genes *vru*(encoding a DNA binding gene regulator), *sub0145* (encoding a putative lactoferrin binding protein), *sub1095* (encoding a collagen-like protein) and *sub1154* (encoding a serine protease with limited homology to C5a peptidase) are important for other host-pathogen interactions.

Soluble factors produced by virulent *S. uberis* 0140J in milk alter the transcriptome in a 94.8% pure PMN population. The transcription of apoptotic mediators is down-regulated and functionally it is seen that PMNs exposed to the supernatant of *S. uberis* milk cultures have a significantly reduced proportion of apoptotic cells compared to those in milk. Transcription from pro-inflammatory cytokines including CXCL8, IL12β and CCL20 are up-regulated and genes involved in cellular structure and metabolism had altered levels of expression in PMNs exposed to soluble factors from *S. uberis*. This project provides initial insights into the mechanisms by which *S. uberis* elicits changes in PMNs that may relate to inhibition the bactericidal function and / or other host pathogen interactions.

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6 Appendix

6.1 Appendix 1 - Reagents.

Re: 2.1.1 - Isolation of Bovine Blood derived Neutrophils (PMNs)

E.D.T.A. (1.5%) in PBS.

EDTA	1.5 g
NaCl	0.7 g
Phosphate buffer (0.132M)	10 ml
Make up to 100 ml with H_2O.	

0.132M Phosphate Buffer, pH6.8

Na_2HPO_4 (mol. wt. 141.96)	18.74 g/l
KH_2PO_4 (mol. wt. 136.09)	17.96 g/l
Mix 1l of each to give pH 6.8	

2.7% NaCl/ PO_4

NaCl	2.7 g
0.132M PO_4	10 ml
Make up to 100 ml with H_2O.	

Filter all solutions through a 0.22µm filter.

Re: 2.5.5 RNA Preparation for Microarray & Hybridization

Medium Stringency buffer (1litre)

20 x SSC Ultra Pure	100ml
10% SDS	10ml
DEPC	890ml

High Stringency buffer (1litre)

20 x SSC Ultra Pure	5ml
10% SDS	5ml
DEPC	990ml

Post Wash buffer (1litre)

20 x SSC Ultra Pure	5ml
DEPC	995ml

1% SDS (1litre)

10% SDS	100ml
DEPC	900ml

Filter all solutions through a 0.22µm filter.

6.2 Appendix 2

List of all Differential expressed Gene ($p<0.05$) in PMNs incubated in the supernatant of *S. uberis* 0140J milk cultures compared to PMNs incubated in Milk

ID	Name	logFC	P.Value	adj.P.Val	Gene Product	PubMed Identifier
TC280060	Bt00008300	2.0522307	0.040084281	0.9999	Bos taurus suppressor of Ty 3 homolog (S. cerevisiae) (SUPT3H),	8464
TC279698	Bt00002046	1.7950908	0.018053657	0.9999	Bos taurus pyridine nucleotide-disulphide oxidoreductase domain 2 (PYROXD2)	84795
TC301115	BLO_ext_00249	1.7280485	0.006239608	0.9999	Bos taurus prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA	5743
TC278334	Bt00000010	1.5980438	0.02904543	0.9999	Bos taurus ATPase, Ca++ transporting, plasma membrane 1 (ATP2B1	490
TC303286	BLO_ext_00813	1.5646392	0.04802556	0.9999	Bos taurus interleukin 12 40kDa subunit (IL12p40) mRNA, complete	3593
TC348871	BLO_ext_00089	1.3904423	0.00476398	0.9999	Bos taurus solute carrier family 7 (cationic amino acid transporter, y+ system), member 5, mRNA	8140
TC320367	BLO_ext_00127	1.3263098	0.046256221	0.9999	PREDICTED: Bos taurus HLA-B associated transcript 2-like, transcript variant 4 (BAT2), mRNA	7916
TC362166	BLO_ext_01361	1.2962611	0.008482366	0.9999	CCL20	6364
TC346213	BLO_ext_00260	1.2773858	0.02618509	0.9999	??? - Homo sapiens carnitine palmitoyltransferase 1A (liver) (CPT1A)	1374
CN787616	BLO_ext_01516	1.2392781	0.006917831	0.9999	Bos taurus interleukin 8 (IL8), mRNA	3576

TC365520	BLO_ext_00097	1.226677	0.006796437	0.9999	PREDICTED: Bos taurus B42b-like (SPIB),	6689
TC264195	Bt00005205	1.1518548	0.009446591	0.9999	PREDICTED: Bos taurus MAP kinase-activated protein kinase 2-like (MAPKAPK2)	9261
TC384398	BLO_ext_01021	1.1334783	0.002169498	0.9999	Bos taurus StAR-related lipid transfer (START) domain containing 7 (STARD7),	56910
TC354007	BLO_ext_01171	1.1197955	0.038996093	0.9999	Bos taurus MAP kinase interacting serine/threonine kinase 1, mRNA	8569
TC334872	BLO_ext_00311	1.1052077	0.005323652	0.9999	Bos taurus BCL2-related protein A1 (BCL2A1),	597
TC349142	BLO_ext_01212	1.0927079	0.018205712	0.9999	8.???	
TC292818	Bt00008064	1.0916935	0.000754145	0.9999	Bos taurus nuclear receptor coactivator 7, mRNA	
TC280954	Bt00001344	1.0915865	0.031019769	0.9999	Bos taurus F-box and WD repeat domain containing 7 (FBXW7),	55294
TC302319	BLO_ext_00786	1.0758957	0.020024842	0.9999	Interleukin-8 precursor (IL-8) (CXCL8), complete	3576
TC292252	Bt00007056	1.0669116	0.011466072	0.9999	Bos taurus heterogeneous nuclear ribonucleoprotein D-like	9987
TC265449	Bt00007449	1.0622141	0.027935873	0.9999	???	
TC265112	Bt00007566	1.0580219	0.011788877	0.9999	Bos taurus zinc finger RAD18 domain-containing protein C1orf124	83932
TC334872	BLO_ext_01835	1.052336	0.006706473	0.9999	Bos taurus BCL2-related protein A1 (BCL2A1),	597
TC265095	Bt00000915	1.0268989	0.032225057	0.9999	Bos taurus Src-like-adaptor (SLA)	6503
TC332609	BLO_ext_01039	1.0190973	0.000469653	0.9999	Bos taurus chemokine (C-C motif) ligand 3 (CCL3), mRNA	6348

