THE UNIVERSITY
of EDINBURGH

Response to Intramammary Challenge with Putatively Host-Adapted and Non-Adapted Strains of *Streptococcus uberis* in Cattle

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A thesis submitted in fulfilment of the requirements for the degree

of Doctor of Philosophy at College of Medicine and Veterinary Medicine

of University of Edinburgh

2014

Abstract

Streptococcus uberis is an important cause of intramammary infection in dairy cattle. Strains of *S. uberis* appear to differ in their ability to cause disease based on previous epidemiological studies. We explored the pathogenicity of 2 strains of *S. uberis*, where one strain represented a putatively host-adapted type based on its ability to cause persistent infection and to spread from cow to cow in a lactating herd. This type was part of a clonal complex that is commonly associated with bovine mastitis. The other strain, which was isolated from a transient infection in a single animal in the same herd and did not belong to any known clonal complex, was selected as putatively non-adapted type. Cows (6 per strain) were experimentally challenged in a single hind quarter and the adjacent hind quarter was used as mock challenged control quarter. Both strains showed an equal ability grow in milk of challenge animals in vitro. All cows that were challenged with the putatively host-adapted strain developed clinical signs of mastitis, including fever and milk yield depression as well as elevated somatic cell count due to influx of polymorphonuclear leucocytes and lymphocytes. The cytokine response followed a specific order, with an increase in IL-1β, IL-6 and IL-8 levels at the time of first SCC elevation, followed by an increase in IL-10, IL-12p40 and TNF-α levels approximately 6 h later. In 4 of 6 animals, IL-17A was detected in milk between 57 and 168 h post challenge. The increase in IL-17A levels coincided with inversion of the pre-challenge $CD4^+$:$CD8^+$ T lymphocyte ratio, and was observed from 96 h post challenge. This was followed by normalisation of the $CD4^+$:$CD8^+$ ratio due to continued increase of the $CD8^+$ concentration up to 312 h post challenge. Spontaneous resolution of infection was observed in 5 animals and coincided with a measurable IL-17A response in 4

animals, suggesting that IL-17 may be involved in the resolution of intramammary infection. To explore the mechanism of action of IL-17A we stimulated bovine PMN and bovine blood derived macrophages with recombinant IL-17A *in vitro*. IL-17A enhanced the killing ability of phagocyte toward the challenge strain. With the exception of minor elevation of IL-8 levels, no clinical, cytological or immunological response was detected in quarters challenged with the non-adapted strain. The observed strain specific pathogenicity was consistent across animals, implying that it is determined by pathogen factors rather than host factors. We further studied in vitro possible mechanisms involved in the differences observed between the two strains such as ability to adhere to the mammary epithelial cells, ability to resist to killing by phagocytes and ability to form biofilm. The adapted strain FSL Z1-048 showed an increased ability to adhere to the epithelial cells and an increase ability to resist to killing of monocyte derived macrophages. These mechanisms thus could potentially explain the *in vivo* observations.

Declaration

I, Riccardo Tassi declare that the work reported in this thesis was conducted under the supervision of Dr. Tom McNeilly, Prof. Ruth Zadoks and Dr. Michael Fontaine at the Moredun Research Institute and the College of Medicine and Veterinary Medicine, University of Edinburgh and it has not been submitted in any form for another degree or professional qualification. All the result presented, unless otherwise stated are the sole work of this author, as is the composition of the thesis.

Signed:

Date:

Acknowledgments

I would like to express my gratitude to my principal supervisors Dr. Tom McNeilly and Prof. Ruth Zadoks for their guidance, patience and support and also to my co-supervisor Dr. Michael Fontaine.

I would like to acknowledge Moredun Research Institute`s Bioservices division, and in particular stockmen for their support during the challenge experiments

Dr. Mara Rocchi for the help in the optimization of FACS protocol, Dr. Christian Delannoy for the help with the genome analysis. Ian Heron for the help in the bacteriology laboratory. Dr. Anja Sipka, Dr. Abhijit Gurjar and Suzanne Klaessig for the help with the PMN killing assay. Dr. Mintu Nath for the advice on the statistical analysis. I would also thank Dr. Keith Ballingall for the support during these years.

I would thank Pfizer Animal Health which financially supported this study.

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Published Work

Tassi, R., T. McNeilly, J. Fitzpatrick, M. Fontaine, D. Reddick, C. Ramage, M. Lutton, Y. Schukken, and R. Zadoks. 2013. Strain-specific pathogenicity of putative host-adapted and non adapted strains of *Streptococcus uberis* in dairy cattle. Journal of Dairy Science 96(8):5129-5145

CHAPTER 1

General Introduction

1. 1 General Introduction

Mastitis is defined as an inflammatory process of the mammary gland. In cattle the most common cause is an ascending bacterial infection. Bacterial pathogens causing mastitis are divided in two categories: Contagious pathogens and environmental/opportunistic pathogens. Contagious pathogens have the infected mammary gland as primary reservoir. Infection spreads from cow to cow during the milking (Radostis *et al*., 1994). Environmental pathogens have the herd environment as reservoir. *Streptococcus agalactiae, Staphylococcus aureus*, and *Mycoplasma bovis* are generally classified as contagious pathogens, whilst *Escherichia coli* and *Klebsiella pneumoniae* are considered environmental pathogens. However, recent data suggests that within certain mastitis pathogen species such as *E. coli* (Dopfer *et al*., 2000), *K. pneumoniae* (Munoz *et al*., 2007) and *S. aureus* (Roberson *et al*., 1994; Smith *et al*., 2005) both pathogen transmission routes may occur.

Streptococcus uberis is a major mastitis pathogen. It is the most common cause of both clinical (32% of the cases) and sub-clinical (13.8% of the cases) mastitis in the UK (Bradley *et al*., 2007). It is often considered an environmental pathogen (Leigh, 1999), although epidemiological, management and recent molecular data show that different strains may have different epidemiology and clinical manifestation (Reviewed by Zadoks, 2007). Control programs for contagious mastitis help to decrease the prevalence and incidence of *S. uberis* mastitis (Neave *et al*., 1969). Studies using strain typing methods such as pulsed field gel electrophoresis (PFGE) (Phuektes *et al*., 2001), and multi locus sequence typing (MLST) (Coffey *et al*.,

2006) show that many *S. uberis* strains are present in the herd environment and the vast majority of mastitis cases are caused by several different strains. Other studies using strain typing showed that most of *S. uberis* mastitis cases are caused by a few strains (Oliver *et al*., 1998; Zadoks *et al*., 2003).

In a longitudinal study an outbreak of *S. uberis* mastitis in a single herd was followed. Results show that most of the infections were caused by a single strain. Other strains were also identified by random amplified polymorphic DNA (RAPD) typing but they were isolated from single cases only. The predominant strain was also isolated from the milking liners suggesting cow to cow transmission during milking. The predominant strain was associated with sub-clinical and chronic infections in milking cows whereas other strains were associated with transient infections in both milking and dry cows. The longer infection caused by the predominant strain would provide the opportunity for the bacteria to spread from cow to cow (Zadoks *et al*., 2003). Based on these data and the literature, a host-adaptation theory has been proposed: *S. uberis* species presents host-adapted and non host-adapted strains. Host-adapted strains are those that cause chronic infections with sub clinical manifestation, they affect lactating cows and they can spread from cow to cow during milking. Non host-adapted strains are those that are found in the herd environment, they cause clinical, transient infections in lactating cows, dry cows and pregnant heifers (Zadoks, 2007).

Despite its importance *S. uberis* pathogenesis is still poorly understood (Leigh, 1999). Two hypotheses have been formulated to explain how *S. uberis* can invade and colonize the mammary gland and cause mastitis. The first hypothesis considers

S. uberis a "replicating irritant" whereas the second hypothesis considers *S. uberis* an adherent and invasive pathogen.

Leigh (1999) considers *S. uberis* a “replicating irritant” which can cause mastitis only if its growth rate in milk overcomes the rate of elimination given by milking and neutrophils. Ability of two strains to resist neutrophils phagocytosis *in vitro* was correlated to ability to cause clinical mastitis (Leigh *et al*., 1990). The resistance to neutrophil phagocytosis was correlated with the presence of the hyaluronic capsule (Leigh and Field, 1994). However, the hyaluronic capsule did not prevent phagocytosis by macrophages *in vitro* (Grant and Finch, 1997). Despite its role, presence of the capsule is not necessary to cause clinical mastitis (Field *et al*., 2003).

Migration of neutrophils in the mammary gland was observed *in vivo* following experimental infections. In those studies the massive migration of neutrophils from blood stream into the mammary gland and then into milk was unable to reduce the number of S. uberis bacteria in milk. (Pedersen *et al*., 2003; Rambeaud *et al*., 2003).

The second hypothesis considers the ability of *S. uberis* to adhere to and invade mammary epithelial cells a virulence factor that would explain the ability of *S. uberis* to persist in the mammary gland (Tamilselvam *et al*., 2006). Adherence of *S. uberis* to the mammary epithelial cells *in vitro* is mediated by a specific protein called *Streptococcus uberis* adhesion molecule (SUAM) (Almeida *et al*., 2006). The adherence is followed by the intracellular invasion of *S. uberis* into the mammary epithelial cells. *In vitro*, *S. uberis* is able to survive up to 120 hours in epithelial cells without loss of their viability. Different strains of *S. uberis* have different abilities to invade and survive in the mammary epithelial cells. UT336 showed a faster rate of

invasion in the first 60 minutes whereas strain UT888 survived better in epithelial cells 8 hours post challenge (Tamilselvam *et al*., 2006). Internalization would explain the poor cure rate after antimicrobial treatment of *S. uberis* mastitis (Zadoks, 2007). Internalization of *S. uberis* in mammary epithelial cells has never been observed *in vivo*. Following an experimental intramammary infection, S. uberis bacteria were observed attached to either healthy or damaged secretory cells of mammary gland (Thomas *et al*., 1994).

Based on these two hypotheses different approaches have been used for vaccine development. Leigh and colleagues attempted to develop a vaccine using as immunogen molecules which mediate essential nutrient acquisition by *S. uberis*. Binding of antibodies to those molecules would block the growth of *S. uberis* in milk. A vaccine using plasminogen activator (PauA) as immunogen was developed (Leigh *et al*, 1999), but further studies showed that PauA is not essential for growth of *S. uberis* (Ward *et al*., 2003).The other approach is to induce an immune response against the molecules which mediate adhesion and invasion of *S. uberis* to the mammary epithelial cells such as SUAM (Almeida *et al*., 2006). Attempts were made in the past using either intramammary or parenteral route of administration of a killed vaccine. In these studies, a protective effect was observed against the infection with the same strain (Finch *et al*., 1994), but not against other strains (Finch *et al*., 1997). Results of these trials shown that increased antibodies in milk do not increase phagocytosis and killing by neutrophils (Finch *et al*., 1994). Further studies in *vitro* suggest that specific antibodies may, in cooperation with complement, inhibit *S. uberis* growth in milk (Fang *et al*., 1998).

The innate immune response to an intramammary infection is responsible for the first recognition of the pathogen. Recognition via Toll like receptors (TLRs) of pathogen-associated molecular patterns (PAMPs) present in the bacterial wall of pathogens promotes the production of proinflammatory cytokines which trigger the inflammatory response (Tizard, 2009). Recent studies show that the innate immune response to the intramammary infections is pathogen species specific (reviewed by Bannerman, 2009). In response to *S. uberis* infection of the mammary gland the first cytokines detected in milk are pro-inflammatory Tumor Necrosis Factor-α (TNF-α) and interleukin-1β (IL-1β) (Bannerman *et al*., 2004; Rambeaud *et al*., 2003). These cytokines have both local effects such as neutrophil recruitment, and systemic effects, such as induction of fever and acute phase protein synthesis by the liver (Tizard, 2009). However, in two challenge studies acute phase protein synthesis and fever were detected before the increase of these two cytokines in milk (Bannerman *et al*., 2004; Rambeaud *et al*., 2003). The response may be due to other cytokines with similar effects such as interleukin-6 (IL-6). In addition, TNF-α and IL-1β might be released by mammary epithelial cells in the interstitial tissue rather than in the alveoli. In this case cytokines would be undetectable in milk (Rambeaud *et al*., 2003). Interleukin-8 (IL-8) is a chemoattractant produced by several cell type and its increase in milk, is coincident with initial increase of somatic cell count. However, difference in magnitude and timing of increase of IL-8 were observed in response to intramammary infection with different strains of *S. uberis* (Bannerman *et al*., 2004; Rambeaud *et al*., 2003).

Interleukin-12 (IL-12) and Interferon-γ (IFN-γ) activate neutrophils and macrophages. IFN-γ is produced by lymphocytes, natural killer cells and monocytes. It enhances neutrophil and macrophage phagocytosis (Ellis and Beaman, 2004). IL-12 is produced by monocytes, dendritic cells and neutrophils and enhances the activity of cytotoxic T-cells and natural killer cells (Langrish *et al*., 2004). IL-12 and IFN-γ promote the Th-1 immune response. *S. uberis* intramammary infection induces the production of both IL-12 and IFN-γ (Bannerman *et al*., 2004).

Interleukin-10 is an anti-inflammatory cytokine produced by Th-2-helper lymphocytes, B-cells and monocytes. It down-regulates the production of pro-inflammatory cytokines and promotes the Th2 immune response (Moore *et al*., 2001). *S. uberis* intramammary infection has been shown to induce a delayed production of this cytokine compared to what has been observed following the infection with gram negative pathogens (Bannerman *et al*., 2004; Bannerman *et al*., 2009). Delayed or absent production of this cytokine might be associated with persistent intramammary infections (Bannerman, 2009).

The aim of this study is to test the hypothesis that intramammary infection with either putatively host-adapted or putatively non host-adapted *S. uberis* strains yields a different host immune response. In addition, the aim is to test the host response to the re-infection with the homologous and the heterologous strain in order to asses if immune memory is present in mammary gland, and if it is strain dependent.

CHAPTER 2

Host Response to Primary Challenge with *Streptococcus uberis*

2. 1 Introduction

Streptococcus uberis is a major mastitis pathogen in dairy cattle. It is one of the most common causes of both clinical and sub-clinical mastitis in the UK (Milne *et al*., 2002; Bradley 2007) and elsewhere (Zadoks, 2007). *S. uberis* causes mastitis in primiparous heifers, dry cows and lactating cows (Hill, 1988; Leigh, 1999). It has been considered an environmental pathogen (Leigh, 1999, Zadoks, 2007) however, based on epidemiological, management and recent molecular data (Reviewed by Zadoks, 2007), a host adaptation theory has been proposed: Host adapted strains are more likely to cause chronic, sub-clinical infections in lactating cows and they can spread cow to cow during milking. Non host adapted strains are more likely to be found in the herd environment and cause clinical, transient infections in lactating cows, dry cows and heifers (Zadoks, 2007).

However, possible mechanisms behind host adaptation and pathogenesis of *S. uberis* mastitis are still unclear (Leigh, 1999; Zadoks, 2007). Ability to grow in milk of *S. uberis* was associated with stage of lactation (Rambeaud *et al*., 2004) or strain type (Kliem *et al*., 2002) however, no association between ability to grow in milk and severity of mastitis *in vivo* was found (Zadoks., 2007). Response to *S. uberis* intramammary infection is characterized by massive influx of neutrophils in the mammary gland (Thomas *et al*., 1994; Pedersen *et al*., 2003; Rambeaud *et al*., 2003; Bannerman *et al*., 2004). Different abilities to resist phagocytosis by neutrophils of two strains of *S. uberis* has been observed *in vitro*. The strain more able to resist phagocytosis was more virulent *in vivo* than the non resistant strain (Hill, 1988).

Ability to resist to phagocytosis was attributed to the presence of a hyaluronic capsule (Leigh *et al*., 1990) but this capsule is not essential for the pathogenesis of *S. uberis* mastitis (Field *et al*., 2003). Despite their massive influx into the mammary gland, neutrophils do not appear to be able to reduce the number of *S. uberis* bacteria (Thomas *et al*., 1994; Rambeaud *et al*., 2003; Bannaerman *et al*., 2004). Following experimental infection *S. uberis* bacteria were observed in macrophages but not in neutrophils (Thomas *et al*., 1994). *In vitro* studies showed the ability of macrophages isolated from dry cow secretions to phagocytise and kill different strains of *S. uberis* (Grant *et al*., 1997) whereas macrophages isolated from milk showed decreased killing activity (Denis *et al*., 2006).

Challenge studies to characterize innate immune response were conducted on major gram positive and gram negative mastitis pathogens (Reviewed by Bannerman, 2009). These studies, focusing on early cytokine response to the infection, demonstrated the specificity of the innate immune response to the major bacterial mastitis pathogens species either gram positive and gram negative , particularly regarding magnitude, timing and production of cytokines in mammary gland.

Response to *S. uberis* is characterized by production in the mammary gland of pro-inflammatory cytokines tumour necrosis factor (TNF-α), interleukin-1β (IL-1β) and chemoattractant interleukin-8 (IL-8) (Rambeaud *et al*., 2003; Bannerman *et al*., 2004). Differences in timing and concentration of IL-8 were observed in response to strain 0140J (Bannerman *et al*., 2004) and UT888 (Rambeaud *et al*., 2003) suggesting a strain specificity in the response. In response to the intramammary

infection, was also observed the production of anti inflammatory cytokine interleukin-10 (IL-10) and T-helper type 1 (Th1) cytokines such as Interferon-γ (INF-γ) and interleukin-12 (IL-12) (Bannerman *et al*., 2004). Lymphocytes are involved in the adaptive immune response to the intramammary infection (Riollet *et al*., 2000). In the healthy lactating mammary gland the majority of lymphocytes are T-cells and αβ $CD8^+$ cells are the predominant subpopulation (Park *et al*., 1992; Taylor *et al*., 1997; Asai *et al*., 1998). An increased percentage of $CD4^+$ cells was observed in the mammary gland of cows naturally infected with either *Staphylococcus aureus* or *Streptococcus spp.* (Taylor *et al*., 1997) suggesting a role in response to intramammary infections. A study conducted on cows naturally infected with *Streptococcus spp*. reported parallel increase in CD4, CD8 and γδ cells in milk (Soltys and Quinn, 1999).

Recent data suggest the importance of IL-17 response in the context of bovine mastitis. Somatic cells obtained from mammary glands with chronic streptococcal or enterococcal infections had elevated levels of mRNA encoding IL-17A compared to cells from uninfected control quarters (Bruno *et al*., 2010). IL-17A also modulates the response of mammary epithelial cells to staphylococci *in vitro* (Bourgarn *et al*., 2011). These studies suggest that an IL-17 response could exist in the mammary gland. In response to IMI however to date, the IL-17A profile of milk during development or resolution of intramammary infection (IMI) with *S. uberis* or other mastitis pathogens has not yet been described.

The aim of this study is to test the hypothesis that intramammary infection in lactating cows with either putatively host-adapted or putatively non host-adapted strains of *S. uberis* yields a different host immune response and investigate some aspects of innate and adaptive immune response to the intramammary infection with *S. uberis* and to characterize local cytokine and immune cell profiles during onset and resolution of infection, with inclusion of IL-17A.

2. 2 Materials and Methods

2. 2. 1 Challenge Strains

Challenge strains of *S. uberis* were selected using epidemiological, clinical and molecular data. Strain FSL Z1-048 was selected to represent characteristics of putatively host-adapted *S. uberis*, such as chronicity of infection and contagious transmission within a herd, whereas strain FSL Z1-124 was selected to represent characteristics of non-adapted *S. uberis*, such as transient infection and environmental origin (Zadoks *et al*., 2003; Zadoks, 2007). The 2 strains were originally isolated from the same herd during the same time period so that differences in clinical manifestation and transmission could not be attributed to differences in management conditions on the farm of origin. FSL Z1-048 belonged to a strain that was isolated from several cases of persistent subclinical mastitis (median duration 50 days) in lactating cows during a mastitis outbreak, whereas FSL Z1-124 belonged to a strain that was isolated from a single case of mastitis of short duration (observed duration < 9 days) in a heifer at calving, i.e. without prior exposure to the milking machine (Zadoks *et al*., 2003). The strains are genetically distinct by a large number of open reading frames (Lang *et al*., 2009). Based on multi-locus sequence typing, FSL Z1-048 belongs to sequence type (ST) 385 and clonal complex 143, a clonal complex that is predominantly associated with subclinical mastitis (Tomita *et al*., 2008). FSL Z1-124 belongs to ST383, which is a singleton, i.e. it is not associated with a known clonal complex (Lang *et al*., 2009; http://pubmlst.org/suberis/).

2. 2. 2 Growth of Challenge Strains in Milk

The ability of both challenge strains to grow in milk was assessed *in vitro*, using milk from the animals enrolled in the challenge study (see below). The bacterial inoculum for the *in vitro* growth experiment was prepared from stock cultures of FSL Z1-048 and FSL Z1-124 stored at -80°C. Stock cultures were thawed, plated on 5% horse blood agar (E&O Laboratories, Bonnybridge, UK) and incubated overnight at 37°C to check for viability and purity. Approximately 5 colonies per strain were then inoculated into 15 mL of brain heart infusion (BHI) broth (Difco, Cambridge, UK) and incubated for 20 h at 37°C. Bacterial concentration of the incubated broth was determined by viable count method as detailed at the end of this section while the remainder was stored at 4ºC, a temperature that does not permit growth of *S. uberis* (Dogan and Boor, 2004). The next day, stored broth cultures were serially diluted in sterile PBS to obtain the target inoculum concentration of 1,000 cfu/mL for each strain. The actual inoculum concentration was checked by viable count method.

To assess the growth potential of challenge strains in milk from study animals, milk was collected aseptically from both hind quarters of each animal just before milking, 1 day prior to experimental challenge. Milk samples were kept on ice and transported to the laboratory for inoculation of culture plates within 1 hour of sample collection. For each cow, equal volumes of milk from both hind quarters were comingled and 10 µl were plated onto horse blood agar (E&O Laboratories) for overnight incubation at 37°C to check for the absence of contaminants and pathogens associated with IMI. For each cow, one 14 mL aliquot of comingled milk was then inoculated with strain FSL Z1-048 and 1 aliquot of 14 mL with FSL Z1-124, using approximately 1,000

cfu of *S. uberis* in 1 mL of PBS (see below). Inoculated milk was incubated for 24 h at 37°C under shaking (200 rpm). At 0, 3, 6, 12 and 24 h post inoculation, 100 µL of milk were taken off and bacterial concentration was determined by viable counting, i.e. 1:10 serial dilution series were prepared in sterile PBS and three 10 µL drops per dilution were plated onto horse blood agar plates (E&O Laboratories) and incubated overnight at 37°C. Colonies were counted when they were in the range of 5 to 50 cfu per spot, and the bacterial concentration for each time point (cfu/mL) was calculated based on average colony counts for the appropriate dilution.

2. 2. 3 Challenge Study Design

Twelve non-pregnant, clinically healthy Holstein cows with no history of clinical mastitis were used in this experiment. Cows were in mid-lactation (60 to 160 DIM on the day of challenge) and parity ranged from 1 to 5. Composite somatic cell count (SCC) as measured during 4-weekly milk recording was < 100,000 cells/mL at the time of purchase, with the exception of 1 animal that had composite SCC of 152,000 cells/mL. This animal tested positive for coagulase negative staphylococci in 1 hind quarter and the culture negative contralateral quarter was used as challenge quarter. On the day before challenge, quarter level SCC was 100,000 cells/mL for 22 of 24 challenge quarters and between 100,000 and 200,000 cells/mL for the remaining 2 quarters. Quarter milk samples from all animals were negative for *S. uberis* prior to purchase and between purchase and challenge. Challenge studies were conducted using 4 cows at a time, with cows housed in loose housing (group 1) or cubicles (groups 2 and 3), with straw bedding in both housing systems. Cows had access to water and grass silage *ad libitum* and concentrate was fed 3 times a day based on

milk yield. Cows were milked twice a day at 6 am and 3 pm using a dedicated milking unit for each cow. After each milking, all teats were dipped in iodine-based teat disinfectant. Each cow was challenged with *S. uberis* in 1 hind quarter and mock challenged with PBS in the other hind quarter as detailed in the next section. In each group of 4 animals, strain-quarter combinations (FSL Z1-048/RH, FSL Z1-124/RH, FSL Z1-048/LH, FSL Z1-124/LH, where RH = right hind and LH = left hind) were allocated to individual cows based on random sampling without replacement. Clinical data and samples were collected for 14 days post challenge. All experiments were conducted at the Moredun Research Institute (Penicuik, UK) with approval of the Institute's Experiments and Ethical Review Committee in accordance with the Animals (Scientific Procedures) Act 1986.

2. 2. 4 Intramammary Challenge

The bacterial inoculum for challenge experiments was prepared as described for the growth curve experiment, with the exception that the target concentration for animal challenge was 100 cfu/mL. Challenge doses were kept at 4°C (in the laboratory) or on ice (in the milking parlour) until infusion. On the day of challenge, cows were milked as per normal procedures, including the use of post milking teat disinfection. Subsequently, teat ends were disinfected by scrubbing with cotton wool swabs with 70% (vol/vol) ethanol. Two mL of inoculum preparation (target dose: 200 cfu/quarter) were infused into each challenge quarter via a J-12 teat infusion cannula (Jorgens Laboratories, Loveland, CO, USA). Control quarters were mock-challenged by infusion of 2 mL of sterile PBS. After infusion, teats were dipped in post-milking teat disinfectant and cows were returned from the milking parlour to their pen, as per

normal procedures. The actual challenge dose for each strain in each challenge round was determined by viable count method using spare doses of the inoculum preparation.

2. 2. 5 Sample and Data Collection

Milk samples and clinical data were collected at various time points following infusion. For the first 48 h, clinical data and milk samples for SCC determination, bacterial culture and cytokine ELISA were collected every 6 h. From 2 to 11 days post-challenge, milk samples for SCC and culture were collected twice a day, and from 11 to 13 days post-challenge once a day. On days 3 and 4 post-challenge, milk samples for cytokine ELISA were collected twice a day and on days 5 through 7 post-challenge, once a day. Additional milk samples for cytokine ELISA were collected on days 10 and 13 post-challenge. Samples for flow cytometric analysis of lymphocyte populations were collected on days 1, 2, 4, 6, 8, 10 and 13 post-challenge. Clinical scores were assigned to each quarter and cow as follows: 0 = no clinical signs; 1 = milk alteration only, such as alteration of colour or presence of clots; 2 = local signs, such as redness of the udder, pain on palpation, or swelling; 3 = systemic signs, e.g. fever, anorexia or lethargy. Quarter milk samples for bacteriological analysis were collected using aseptic technique (National Mastitis Council, 1999) and stored on ice until refrigeration at 4°C. Separate receptacles were used to collect quarter milk samples for SCC measurement, cytokine ELISA and flow cytometry. The vast majority of data were recorded and samples were collected by Prof. Ruth Zadoks (Moredun Research Institute) and David Reddick (Moredun Scientific Ltd, Penicuik, UK)

2. 2. 6 Bacteriological Analysis

For qualitative analysis, 10 μL of milk from each quarter (challenged, mock-challenged and unchallenged quarters) were plated on horse blood agar (E&O Laboratories) and incubated at 37°C for 24 h. Colonies were identified based on morphology and standard biochemical tests (Gram staining, catalase test, esculin splitting) (National Mastitis Council, 1999). For quantitative analysis, milk samples from challenged quarters were serially diluted with 10-fold dilutions from neat to 10^{-8} in sterile PBS. Triplicate 10 μL aliquots of each dilution were spotted on blood agar plates (E&O Laboratories), allowed to air dry and incubated overnight at 37°C. Colonies with morphology consistent with *S. uberis* were counted, if possible in the range of 5 to 50 cfu per spot, and the bacterial concentration in milk (cfu/mL) was calculated based on average colony counts for appropriate dilutions. Qualitative and quantitative bacteriological analysis was performed by Cliff Ramage (Moredun Scientific Ltd.).

2. 2. 6 Molecular Typing

Polymerase chain reaction and pulsed-field gel electrophoresis (PFGE) were used to confirm species and strain identity, respectively. Where possible, 3 *S. uberis* isolates from each challenged quarter were analysed, i.e. 1 isolate each from the first and last positive sample post challenge and from the time point with peak bacterial shedding. A total of 18 isolates were analysed from the 6 cows challenged with strain FSL Z1-048, with each cow represented by 3 isolates. Five isolates, representing 3 cows, were available from animals challenged with strain FSL Z1-124. Species identity of isolates was confirmed using PCR primers and parameter settings as described by

Phuektes and colleagues (2001b). For PFGE of confirmed *S. uberis* isolates, 1.5 mL of overnight culture in BHI was centrifuged for 10 minutes at 13,400 × g. Supernatants were discarded and cell pellets were resuspended in 0.5 mL of TE buffer (10 m*M* tris hydrocloridric acid, 1 m*M* EDTA, pH 8). Bacterial suspensions were mixed with 2% (wt/vol) low melting-point agarose (BioRad Laboratories, Hertfordshire, UK) in TE buffer at 56 °C. Plugs were allowed to solidify and incubated overnight at 37°C in 2 mL of TE buffer containing 4 mg/mL lysozyme (Sigma-Aldrich, Dorset, UK). Supernatants were removed and plugs were incubated for 48 h at 56 °C in 2 mL of 0.5 *M* EDTA containing 2 mg/mL proteinase K (Sigma-Aldrich) and 1% wt/vol N-laurylsarcosine sodium. Proteinase K solution was removed and plugs were washed for 1 h in 36 mL of TE buffer under shaking (100 rpm). Washing was repeated 6 times. Plugs were pre-incubated in 300 µL of reaction buffer containing (1% wt/vol) BSA, 20 m*M* Tris-acetate, 50 m*M* potassium acetate, 10 m*M* magnesium acetate, and 1 m*M* dithiothreitol (pH 7.9) for 1 h at 37°C. Reaction buffer was removed, 100 µL of enzyme buffer containing 30 units of restriction enzyme *Sma*I (New England Biolabs, Hitchin, UK) were added to each plug and plugs were incubated overnight at 25°C. Plugs were then inserted into a gel prepared from 1% (wt/vol) PFGE certified agarose (BioRad Laboratories) in 0.5 × TBE buffer (45 m*M* Tris base, 45 m*M* boric acid, 1 m*M* EDTA, pH 8.0). Restriction fragments were separated by PFGE at 14°C for 24 h, with initial switch time 3 s, final switch time 55 s, and linear ramp using a CHEF mapper (BioRad Laboratories). Gels were stained with SYBR Gold stain (Invitrogen, Paisley, UK) according manufacturer's instruction, rinsed and photographed under UV light. Molecular typing was performed by Mark Lutton (Moredun Research Institute).

2. 2. 7 Somatic Cell Counting and Flow Cytometry

Approximately 25 mL of milk were collected in plastic vials pre-loaded with preservative and shipped to National Milk Laboratories (Hillington Park, Glasgow, UK) for determination of SCC by Fossomatic milk cell counter (Foss Electronic, Hillerød, Denmark). For flow cytometry, approximately 50 mL of milk per quarter were collected in plastic Falcon vials (BD Bioscience, Oxford, UK). Milk was kept on ice and transported to the laboratory within 30 minutes, where it was diluted with an equal volume of cold PBS and centrifuged at 800 × *g* for 20 min. Using a sterile pastette, cell pellets were transferred into 30 mL of PBS and resuspended. Cell suspensions were filtered through a 70 μm cell strainer (BD Bioscience) and centrifuged at 400 × *g* for 15 min. Supernatants were discarded and cell pellets resuspended in 25 mL of PBS. Washing was repeated once and pellets were resuspended in 1 mL of FACS buffer (PBS supplemented with 1% wt/vol BSA). Ten μL of cell suspension were stained with an equal volume of trypan blue (Sigma-Aldrich) and viable cell concentration was determined using a microscope cell counting chamber, with cell concentration adjusted to $1x10^6$ cells/mL. Aliquots of milk cells were labelled with a panel of monoclonal antibodies (Table 2. 1) to identify T-lymphocyte populations and subpopulations, i.e. CD4, CD8 and γδ T cells. Fifty microliters of cell suspension were mixed with 50 μL of the appropriate blocking buffer (20% vol/vol heat inactivated normal mouse serum in FACS buffer for CD4 or CD8 staining; 20% vol/vol heat inactivated normal goat serum in FACS buffer for CD3 or γδ staining). Samples were incubated for 30 min at room temperature and subsequently centrifuged at 700 × *g* for 1 min. Supernatants were discarded and 100 μL of antibody diluted with the appropriate buffer (10% vol/vol

heat inactivated normal mouse serum in FACS buffer for CD4 or CD8 staining; 10% vol/vol heat inactivated normal goat serum in FACS buffer for CD3 or γδ staining) were added. Samples were incubated for 30 min at 4°C. After staining with anti-CD4 or anti-CD8 antibody, cells were washed twice with FACS buffer and once with PBS. Finally, they were re-suspended in PBS with 1% wt/vol paraformaldehyde for 10 min. Cells stained with anti-CD3 or anti-γδ antibodies were washed twice with FACS buffer; 100 µL of a 1µg/mL dilution of goat anti- mouse IgG conjugated to Alexa Fluor 488 (Invitrogen) was added and the cells were incubated at 4°C for 30 min. After staining, cells were washed and fixed in PBS with paraformaldehyde as described for CD4 and CD8 staining. Cells were acquired with a 2 laser Cyan flow cytometer (Beckman Coulter, High Wycombe, UK). Cell debris was differentiated from cells based on forward and side scatter (Figure 2. 1A) and excluded from analysis. Data were collected for a minimum of 10,000 cells for each sample. Data were analysed using FlowJo software (Tree Star, Ashland, OR, USA). The lymphocyte population was gated based on forward and side scatter and the percentage of positive cells for each staining (CD3, CD4, CD8 and γδ) was calculated within the lymphocyte gate (Example showed in Figure 2. 1B and C). Cytocentrifuge preparations of selected samples were prepared using a Shandon Cytospin 4 cytocentrifuge (Thermo Electron Corporation, Milford, MA, USA) and stained using a REASTAIN Quick-Diff Kit (Reagena, Toivala, Finland) for subsequent differential cell counting. Absolute concentration (i.e. cells/mL) of lymphocyte sub-populations was calculated based on SCC and the percentage of lymphocytes as determined by flow cytometry, by multiplying the percentage of positive cells by total number of cell (SCC).

Table 2. 1 Details of mouse monoclonal antibodies used for flow cytometry.

Clone	Specificity	Isotype	Conjugation	Cellular expression	Source
CC8	Bovine CD4	IgG2a	Alexa Fluor® 647	Helper T cells	AbD Serotec[4]
CC63	Bovine CD8	IgG2a	FITC	Cytotoxic T cells	AbD Serotec[4]
MM1A	Bovine CD3	IgG1	n/a	Pan-T cells	VMRD Inc.[5]
GB21A	Bovine γδ TCR[1]	IgG2b	n/a	Pan-γδ T cells	VMRD Inc.[5]
eBM2A	n/a[2]	IgG2a	Alexa Fluor® 647/FITC	Isotype control	eBioscience[6]
VPM21	BDV[3] p125/p80	IgG1	n/a	Isotype control	MRI[7]
VPM22	BDV p125/p80	IgG2b	n/a	Isotype control	MRI[7]

[1] TCR = T cell receptor; [2] n/a = not applicable; [3] BDV = border disease virus (Entrican *et al*., 1995); [4]AbD Serotec, Kidlington, UK; [5] VMRD Inc., Pullman, WA, USA; [6] eBioscience, Hatfield, UK; [7] Kindly provided by Sean Wattegedera, Moredun Research Institute, Penicuik, UK.

Table 2. 2 Details of antibodies used for cytokine ELISAs.

Specificity	Antibody	Isotype, species	Conce. (μg/mL)	Source
IL-1β Coating	Polyclonal	n/a[2], rabbit	5	AbD Serotec[3]
IL-1β Detection	Biotinylated polyclonal	n/a, rabbit	1	AbD Serotec
IL-8 Coating	8M6	IgG2a, mouse	5	AbD Serotec
IL-8 Secondary	Polyclonal	IgG, rabbit	2	AbD Serotec
IL-10 Coating	CC318	IgG2b, mouse	4	AbD Serotec
IL-10 Detection	Biotinylated CC320	IgG1, mouse	1	AbD Serotec
IL-12p40 Coating[1]	CC301	IgG2a, mouse	1	AbD Serotec
IL-12p40 Detection[1]	Biotinylated CC326	IgG2b, mouse	2	AbD Serotec
TNF-α Coating	CC327	IgG2b, mouse	2	AbD Serotec
TNF-α Detection	Biotinylated CC328	IgG2a, mouse	1	AbD Serotec

[1] IL-12p40 is the subunit that IL-12 has in common with IL-23 (Oppmann *et al*., 2000); [2] n/a = not applicable; [3]AbD Serotec, Kidlington, UK.

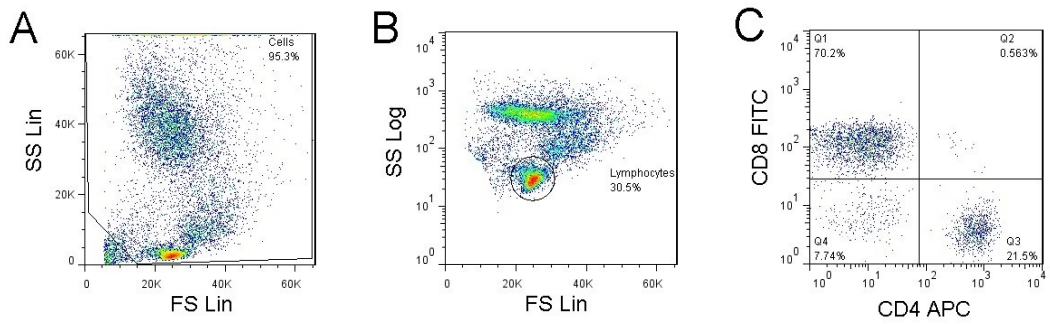

Figure 2. 1 Example of flow cytometry analysis of cells from milk. Cell debris was excluded from analysis (A). The lymphocyte population was identified by forward and side scatter (B). Within the lymphocyte population sub populations were identified by staining with specific antibodies for CD3 (not shown), CD4 (C), CD8 (C) and γδ (not shown).

2. 2. 8 Cytokine Measurements

Milk samples for cytokine measurement were collected in sterile 50 mL plastic Falcon tubes. Skim milk was prepared by centrifuging 50 mL of milk at 2,800 × *g* at 4°C for 20 minutes. The fat layer was discarded and the supernatant was transferred to a new 50 mL Falcon tube. Centrifugation was repeated and the supernatant was stored at -20°C until analysis. Microtitre plates (Immunolon 2 HB, Thermo Electron Corporation, Langenselbold, Germany) were coated overnight at 4°C with 100 µL/well of the appropriate coating antibody (Table 2. 2) diluted in 0.5 *M* carbonate buffer (0.5 *M* Na_2CO_3, 0.5 *M* $NaHCO_3$, pH 9.6). Wells were washed with washing buffer (PBS at pH 7.4 with 0.05% vol/vol Tween 20 (Sigma-Aldrich) and non-specific binding sites were blocked at room temperature for 1 hour with 300 µL/well of PBS containing 3% (wt/vol) BSA and 0.05% (vol/vol) Tween 20 (Sigma-Aldrich). Plates were washed again with washing buffer and incubated for 1 h at room temperature with 100 µL/well of skim milk. Each sample was tested in duplicate. When necessary, samples were diluted as appropriate with PBS supplemented with 0.05% (vol/vol) Tween 20 (Sigma-Aldrich) and 1% (wt/vol) BSA (reagent diluent). A standard curve of known cytokine concentrations was assayed for each plate using appropriate standard diluted in reagent diluent. Recombinant bovine standards were used for IL-1β (AbD Serotec, Kidlington, UK), IL-8 (Kingfisher Biotech, Upper Heyford, UK), and TNF-α (R&D systems, Minneapolis, MD, USA). For IL-10 and IL-12p40, recombinant ovine standards were used (provided by Sean Wattegedera, Moredun Research Institute, Penicuik, UK). Plates were washed with washing buffer and 100 µL/well of appropriate detection antibody (Table 2. 2) in reagent diluent were added, followed by incubation for 1 h at room

temperature. For IL-1β, IL-10, IL-12p40 and TNF-α plates, 100 µL/well of horseradish peroxidase (HRP)-streptavidin diluted 1:500 in reagent diluent (Sigma-Aldrich) were added, followed by incubation for 45 minutes at room temperature. IL-8 plates were incubated with 100 µl/well of HRP-conjugated polyclonal goat anti-rabbit immunoglobulins (Dako, Ely, UK) diluted 1:1,000 in reagent diluent for 1 hour at room temperature. After incubation with HRP-streptavidin or HRP-conjugated antibody, plates were washed and incubated for 20 minutes at room temperature with 100 µL per well of o-Phenylenediamine dihydrochloride substrate (Sigma-Aldrich). The reaction was stopped with 25 µL per well of 2.5 *M* H_2SO_4 and optical density was measured at 492 nm using a sunrise absorbance reader (Tecan, Theale, UK). Cytokine concentrations in skim milk samples were calculated from the standard curve. Concentrations of IFN-γ, IL-6 and IL-17A in milk samples were measured with commercial kits (R&D Systems; Pierce biotechnology, Rockford, IL, USA and Kingfisher Biotech, respectively) according to the manufacturers' instructions.

2. 2 .9 Statistical Analysis

All data were entered into a database and checked for outliers and missing values using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Outliers were re-evaluated where necessary but no outliners were removed from the data set. Simple descriptive and graphical analyses were performed for all parameters. Where necessary, transformations were performed to normalize the data, e.g. for SCC and cfu counts. To evaluate the impact of strain and time post infusion (PI) on any of the measured

parameters, a generalized linear mixed model was fit to the data. The general format of the model was:

$$f(y) = incpt + time + strain + time * strain + Cow + Re$$

where *f(y)* is a function of the parameter of interest, i.e. a transformation or identity function, as appropriate; *incpt* is the intercept; time is the time PI; *strain* is an indicator variable indicating either FSL Z1-048 or FSL Z1-124; and *time * strain* is the interaction between time and strain. *Cow* is a random cow effect, *Re* is a complex error term where *R* indicates the within quarter correlation of the repeated measures and e indicates a random error term, assumed to be normally distributed with mean 0 and variance σ^2. The model fit was assessed using the ratio of the deviance to the remaining degrees of freedom. Least square means from the models were calculated and used for graphing of the data. Significance testing was done to evaluate the differences between strains over time using contrasts of the least square means. A Bonferroni correction was used in case of comparisons against pre-challenge values in infected and control quarters at multiple time points post challenge. Statistical significance was declared at $P < 0.05$. Post-inoculation least square mean values were compared to the pre-inoculation (Time 0) values, with the exception of lymphocyte concentrations and ratios. The latter were compared between challenge quarters and control quarters within time points to allow for significance testing despite lack of complete data for individual quarters. Statistical analysis was performed by Prof. Ynte. Schukken (Department of Population Medicine and

Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA.