TC270521	Bt00001042	1.0069137	0.012528668	0.9999	PREDICTED:Bos taurus class E basic helix-loop-helix protein 22-like (LOC100438493)	525275
TC346994	BLO_ext_01667	1.0054223	0.00866878	0.9999	Bos taurus acetyl-CoA acetyltransferase 1 (ACAT1), nuclear gene encoding mitochondrial protein, mRNA	38
TC288064	Bt00005066	1.0040587	0.016166023	0.9999	Bos taurus serine/arginine-rich splicing factor 5 (SRSF5)	6430
TC288300	Bt00001362	0.9767791	0.011219578	0.9999	Bos taurus cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A)	1026
TC261806	Bt00007981	0.9336074	0.02414013	0.9999	Other spp similar to casein kinase 1, alpha 1	1452
TC278999	Bt00006894	0.9315588	0.001122346	0.9999	Bos taurus casein kinase 1, alpha 1, mRNA (cDNA clone MGC:133860 IMAGE:8051424), complete cds	120321
TC332683	BLO_ext_00608	0.9274987	0.033139069	0.9999	Bos taurus nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA)	4792
TC262404	Bt00002862	0.9254754	0.019746054	0.9999	PREDICTED: Bos taurus sorting nexin 9-like (SNX9)	51429
TC277342	Bt00001336	0.9145378	0.015380305	0.9999	Bos taurus v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian) (MAFF)	23764
TC279100	Bt00004054	0.9050894	0.002262304	0.9999	Bos taurus transglutaminase 3	343641
TC319160	BLO_ext_01071	0.8924126	0.009932955	0.9999	Homo sapiens nuclear casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1)	64710
TC277780	Bt00002608	0.8859808	0.002137444	0.9999	Bos taurus non-SMC element 1 homolog (S. cerevisiae) (NSMCE1)	197370
TC304649	BLO_ext_01821	0.8802318	0.012424566	0.9999	Bos taurus golgin A4 (GOLGA4)	2803

3X SSC	3X SSC	0.8695549	0.044369335	0.9999		
TC291688	Bt00000692	0.8334175	0.006691278	0.9999	Bos taurus chemokine (C-X-C motif) ligand 5, mRNA	6374
TC378794	BLO_ext_01689	0.8318371	0.022507824	0.9999	Bos taurus casein alpha s1,	1446
TC263414	Bt00004612	0.8313134	0.01750988	0.9999	PREDICTED: Pan troglodytes zinc finger protein 253, transcript variant 2 (ZNF253)	56242
TC359843	BLO_ext_00151	0.8095987	0.016634319	0.9999	Bos taurus retinoblastoma-like 1 (p107) (RBL1)	5933
TC266431	Bt00007563	0.8081819	0.010549532	0.9999	92% Smad nuclear interacting protein 1	79753
TC359669	BLO_ext_00845	0.806265	0.0069572	0.9999	voltage-dependent anion channel 3 [Bos taurus] /Bos taurus voltage-dependent anion channel 5, pseudogene	7419
TC317663	BLO_ext_00877	0.8024091	0.0425046	0.9999	PREDICTED: Bos taurus Monofunctional C1-tetrahydrofolate synthase, mitochondrial-like	
TC298822	Bt00008022	0.7930591	0.0280594	0.9999	Bos taurus diacylglycerol kinase, eta (DGKH)	160851
TC276581	Bt00007282	0.7852402	0.00051026	0.9999	NADH dehydrogenase subunit 2 [Bos taurus]/ complete mitochondrion genome seq	4729
TC326824	BLO_ext_01169	0.7680174	0.008777257	0.9999	Bos taurus major histocompatibility complex, class II, DQ beta (BOLA-DQB), mRNA	282495
TC289830	Bt00003423	0.755799	0.003740602	0.9999	Bos taurus RAB14, member RAS oncogene family (RAB14),	51552
TC326320	BLO_ext_00930	0.7421851	0.000917231	0.9999	???	
TC278934	Bt00004972	0.720156	0.039009542	0.9999	PREDICTED: Bos taurus Nipped-B homolog (Drosophila) (NIPBL)	25836

TC262379	Bt00007677	0.7160329	0.001282366	0.9999	Bos taurus proteasome (prosome, macropain) 26S subunit, non-ATPase, 3 (PSMD3), mRNA	5709
TC277642	Bt00001170	0.7160173	0.001726741	0.9999	Bos taurus sushi-repeat-containing protein, X-linked	27286
TC279132	Bt00008005	0.7094219	0.012421132	0.9999	Bos taurus RAN binding protein 2 (RANBP2)	5903
TC305737	BLO_ext_00124	0.7024293	0.037976841	0.9999	Bos taurus signal recognition particle 19kDa (SRP19)	6728
TC366499	BLO_ext_00946	0.688937	0.021677628	0.9999	rnmt	8731
TC306072	BLO_ext_00612	0.6881203	0.012169581	0.9999	Bos taurus solute carrier family 38, member 2 (SLC38A2)	54407
TC260344	Bt00001246	0.684884	0.038892513	0.9999	Bos taurus erythrocyte membrane protein band 4.9 (dematin)	2039
TC291405	Bt00002559	0.6841295	0.015152115	0.9999	PREDICTED: Bos taurus protein tyrosine phosphatase type IVA, member 1, transcript variant 2 (PTP4A1)	7803
TC347387	BLO_ext_00456	0.6810544	0.017158257	0.9999	Bos taurus K(lysine) acetyltransferase 5 (KAT5)	10524
TC298968	Bt00003685	0.6581573	0.015263877	0.9999	71% similar to: Canis familiaris chromosome 26, clone XX-367D6, complete sequence	340654
TC313034	BLO_ext_01275	0.6509534	0.021389524	0.9999	Bos taurus ubiquitin specific peptidase 11	8237
TC260940	Bt00007469	0.6498008	0.041152369	0.9999	Bos taurus potassium channel tetramerisation domain containing 15 (KCTD15)	79047
TC362744	BLO_ext_01658	0.6488661	0.012155731	0.9999	Homo sapiens SERTA domain containing 2 (SERTAD2)	9792
TC267196	Bt00004993	0.647851	0.049748232	0.9999	PREDICTED: Bos taurus homer protein homolog 3-like (LOC508124)	508124

TC323641	BLO_ext_00465	0.6360772	0.032382225	0.9999	PREDICTED: Bos taurus ARF GTPase-activating protein GIT1-like (GIT1)	28964
TC264549	Bt00006500	0.63593	0.015205072	0.9999	Bos taurus BCL2-related protein A1 (BCL2A1)	597
TC289705	Bt00003058	0.6321312	0.018169028	0.9999	Bos taurus mitochondrial succinate dehydrogenase complex subunit D (SDHD)	6392
TC263726	Bt00006196	0.6290897	0.028248065	0.9999	Bos taurus chromosome 12 open reading frame 57 ortholog (C5H12ORF57)	511545
TC283458	Bt00007526	0.6254424	0.009216017	0.9999	82% Homo sapiens forkhead transcription factor (FREAC2) gene	
TC321111	BLO_ext_01808	0.6198772	0.047385229	0.9999	PREDICTED: Bos taurus chromodomain helicase DNA binding protein 4 (CHD4)	1108
TC277709	Bt00006694	0.6173853	0.00180441	0.9999	PREDICTED: Bos taurus lysine (K)-specific demethylase 1A, transcript variant 2 (KDM1A), mRNA	23028
TC307794	BLO_ext_00022	0.6161659	0.011491206	0.9999	similar to UP|CDNA_FELCA (O19002) Cyclin-dependent kinase inhibitor 1 (p21) (CDK-interacting protein 1), partial (98%)	1026
TC277533	Bt00000093	0.6147841	0.016434199	0.9999	Bos taurus thioredoxin reductase	7296
TC276739	Bt00007335	0.6066081	0.025945593	0.9999	Bos taurus legumain	5641
TC263382	Bt00006019	0.5990663	0.037692544	0.9999	Bos taurus solute carrier family 38, member 2 (SLC38A2)	54407
TC260762	Bt00002208	0.5974352	0.034369367	0.9999	Homo sapiens SERTA domain containing 2 (SERTAD2),	9792
DV918751	BLO_ext_01547	0.5906311	0.033918712	0.9999	PREDICTED: Bos taurus ataxia telangiectasia mutated-like (ATM)	472

TC296578	Bt00006431	0.5871703	0.034103824	0.9999	Bos taurus transmembrane protein 102 (TMEM102)	284114
TC289042	Bt00003852	0.5847657	0.018074799	0.9999	PREDICTED: Bos taurus zinc finger and BTB domain containing 3-like (LOC508118)	79842
TC280297	Bt00004505	0.581536	0.003986521	0.9999	PREDICTED: Bos taurus protein tyrosine phosphatase, receptor type, E, transcript variant 2 (PTPRE)	5791
TC301906	BLO_ext_01328	0.5795819	0.001566504	0.9999	Bos taurus interleukin 1 receptor antagonist (IL1RN),	3557
TC265123	Bt00004614	0.574353	0.020646968	0.9999	Other spp. myosin light chain kinase	4638
TC293956	Bt00005119	0.5736674	0.013978743	0.9999	ankyrin repeat and BTB (POZ) domain containing 2 [Homo sapiens]	25841
TC294454	Bt00000791	0.5669323	0.036388279	0.9999	???	
TC290846	Bt00004551	0.5609159	0.036052021	0.9999	PREDICTED: Bos taurus FGFR1 oncogene partner-like (LOC100335363)	11116
TC292089	Bt00005055	0.5581411	0.007360991	0.9999	Bos taurus solute carrier organic anion transporter family, member 2A1	6578
TC304070	BLO_ext_00869	0.5510144	0.047726196	0.9999	Other sppPREDICTED: Sus scrofa extracellular related kinase 3 (SUSERK3),	5597
TC265010	Bt00004149	0.5487082	0.035476272	0.9999	Bos taurus MAX interactor 1 (MXI1)	4601
TC274338	Bt00002597	0.5472316	0.011889706	0.9999	Bos taurus collagen, type XVIII, alpha 1	80781
TC291913	Bt00004741	0.5462598	0.005573802	0.9999	5.???	
TC294158	Bt00005118	0.543254	0.019178307	0.9999	Bos taurus solute carrier 11A1 (SLC11A1) gene	6556
TC320323	BLO_ext_00112	0.5401201	0.012077176	0.9999	Bos taurus bone morphogenetic protein 2	656

CK773489	Bt00002391	0.5373134	0.005987278	0.9999	PREDICTED: Bos taurus Deafness, autosomal recessive 31-like (LOC533033), / CASK-interacting protein CIP98 [Bos taurus]	25861
TC265064	Bt00007559	0.5368674	0.018137527	0.9999	PREDICTED: Bos taurus tubulin folding cofactor E-like (TBCEL)	219899
TC293460	Bt00008147	0.5308719	0.004260563	0.9999	RNF41	10193
CK770390	Bt00000685	0.5287193	0.040274386	0.9999	Bos taurus CD34 molecule (CD34),	947
TC290083	Bt00005798	0.5274001	0.022604405	0.9999	Bos taurus osteoclast stimulating factor 1	1435
TC294150	Bt00006108	0.5240385	0.023787474	0.9999	Bos taurus transmembrane protein 177 (TMEM177)	80775
TC314145	BLO_ext_01339	0.5238979	0.028721657	0.9999	Bos taurus hypothetical LOC510651	510651
TC369042	BLO_ext_01249	0.5238581	0.043110665	0.9999	???	
TC307162	BLO_ext_00327	0.5146834	0.003662182	0.9999	Bos taurus insulin induced gene 1 (INSIG1)	3638
TC289830	Bt00007491	0.5085601	0.004191556	0.9999	Bos taurus RAB14, member RAS oncogene family (RAB14),	51552
TC290924	Bt00002022	0.490484	0.029481937	0.9999	Bos taurus plasminogen activator, urokinase receptor (PLAUR)	5329
TC290573	Bt00007320	0.4904174	0.039275487	0.9999	Bos taurus SEC11 homolog A (S. cerevisiae)	23478
TC281731	Bt00004401	0.489582	0.013922257	0.9999	PREDICTED: Canis familiaris similar to nuclear receptor co-repressor/HDAC3 complex subunit, transcript variant 1 (LOC488176)	8841
TC278673	Bt00008280	0.4886953	0.033155971	0.9999	Bos taurus cadherin 5, type 2 (vascular endothelium) (CDH5)	1003
TC279228	Bt00006291	0.4755307	0.031682125	0.9999	Bos taurus cannabinoid receptor interacting protein 1 (CNRIP1)	25927