2. 3 Results

2. 3. 1 *In-vitro* Growth of Challenge Strains in Milk

The ability of strains FSL Z1-048 (putatively host-adapted) and FSL Z1-124 (non-adapted) to grow in fresh milk *in vitro* was tested using milk samples from 8 individual animals. Results from 1 sample inoculated with strain FSL Z1-048 were discarded because of bacterial contamination of the sample. The actual inoculum dose ranged between 700 and 1,280 cfu of *S. uberis* per sample. Both challenge strains were able to grow in fresh milk *in vitro* with significantly higher levels of bacterial growth observed with strain FSL Z1-124 compared to strain FSL Z1-048 at 3, 6 and 12 h post-inoculation (Figure 2. 2A). FSL Z1-124 was first detected at 3 h post inoculation, with more than half of the milk samples testing positive, whereas strain FSL Z1-048 was first detected in milk at 6 h post inoculation, with only 1 sample testing positive (Figure 2. 2B). At 24 h post inoculation, all milk samples tested positive. The maximal bacterial concentration at 24 h post inoculation did not differ between FSL Z1-048 and FSL Z1-124 (7.68 ± 0.13 and 6.68 ± 0.19 Log_{10} cfu/mL respectively).

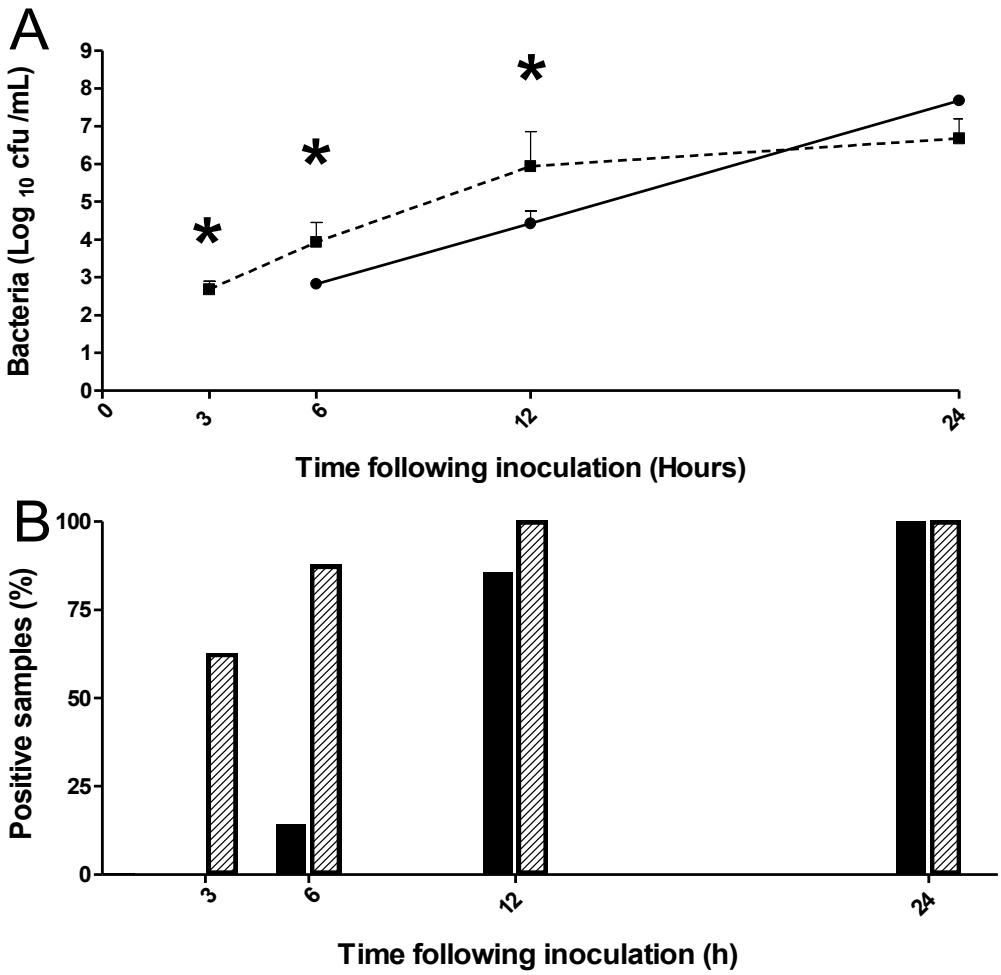

Figure 2. 2 (A) *In vitro* growth curves of *S. uberis* strain FSL Z1-048 (full line) and FSL Z1-124 (dashed line) in milk from individual cows. Mean bacterial concentrations and SEM are shown for culture positive samples. Stars indicate significant differences between strains. (B) Proportion of milk samples from individual cows inoculated with *S. uberis* strain FSL Z1-048 (solid bars; n = 7) or FSL Z1-124 (hashed bars; n = 8) in which bacteria were detected (detection limit 333 cfu/mL).

2. 3. 2 Clinical Response

All 6 cows challenged with *S. uberis* strain FSL Z1-048 developed clinical mastitis. In contrast, none of the 6 cows challenged with strain FSL Z1-124 developed clinical signs of mastitis. Clinical signs were first observed 30 to 48 h PI (Table 2. 3). The peak in average clinical score (2.2 ± 0.4) occurred between 48 and 57 h PI (Figure 2. 3A). Increased rectal temperature was observed in all animals challenged with strain FSL Z1-048. The first increase in rectal temperature was detected between 24 and 72 h PI (Table 2. 3). The average temperature reached a peak of 40.0 ± 1.0°C at 36 h PI (Figure 2. 3B) and remained elevated at 48 and 57 h PI ($P < 0.05$). At 72 h, average body temperature was not significantly different from pre-challenge levels, despite delayed onset of fever in 1 individual (Tab 2. 3, Figure 2. 3B). The body temperature of cows challenged with FSL Z1-124 remained at pre-challenge level throughout the study. Milk production decreased in all animals challenged with strain FSL Z1-048. Mean production decreased by almost 50% on the second day PI and remained significantly depressed on day 3 PI ($P < 0.05$) (Figure 2. 3C). Milk production started to increase again at day 4 PI. No change in milk output was observed in animals challenged with strain FSL Z1-124 (Figure 2. 3C).

Table 2. 3 Time (hours post infusion) of first detection (bacteria; clinical signs other than fever) or first detected increase (temperature, SCC, cytokine levels) of several parameters in 6 cows challenged in one mammary quarter with *Streptococcus uberis* strain FSL Z1-048. Apart from body temperature, all parameters were observed or measured at quarter level.

	Cow identification					
	1	**2**	**3**	**4**	**5**	**6**
Clinical signs	30	30	36	36	48	36
Temperature	30	24	24	30	72	36
SCC	30	30	30	30	42	36
Bacteria	12	18	12	12	18	18
IL-1β	30	36	30	30	42	36
IL-6	30	36	30	30	42	30
IL-8	30	30	30	30	42	36
IL-10	36	36	30	36	48	42
IL-12p40	36	36	36	36	48	42
TNF-α	36	36	42	36	48	36
IL-17A	144	ND	72	57	ND	72

ND. = not detected.

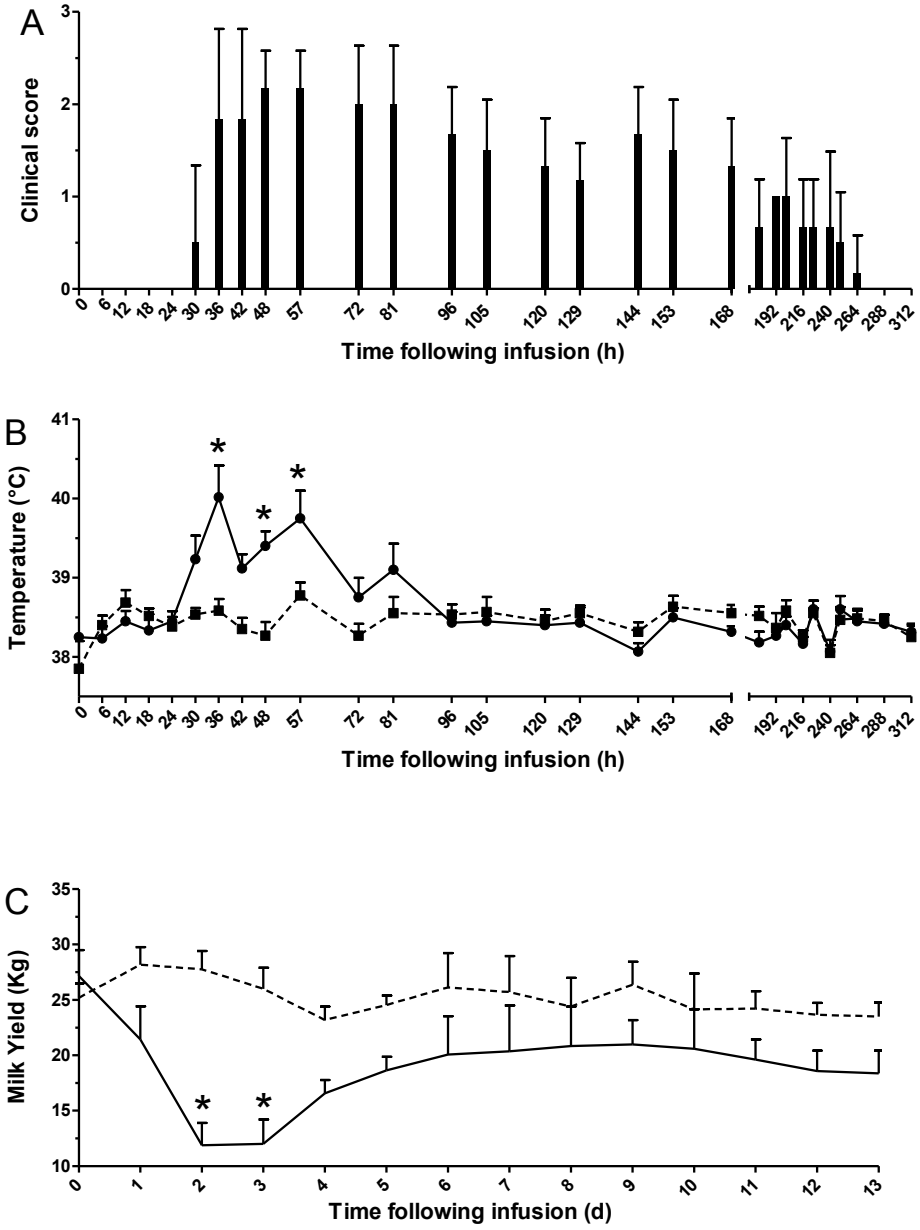

Figure 2. 3 (A) Clinical score in response to intramammary challenge with *S. uberis* strain FSL Z1-048. Mean and SEM for 6 cows. 0 = no clinical signs; 1= abnormalities in milk, e.g. clots or discoloration; 2 = abnormalities in udder, e.g. swelling or pain; 3 = systemic signs, e.g. fever. None of the cows (n = 6) challenged with strain FSL Z1-124 showed clinical signs. (B) Rectal temperature in response to intramammary challenge with FSL Z1-048 (full line) or FSL Z1-124 (dashed line). Mean and SEM are shown. (C) Daily milk yield in response to intramammary challenge with *S. uberis* strain FSL Z1-048 (full line) or FSL Z1-124 (dashed line). Daily milk yield was calculated as the sum of morning and afternoon milking. Mean and SEM are shown for 6 cows per strain. Values marked by an asterisk are significantly different from those at t = 0 ($P < 0.05$).

2. 3. 3 Bacterial Culture and Molecular Typing

Culture of unused doses of the challenge inoculum showed that quarters had been infused with 53 to 712 cfu of strain FSL Z1-048 or 80 to 700 cfu of FSL Z1-124. In the last round of the experiment, the dose of FSL Z1-124 was increased approximately 100-fold, to 14,800 cfu, to determine whether a high inoculum dose would induce a clinical response, however, no difference in response was observed between animals challenged with either the low or the high dose of this strain. Viable *S. uberis* was isolated from milk from all mammary quarters (n = 6) infused with FSL Z1-048 (Figure 2. 4A). Bacteria were first detected at 12 h PI and all quarters were positive at 18 h PI (Figure 2. 3B). By 81 h PI, the number of culture positive quarters started to fluctuate. Five of 6 animals had cleared the infection spontaneously by the end of the study at 312 h post challenge (Figure 2. 4B), as confirmed by culture of milk samples collected on a daily basis for 10 days after the end of the study. The maximum average concentration of *S. uberis* in positive quarters was observed at 36 h PI (7.67 ± 0.35 Log_{10} cfu/mL) (Figure 2. 4A). By 96 h PI, bacterial concentration had decreased to 3.49 ± 0.54 Log_{10} cfu/mL and it remained at a similar level in culture positive quarters for the rest of the follow-up period (Figure 2. 4A). *S. uberis* was recovered in milk from 4 quarters challenged with strain FSL Z1-124 between 12 and 48 h PI, of which 3 had been challenged with the low dose and 1 with the high dose. No more than 2 quarters were positive at any one time (Figure 2. 4B). Average concentration in positive quarters reached its maximum at 24 h post challenge (2.91 ± 0.55 Log_{10} cfu/mL) (Figure 2. 4A) and all quarters were negative for FSL Z1-124 by 57 h PI.

From each quarter challenged with strain FSL Z1-048, 3 isolates were used for PCR and PFGE, i.e. 1 isolate representing the first isolation, peak concentration and last isolation from each quarter, respectively, for a total of 18 isolates. All isolates were confirmed to be *S. uberis* by PCR. PFGE patterns of all isolates matched that of the challenge strain (Figure 2. 5). Only a limited number of isolates from quarters challenged with FSL Z1-124 were available for analysis, i.e. 1 isolate from 1 quarter and 3 isolates obtained at 3 different time points (12, 18, 24 h PI) from a different quarter. PCR using species-specific primers yielded a band of 470 bp rather than the expected 330 bp. Sequence analysis of the PCR amplicon revealed a 160 bp insertion in the 16S-23S rRNA intergenic spacer region. The insertion fragment which showed 99% sequence homology with the 16S-23S intergenic spacer region of *Streptococcus porcinus* (ATCC35647). Otherwise, the 16S-23S rRNA intergenic spacer amplicon showed 100% sequence homogeneity with that of *S. uberis* reference strains O140J, ATCC70047 and ATCC19436. Sequencing of a 740 bp fragment of a second housekeeping gene, *rpoB* (Drancourt *et al*., 2004), confirmed the species identity of FSL Z1-124 as *S. uberis*. PFGE patterns of isolates from quarters challenged with FSL Z1-124 were indistinguishable from the PFGE pattern of the challenge strain (Figure 2. 5).

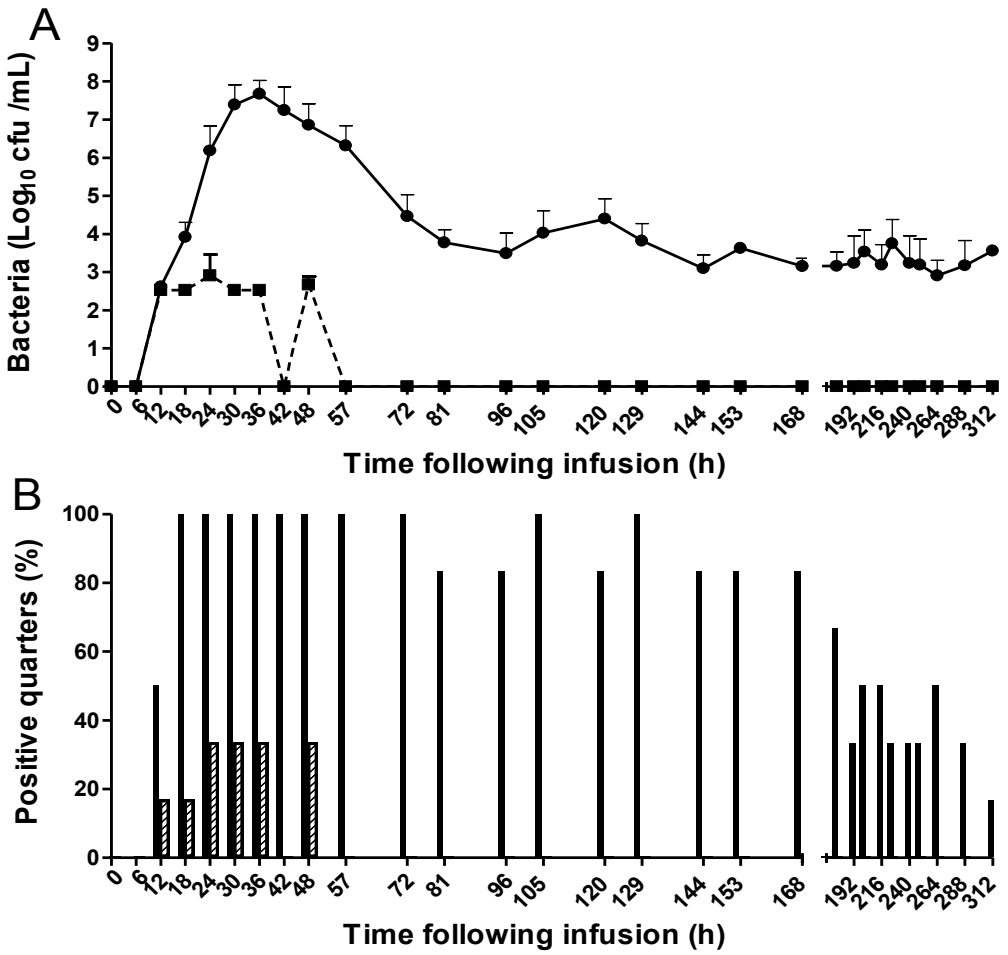

Figure 2. 4 (A) Bacteria counts in milk of quarters excreting *S. uberis* strain FSL Z1-048 (full line) or FSL Z1-124 (dashed line). Mean and SEM are shown. (B) Proportion of quarters challenged with *S. uberis* strain FSL Z1-048 (full bars) or FSL Z1-124 (hashed bars) from which *S. uberis* was recovered. Six quarters (1 per cow) were challenged per strain.

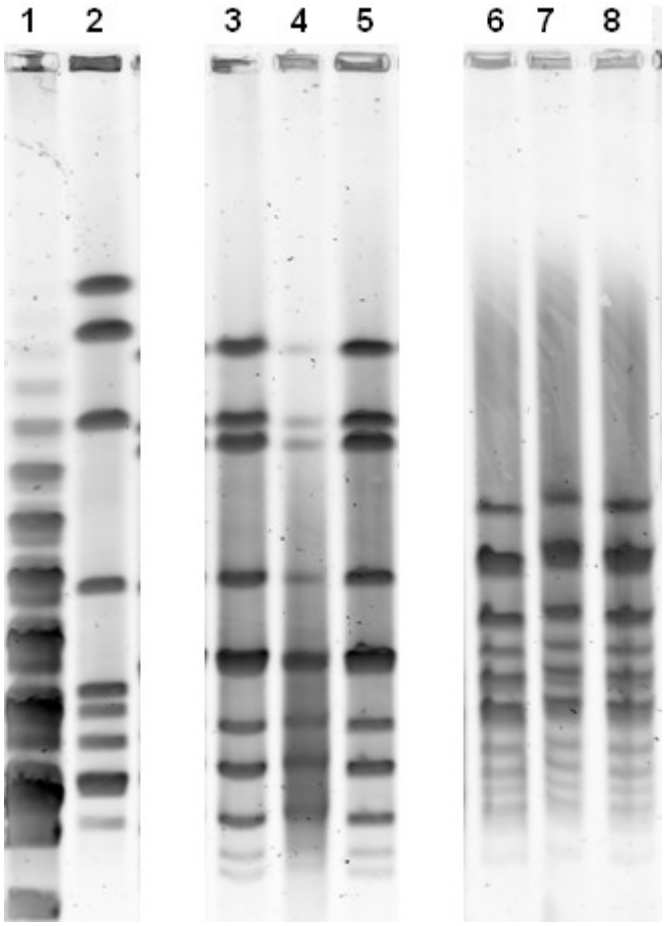

Figure 2. 5 Examples of pulsed field gel electrophoresis patterns of *S. uberis* isolates from quarters challenged with strain FSL Z1-048 (lanes 6 and 8) or FSL Z1-124 (lanes 3 and 5). The respective challenge strains were included for comparison (lane 4 and lane 7). Lanes 1 and 2 show a DNA ladder (concatemers of λ DNA) and a marker strain (*Streptococcus agalactiae* STIR-CD-25), respectively.

2. 3. 4 Somatic Cell Count and Flow Cytometry

All quarters challenged with strain FSL Z1-048 showed an increase in SCC. In individual quarters, elevation of SCC was first observed 30 to 42 h PI (Table 2. 3). Mean SCC reached its peak (7.40 ± 0.07 Log_{10} cells/mL) at 42 h PI and remained elevated throughout the study (Figure 2. 6). PBS-infused control quarters from animals challenged with FSL Z1-048 showed a significant increase in SCC compared to pre-challenge levels at several time points post-challenge, e.g. during peak milk

yield depression and towards the end of the study (Figure 2. 6). SCC elevation was observed in animals challenged with strain FSL Z1-124, in quarters infused with bacteria (Figure 2. 6) but not control quarters infused with PBS (data not shown).

A large influx of polymorphonuclear leukocytes (PMNL) in the early stage of the infection, confirmed by differential cell counts of cytocentrifuge preparations, affected the ability to quantify T-cell populations by flow cytometry, resulting in incomplete data on T-cell subsets for some time points (e.g. 96 h PI). In milk from quarters infused with strain FSL Z1-048, the mean concentration of $CD3^+$ cells was significantly elevated from 96 h PI onwards, reaching its highest level at 312 h PI ($8.18 \times 10^5 \pm 1.6 \times 10^5$ cells/mL) (Figure 2. 7A). The concentration of $CD4^+$ and $CD8^+$ lymphocyte subsets also increased from 96 h PI, with higher concentrations of $CD4^+$ lymphocytes compared to $CD8^+$ lymphocytes present in initial stages of infection. The concentration of $CD4^+$ cells stabilised after the initial increase whereas the concentration of $CD8^+$ cells increased from 144 to 312 h PI (Figure 2. 7B-C). As a result, the mean ratio between the 2 populations changed over time, from 0.69 at time 0 to peak at 2.8 at 96 h PI (Figure 2. 8). The ratio remained inverted compared to the PBS infused control quarters until 240 h PI ($P < 0.05$) and returned to normal at 312 h PI. The concentration of γδ cells increased from 144 h PI, increasing from 194 ± 171 cells/mL at time 0 to $4.6 \times 10^4 \pm 2.7 \times 10^4$ cells/mL at 144 h PI and remained elevated compared to the control quarters until the end of the study ($4.4 \times 10^4 \pm 3.1 \times 10^4$ cells/ mL at 312 h) (Figure 2. 7D). Concentrations of $CD3^+$ cells and $CD4^+$, $CD8^+$ or γδ subsets did not change in milk from cows challenged with strain FSL Z1-124 (data not shown).

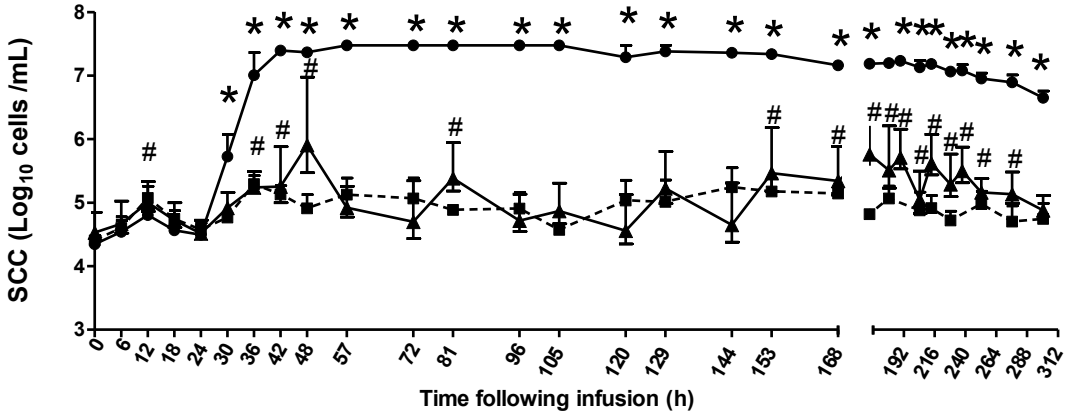

Figure 2. 6 SCC in response to challenge with *S. uberis* strain FSL Z1-048 (full lines) or FSL Z1-124 (dashed line). Main and SEM are shown for challenge quarters (circles), and for PBS infused control quarters (triangles) of animals challenged with strain FSL Z1-048. PBS infused control quarters of animals challenged with FSL Z1-124 did not show elevation of SCC compared to pre-challenge levels (data not shown). Significant differences ($P < 0.05$) between pre- and post-challenge levels are shown for challenge and PBS-infused control quarters of animals challenged with FSL Z1-048 (* and #, respectively).

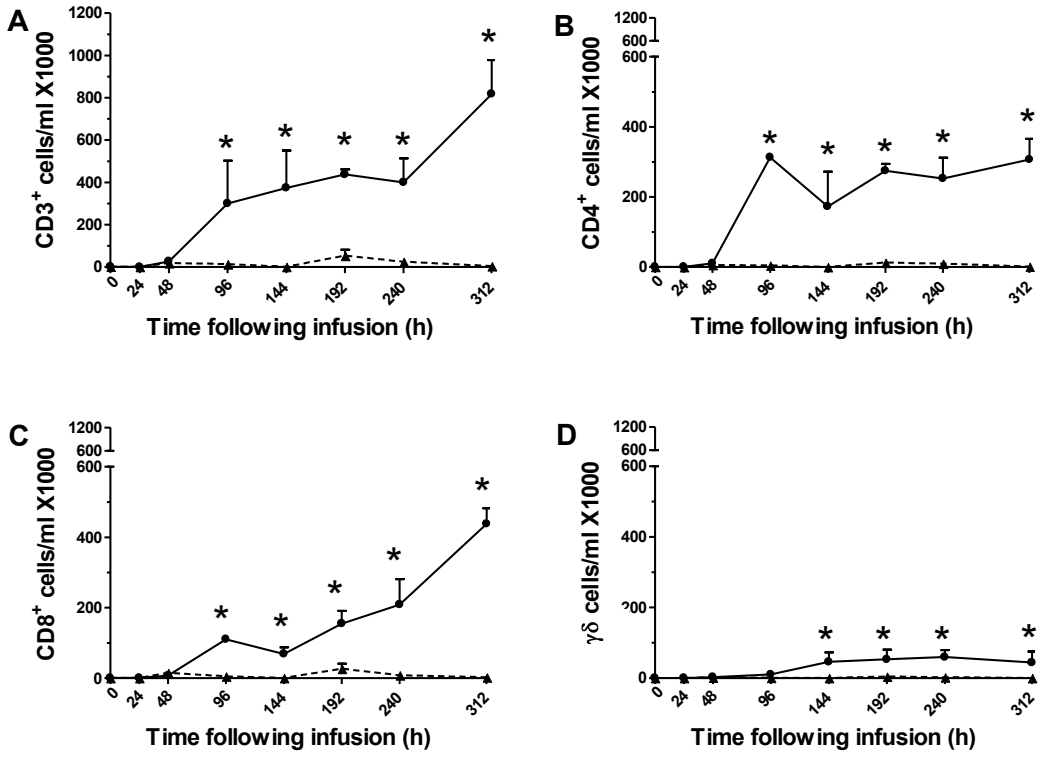

Figure 2. 7 Concentration in milk of $CD3^+$ (A), $CD4^+$ (B), $CD8^+$ l (C) and γδ (D) lymphocytes in milk. Mean and SEM are shown for quarters infused with strain FSL Z1-048 (full line) and PBS infused control quarters (dashed line) of the same animals. Stars indicate significant differences between challenged quarters and PBS infused control quarters. Models for γδ lymphocytes did not converge.

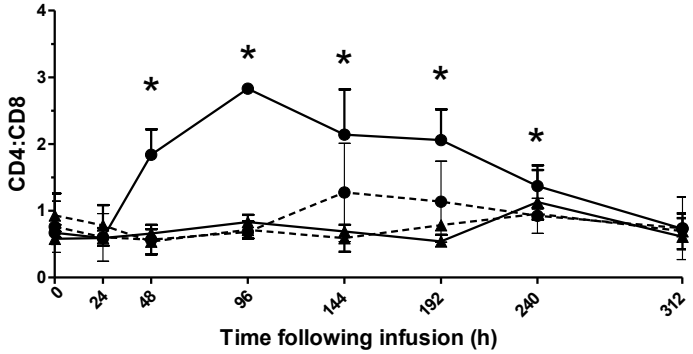

Figure 2. 8 $CD4^+$: $CD8^+$ ratio in milk from cows challenged with *S. uberis* strain FSL Z1-048 (full lines) or FSL Z1-124 (dashed lines). Mean and, where possible, SEM are shown for challenged quarters (circles) and PBS infused control quarters (triangles). Data were available for 5 or 6 quarters per strain at times 0 and 24, for 1 or 2 quarters per strain at time 96 and for 3 or 5 quarters per strain for other time points. Stars indicate significant differences between challenge quarters and PBS infused control quarters for animals challenged with FSL Z1-048.

2. 3. 5 Cytokine Response

All quarters challenged with FSL Z1-048 showed an increase in concentration of IL-1β, TNF-α, IL-8, IL-6, IL-12p40 and IL10, and 4 of 6 quarters showed an increase in concentration of IL-17A (Table 2. 3). IL-1β was first detected in challenged quarters at 30 to 42 h PI (Table 2. 3), whereas no increase was found in control quarters infused with PBS. Mean IL-1β concentration reached its highest level (327 ± 102 pg/mL) at 48 h PI (Figure 2. 9A). TNF-α was first detected between 36 and 48 h PI and the highest mean concentration (1,219 ± 599 pg/mL) was observed at 36 h PI (Figure 2. 9B). IL-8 was first detected at 30 to 42 h PI (Table 2. 3) and the highest mean IL-8 concentration (4.2 ± 0.2 Log10 pg/mL) was reached at 48 h PI (Figure 2. 9C). Five of 6 control quarters in FSL Z1-048 challenged animals showed a moderate increase in IL-8 concentration (peak of mean concentration 1.72 ± 0.36 Log_{10} pg/mL), which coincided with peak concentrations in challenged quarters (Figure 2. 9C). IL-6 was first detected at 30 to 42 h PI (Table 2. 3). The IL-6 curve was bimodal, with peaks at 48 and 72 h PI (11.3 ± 2.8 and 11.2 ± 2.9 ng/mL respectively) (Figure 2. 9D). Increased IL-12p40 concentration was first detected at 30 to 48 h PI (Table 2. 3). Mean IL-12p40 concentration peaked at 48 h PI (124 ± 35 bU/mL) (Figure 2. 9E). Increased IL-10 concentration was first detected at 30 to 42 h PI (Table 2. 3) and the peak in mean concentration (35.6 ± 8.2 bU/mL) occurred at 48 h PI (Figure 2. 9F). Interferon-γ was detected at 1 or 2 isolated time points in milk from some challenged quarters whilst in other quarters the concentration was elevated for up to 10 consecutive time points. This variability was also observed in PBS infused control quarters (Figure 2. 9H). The first increase in IL-17A concentration was detected 57 to 144 h PI (Table 2. 3) and mean IL-17A

concentration reached its peak (463 ± 345 pg/mL) at 81 h post challenge (Figure 2. 9G). The elevation in concentration of IL-17A coincided with the decrease in concentration of *S. uberis* bacteria in milk from infected quarters (Figure 2. 10). All 4 animals which showed an increase in levels of milk IL-17A cleared the infection before the end of the study, whereas 1 of 2 animals without a detectable IL-17A response did not clear the infection spontaneously.

For quarters challenged with strain FSL Z1-124, cytokines were measured in milk samples collected at all time-points for 1 animal, and at time-points up to 81 h PI for the remaining 5 animals. No increase in concentration of IL-1β, TNF-α, IL-6, IL-10, IL-12p40 or IL-17A was observed in challenged or control quarters of cows challenged with strain FSL Z1-124. Low levels of IL-8 were detected in 3 of 6 quarters challenged with strain FSL Z1-124 (data not shown). Elevations in IL-8 concentration were observed between 12 and 48 h PI and ranged from 32.2 to 72.6 pg/mL and only occurred in quarters in which the challenge strain was recovered.

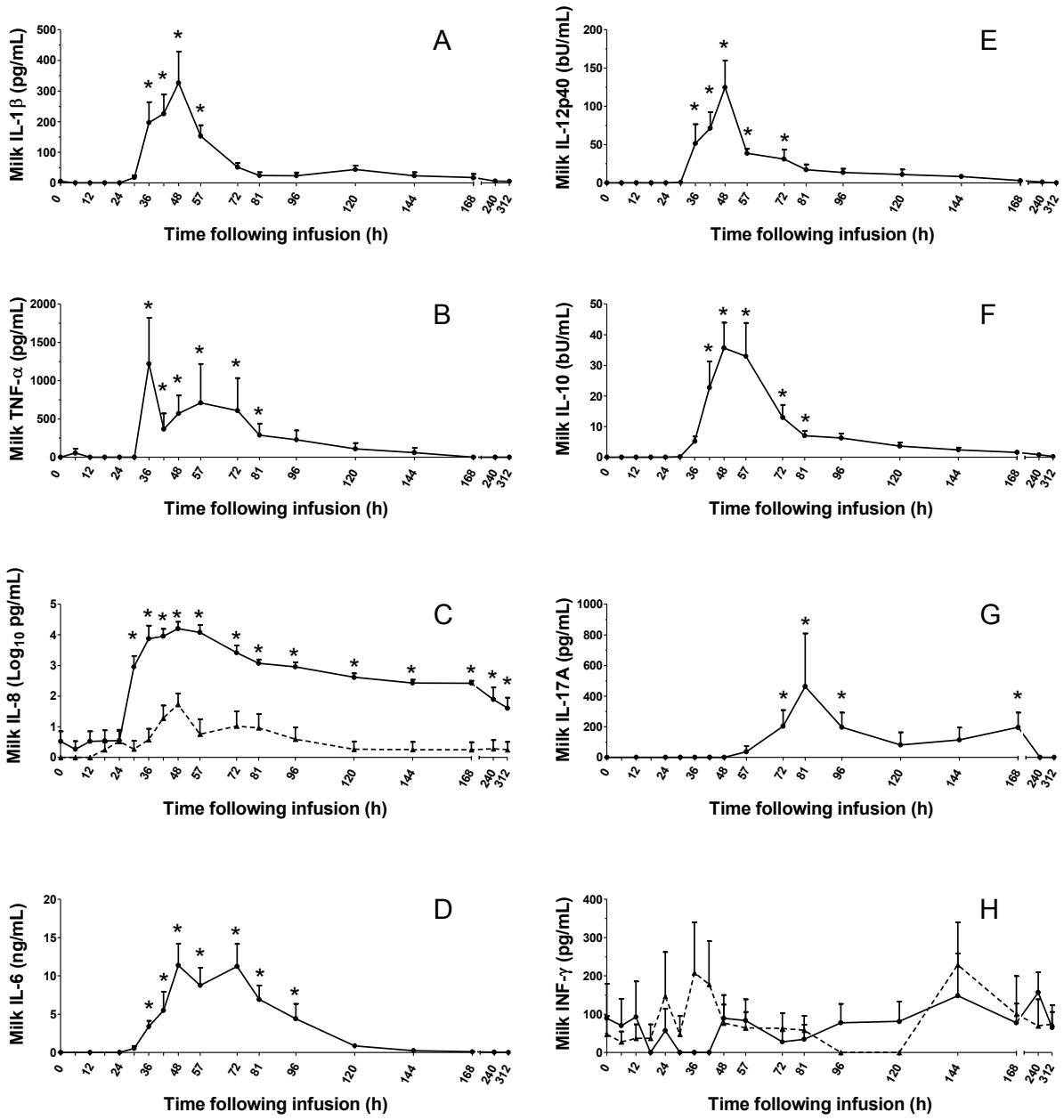

Figure 2. 9 Cytokine concentration in milk from cows challenged with *S. uberis* strain FSL Z1-048. Mean and SEM are shown for challenge quarters (full line) and, where post-challenge levels were above the detection limit, for PBS infused control quarters (dashed line). (A) Concentration of IL-1β (detection limit 31.25 pg/mL). (B) Concentration of TNF-α (detection limit 125 pg/mL). (C) Concentration of IL-8 (detection limit 0.031 ng/mL) (D) Concentration of IL-6 (detection limit 78.25 pg/mL). (E) Concentration of IL-12p40 (detection limit 0.366 bU/mL). (F) Concentration of IL-10 (detection limit 0.825 bU). (G) Concentration of IL-17A (detection limit 188 pg/mL). (H) Concentration of IFN-γ (detection limit 156.125 pg/mL). Stars indicate significant elevation compared to pre-challenge levels ($P < 0.05$).

2. 4 Discussion[1]

Clear differences in ability to cause mastitis in lactating cows were observed between the *S. uberis* putatively host-adapted strain FSL Z1-048 and the non-adapted strain FSL Z1-124. The adapted strain FSL Z1-048 was able to cause clinical mastitis in lactating cows in all the 6 animals challenged whereas none of the cows challenged with the non-adapted strain FSL Z1-124 developed mastitis even using a challenge dose 100 fold higher. These findings support the hypothesis that putatively host adapted strains identified by epidemiological and molecular data such as FSL Z1-048 are more adapted to colonize the mammary gland and thus cause IMI than the non adapted strains, explaining the predominance of adapted strains within the herds, where non adapted strains cause mastitis sporadically. Thus, differences in incidence of strain specific IMI are not simply due to different levels of exposure but dependant by characteristics of the strains. Surprisingly in this study IMI caused by the putatively host adapted strain caused a clinical mastitis which self cured in 5/6 animals whereas this strain was previously associated with sub-clinical persistent mastitis in field. This outcome was indicated as characteristic of the host adapted strains (Zadoks *et al*., 2003; Zadoks *et al*., 2007). Differences in the host response between natural and experimental IMI could be at least partially explained by challenge dose and method of exposure, e.g. deposition of bacteria in the teat cistern via a cannula.

[1] Prof. R. Zadoks (Moredun Research Institute) contributed to the writing of this section

The challenge with strain FSL Z1-124 did not result in a significant cellular response and with the exception of low levels of IL-8 which were detected in 3 of the 4 challenged quarters from which FSL Z1-124 was recovered. This suggests that clearance of this strain after the challenge occurs at early stages after the deposition of the bacteria in the mammary gland and it is due to is due to resident cells, molecules or mechanisms, rather than to influx of cells into the mammary gland Several mechanisms described by literature could explain the differences observed. These include ability to grow in milk, epithelial adhesion and invasion, resistance to killing by phagocytes.

Ability of *S. uberis* to grow in milk was investigated in the past as possible virulence factor (Leigh, 1999; Kliem *et al*., 2002; Rambeaud *et al*., 2004) because a rapid growth would enable bacteria to replicate within the mammary gland at a rate exceeding that by which they are cleared via milking and by the activity of the phagocytes such as macrophages and PMNL (Leigh, 1999). However, no clear relation between ability of *S. uberis* to grow in milk and virulence *in vivo* was found as is the case for *E. coli* (Kornalijnslijper *et al*., 2004; Zadoks, 2007). In the present study the ability to grow in milk of the two strains used in the challenge study was investigated. Both strains were able to grow in milk from the challenge animals, thus it is unlikely that different pathogenicity observed between the two strains is due to the growth in milk.

The differences observed between the strains may be due to a different ability to adhere or to invade the mammary epithelial cells which has been suggested to be

important for colonization of the mammary gland by *S. uberis* (Matthews *et al*., 1994). Alternatively or additionally, the strains may have different abilities to resist killing by phagocytes present in the mammary gland such as PMNL or macrophages, although the role of those cell types in the clearance of *S. uberis* IMI is not clear (Leigh, 1999, Pedersen *et al*, 2003, Bannermann *et al*., 2004, Denis *et al*., 2006).

Challenge with the putatively host-adapted strain, FSL Z1-048, resulted in a significant cellular and cytokine response in the mammary gland. The onset of clinical, bacteriological, cellular and cytokine events within the mammary gland relative to challenge with strain FSL Z1-048 differed between animals but the sequence and timing of these events within animals was relatively constant.

The increase in cfu count was detected at 12 to 18 h PI followed by a detectable increase in SCC and preceded detectable increases in SCC by 12 to 24 h. The initial increase in SCC was largely due to influx of neutrophils in the mammary gland and SCC reached its highest level at 36 hours PI, which coincided with peak cfu level. After that time point, SCC remained elevated until the end of the experiment whereas the concentration of *S .uberis* in milk from infected quarters decreased approximately 10^4 fold. This finding suggests that neutrophils play a role in the initial decrease of bacterial concentration. This is in agreement with results from previous *S. uberis* challenge studies describing temporal coincidence of influx of neutrophils and decrease in bacteria in milk (Hill, 1988; Pedersen *et al*., 2003). In other challenge studies, in contrast, the influx of neutrophils did not result in a decrease of the bacterial count (Rambeaud *et al*., 2003; Bannermann *et al*., 2004).

The initial increase in SCC in this study coincides with the first detectable increase in IL-8 in all quarters. In a challenge model using *S. uberis* strain O140J, SCC increases also coincided with increase in IL-8, and both were detected at 30 h post challenge (Bannerman *et al*., 2004a). These observations are consistent with the role of IL-8 in neutrophil chemotaxis (Watanabe *et al*., 2008). In a third challenge study, SCC increase preceded the first detected increase in IL-8 by approximately 6 hours (Rambeaud *et al*., 2003). In all 3 challenge studies with *S. uberis*, the increase in IL-8 levels was sustained for days. The slow but constant reduction of IL-8 concentration after the peak observed in our challenge model may reflect the reduction of bacterial concentration within the mammary gland. Nevertheless the reduction of IL-8 levels was not associated with a parallel reduction in SCC which remained at their maximal levels until the end of the study. The major source of this chemoattractant in the mammary gland is thought to be the secretory epithelium and its expression is dependent on the concentration of the pathogen (Gunther *et al*., 2010).

The early inflammatory response was also characterised by increased levels of the pro-inflammatory cytokines IL-1β and IL-6, which were first detected at 30 to 42 h PI, whereas TNF-α , another pro-inflammatory cytokine was detected 6 h later . IL-1β, which is both pro-inflammatory and pyrogenic, reached a peak and remained significantly elevated until 57 h PI, which coincided with the febrile response observed in our study. Elevations of these cytokines were in our study earlier and shorter than elevations observed in response to other *S. uberis* strains (Rambeaud *et*

al., 2003; Bannerman *et al*., 2004a) suggesting that host immune response may differ between bacterial strains as it differs between bacterial species (Bannerman, 2009).