TC279696	Bt00008215	0.4752285	0.00859445	0.9999	Bos taurus nudE nuclear distribution gene E homolog (A. nidulans)-like 1 (NDEL1), mRNA	81565
DT820236	BLO_ext_01531	0.4734749	0.015547697	0.9999	PREDICTED: Bos taurus ataxia telangiectasia and Rad3 related (ATR)	545
TC277492	Bt00006592	0.4552978	0.023020835	0.9999	Bos taurus myotubularin related protein 3 (MTMR3)	8897
TC305384	BLO_ext_00658	0.4532279	0.042194582	0.9999	PREDICTED: Bos taurus hypothetical protein LOC613429	613429
TC262059	Bt00003062	0.4505764	0.034122291	0.9999	Bos taurus lamin B1 (LMNB1)	4001
TC284094	Bt00003543	0.448504	0.048112873	0.9999	Bos taurus G protein-coupled receptor 18 (GPR18)	2841
TC268008	Bt00007440	0.4478432	0.029033915	0.9999	Bos taurus zinc finger protein 18 (KOX 11) (ZNF18),	7566
TC263932	Bt00006407	0.4466628	0.023951848	0.9999	Bos taurus cyclin-dependent kinase 2 interacting protein (CINP)	51550
TC349688	BLO_ext_01240	0.4429201	0.049297324	0.9999	PREDICTED: Bos taurus proprotein convertase subtilisin/kexin type 6 (PCSK6)	5046
BM251455	BLO_ext_01575	0.4393582	0.010285485	0.9999	Bos taurus protein tyrosine phosphatase, receptor type, C-associated protein (PTPRCAP)	5790
TC263033	Bt00006867	0.4380581	0.02023723	0.9999	Bos taurus UBA domain containing 1 (UBAC1)	10422
TC263222	Bt00006950	0.4377182	0.049323562	0.9999	PREDICTED: Bos taurus transcription elongation factor A protein-like 8-like (LOC100335186)	100335186
TC262573	Bt00008277	0.4347916	0.013965235	0.9999	Bos taurus olfactomedin-like 3 (OLFML3),	56944
TC270316	Bt00001549	0.4336504	0.031607555	0.9999	Bos taurus sex comb on midleg-like 4 (Drosophila) (SCML4)	256380

TC276127	Bt00007155	0.4322309	0.016522082	0.9999	Bos taurus coatomer protein complex, subunit alpha (COPA)	1314
TC278493	Bt00000165	0.4321336	0.033993263	0.9999	Bos taurus spectrin repeat containing, nuclear envelope 1,	23345
TC296368	Bt00000034	0.4286303	0.040573844	0.9999	PREDICTED: Bos taurus hypothetical LOC507299 (LOC507299)	507299
TC301109	BLO_ext_01778	0.4267886	0.045047759	0.9999	Bos taurus toll-like receptor 2 (TLR2)	7097
TC261604	Bt00000750	0.4227565	0.01460475	0.9999	PREDICTED: Bos taurus protein disulfide isomerase family A, member 6 (PDIA6),	10130
TC264274	Bt00000252	0.4200305	0.032406568	0.9999	Bos taurus chromosome 3 open reading frame 39 (C3orf39)/ glycosyltransferase [Bos taurus]	84892
TC291993	Bt00005117	0.4182977	0.02275651	0.9999	Bos taurus mannosidase, alpha, class 2C, member 1 (MAN2C1)	4123
TC312374	BLO_ext_00131	0.4174013	0.045543371	0.9999	PREDICTED: Bos taurus FLJ00400 protein-like (TMC8)	147138
TC280157	Bt00002801	0.4169272	0.040063378	0.9999	PREDICTED: Bos taurus tetratricopeptide repeat domain 28 (TTC28)	23331
TC281731	Bt00007917	0.416927	0.031671762	0.9999	PREDICTED: Canis familiaris similar to nuclear receptor co-repressor/HDAC3 complex subunit, transcript variant 1 (LOC488176)	79718
TC277255	Bt00005123	0.4152404	0.024357395	0.9999	Bos taurus COMM domain containing 3 (COMMD3)	23412
TC294239	Bt00006180	0.4125064	0.040376154	0.9999	Bos taurus nuclear fragile X mental retardation protein interacting protein 1 (NUFIP1)	26747
TC262843	Bt00006904	0.4074794	0.024155087	0.9999	Bos taurus OCIA domain containing 1	54940

TC262230	Bt00007369	0.4074427	0.025768525	0.9999	Bos taurus solute carrier family 37 (glycerol-3-phosphate transporter), member 3 (SLC37A3)	84255
TC263467	Bt00001189	0.4062924	0.048663943	0.9999	Bos taurus G protein beta subunit-like	64223
TC309408	BLO_ext_00350	0.4046797	0.035454655	0.9999	Bos taurus secreted frizzled-related protein 1 (SFRP1)	6422
TC279126	Bt00006978	0.4020171	0.020740456	0.9999	PREDICTED: Bos taurus ash2-like (ASH2L)	9070
TC284722	Bt00003124	0.3992534	0.039188484	0.9999	???	
TC264888	Bt00006779	0.3956723	0.032487294	0.9999	Bos taurus ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein, 1 (Drosophila) (ARIH1)	25820
TC284191	Bt00002937	0.3887799	0.048124581	0.9999	Bos taurus G protein-coupled receptor 19 (GPR19)	2842
TC341560	BLO_ext_00533	0.3834356	0.020327497	0.9999	PREDICTED: Bos taurus TNFAIP3 interacting protein 2-like (LOC100336382)	79155
TC292506	Bt00000646	0.3831918	0.036502415	0.9999	PREDICTED: Bos taurus malignant fibrous histiocytoma amplified sequence 1-like (LOC782395)	9258
TC281577	Bt00004382	0.3792729	0.020099955	0.9999	Bos taurus zinc finger CCCH-type containing 12A (ZC3H12A)	80149
TC269681	Bt00007124	0.3748167	0.046639122	0.9999	Bos taurus moesin (MSN)	4478
TC353647	BLO_ext_00966	0.3728141	0.032994735	0.9999	Bos taurus eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3)	9451
TC265861	Bt00005515	0.372731	0.028168676	0.9999	PREDICTED: Bos taurus kinase suppressor of ras-like (KSR1)	8844
TC274336	Bt00008323	0.3711971	0.036557084	0.9999	Bos taurus cleft lip and palate associated transmembrane protein 1	1209