Concentration of IL-6 showed a bimodal peak at 48 and 72 h PI, and remained elevated until 96 h PI. IL-6 peaked relatively later and remained elevated longer than pro-inflammatory cytokines IL-1β and TNF-α. This may reflect its role of both pro-inflammatory and anti-inflammatory cytokine which plays a crucial role in the transition from innate to adaptive immunity, for example by inducing the production of antagonists of IL-1β and TNF-α thus reducing the inflammatory response (Weissenbach *et al*., 2004). Moreover, IL-6 modulates T cell polarisation and promotes T cell trafficking into tissues (Jones, 2005). IL-6 was detected in cows with clinical mastitis due to streptococci, staphylococci or *E. coli* (Taylor *et al*., 1997). Up-regulation of IL-6 mRNA has been reported in blood PMNL after intramammary challenge with *S. uberis* (Moyes *et al*., 2010) thus detection in milk may be due to migration of these cells to the mammary gland. Bovine mammary epithelial cells can express IL-6 *in vitro* (Bougran *et al*., 2011) suggesting that production of this cytokine in the mammary gland may also occur locally. In humans and mice, IL-6 promotes the migration of lymphocytes to the site of inflammation (Weissenbach *et al*., 2004; McLoughlin *et al*., 2005). In our study IL-6 levels increased before the lymphocyte influx, suggesting that IL-6 may have a similar function in cattle.

First increase in levels of IL-10 and IL-12p40 was detected 6 h after the increase in IL-8 (Table 2. 3) and concentration of these cytokines remained elevated respectively up to 72 and 81 h PI .The observed increase in IL-12p40 levels may indicate the

presence of IL-12 or IL-23, because our ELISA assay is based on the detection of the p40 subunit which is shared by these two cytokines (Oppmann *et al*., 2000).

After challenge with *S. uberis* strain O140J in a separate study, IL-10 and IL-12p40 levels increased at the same time as IL-8 levels, and remained elevated for up to 168 h (Bannerman *et al*., 2004a). The sustained levels of these cytokines observed by Bannerman and colleagues may reflect the fact that in their challenge model bacterial concentration continued to increase until the end of the study whereas in our study concentration of bacteria after a peak observed at 36 h PI decreased by approximately 10^4 fold by 72 to 81 h PI then concentration remained relatively stable, until spontaneous resolution which occurred in 5 animals (Figure 2. 4).

First detection of IL-17A occurred at 72 h PI and the rise of the concentration of this cytokine coincided with the influx of $CD4^+$ and $CD8^+$ T lymphocytes, although the exact time of onset of lymphocyte influx was not measured (Figure 2. 7, 2. 9). IL-17A is produced mainly by $CD4^+$ Th17 cells. *In vitro*, the differentiation of naive T-cells into $CD4^+$ Th17 requires the presence of IL-6 and TGF-β1. The differentiation of naive T cells is further supported by IL-1β and TNF-α, (Stockinger and Veldhoen, 2007) and subsequent maintenance of differentiated Th17 cells is promoted by IL-23 (Stritesky *et al*., 2008). In our study elevation of IL-1β and IL-6 levels was followed by elevation of IL-12p40/IL-23p40 and TNF-α levels which preceded elevation of the IL-17A level. The elevation of IL-17A levels coincided with the influx of $CD4^+$ cells which increased their levels more rapidly than $CD8^+$ cell population, resulting in an inversion of the CD4:CD8 ratio (Figure 2. 7). Inversion of the CD4:CD8 ratio has been observed in cows with naturally acquired mastitis due to *Streptococcus spp*.

(Taylor *et al*., 1997; Soltys and Quinn, 1999). Although the concentration of $CD4^+$ cells remained high the ratio normalized after 240 h PI due to sustained influx of $CD8^+$ cells. These findings together support the hypothesis that differentiation of naive T-cells into Th17 cells may occur in response to the IMI with *S. uberis*. Temporal association of increase of IL-17A and influx of $CD4^+$ suggests moreover that this cell population may be the source of IL-17A detected in the milk in our challenge study.

Spontaneous resolution of infection occurred in 5 of 6 animals. Resolution was preceded by increased levels of IL-17A in 4 of 6 animals, and was temporally associated with influx of lymphocyte, moreover reduction of bacterial concentration in infected quarters coincided with the rise of this cytokine (Figure 2. 10). IL-17 is considered important for control of extracellular bacterial infections (Curtis and Way, 2009). In mice, IL-17A mediates acquired immunity to streptococcal colonization and infection, by stimulating pneumococcal killing by PMNL (Lu *et al*., 2008). Moreover, *in vitro*, IL-17 up-regulates genes that encode antimicrobial proteins in bovine mammary epithelial cells. These compounds may have a role in bacterial killing in the mammary gland (Bougarn *et al*., 2011). Our findings suggest that IL-17A may play a role in control and resolution of *S. uberis* intramammary infection.

Resolution of the infection may also be mediated by $CD8^+$ lymphocytes. A recent study showed that *S. uberis* specific $CD8^+$ memory cells are present in most cows, regardless of prior intramammary infection with *S. uberis*, and these cell population

is has a direct killing activity against *S. uberis in vitro* (Denis *et al*., 2011). This study together with the observation that clearance of infection occur in our study after the increase of $CD8^+$ support the hypothesis that $CD8^+$ lymphocytes play a role in response to *S. uberis* IMI.

In conclusion, our study demonstrates that putatively host adapted strain FSL Z1-048 and non-adapted strain FSL Z1-124, which caused IMI with different epidemiological patterns in a field study, elicit distinct clinical and immune responses after experimental challenge of lactating dairy cows. Moreover, this study suggests that neutrophils, lymphocytes and IL-17A may play important roles in reduction of bacterial load in the mammary gland and in clearance of *S. uberis* IMI.

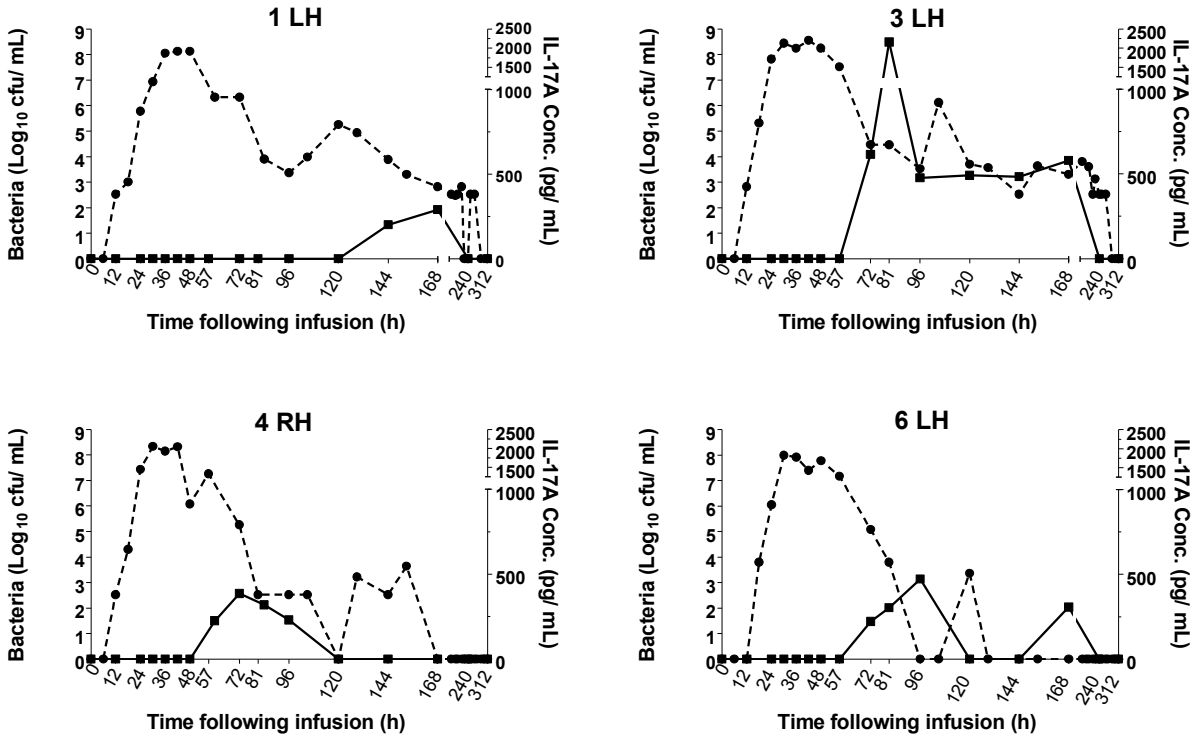

Figure 2. 10 Concentration of *S. uberis* bacteria (dashed line) and IL-17A (full line) in milk from mammary quarters challenged with strain FSL Z1-048. Quarters with detectable IL-17A are shown. Elevation of IL-17A coincided with decreased concentration of bacteria after the initial peak. All the quarters with detectable IL-17A cleared the infection by the end of the study (312 h PI). Cow identification numbers match those in Table 3. LH = left hind quarter; RH = right hind quarter.

CHAPTER 3

Host Response to Secondary Challenge with *Streptococcus uberis*

3. 1 Introduction

In the past decades implementation of control plans and hygienic measures have decreased prevalence of mastitis. However, it is still the most costly disease on dairy farms. Beside control programs and hygienic prevention, a vaccine against *S. uberis* would help to reduce the number of mastitis cases (Leigh, 1999; Zadoks, 2007; Denis *et al*., 2009).

In the past years different approaches have been taken to develop a vaccine against *S. uberis.* Adhesion and invasion of the epithelial cells is considered a crucial step in the colonization of the mammary gland by *S. uberis* (Prado *et al*., 2011). *S. uberis* adhesion molecule (SUAM), a protein involved in the adhesion and invasion of epithelial cells *in vitro* was identified (Chen *et al*., 2011). Serum of cows immunized with recombinant SUAM contained SUAM-specific antibodies and reduced the adhesion and the internalization of *S. uberis* to mammary epithelial cells *in vitro* but the efficacy of this vaccine is yet to be tested *in vivo* (Prado *et al*., 2011). A similar approach was taken to develop a vaccine using recombinant GapC protein as immunogen (Fontaine *et al*., 2002), a surface associated molecule common to several species of *Streptococci* involved in the adhesion to the host cells, but *in vivo* efficacy has to be further tested (Leigh, 2002). A different approach was used in the development of a vaccine targeting the plasminogen activator (PauA), a protein involved in the acquisition of nutrients by *S. uberis*. The aim of this vaccine was to impair the ability of the bacteria to acquire nutrients, reducing the growth rate of the bacteria in the mammary gland. A reduction of number of bacteria in the mammary

gland would reduce the inflammatory response of the host (Leigh, 1999). Subsequent studies showed that PauA is not necessary for the growth of *S. uberis* in milk (Ward *et al*., 2003), moreover strains lacking the gene encoding for PauA have been described (Gilchrist *et al*., 2013)

Although promising, studies conducted using vaccines targeting specific components or products of *S. uberis* did not show evidence of successful protection *in vivo* or, in the case of SUAM, descriptions of such studies are lacking. In other studies, rather than using selected targets, the whole organism was used and in these studies some evidence of protection was observed *in vivo*. Subcutaneous vaccination with killed *S. uberis* or intra mammary inoculation with killed *S. uberis* was shown to protect cows from intramammary infection with the same strain (Finch *et al*., 1994, 1997). None of the animals vaccinated by the intramammary route showed clinical disease or bacteria in milk after the subsequent challenge, whereas animals vaccinated subcutaneously showed a reduced bacterial concentration in milk compared to the control animals but developed clinical signs of mastitis. In these studies, protection was significant only against the homologous strain (Finch *et al*, 1994; Finch *et al*., 1997) whereas only a small decrease of bacterial concentration in milk was observed in quarters challenged with a heterologous strain of *S. uberis* (Finch *et al*., 1997). Due to the large number of different strains (Zadoks, 2007) the lack of protection against heterologous strains observed in these studies represents an important problem in the development of a vaccine against *S. uberis*.

Specific milk antibodies against *S. uberis* were raised after the vaccination with killed *S. uberis* and were different in isotype composition depending on the route of administration. With intramammary vaccination, IgG1, IgG2 and IgM responses were observed, whereas with subcutaneous vaccination only IgG1 and IgG2 responses were observed. These antibodies, when tested *in vitro*, did not show an enhanced opsonising and did not increase the ability of neutrophils to kill *S. uberis* (Finch *et al*., 1994). These data suggest that the humoral immune response may not be protective against intramammary infection with *S. uberis*, although the hypothesis that antibodies were protective by preventing or reducing the adhesion of bacteria to the mammary gland cells cannot be ruled out (Leigh, 1999). Specific antibodies could theoretically bind the bacterial adhesins involved in the process of adhesion and invasion of the epithelial cells, impairing the ability of the bacteria to adhere and invade the host cells thus reducing the pathogenicity.

In addition to a humoral response, intramammary vaccination with killed *S. uberis* evoked a strong cellular immune response. Lymphocytes isolated from blood and dry period mammary gland secretions of vaccinated animals showed a proliferative response to *S. uberis in vitro* (Finch *et al*., 1994). Recent data suggest a possible involvement of lymphocytes in control of *S. uberis* mastitis. $CD8^+$ cells specific to *S. uberis* isolated from blood of dairy cows expressed the memory cell repertoire antigen ($CD45RO^+$) regardless of exposure history with *S. uberi*s. These cells responded to the stimulation with *S. uberis in vitro* by proliferating. The vast majority of the cells were of the Th1 profile producing IFN-γ and were also able to directly kill *S. uberis in vitro* (Denis *et al*., 2011). Based on these findings authors

speculated that an effective vaccine against *S.uberis* would ideally generate *S. uberis* specific $CD8^+$ memory cells in the mammary gland.

Vaccines are based on the induction of an immunological memory, which is the ability of the adaptive immune system to respond to each subsequent encounter with a given antigen in a faster and more effective manner (Jenaway *et al*., 2001). Little is known about immunological memory following natural exposure of the mammary gland with *S. uberis*. Protective effect of previous infection with *S. uberis* was observed in cows intramammarily challenged with the live bacteria of the homologous strain, showing a reduction in the number of susceptible quarters between the first and the secondary challenge (Hill, 1988) although the possible mechanism behind protection was not investigated.

Recently, epidemiological studies have been conducted. A longitudinal study showed an increased rate of *S. uberis* intramammary infection in quarters that had recovered from mastitis caused by the same pathogen in the past (Zadoks *et al*., 2001). Schukken *et al*. (2009) found that clinical mastitis with *Streptococcus spp*. did not reduce the severity, measured by milk loss, of subsequent clinical cases caused by the same pathogen. These epidemiological studies suggest that intramammary infection is not protective to a subsequent infection with the same pathogen, and that protective immunological memory is not induced.

The aim of the present work is to characterize the host response of lactating cows previously challenged with *S. uberis* to the secondary challenge with a homologous

or heterologous strain to test the hypothesis that immunological memory exists following intramammary infection with *S. uberis*. Moreover the host response was measured at quarter level to test the hypothesis that, if memory responses are elicited, local (quarter level) immunological memory differs from systemic (mammary gland level) immunological memory. As for the response to the primary challenge described in the Chapter 2, clinical response, cellular subsets and cytokines involved in the response were characterized.

3. 2 Materials and Methods

3. 2. 1 Study Design

In total, 12 clinically healthy Holstein cows previously challenged with *S. uberis* strain FSL Z1-048 or FSL Z1-124 were used in this experiment (Table 3. 1). As for the primary challenge, the secondary challenge experiments were conducted using four cows at time. After the primary challenge (See Chapter 2 for details) one cow primary challenged with strain FSL Z1-048 and still infected with the challenge strain at the end of the initial follow-up period (14 days) was treated with 4 daily administrations of penethamate hydrodide (Mamyzin 10 g intramouscular injection, Boehringer Ingelheim, Bracknell, UK), an antimicrobial tested before as effective against strain FSL Z1-048 (Prof. R. Zadoks, personal communication). Cows were allowed to rest for 14 d and bacteriological milk examination was performed prior the secondary challenge to ensure that all the mammary quarters were free of infection.

At secondary challenge cows were challenged in uninfected quarters. To test the hypothesis that immunological memory against intramammary infection with *S. uberis* exists and that it is strain specific, half of the cows received the same strain as at the primary challenge (homologous strain) whereas half received the other strain (heterologous strain). To test the hypothesis that local (quarter level) immunological memory differs to systemic (mammary level) immunological memory, two quarters per cow were challenged: the quarter that had been inoculated at primary challenge (previously challenged quarter) and the contralateral or the diagonally opposed

quarter (naive quarter) were challenged. No mock challenged control quarters were used. Allocation of homologous or heterologous secondary challenge was randomized. An example of one round of the experiment is shown in Figure 3. 1. In two cases, complete randomization was not possible because quarters that were mock challenged in the primary challenge experiment acquired a spontaneous infection during the resting period. The number of animals and quarters for each challenge is summarized in Table 3. 1.

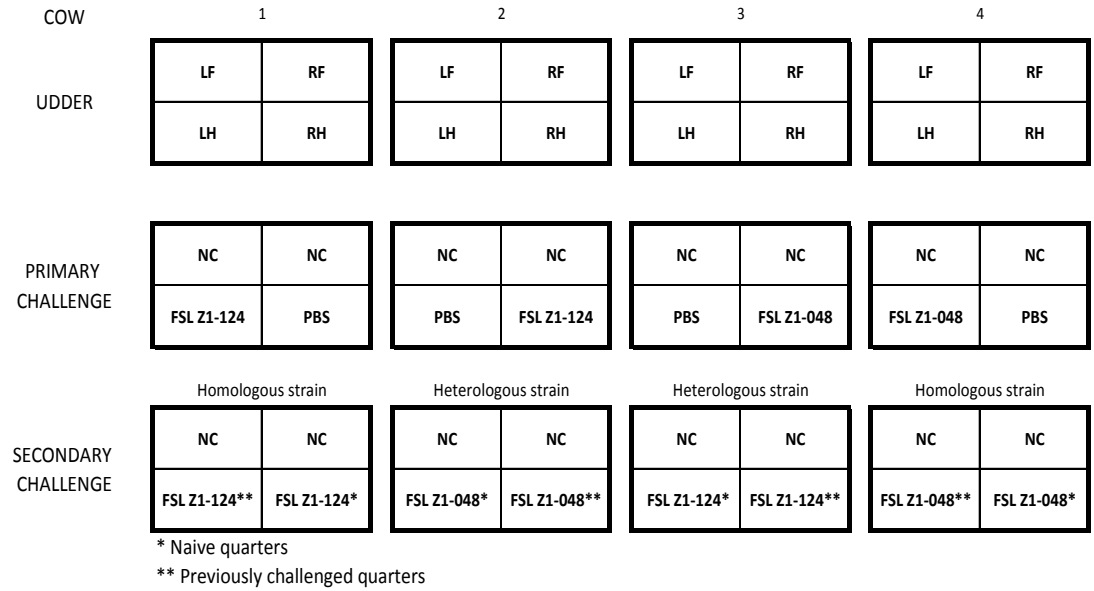

Figure 3. 1 Example of experimental design. Large squares represent the udder of 4 cows used in each of three rounds of infection. Udders are divided in the 4 mammary quarters (LF left front, RF right front, LH left hind, RH right hind). In the primary challenge experiment half of the cows were challenged with strain FSL Z1-048 and half with strain FSL Z1-124. Each cow was infused with the challenge strain in one rear quarter whereas the other rear quarter was infused with PBS as control. In the secondary challenge experiment half of the cows were re-challenged with the same strain (homologous) as the primary challenge (either FSL Z1-048 or FSL Z1-124). Half were challenged with the other (heterologous) strain. For each cow one quarter received both a primary and secondary bacterial challenge (previously challenged quarters). A second quarter received PBS in the primary challenge as control and *S. uberis* bacteria in the secondary challenge (naive quarters). The remaining quarters were not challenged (NC).

Table 3. 1 Number of animals and mammary quarters used for secondary challenge with *Streptococcus uberis*. Cows were previously challenged with FSL Z1-048 or FSL Z1-124 and received a secondary challenge with the homologous or heterologous strain. Challenged quarters had been used for the primary challenge (previously challenged) or were challenged for the first time on this occasion (naive).

Strain	Cows		Quarters	
Strain FSL Z1-048	n=6		n=12	
	as Homologous	n=3	n=6	
			Previously challenged	n=3
			Naive	n=3
	as Heterologous	n=3	n=6	
			Previously challenged	n=3
			Naive	n=3
Strain FSL Z1-124	n=6		n=12	
	as Homologous	n=3	n=6	
			Previously challenged	n=3
			Naive	n=3
	as Heterologous	n=3	n=6	
			Previously challenged	n=3
			Naive	n=3

3. 2. 2 Intramammary Challenge

The bacterial inoculum for challenge experiments was prepared as described for the primary challenge (see section 2. 2. 4 for the details).

3. 2. 3 Sample and Data Collection

Milk samples and data were collected with the same frequency as in the primary challenge study (see section 2. 2. 8 for the details). Cows were followed up to 7 days or 168 h post infusion (PI). At completion of the follow-up, cows were culled. All experiments were conducted at the Moredun Research Institute (Penicuik, UK) with approval of the Institute's Experiments and Ethical Review Committee in accordance with the Animals (Scientific Procedures) Act 1986. The vast majority of data were recorded and samples were collected by Prof. Ruth Zadoks (Moredun Research Institute) and David Reddick (Moredun Scientific Ltd, Penicuik, UK)

3. 2. 4 Bacteriological Analysis and Molecular Typing

Milk for quantitative and qualitative bacteriology analysis was collected every 6 h for the first 2 d post infusion and twice a day from 3 to 7 d PI. Species and strain identity of the *S. uberis* isolates were confirmed by PCR and PFGE respectively (for detailed protocols see section 2. 2. 6 and 2. 2. 7). A total of 39 isolates from animals challenged with strain FSL Z1-048 and 5 isolates from animals challenged with strain FSL Z1-124 were available for strain typing. Qualitative and quantitative bacteriological analysis was performed by Cliff. Ramage (Moredun Scientific Ltd.). Molecular typing was performed by Mark Lutton (Moredun Research Institute).