					(CLPTM1)	
TC266323	Bt00004984	0.3693119	0.047191565	0.9999	Bos taurus exosome component 2 (EXOSC2)	23404
TC263575	Bt00007132	0.3684037	0.039843822	0.9999	HLA-B associated transcript 5 [Bos taurus]	7920
TC263160	Bt00006997	0.3616417	0.034469965	0.9999	Bos taurus phenylalanine hydroxylase (PAH)	5053
CK776781	Bt00004226	0.3551084	0.049082729	0.9999	Bos taurus LIM homeobox 2 (LHX2)	9355
TC278440	Bt00005750	0.3545765	0.035858413	0.9999	Bos taurus hypothetical protein LOC617407 (MYEOV2)	150678
TC290661	Bt00000804	0.3498708	0.026883825	0.9999	Bos taurus selenoprotein S (SELS)	55829
TC266895	Bt00004006	0.3477441	0.043885141	0.9999	Bos taurus aminolevulinate, delta-, synthase 2 (ALAS2), nuclear gene encoding mitochondrial protein	212
TC294182	Bt00003131	0.3381972	0.043843573	0.9999	PREDICTED: Bos taurus Ephrin type-A receptor 2-like (EPHA2)	1969
TC276619	Bt00001026	-0.3007036	0.046330664	0.9999	Bos taurus proteasome activator 28 alpha subunit (PSME1)	5720
TC271006	Bt00000309	-0.308377	0.049769906	0.9999	PREDICTED: Bos taurus Protein SPT2 homolog (SPTY2D1)	144108
TC277336	Bt00001294	-0.3109363	0.046404978	0.9999	Bos taurus Protein FAM177A1 (FAM177A1)	283635
TC266737	Bt00001722	-0.3232869	0.043536817	0.9999	Bos taurus retinol dehydrogenase 5 (11-cis/9-cis) (RDH5)	5959
TC264579	Bt00000415	-0.3307699	0.044876914	0.9999	Bos taurus dual specificity phosphatase 26 (putative) (DUSP26)	78986
TC277732	Bt00007545	-0.340751	0.041944263	0.9999	similar to -linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-	8473

					acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	
TC292021	Bt00004996	-0.3509704	0.032038455	0.9999	Bos taurus LSM2 homolog, U6 small nuclear RNA associated (S. cerevisiae) (LSM2), mRNA	57819
TC290641	Bt00001448	-0.354034	0.043787281	0.9999	Bos taurus intraflagellar transport 20 homolog (Chlamydomonas), mRNA (cDNA clone :128507 IMAGE:7984736), complete cds Length=1116	90410
TC295191	Bt00002594	-0.3595893	0.029622981	0.9999	Bos taurus DEAD (Asp-Glu-Ala-Asp) box polypeptide 25 (DDX25),	29118
TC266660	Bt00000024	-0.3677349	0.028329748	0.9999	Bos taurus megakaryocyte-associated tyrosine kinase (MATK), mRNA	4145
CK772121	Bt00001655	-0.3746503	0.038865869	0.9999	Bos taurus ELK4, ETS-domain protein (SRF accessory protein 1) (ELK4), mRNA	2005
TC262137	Bt00007214	-0.3761254	0.041268053	0.9999	homologue to UP|O77972 (O77972) MHC class I antigen precursor, partial (94%)	515712
CK846934	Bt00005947	-0.3820454	0.048206147	0.9999	???	
TC291215	Bt00000788	-0.391137	0.040626126	0.9999	Bos taurus XIAP associated factor 1, mRNA (cDNA clone MGC:152158 IMAGE:8401538), complete cds	54739
TC264026	Bt00000036	-0.3954973	0.022224638	0.9999	Bos taurus YY1 transcription factor (YY1), mRNA	7528
TC260259	Bt00007261	-0.3964111	0.045228977	0.9999	similar to UP|Q96AQ3 (Q96AQ3) Activating transcription factor 4 (ATF4 protein), partial (97%)	468

TC274694	Bt00000319	-0.3995986	0.035331919	0.9999	Bos taurus pre-B-cell leukemia homeobox interacting protein 1 (PBXIP1), mRNA	57326
TC280483	Bt00001023	-0.4003575	0.023447415	0.9999	Bos taurus mitogen-activated protein kinase 10 (MAPK10), mRNA	5602
TC291256	Bt00006331	-0.4016636	0.030936768	0.9999	Bos taurus cDNA clone IMAGE:8204390, **** WARNING: chimeric clone ****/neuroplastin[Bos taurus]	27020
TC332973	BLO_ext_00373	-0.4049599	0.045710582	0.9999	???	
TC323485	BLO_ext_01377	-0.4067972	0.029863043	0.9999	???	
TC375575	BLO_ext_01721	-0.4072243	0.039824431	0.9999	Bos taurus thyroid hormone receptor interactor 12 (TRIP12), mRNA	9320
TC289232	Bt00006991	-0.4086506	0.049622477	0.9999	PREDICTED: Bos taurus glyceraldehyde-3-phosphate dehydrogenase-like, transcript variant 2 GAPDH), mRNA Length=1321	2597
TC290662	Bt00008157	-0.4103671	0.022377858	0.9999	Bos taurus DDB1 and CUL4 associated factor 12 (DCAF12), mRNA Length=3606	25853
TC294903	Bt00001534	-0.4108721	0.04071321	0.9999	PREDICTED: Bos taurus endoplasmic reticulum metallopeptidase 1 ERMP1),mRNALength=3131	79956
TC293332	Bt00005116	-0.4156938	0.031938221	0.9999	Bos taurus tetraspanin 2 (TSPAN2), mRNA	10100
TC263111	Bt00005032	-0.4236258	0.030302973	0.9999	Bos taurus selenoprotein W, 1 (SEPW1), mRNA Length=975	6415

TC276886	Bt00000226	-0.4238415	0.014749543	0.9999	B.taurus CI-19 mRNA for 19 kDa subunit of NADH:ubiquinone oxidoreductase complex complex I) Length=793	4702
TC290892	Bt00007027	-0.4240478	0.040408732	0.9999	PREDICTED: Bos taurus cyclin L2 (CCNL2), mRNA Length=2268	81669
TC261318	Bt00007055	-0.4264981	0.046785748	0.9999	similar to GB|AAC50817.1|1669498|HSU63420 Sp140 protein {Homo sapiens;} , partial (36%)	
TC288347	Bt00003725	-0.4292927	0.04228924	0.9999	similar to UP|RT12_MOUSE (O35680) 28S ribosomal protein S12, mitochondrial precursor (MPR-S12) (MT-RPS12), partial (79%)	
TC299695	Bt00001133	-0.4326274	0.026075278	0.9999	Bos taurus enhancer of mRNA decapping 3 homolog (S. cerevisiae) (EDC3), mRNA	80153
TC279941	Bt00001258	-0.4419895	0.04244383	0.9999	PREDICTED: Bos taurus arrestin, beta 2 (ARRB2), mRNA Length=2027	409
TC281108	Bt00002895	-0.4440237	0.007585969	0.9999	PREDICTED: Bos taurus cartilage intermediate layer protein, nucleotide pyrophosphohydrolase-like (LOC100336614), miscRNA Length=4405	8483
TC260532	Bt00002710	-0.4442126	0.012324431	0.9999	Bos taurus BCL2-associated X protein (BAX), mRNA	581
TC277236	Bt00006928	-0.4499226	0.029739448	0.9999	Bos taurus fucosidase, alpha-L- 1, tissue (FUCA1), mRNA	2517
TC275397	Bt00005299	-0.4522479	0.027024798	0.9999	Bos taurus damage-specific DNA binding protein 2, 48kDa (DDB2)	1643
TC336987	BLO_ext_00935	-	0.033314767	0.9999	???	