3. 2. 5 Somatic Cell Counting and Flow Cytometry

Milk samples for SCC determination were collected every 6 h for the first 2 d PI and twice a day from 3 to 7 d PI. Milk samples for flow cytometric analysis were collected on days 1, 2, 4 and 6 PI. Samples to analyze the T lymphocyte populations cells isolated from milk were stained with CD3, CD4, CD8 and γδ antibodies (for detailed protocols see section 2. 2. 8).

3. 2. 6 Cytokine Measurements

Milk samples for cytokine measurements were collected every 6 h for the first 2 d PI, twice a day from 3 to 5 d PI and once a day from 6 to 7 d PI. ELISA tests were used to determine the concentration of TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-12p40, IL-17A and IFN-γ (for detailed protocols see section 2. 2. 9).

3. 2. 7 Statistical Analysis

Due to the complexity of the study design and the number of the hypotheses being tested, the number of observations for each treatment was extremely low (n= 3, Table 3. 1). As a result, the power of the study was very low even where numerical results were clearly different between groups. No statistical significance was attained comparing groups with un-paired t-tests. Therefore, no further significance testing was undertaken and results were reported in a qualitative manner.

3. 3 RESULTS

3. 3. 1 Clinical data

3. 3. 1. 1 Clinical Response to Secondary Challenge with Strain FSL Z1-048

Considering clinical data collected at cow level such as fever, anorexia and lethargy, all 6 cows that received *S. uberis* FSL Z1-048 at secondary challenge developed clinical mastitis. At quarter level, differences were observed between cows that received it as the homologous challenge (e.g. animals that also received strain FSL Z1-048 at primary challenge) and those that received it as the heterologous challenge (e.g. animals that were primary challenged with strain FSL Z1-124). In animals receiving FSL Z1-048 as homologous challenge, local signs of mastitis at quarter level were observed in all 3 naive quarters whereas only one out of 3 previously challenged quarters showed visible abnormalities (Table 3. 2). In animals receiving FSL Z1-048 as heterologous challenge, clinical signs at quarter level were observed in all 6 quarters regardless of whether they were naive (i.e. quarters that were mock challenged at primary challenge experiment) or previously challenged (i.e. quarters that were challenged at primary challenge experiment) (Table 3. 3). Because of severe clinical conditions, two animals which received strain FSL Z1-048 as heterologous challenge were treated with antimicrobials and eliminated from the study (respectively at 48 and 81 h PI). The results for this challenge group are presented for 3 animals until 48 h PI, 2 animals between 57 and 81 h PI and 1 animal between 96 and 168 h PI.

Clinical signs were first detected in individual cows challenged with FSL Z1-048 as homologous strain between 36 and 42 h PI and between 30 and 57 h PI in cows challenged with FSL Z1-048 as the heterologous strain. Mean clinical score peaked at 72 h PI in cows challenged with FSL Z1-048 as the homologous strain and at 57 h PI in cows challenged with FSL Z1-048 as the heterologous strain (Figure 3.2).

Body temperature increased in 3 out of 3 cows challenged with FSL Z1-048 as homologous strain as well as in 3 out of 3 cows challenged with FSL Z1-048 as heterologous strain. The first increase in body temperature in individual animals was detected between 36 and 57 h PI both groups, regardless of strain homology. The mean temperature peak was observed 57 h PI in cows receiving FLS Z1-048 as the homologous or the heterologous secondary challenge and was 40.1 ± 1.3 and 40.6 ± 0.6 °C, respectively (Figure 3. 3).

Milk production decreased in all the cows in response to the secondary challenge with strain FSL Z1-048. The decrease started the first day following the infusion and continued until the third day (Figure 3. 4). Daily milk yield was reduced by approximately 50% by the fourth day PI in cows challenged with FSL Z1-048 as homologous strain whereas in cows which received it as the heterologous strain milk yield was reduced by approximately 80% on the fourth day PI.

3. 3. 1. 2 Clinical Response to Challenge with Strain FSL Z1-124

None of the cows challenged with strain FSL Z1-124 either as homologous or heterologous strain developed clinical mastitis. Body temperature remained on the baseline, and no reduction in daily milk yield was observed (Figure 3. 4).

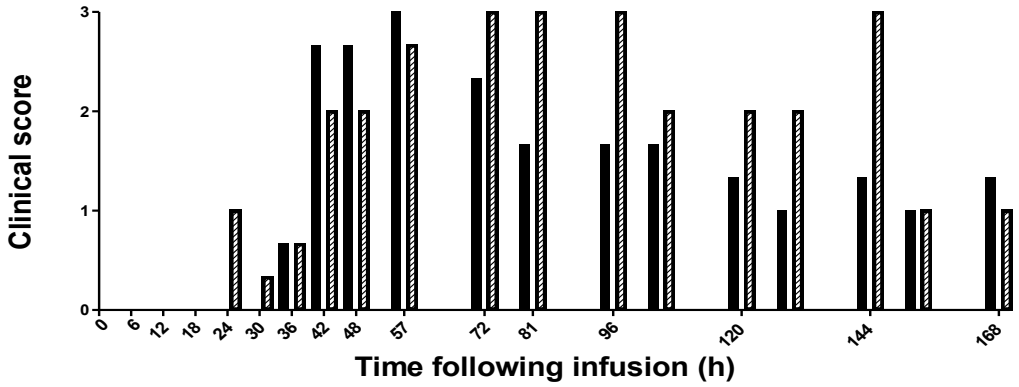

Figure 3. 2 Clinical score in cows after secondary challenge with FSL Z1-048 either as heterologous (hashed bars, n=3) or homologous strain (solid bars, n=3). Mean and SD are shown. 0 = no clinical signs; 1= abnormalities in milk, e.g. clots or discoloration; 2 = abnormalities in udder, e.g. swelling or pain; 3 =systemic signs, e.g. fever. Two animals that received FSL Z1-048 as heterologous secondary challenge were removed from the study after 48 h and 81 h PI respectively.

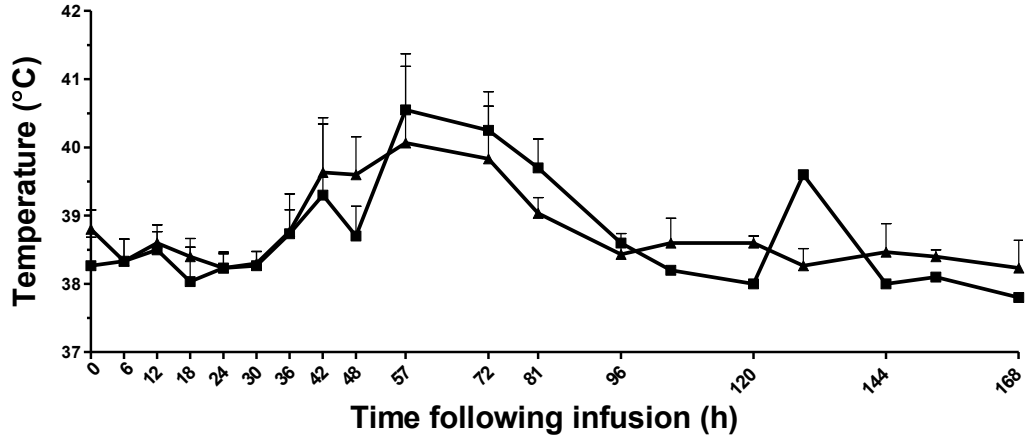

Figure 3. 3 Body temperature in cows secondary challenged with FSL Z1-048 either as heterologous (squares, n=3) or homologous strain (triangles, n=3). Mean and standard deviation are shown. Two animals that received FSL Z1-048 as heterologous secondary challenge were removed from the study after 48 h and 81 h PI respectively

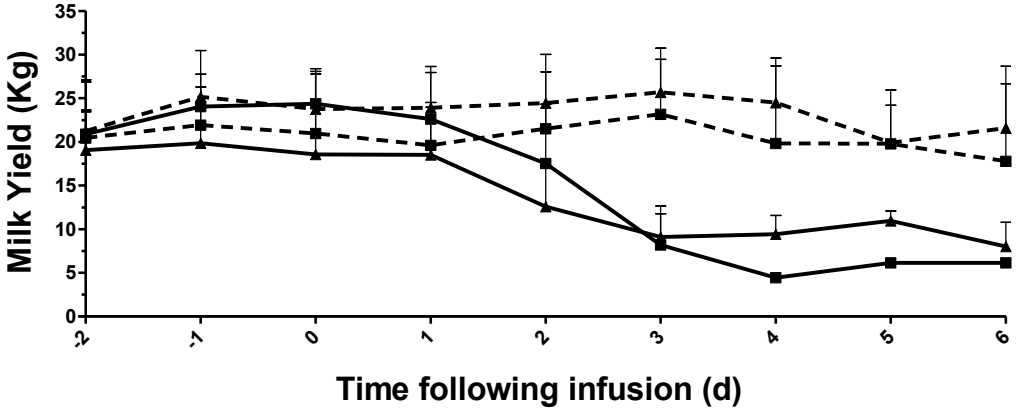

Figure 3. 4 Daily milk yield in cows secondary challenged with strain FSL Z1-048 (solid lines) or FSL Z1-124 dashed lines. Mean and standard deviation are shown for the cows which received the homologous strain (triangles) and the heterologous strain (squares).Three cows per group are shown except for the group that received FSL Z1-048 as heterologous secondary challenge (n=3 up to and including 2 d PI, n=2 between 2 and 4 d PI and n=1 until 6 d PI)

3. 3. 2 Bacterial Culture and Molecular Typing

Culture of unused doses of the challenge inoculum showed that quarters had been infused with 53 to 160 cfu of strain FSL Z1-048 or 80 to 143 cfu of strain FSL Z1-124. In the last round of the experiment, the challenge dose of strain FSL Z1-124 was increased to 20,660 cfu to determine whether a higher inoculum dose would induce a clinical response. Bacteriological analysis on milk from quarters was performed in cows after secondary challenge with *S. uberis*.

3. 3. 2. 1 Strain FSL Z1-048

S. uberis was isolated in milk from all cows that received strain FSL Z1-048 at secondary challenge either as homologous or heterologous strain (Figure 3. 5 and 3. 6). Bacteria were detected in milk of 3 of 3 naive quarters in cows that received this strain as homologous secondary challenge. All 3 quarters were positive between 30 and 168 h PI (Figure 3. 5). *S. uberis* was isolated in milk from 2 of 3 previously challenged quarters that received the strain as homologous secondary challenge. Quarter 5RH was tested positive continuously from 18 to 168 h PI whereas quarter 4RH was positive at 18, 42, 48 and 96 h PI (Figure 3. 5, Table 3. 2). Mean concentration in previously challenged quarters which received FSL Z1-048 as homologous strain reached its peak (3.38 ± 3.21 Log_{10} cfu/mL) at 48 h PI. In naive quarters the peak (7.31 ± 1 Log_{10} cfu/mL) was much higher and observed at 42 h PI (Figure 3. 7). *S. uberis* was isolated in milk from all the cows challenged with strain FSL Z1-048 as heterologous challenge either in naive (n=3) or previously challenged (n=3) quarters. *S. uberis* was first detected in quarters between 18 and 24 h in previously challenged quarters and between 24 and 30 h in naive quarters (Table 2. 3). Thereafter, the quarters remained positive until the end of the study (n=1) or the antimicrobial treatment (n=2, Figure 3.6). Maximal bacterial concentrations in both naive and previously challenged quarters were observed between 57 and 72 h PI (8.1 ± 0.14 Log_{10} cfu/mL and 7.79 ± 0.59 Log_{10} cfu/mL respectively, Figure 3.8).

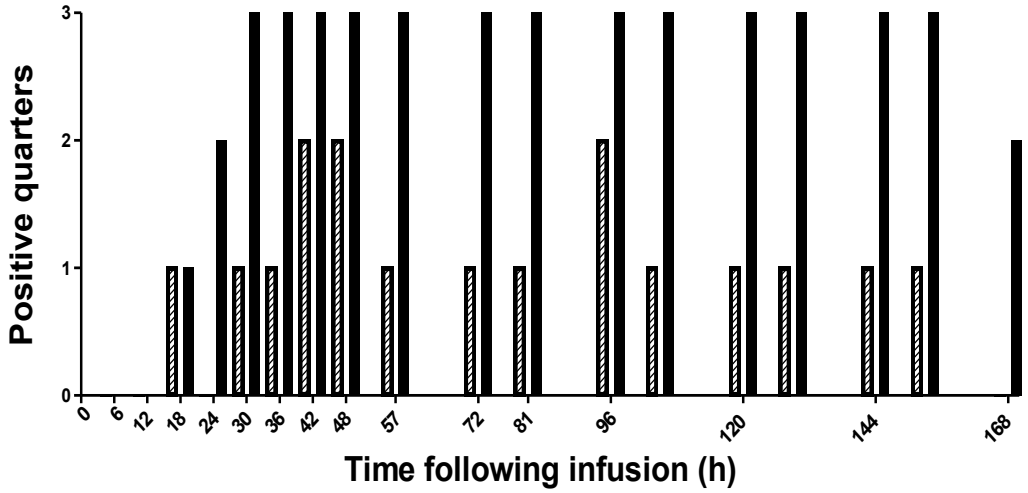

Figure 3. 5 Number of bacteriologically positive quarters after secondary challenge with FSL Z1-048 as homologous strain. Naive (solid bars, n=3) and previously challenged quarters (hashed bars, n=3) are shown.

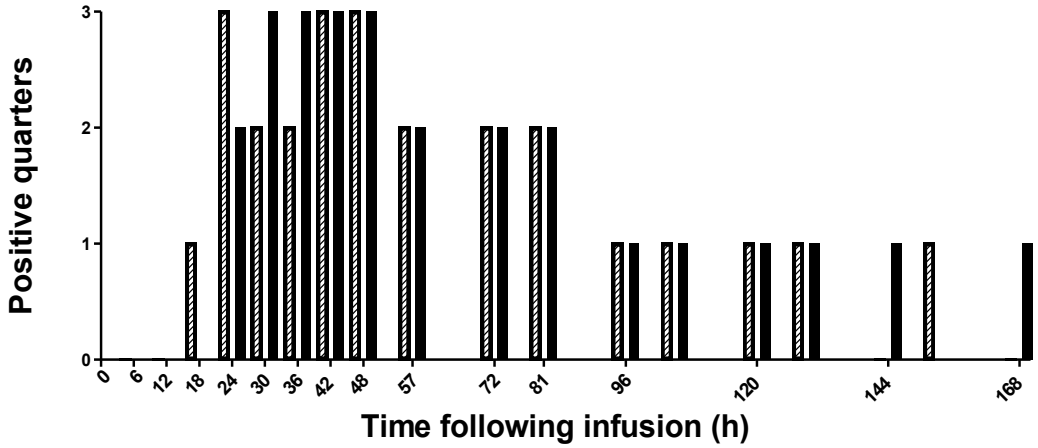

Figure 3. 6 Number of bacteriologically positive quarters after secondary challenge with FSL Z1-048 as heterologous strain. Naive (solid bars, n=3) and previously challenged quarters (hashed bars n=3) are shown. After 48 h and 81 h PI animals were removed from the study, reducing the number of quarters per category to 2 and 1 respectively.

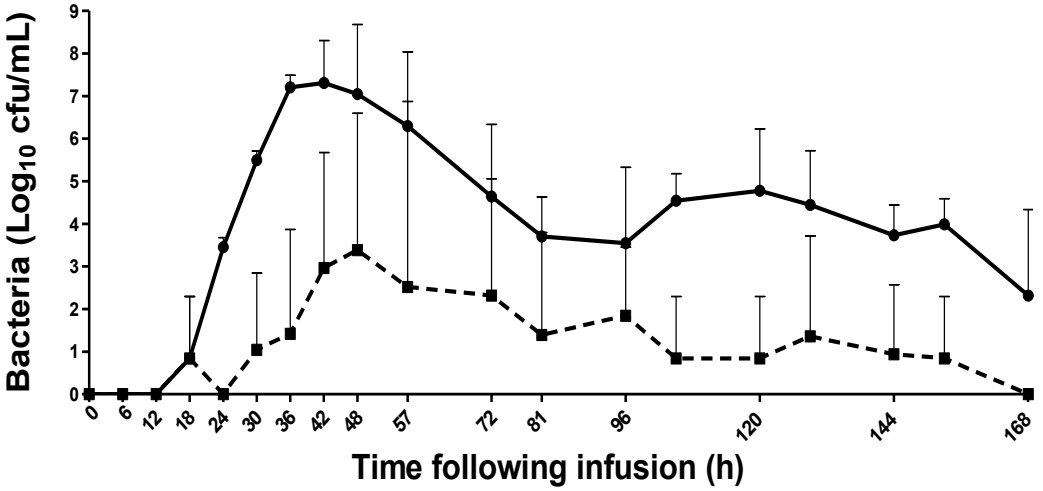

Figure 3. 7 Concentration of *S. uberis* bacteria in milk after secondary challenge with FSL Z1-048 as homologous strain. Mean and standard deviation are shown for naive (solid lines, n=3) and previously challenged (dashed lines, n=2 quarters).

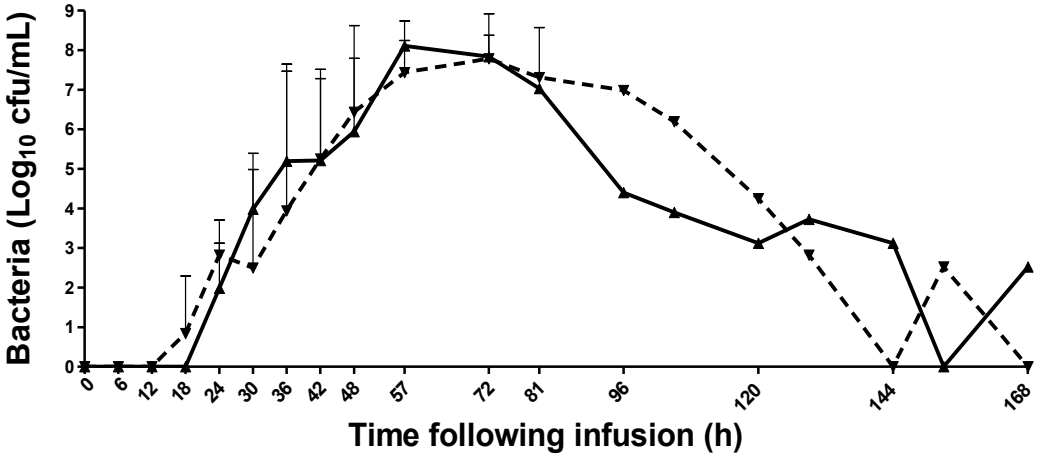

Figure 3. 8 Concentration of *S. uberis* bacteria in milk of cows secondary challenged with FSL Z1-048 as heterologous strain. Mean and standard deviation are shown for naive (solid lines) and previously challenged (dashed lines) quarters. 3 quarters per group are shown up to and including 48 h, 2 from 57 to 81 h PI and 1quarter only after 81 h PI.

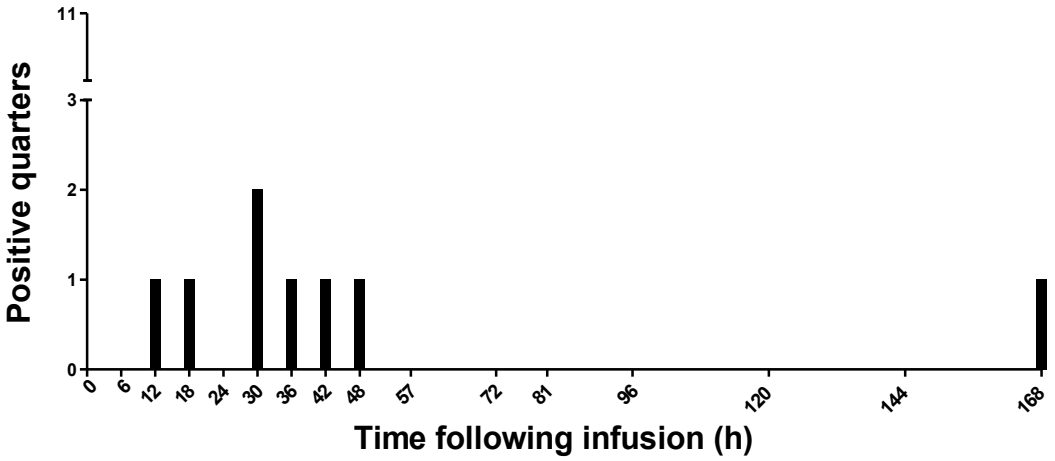

Figure 3. 9 Number of positive quarters of cows after secondary challenge with FSL Z1-124 either as homologous or heterologous strain. 11 quarters were infused with strain FSL Z1-124 in total in the secondary challenge. Four quarters and 3 cows became positive, a single animal was positive in both two challenged quarters.

Table 3. 2 Time (hours post infusion) of first detection (bacteria) or first detected increase (SCC, cytokine levels) and maximal concentration of several parameters in milk from mammary quarters of cows after secondary challenge with *Streptococcus uberis* strain FSL Z1-048 as homologous strain. Each animal was challenged in two quarters, one naive and one previously challenged with the same strain.

	Homologous naive						Homologous previously challenged					
Animal/Quarter	2LH		4LH		5LH		2RH		4RH		5RH	
	Time	Conc.	Time	Conc.	Time	Conc.	Time	Conc.	Time	Conc.	Time	Conc.
Clinical signs (quarter)	36	NA	42	NA	42	NA	ND	NA	ND	NA	72	NA
Bacteria (Log_{10} cfu/mL)	18	7.25	24	8.9	24	7.1	ND	-	18	3.75	30	7.55
SCC (Log_{10} cell/mL)	36	7.48	36	7.48	36	7.48	57	6.7	57	6.94	48	7.48
IL-1β (pg/mL)	42	135.3	42	189.8	42	153.3	ND	-	ND	-	57	301.1
IL-8 (ng/mL)	36	10.18	36	40	36	19.55	48	0.369	48	1.271	48	39.39
TNF-α (pg/mL)	42	3762	42	8000	72	3130	ND	-	57	823.5	48	5039
IL-6 (ng/mL)	42	15.17	42	18.09	42	3.18	57	0.086	48	0.437	57	3.755
IL-10 (bU/mL)	42	24.85	42	21	42	19.04	48	1.763	48	1.385	57	16.27
IL-12p40 (bU/mL)	42	27.61	42	132.7	48	29.38	ND	-	ND	-	57	114.5
IL-17A (pg/mL)	ND	-	57	633.2	72	279.9	ND	-	ND	-	57	878.1

ND not detected, NA not applicable

Table 3. 3 Time (hours post infusion) of first detection (bacteria) or first detected increase (SCC, cytokine levels) and maximal concentration of several parameters in milk from mammary quarters of cows after secondary challenge with *Streptococcus uberis* strain FSL Z1-048 as heterologous strain. Each animal was secondary challenged in two quarters, one naive and one previously challenged with a different strain.

	Heterologous naive						Heterologous previously challenged					
Animal/Quarter	7RH		8RF*		9RF**		7LH		8LH*		9LH**	
	Time	Conc.	Time	Conc.	Time	Conc.	Time	Conc.	Time	Conc.	Time	Conc.
Clinical signs (quarter)	81	NA	36	NA	57	NA	96	NA	36	NA	57	NA
Bacteria (Log_{10} cfu/mL)	24	7.44	24	8.02	18	8.36	30	8.01	24	8.01	24	8.02
SCC (Log_{10} cell/mL)	57	7.48	42	7.48	42	7.19	57	7.48	48	7.48	42	7.48
IL-1β (pg/mL)	81	111.4	48	93.18	57	111.2	72	203.1	42	227.8	81	71.89
IL-8 (ng/mL)	120	3.400	48	40	57	15.40	81	40	48	4.113	48	40
TNF-α (pg/mL)	42	4189	48	1921	36	705.8	57	578.4	42	1705	48	1055
IL-6 (ng/mL)	96	4.982	48	1.396	81	4.877	81	8.893	48	4.847	81	4.113
IL-10 (bU/mL)	120	15.08	48	3.547	81	13.07	81	26.4	48	6.303	81	9.844
IL-12p40 (bU/mL)	96	63.49	48	14.28	57	127.2	72	55.92	48	88.28	48	86.38
IL-17A (pg/mL)	ND	-	ND	-	81	339.1	ND	-	48	393.3	81	223.3

* treated with antimicrobials at 48 h PI
** treated with antimicrobials at 81 h PI
ND not detected, NA not applicable

3. 3. 2. 2 Strain FSL Z1-124

S. uberis was isolated in milk from only 3 of 6 cows after secondary challenge with strain FSL Z1-124 either as homologous or heterologous strain. Bacteria were isolated in both the previously challenged and the naive quarter in one individual cow which received strain FSL Z1-124 as heterologous challenge (quarters 1RH and 1LH respectively) and from one quarter in other two cows 1 (RH, naive quarter) and 12 (LH, previously challenged quarter) which received strain FSL Z1-124 as homologous challenge (Figure 3. 9). Concentration of bacteria in milk in individual quarters ranged from 2.52 to 3 Log10 cfu/mL.

3. 3. 3 Strain Typing

From each quarter challenged with strain FSL Z1-048, 3 isolates were used for PCR and PFGE, i.e. one isolate representing the first isolation, peak concentration and last isolation, respectively, for a total of 36 isolates. All isolates were confirmed to be *S. uberis* by PCR and PFGE patterns of all isolates matched that of the challenge strain. Only 7 isolates were available from cows secondary challenged with strain FSL Z1-124. Five isolates from quarters challenged with FSL Z1-124 were confirmed to belong to the challenge strain, i.e. 2 isolates from quarter 10RH obtained at 96 and 168 h PI and 3 isolates obtained from quarter 12RH at 18, 42 and 48 h PI.

3. 3. 4 Somatic Cell Count

Somatic cell count was measured in mammary quarters during the resting period between primary and secondary challenge and after secondary challenge with *S. uberis*.

After secondary challenge, elevation in SCC was observed in all the previously challenged quarters which received FSL Z1-048 as homologous strain, however, SCC in individual quarters varied considerably amongst the quarters. First elevation was detected in individual quarters between 48 and 57 h PI (Table 3. 3) and mean concentration reached the maximal level (6.87 ± 0.15 Log_{10} cell/mL) by 57 h PI (Figure 3. 10).

Elevations in SCC were observed in all the naive quarters after secondary challenge with FSL Z1-048 as homologous strain. First elevation in individual quarters was detected at 36 h PI (Table 3. 2) and maximal mean concentration was observed 42 h PI (7.31 ± 0.23 Log_{10} cell/mL). Elevation in SCC was also observed in all cows challenged with strain FSL Z1-048 as heterologous strain. In previously challenged quarters, the first elevation was detected in individual quarters between 42 and 57 h PI (Table 3. 3). The peak in mean concentration (7.25 Log_{10} cell/mL) was observed 81 h PI (Figure 3. 10). First increase was detected in naive quarters between 42 and 57 h PI (Table 3. 3) and maximal elevation of mean concentration (7.24 ± 0.08 Log_{10} cell/mL) was reached by 57 h PI (Figure 3. 10).

No increase in SCC was observed in previously challenged or naive quarters after secondary challenge with strain FSL Z1-124, either in cows which received the strain as homologous or heterologous challenge (data not shown). SCC was increased at time 0 in previously challenged quarters of cows which received FSL Z1-124 as heterologous strain, i.e. quarters that had received strain FSL Z1-048 at primary challenge.

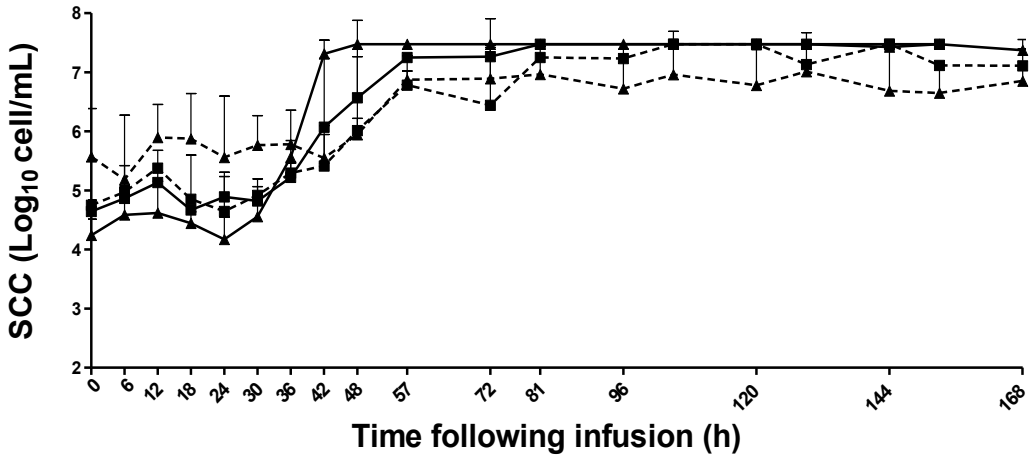

Figure 3. 10 SCC in cows after secondary challenge with FSL Z1-048 as homologous (triangles) or heterologous (squares) strain. Mean and standard deviation for naive (solid lines) and previously challenged (dashed lines) quarters are shown. 3 quarters per group are shown for cows that received the homologous strain, 3 quarters per group are shown up to 48 h, 2 from 57 to 81 h PI and 1quarter only after 81 h PI for cows that received FSL Z1-048 as heterologous strain.

3. 3. 5 Milk Concentrations of T Cell Subsets as Determined by Flow Cytometry

Concentrations of T-cell subsets were measured in milk from quarters after secondary challenge of cows with strain FSL ZI-048 or FSL Z1-124. Cells expressing CD3 (pan T cell marker), CD4, CD8 and γδ TCR were assessed.

3. 3. 5. 1 Concentration of T cell Subsets in Cows After Secondary Challenge with Strain FSL Z1-048

Concentration of $CD3^+$ cells was elevated in all the quarters after challenge with FSL Z1-048 as homologous strain, regardless of whether they were previously challenged or naive. Mean concentration in previously challenged quarters increased from 276 ± 254 × 10^3 cell/mL at 0 h (pre- infusion) to 1,243 ± 1,245 × 10^3 cell/mL 96 h PI (Figure 3. 11). Mean concentration in naive quarters increased from 1.22 ± 1.27 × 10^3 cell/mL at 0 h to 364 ± 19 × 10^3 cell/mL 96 h PI (Figure 3. 11).

All the T cell subsets (CD4, CD8 and γδ) were increased after the challenge with FSL Z1-048 as homologous strain (Figures 3. 12, 3. 14). The increase of $CD4^+$ cells preceded the increase of $CD8^+$ cells in naive quarters. In fact, CD4:CD8 ratio changed in naive quarters from 0.68 ± 0.03 to 1.71 ± 0.32 at 96 h PI. In contrast, the CD4:CD8 ratio in previously challenged quarters remained below 1 until the end of the study reaching the maximal increase at 96 h PI (0.77 ± 0.49, Figure 3. 15).

Elevation in $CD3^+$ milk cells was also observed in cows challenged with FSL Z1-048 as heterologous strain. Mean concentration in previously challenged quarters

increased from 2.6 ± 3.0 × 10^3 cell/mL at time 0 to 74 ± 78 × 10^3 cell/mL 48 h PI (Figure 3. 11). Mean concentration increased in naive quarters from 1.4 ± 1.3 × 10^3 cell/mL at time 0 to 4.5 ± 3.4 × 10^3 cell/mL 48 h PI. All the T cell subsets increased in concentration by the end of the study. Mean CD4:CD8 ratio was inverted at 144 h PI (Figure 3. 14). It increased from 0.72 ± 0.27 at time 0 to 1.73 at 144 h PI in previously challenged quarters and from 0.66 ± 0.25 at time 0 to 1.29 at 144 h PI in naive quarters.

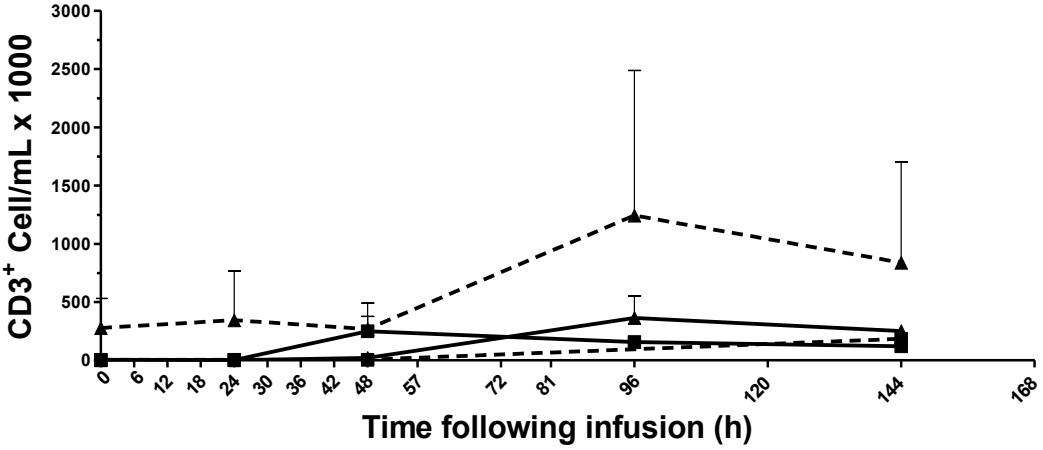

Figure 3. 11 Milk $CD3^+$ cell concentration after secondary challenge with FSL Z1-048 as homologous (triangles) or heterologous (squares) strain. Mean and, where possible, standard deviation for naive (solid lines) and previously challenged (dashed lines) quarters are shown. Three quarters per group were available from cows that received FSL Z1-048 as the homologous strain. Three quarters per group are shown up to 48 h, 1 at 96 and 144 h PI and 1quarter only after 81 h PI for cows that received FSL Z1-048 as the heterologous strain. No quarters were available at 96 h PI for previously challenged quarters that received FSL Z1-048 as heterologous strain. The same number of quarters per group was available for $CD4^+$, $CD8^+$, γδ cell concentrations and CD4:CD8 ratio (Figure 3. 12, 3. 13, 3. 14 and 3. 15 respectively).

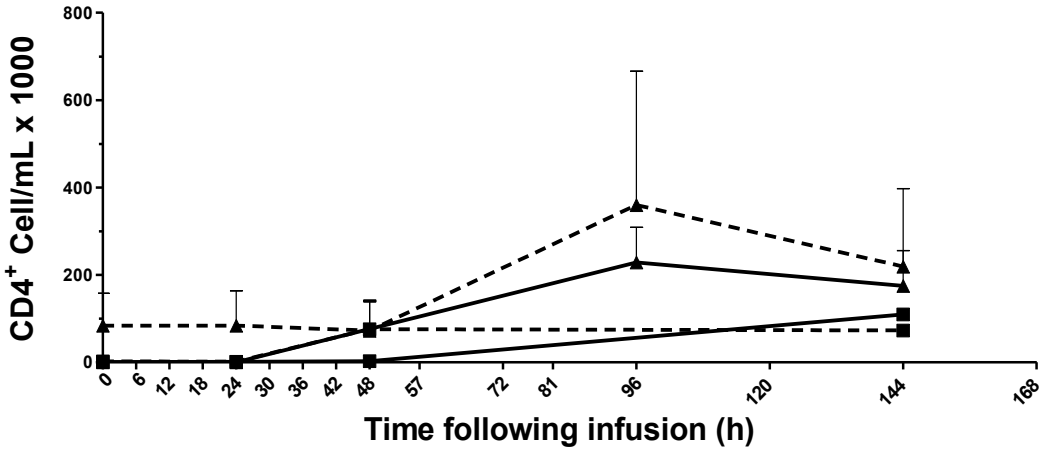

Figure 3. 12 Milk $CD4^+$ cell concentration in cows after secondary challenge with FSL Z1-048 as homologous (triangles) or heterologous (squares) strain. Mean and, where possible, standard deviation for naive (solid lines) and previously challenged (dashed lines) quarters are shown.

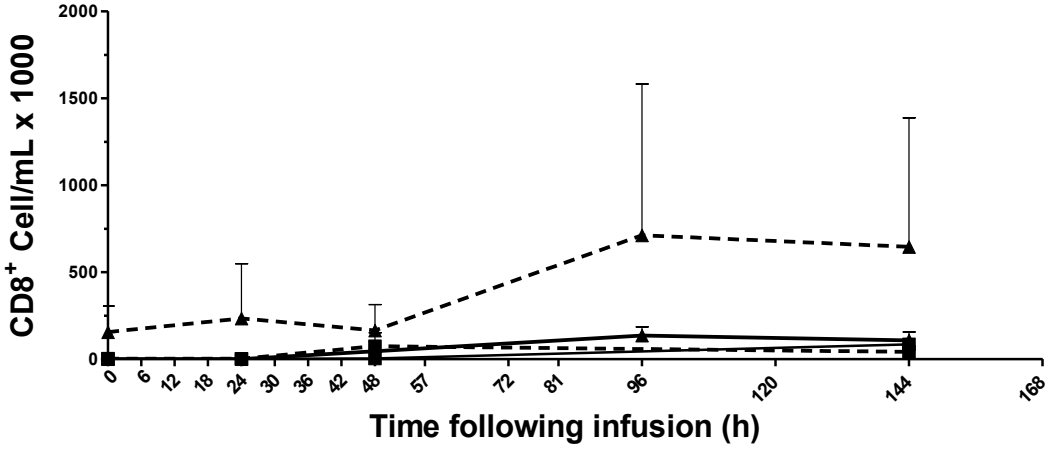

Figure 3. 13 Milk $CD8^+$ cells in cows after secondary challenge with FSL Z1-048 as homologous (triangles) or heterologous (squares) strain. Mean and, where possible, standard deviation for naive (solid lines) and previously challenged (dashed lines) quarters are shown.

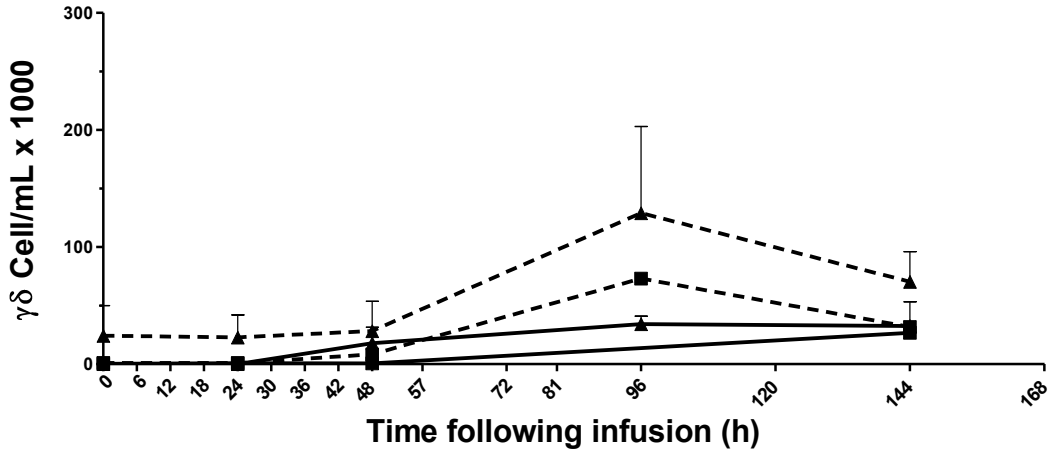

Figure 3. 14 Milk γδ cells in cows after secondary challenge with FSL Z1-048 as homologous (triangles) or heterologous (squares) strain. Mean and, where possible, standard deviation for naive (solid lines) and previously challenged (dashed lines) quarters are shown.

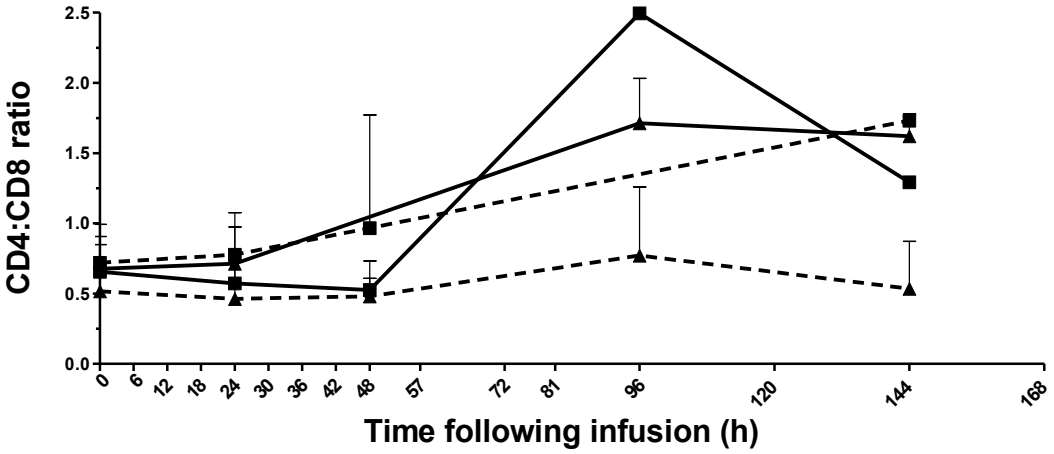

Figure 3. 15 Milk CD4:CD8 ratio in cows after secondary challenge with FSL Z1-048 as homologous (triangles) or heterologous (squares) strain. Mean and, where possible, standard deviation for naive (solid lines) and previously challenged (dashed lines) quarters are shown.

3. 3. 5. 2 Concentration of T Cell Subsets in Cows After Secondary Challenge with Strain FSL Z1-124

No elevation in milk T cell populations was observed in cows after secondary challenge with FSL Z1-124 either as homologous or heterologous strain. Mean concentrations of T cells remained at the pre-infusion levels both in previously challenged and in naive quarters. Ratio between $CD4^+$ and $CD8^+$ cells in previously challenged and naive quarters remained at pre-infusion levels throughout the study (data not shown).

3. 3. 6. 5 Milk Cytokines

3. 3. 6. 1 Concentration of Milk Cytokines in Cows after Secondary Challenge with strain FSL Z1-048

The concentrations of several cytokines (IL-1β, IL-8, TNF-α, IL-6, IL-10, IL-12p40, IL-17A, and IFN-γ) were measured in milk from cows after secondary challenge with *S. uberis* strains FSL Z1-048 or FSL Z1-124. First time of detection and maximal concentrations of cytokines in milk after secondary challenge with strain FSL Z1-048 are summarized in Table 3. 2 for cows receiving the strain as homologous secondary challenge and in Table 3. 3 for cows receiving the strain as heterologous secondary challenge.