		0.4562459				
TC316499	BLO_ext_01712	-0.4573851	0.026340669	0.9999	Bos taurus complement factor properdin (CFP), mRNA	5199
TC289844	Bt00000641	-0.4574335	0.037837545	0.9999	???	
TC290803	Bt00000321	-0.4600286	0.018210341	0.9999	Bos taurus nucleoporin 93kDa (NUP93), mRNA	9688
TC302380	BLO_ext_00716	-0.4645305	0.026005616	0.9999	Bos taurus RAB11B, member RAS oncogene family (RAB11B), mRNA	9230
TC278232	Bt00005769	-0.4652662	0.049932706	0.9999	Bos taurus chromosome 17 open reading frame 37 ortholog (C19H17orf37), mRNA	505710
TC274907	Bt00006659	-0.4658035	0.008099921	0.9999	Bos taurus tubulin, alpha 4a (TUBA4A), mRNA	7277
TC282581	Bt00005907	-0.4692216	0.019201868	0.9999	Bos taurus protein arginine methyltransferase 3 (PRMT3), mRNA	10196
TC278690	Bt00003759	-0.4784624	0.030574211	0.9999	Bos taurus NudC domain containing 2, mRNA (cDNA clone MGC:157142 IMAGE:8445848), complete cds Length=928	134492
TC279191	Bt00003812	-0.4837959	0.043958926	0.9999	Bos taurus S100 calcium binding protein A8 (S100A8), mRNA	6279
TC262912	Bt00001175	-0.4841372	0.012296855	0.9999	Bos taurus GABA(A) receptor-associated protein (GABARAP), mRNA	11337
TC352430	BLO_ext_01203	-0.4848352	0.004113953	0.9999	Bos taurus nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, mRNA (cDNA clone MGC:159793 IMAGE:8566028), complete cds Length=4006	4790

TC289914	Bt00005525	-0.4891006	0.037696196	0.9999	PREDICTED: Bos taurus methyl-CpG binding domain protein 2-like (LOC100337206), mRNA Length=1186	8932
TC278851	Bt00003119	-0.4938317	0.015021413	0.9999	Bos taurus MID1 interacting protein 1 (gastrulation specific G12 homolog (zebrafish)) (MID1IP1), mRNA	58526
TC274870	Bt00001766	-0.4971227	0.037268566	0.9999	PREDICTED: Bos taurus hypothetical protein LOC507475 (C2orf17), mRNA Length=2830	79137
TC302757	BLO_ext_00973	-0.4978186	0.037386555	0.9999	PREDICTED: Bos taurus high-mobility group box 2-like (LOC618297), mRNA Length=1224	3148
TC277381	Bt00002702	-0.5108908	0.011758632	0.9999	Bos taurus glutamate-cysteine ligase catalytic subunit (GCLC), MHC class II antigen	2729
TC265296	Bt00003835	-0.5112298	0.009942694	0.9999	thymosin beta 15a (TMSB15A)	11013
TC340441	BLO_ext_00298	-0.5152751	0.030607327	0.9999	chk again	4067
TC264146	Bt00001614	-0.5162334	0.020431182	0.9999	Bos taurus GDP dissociation inhibitor 1 (GDI1), mRNA	2664
TC260186	Bt00006414	-0.5177554	0.010525536	0.9999	Bos taurus DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 (DDX5), mRNA Length=2460	1655
3X SSC	3X SSC	-0.5195017	0.037082798	0.9999		
TC294081	Bt00005512	-0.5212872	0.003411415	0.9999	Bos taurus empty spiracles homeobox 2 (EMX2), mRNA	2018
TC331799	BLO_ext_00405	-0.5218898	0.025764253	0.9999	Bos taurus inositol 1,4,5-triphosphate receptor interacting protein (ITPRIP), mRNA	85450

TC261379	Bt00005603	-0.5219759	0.031048512	0.9999	Bos taurus chromosome 10 open reading frame 58 ortholog (C28H10orf58), mRNA	534049
TC275669	Bt00006629	-0.5281892	0.035513335	0.9999	Bos taurus dystrobrevin, beta (DTNB), mRNA Length=2236	1838
TC290180	Bt00000320	-0.5326378	0.016398512	0.9999	Bos taurus GLI pathogenesis-related 2 (GLIPR2), mRNA	152007
CK778581	Bt00005131	-0.5455767	0.049744792	0.9999	p25/p35=Cyclin-dependent kinase 5 activator [cattle, brain, mRNA,	8851
TC261722	Bt00003466	-0.5465393	0.002999324	0.9999	PREDICTED: Bos taurus interferon gamma receptor 2 (interferon gamma transducer 1) (IFNGR2), mRNA Length=2450	3460
TC276804	Bt00000048	-0.5570155	0.035704122	0.9999	Bos taurus FK506 binding protein 3, 25kDa (FKBP3), mRNALength=965	2287
TC278620	Bt00000858	-0.5594772	0.002686588	0.9999	PREDICTED: Bos taurus transducer of ERBB2, 1, transcript variant 1 (TOB1), mRNA Length=1624	10140
TC363120	BLO_ext_00141	-0.5619924	0.032330248	0.9999	Bos taurus ribosomal protein SA, mRNA (cDNA clone MGC:127394 IMAGE:7952265), complete cds	3921
TC277432	Bt00006028	-0.5625449	0.031165223	0.9999	Bos taurus stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein) (STIP1), mRNA	10963
3X SSC	3X SSC	-0.5635035	0.03133276	0.9999		
TC290563	Bt00001647	-0.5675191	0.013714313	0.9999	chk again - not on BLAST but on gene list as Yippee-like pr3/no match after SW chk	
TC266345	Bt00000060	-0.5701487	0.002729738	0.9999	Bos taurus transducin (beta)-like 2 (TBL2), mRNA	26608

TC355595	BLO_ext_01714	-0.570866	0.047894305	0.9999	Bos taurus heat-shock 70-kilodalton protein 1A (HSPA1A) and heat-shock 70-kilodalton protein 1B (HSPA1B) genes, complete	3303
TC292929	Bt00003987	-0.5803721	0.030769865	0.9999	Bos taurus tRNA splicing endonuclease 2 homolog (S. cerevisiae), mRNA (cDNA clone MGC:166394 IMAGE:8479061), complete cds Length=2084	80746
TC292492	Bt00001330	-0.585259	0.038474826	0.9999	PREDICTED: Bos taurus immediate early response 2-like (LOC100335829), mRNA Length=2143	9592
TC275351	Bt00000450	-0.5903565	0.021857794	0.9999	3.???	
TC318087	BLO_ext_01656	-0.5914872	0.03526453	0.9999	Bos taurus alpha-2-macroglobulin (A2M), mRNA	2
TC276744	Bt00005805	-0.5958061	0.034929901	0.9999	PREDICTED: Bos taurus calmodulin 2-like (LOC100297240), mRNA Length=1144	805
TC263098	Bt00005763	-0.5974019	0.032971675	0.9999	Bos taurus citrate synthase (CS), nuclear gene encoding mitochondrial protein, mRNA	1431
TC289511	Bt00007216	-0.6038192	0.033482297	0.9999	Bos taurus heat shock 70kDa protein 1A (HSPA1A), mRNA	3303
TC275352	Bt00004613	-0.6047629	0.02004654	0.9999	PREDICTED: Bos taurus apolipoprotein L4-like (LOC518495), miscRNA Length=3360	
TC263733	Bt00001079	-0.6059492	0.025112101	0.9999	Bos taurus gamma-inducible protein 30 (IFI30), mRNA	10437
TC274428	Bt00001352	-0.6067946	0.022800704	0.9999	Bos taurus phosphatidylglycerophosphate	9489

					synthase 1 (PGS1), mRNA Length=3584	
TC278133	Bt00002317	-0.6095712	0.029590218	0.9999	Bos taurus CD47 molecule (CD47), mRNA	961
TC264915	Bt00002146	-0.6118817	0.021423217	0.9999	2.???	
TC314950	BLO_ext_00459	-0.6178177	0.024966141	0.9999	Bos taurus dCTP pyrophosphatase 1 (DCTPP1), mRNA	79077
TC367570	BLO_ext_01075	-0.6216882	0.010359661	0.9999	Bos taurus golgi to ER traffic protein 4 homolog (S. cerevisiae) (GET4), mRNA	51608
TC304483	BLO_ext_01612	-0.6245521	0.032378853	0.9999	Bos taurus amyloid beta (A4) precursor protein-binding, family B, member 3	10307
TC291119	Bt00000333	-0.6284391	0.002451886	0.9999	AT-hook transcription factor [Homo sapiens]	80709
TC265246	Bt00004431	-0.6296791	0.040440466	0.9999	similar to DVL-binding protein DAPLE, transcript variant 1	
TC293478	Bt00003548	-0.6303222	0.012003873	0.9999	Bos taurus myogenic factor 6 (herculin), mRNA	4618
TC267031	Bt00005887	-0.6310142	0.030484584	0.9999	Bos taurus eukaryotic translation initiation factor 2C, 3 (EIF2C3)	
TC263181	Bt00007790	-0.6436186	0.049963773	0.9999	PREDICTED: Bos taurus regulator of G-protein signaling 2-like (RGS2), mRNA	5997
TC263963	Bt00000584	-0.6454735	0.0043014	0.9999	Bos taurus torsin family 1, member A (torsin A) (TOR1A),	1861
TC265651	Bt00004882	-0.6551844	0.001143084	0.9999	PREDICTED: Bos taurus retinoic acid receptor, alpha (RARA), mRNA	5914
TC289553	Bt00003687	-0.6642732	0.025201282	0.9999	os taurus retinoic acid receptor responder (tazarotene induced) 2	5919

					(RARRES2),	
TC263767	Bt00004062	-0.6675882	0.023681324	0.9999	PREDICTED: Bos taurus kinesin light chain 4 (KLC4), mRNA	89953
TC285244	Bt00003573	-0.6704754	0.02273841	0.9999	Bos taurus armadillo repeat containing 2 (ARMC2	84071
Rn_Control_09	Rn_Control_09	-0.6705993	0.031543978	0.9999		
TC260214	Bt00003180	-0.6737341	0.035877591	0.9999	???	
TC367139	BLO_ext_01030	-0.6738599	0.019160163	0.9999	Bos taurus growth arrest and DNA-damage-inducible, alpha,	1647
TC267448	Bt00003475	-0.6801215	0.010472484	0.9999	PREDICTED: Bos taurus zinc finger protein 76 (expressed in testis)-like (LOC100337287), mRNA	7629
TC278438	Bt00001802	-0.6827445	0.013894496	0.9999	Bos taurus early growth response 1, mRNA	1958
CK769831	Bt00000352	-0.682837	0.030741375	0.9999	???	
TC300762	Bt00003550	-0.684194	0.047569939	0.9999	Bos taurus major facilitator superfamily domain containing 10-like	
TC277110	Bt00003303	-0.6847696	0.001604192	0.9999	Bos taurus chromosome 2 open reading frame 24 ortholog	507473
TC274295	Bt00001718	-0.6926451	0.008129825	0.9999	Bos taurus sorcin (SRI), mRNA	6717
TC294544	Bt00005535	-0.6930836	0.034497667	0.9999	Bos taurus alanyl-tRNA synthetase 2, mitochondrial (putative) (AARS2), nuclear gene encoding mitochondrial protein, mRNA Length=3783	57505
TC351653	BLO_ext_00694	-0.6976877	0.00544446	0.9999	MLL	4297

TC333673	BLO_ext_00841	-0.7034666	0.00564252	0.9999	4.???	
TC282695	Bt00003954	-0.7067364	0.012119552	0.9999	Bos taurus exocyst complex component 8 (EXOC8), mRNA	149371
TC278332	Bt00001038	-0.7127656	0.033893797	0.9999	Bos taurus regulator of G-protein signaling 19 (RGS19), mRNA	10287
TC321922	BLO_ext_01623	-0.7181405	0.025404722	0.9999	Bos taurus CCAAT/enhancer binding protein (C/EBP), delta (CEBPD), mRNA	1052
TC277480	Bt00005182	-0.7304145	0.006278031	0.9999	PREDICTED: Bos taurus erbb2 interacting protein, transcript variant 2 (ERBB2IP), mRNA	55914
TC292630	Bt00005279	-0.7383412	0.03743593	0.9999	Bos taurus uridine phosphorylase 1 (UPP1), mRNA	7378
TC279624	Bt00006409	-0.753558	0.007189578	0.9999	Bos taurus sorting nexin 10 (SNX10), mRNA	29887
TC278443	Bt00002186	-0.7664786	0.026305696	0.9999	Bos taurus v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), mRNA, complete cds Length=2097	2353
TC276824	Bt00001530	-0.7665316	0.017859635	0.9999	Bos taurus dual specificity phosphatase 1 (DUSP1), mRNA	1843
TC367459	BLO_ext_00836	-0.7675945	0.007114349	0.9999	Bos taurus amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein (APBB1IP), mRNA	54518
TC278936	Bt00007536	-0.7687546	0.020227269	0.9999	Bos taurus ubiquitin-conjugating enzyme E2G 2 (UBC7 homolog, yeast) (UBE2G2), mRNA, complete cds Length=2159	7327
TC261637	Bt00003698	-0.7715723	0.007104173	0.9999	Bos taurus egl nine homolog 2 (C. elegans) (EGLN2), mRNA, complete	112398

					cds Length=1818	
TC265527	Bt00002154	-0.7866212	0.000303215	0.9999	Bos taurus hemopoietic cell kinase (HCK), transcript variant 1, mRNA Length=2046	3055
TC357443	BLO_ext_00927	-0.7917521	0.004351336	0.9999	PREDICTED: Bos taurus lymphotoxin beta (TNF superfamily, member 3) (LTB), mRNA	4050
TC334244	BLO_ext_00079	-0.7933595	0.0440944	0.9999	Bos taurus cDNA clone IMAGE:8452392 Length=1216	7326
TC299298	Bt00006297	-0.8040281	0.025167653	0.9999	PREDICTED: Bos taurus C2 calcium-dependent domain containing 3 (C2CD3), partial mRNA	26005
TC290822	Bt00004639	-0.8073241	0.011007653	0.9999	Bos taurus ubiquitin-conjugating enzyme E2G 1 (UBC7 homolog, yeast) (UBE2G1)	7326
TC263181	Bt00001594	-0.8129252	0.043891789	0.9999	PREDICTED: Bos taurus regulator of G-protein signaling 2-like (RGS2), mRNA	5997
TC361856	BLO_ext_00349	-0.8144223	0.005791424	0.9999	Bos taurus Parkinson disease 7 domain containing 1 (PDDC1)	347862
TC277688	Bt00004637	-0.8218049	0.003215404	0.9999	PREDICTED: Bos taurus Rho GTPase activating protein 9-like (ARHGAP9), mRNA Length=2727	64333
3X SSC	3X SSC	-0.8222615	0.018442282	0.9999		
3X SSC	3X SSC	-0.8351846	0.01818489	0.9999		
TC295250	Bt00006737	-0.8392626	0.045216896	0.9999	Bos taurus vanin 2 (VNN2	8875
TC310648	BLO_ext_01200	-0.8574485	0.001239908	0.9999	Bos taurus serine dehydratase (SDS)	10993

3X SSC	3X SSC	-0.888731	0.028668544	0.9999		
TC275353	Bt00006525	-0.889815	0.035214426	0.9999	PREDICTED: Bos taurus N-myc-interactor-like, transcript variant 2 (LOC100336259), mRNA	9111
TC290705	Bt00005818	-0.8944422	0.020577536	0.9999	Bos taurus mitochondrial ribosomal protein L34 (MRPL34), nuclear gene encoding mitochondrial protein, mRNA	64981
TC275219	Bt00000176	-0.8974086	0.001075358	0.9999	PREDICTED: Bos taurus eIF4GI protein, transcript variant 2 (EIF4GI), mRNA	1981
TC278835	Bt00003669	-0.8977516	0.020381768	0.9999	PREDICTED: Bos taurus thioredoxin domain containing 17 (TXNDC17),	84817
TC261047	Bt00005723	-0.9102193	0.038651376	0.9999	Bos taurus mitochondrial translational release factor 1-like (MTRF1L), nuclear gene encoding mitochondrial protein, mRNA	54516
TC278090	Bt00005520	-0.9316696	0.024920119	0.9999	PREDICTED: Bos taurus solute carrier family 40 (iron-regulated transporter), member 1-like (LOC100335220), mRNA	30061
TC277881	Bt00000391	-0.9544988	0.00232998	0.9999	PREDICTED: Bos taurus lymphotoxin beta (TNF superfamily, member 3) (LTB), mRNA	4050
TC319937	BLO_ext_01352	-0.9784217	0.002317773	0.9999	Bos taurus fibrinogen-like 2 (FGL2), mRNA	10875
TC368847	BLO_ext_00091	-0.9893231	0.048289121	0.9999	???	
TC289789	Bt00002906	-0.994158	0.037249807	0.9999	Bos taurus solute carrier family 39 (zinc transporter), member 1 (SLC39A1), mRNA	27173

TC375717	BLO_ext_00404	-0.9951563	0.000509981	0.9999	Bos taurus uncoupling protein 2 (mitochondrial, proton carrier) (UCP2), nuclear gene encoding mitochondrial protein, mRNA	7351
TC99448	TC99448	-1.0055053	0.025461097	0.9999	Bos taurus glucuronidase, beta (GUSB), mRNA	2990
TC343755	BLO_ext_00820	-1.0254509	0.012156463	0.9999	DCN1, defective in cullin neddylation 1, domain containing 5	84259
TC279244	Bt00001997	-1.0421358	0.006543508	0.9999	Bos taurus 1-acylglycerol-3-phosphate O-acyltransferase 3 (AGPAT3), mRNA, incomplete 5' cds Length=699	56894
TC267945	Bt00006569	-1.043036	0.016981817	0.9999	Bos taurus cytochrome b-561 domain containing 1 (CYB561D1), mRNA	284613
TC290878	Bt00005523	-1.0672874	0.020615908	0.9999	Bos taurus microtubule associated monoxygenase, calponin and LIM domain containing 1 (MICAL1), mRNA	64780
TC375950	BLO_ext_00587	-1.0956933	0.013116818	0.9999	UP|MK09_HUMAN (P45984) Mitogen-activated protein kinase 9 (Stress-activated protein kinase JNK2) (c-Jun N-terminal kinase 2) (JNK-55) , partial (80%)	5601
TC335505	BLO_ext_01400	-1.102648	0.002014228	0.9999	Bos taurus ring finger protein 145 (RNF145), mRNA >gb|BC148080.1| Bos taurus ring finger protein 145, mRNA (cDNA clone MGC:152141 IMAGE:8398633), complete cds	153830
TC301648	BLO_ext_01747	-1.1347712	0.002939321	0.9999	Bos taurus DNA fragmentation factor, 40kDa, beta polypeptide (caspase-activated DNase) (DFFB), mRNA	1677

TC99448	TC99448	-1.1724083	0.016557485	0.9999	Bos taurus glucuronidase, beta (GUSB), mRNA	2990
TC302370	BLO_ext_00125	-1.2355543	0.002240387	0.9999	Bos taurus v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), mRNA, complete cds	2353