The concentration of IL-1β increased in milk of all naive quarters of cows that received FSL Z1-048 as homologous secondary challenge. The first increase in these quarters was detected 42 h PI and mean concentration reached a peak of 144.3 ± 41.7

pg/mL (n=3) at 42 h PI. IL-1β was detected only in one previously. challenged quarter that received FSL Z1-048 as homologous secondary challenge. In this quarter first detection and peak of concentration (301.1 pg/mL) was at 57 h PI (Table 3. 2).

IL-1β increased in all naive and previously challenged quarters of cows that received FSL Z1-048 as heterologous strain. In previously naive quarters, IL-1β was first detected between 42 and 57 h PI and peak mean concentration (118.3 ± 120 pg/mL, n=2) was observed at 72 h PI (Figure 3. 16). In previously challenged quarters, IL-1β was first detected between 48 and 72 h PI. Mean maximal concentration (102.3 ± 12.9 pg/mL, n=2) was observed 81 h PI (Figure 3. 16).

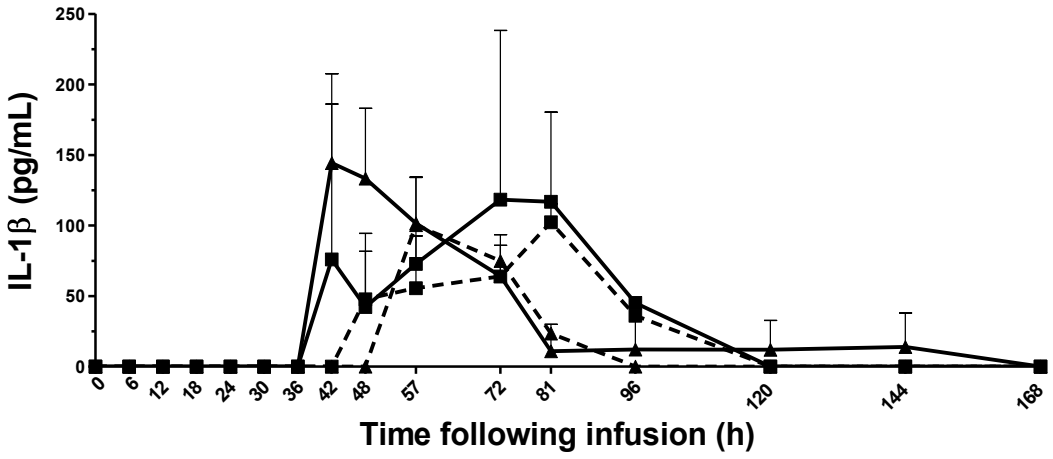

Figure 3. 16 Concentration of milk IL-1β in cows after secondary challenge with FSL Z1-048 as homologous (triangles) or heterologous (squares) strain. Mean and, where possible, standard deviation for naive (solid lines) and previously challenged (dashed lines) quarters are shown. Three quarters for each group are shown for cows after secondary challenge with FSL Z1-048 as homologous strain. Three quarters for each group are shown for cows after secondary challenge with FSL Z1-048 as heterologous strain up to and including 48 h PI, 2 quarters from 57 to 81 h PI and 1 quarter from 96 h PI to the end of the study (168 h PI). The same number of quarters was available for measurement of IL-8, IL-6, TNF-α, IL-10, IL-12p40 and IL-17A (Figure 3. 17 to 3. 22).

IL-8 was first detected at 36 h in all three naive quarters challenged with FSL Z1-048 as homologous strain, with peak mean concentration at 42 h 20.35 ± 17.12 ng/mL. Concentration of milk IL-8 also increased in all quarters after secondary challenge with FSL Z1-048 as homologous strain. In these quarters, the first elevation of IL-8 was detected 48 h PI, with peak mean concentration of IL-8 (13.4 ± 22.5 ng/mL) occurring at 72 h PI (Figure 3. 17). The maximal concentration of IL-8 was highly variable between individual animals: in quarter 2 RH, the maximum concentration was ca. 10 fold lower than the maximal concentration observed in quarter 4RH and ca. 100 fold lower than the maximal concentration observed in quarter 5RH.

Concentration of IL-8 was also increased in all cows after secondary challenge with FSL Z1-048 as heterologous strain. In previously challenged quarters first elevation was observed between 36 and 57 h PI, with maximal mean concentration (16. ± 21 ng/mL) occurring at 48 h PI (Figure 3. 17). In naive quarters, first elevation was observed between 36 and 57 h PI and mean concentration in these quarters (39.92 ± 0.11 ng/mL) peaked 72 h PI (Figure 3. 16).

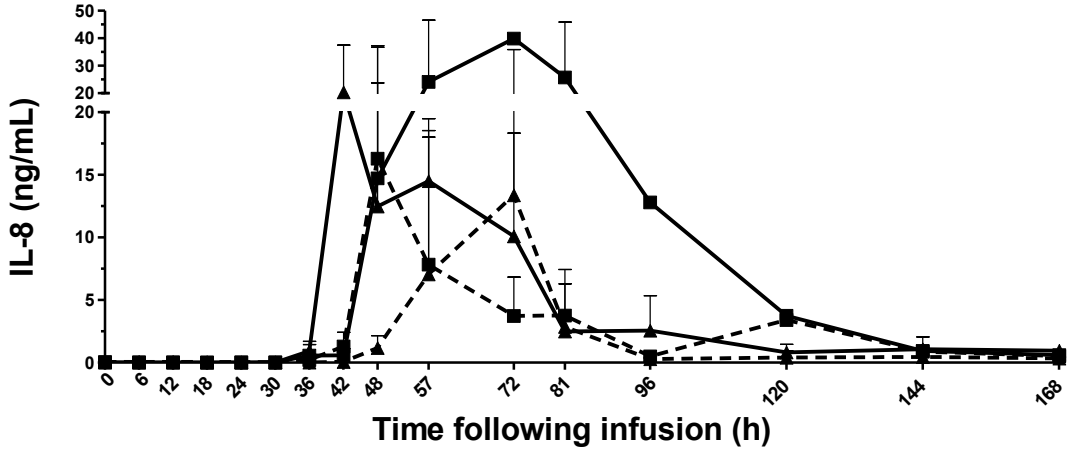

Figure 3. 17 Concentration of milk IL-8 in cows after secondary challenge with FSL Z1-048 as homologous (triangles) or heterologous (squares) strain. Mean and, where possible, standard deviation for naive (solid lines) and previously challenged (dashed lines) quarters are shown.

Concentration of TNF-α was increased in all the naive quarters after secondary challenge with FSL Z1-048 as homologous strain. In individual quarters, first elevation was detected between 42 and 72 h PI and maximal mean concentration (3,344 ± 4,035 pg/mL) was observed 81 h PI (Figure 3. 18). Concentration of TNF-α was increased in 2 of the 3 previously challenged quarters which received FSL Z1-048 as homologous strain. First elevation was detected in individual quarters at 57 h PI. Maximal mean concentration (1,954.31 ± 2,703.31 pg/mL) was observed at 81 h PI (Figure 3. 18). Maximal concentration observed in quarter 5RH was 5-fold that observed in quarter 4RH.

Concentration of TNF-α was elevated in all quarters that received FSL Z1-048 as heterologous strain, either in naive or previously challenged quarters. In individual naive quarters elevation of TNF-α was first detected between 42 and 48 h PI and maximal mean concentration (1,705.90 pg/mL) was observed 96 h PI (Figure 3. 18).

In individual previously challenged quarters first elevation was detected between 36 and 48 h PI and maximal mean concentration (1,704 ± 2,153 pg/mL) was observed at 48 h PI (Figure 3. 18)

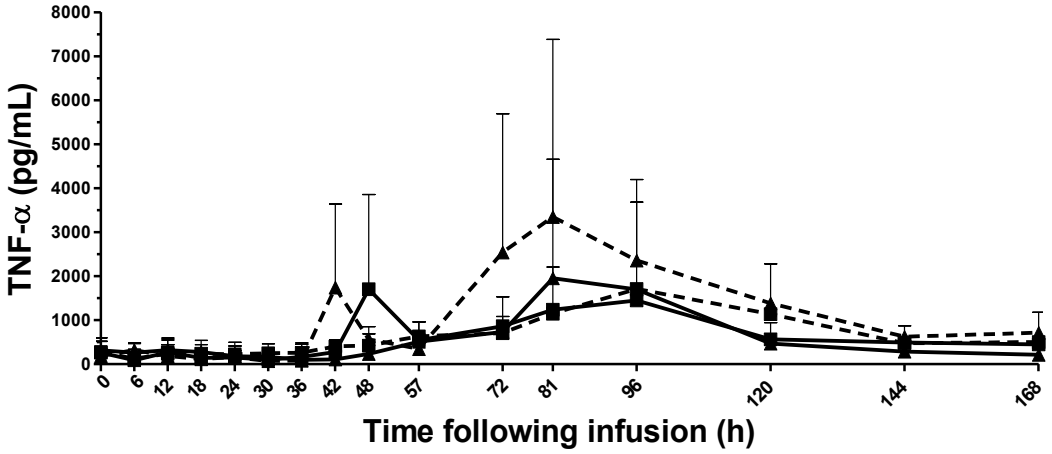

Figure 3. 18 Concentration of milk TNF-α in cows after secondary challenge with FSL Z1-048 as homologous (triangles) or heterologous (squares) strain. Mean and, where possible, standard deviation for naive (solid lines) and previously challenged (dashed lines) quarters are shown.

Concentration of IL-6 was increased in all the quarters after secondary challenge with FSL Z1-048 as homologous strain, whether naive or previously challenged. Elevation in IL-6 concentration in naive quarters was first detected at 42 h PI and maximal mean concentration (11.6 ± 8.8 ng/mL) was detected at 57 h PI (Figure 3. 19). Elevation in IL-6 concentration was first detected in previously challenged quarters between 48 and 57 h PI and maximal mean concentration (1.3 ± 2.1 ng/mL) was observed at72 h PI (Figure 3. 19). Concentration in two individual previously challenged quarters (2RH and 4RH) was elevated at only one and three time points, respectively.

Concentration of IL-6 was elevated in all the quarters after secondary challenge with FSL Z1-048 as heterologous strain. First elevation was detected in individual naive quarters between 42 and 57 h PI and maximal mean concentration (1.9 ± 2.6 ng/mL) was detected at 48 h PI. First elevation was detected in individual previously challenged quarters between 42 and 72 h PI and maximal mean concentration (2.5 ± 2.1 ng/mL) was detected 72 h PI (Figure 3. 19).

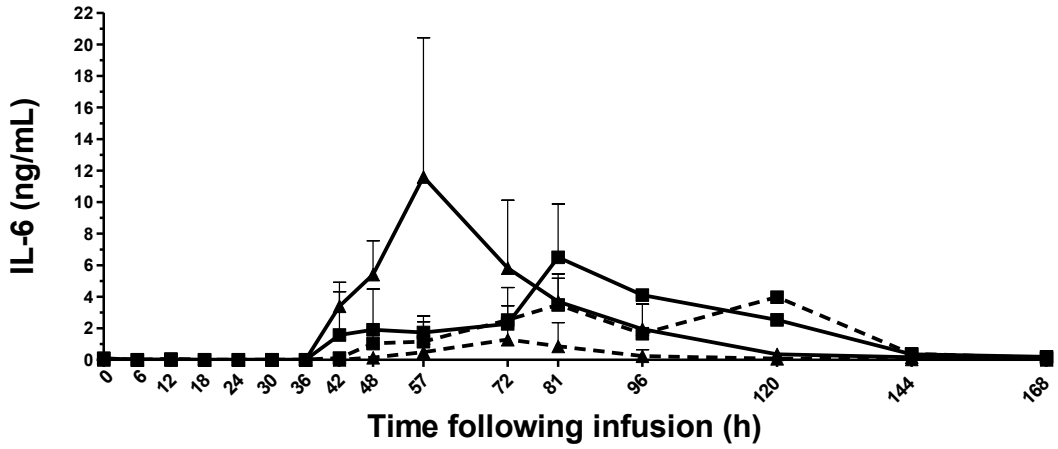

Figure 3. 19 Concentration of milk IL-6 in cows after secondary challenge with FSL Z1-048 as homologous (triangles) or heterologous (squares) strain. Mean and, where possible, standard deviation for naive (solid lines) and previously challenged (dashed lines) quarters are shown.

Concentration of IL-10 was elevated in all the quarters after secondary challenge with FSL Z1-048 as homologous strain. First elevation in individual naive quarters was detected at 42 h PI, and maximal mean concentration (17.5 ± 7.8 bU/mL) was detected at 48 h PI (Figure 3. 20). First detection of IL-10 in individual previously challenged quarters was between 48 and 57 h PI. Maximal mean concentration (6.4 ± 8.6 bU/mL) was observed at 81 h PI (Figure 3. 20).

Concentration of IL-10 was increased in all the quarters that received FSL Z1-048 as heterologous strain at secondary challenge. First elevation of IL-10 concentration was detected between 42 and 57 h PI in naive quarters and maximal mean concentration (18.1 ± 11.7 bU/mL) was observed at 81 h PI (Figure 3. 20). First elevation of IL-10 was detected in previously challenged quarters between 48 and 72 h PI and maximal mean concentration (15 bU/mL) was observed at 120 h PI (Figure 3. 20).

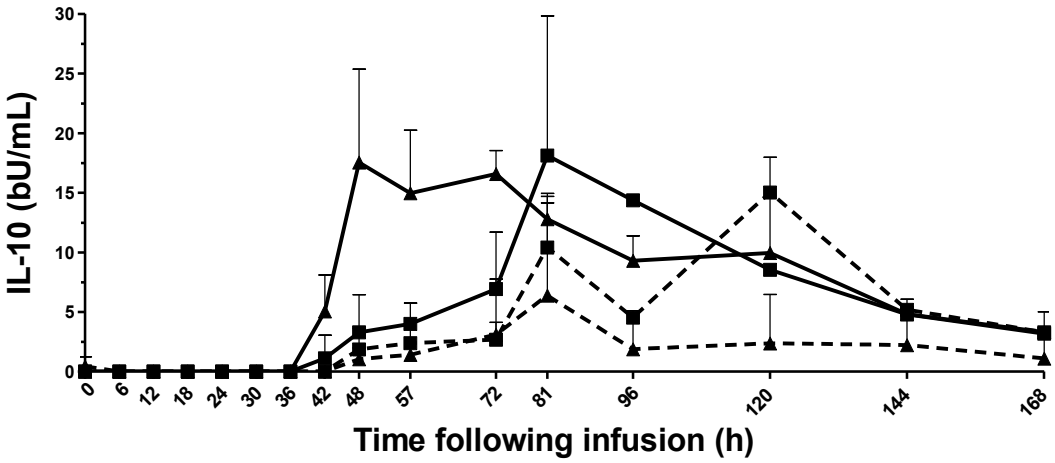

Figure 3. 20 Concentration of milk IL-10 in cows after secondary challenge with FSL Z1-048 as homologous (triangles) or heterologous (squares) strain. Mean and, where possible, standard deviation for naive (solid lines) and previously challenged (dashed lines) quarters are shown.

Concentration of IL-12p40 was increased in all the naive quarters which received FSL Z1-048 as homologous strain. First increase was detected between 42 and 48 h PI. Maximal mean concentration (62 ± 60 bU/mL) was observed 48 h PI (Figure 3. 21).

Concentration of IL-12p40 was increased in one out of three previously challenged quarters that received FSL Z1-048 as homologous strain. IL-12p40 was first detected in quarter 7RH 57 h PI, and reached the peak (114 bU/mL) at 72 h PI (Figure 3. 21).

Concentration of IL-12p40 was increased in all the quarters after secondary challenge with FSL Z1-048 as heterologous strain, whether naive or previously challenged. First increase in individual naive quarters was detected 42 to 57 h PI and maximal mean concentration (58.2 ± 50.4 bU/mL) was observed at 48 h PI (Figure 3. 21). First increase in individual previously challenged quarters was detected between 48 and 72 h PI and maximal mean concentration (64 ± 90 bU/mL) was observed 57 h PI (Figure 3. 21).

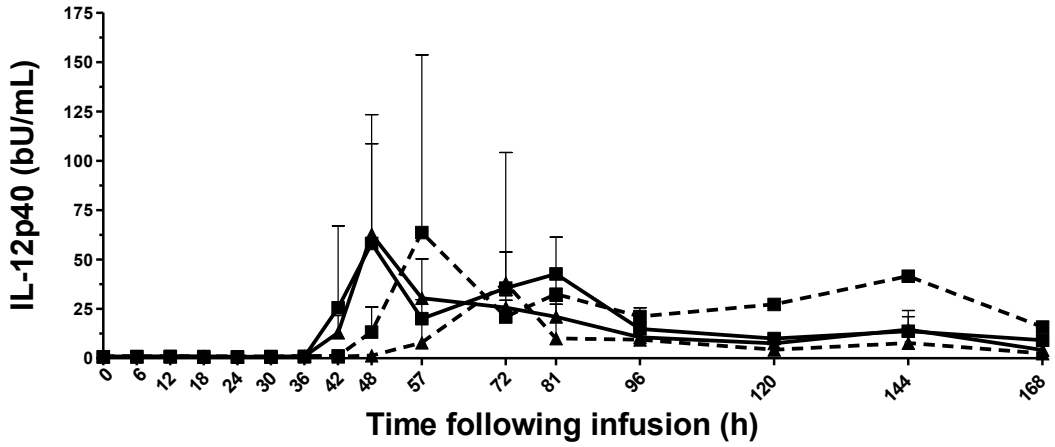

Figure 3. 21 Concentration of milk IL-12p40 in cows after secondary challenge with FSL Z1-048 as homologous (triangles) or heterologous (squares) strain. Mean and, where possible, standard deviation for naive (solid lines) and previously challenged (dashed lines) quarters are shown.

Concentration of IL-17A was increased in 2 of 3 naive quarters that received FSL Z1-048 as homologous strain. Elevation was first detected in individual naive quarters between 57 and 81 h PI and maximal mean concentration was observed at 72 and 144 h PI (211 ± 366 and 211 ± 187 pg/mL, respectively, Figure 3. 21). IL-17A was elevated only in one previously- challenged quarter that received FSL Z1-048 as homologous strain. First elevation in that quarter was observed at 57 h PI and concentration reached the peak (878 pg/mL) at 72 h PI (Figure 3. 22).

IL-17A was first detected at 48 and 81 h PI in 2 of 3 previously challenged quarters that received FSL Z1-048 as homologous strain and reached a maximal mean concentration of 131 ± 127 pg/mL . Concentration of IL-17A was elevated in 1 of 3 previously naive quarters that received FSL Z1-048 as heterologous strain. First elevation in quarter 9RH was observed at 72 h PI and maximal concentration (339 pg/mL) was observed at 81 h PI (Figure 3. 22, Table 3. 2).

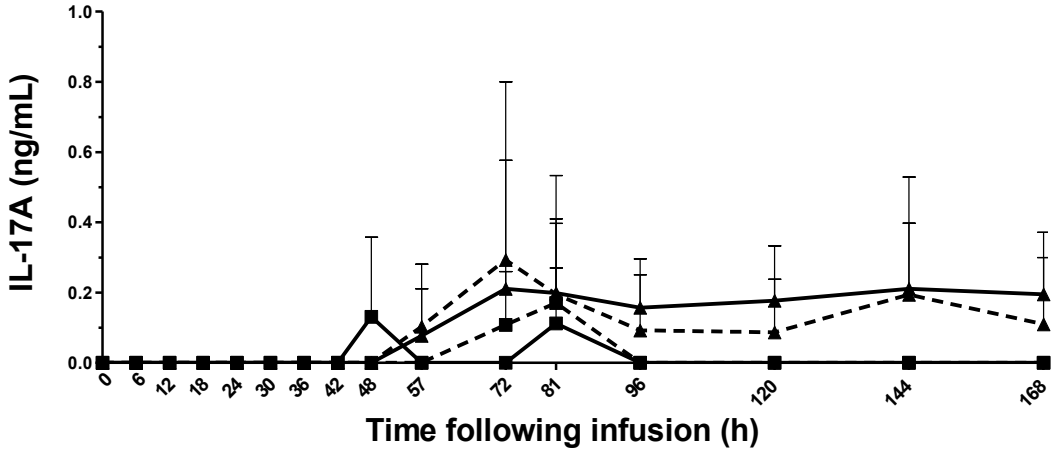

Figure 3. 22 Concentration of milk IL-17A in cows after secondary challenge with FSL Z1-048 as homologous (triangles) or heterologous (squares) strain. Mean and, where possible, standard deviation for naive (solid lines) and previously challenged (dashed lines) quarters are shown.

3. 3. 6. 2 Concentration of Milk Cytokines in Cows After Secondary Challenge with strain FSL Z1-124

No increase in concentration of any of the cytokine measured was observed in milk after secondary challenge with strain FSL Z1-124 (data not shown).

3. 3. 7 Comparison between Primary and Secondary Challenge Response

In order to allow a comparison between the host response to primary and secondary challenge with strain FSL Z1-048, relevant data from the primary (Chapter 2) and secondary challenge experiments were plotted together. The clinical, bacteriological and SCC responses to the secondary challenge with *S. uberis* were very similar to the host response following the primary challenge described in Chapter 2. The temperature profile (Figure 3. 23) was comparable between primary and secondary challenge although the peak of the average temperature was delayed following the secondary challenge by approximately 20 h compared to the response to the primary challenge. A similar delay was observed for concentration of bacteria in milk (Figure 3. 24) and SCC (Figure 3. 25). The delayed response was observed both in cows that received strain FSL Z1-048 as homologous secondary challenge and in cows that received it as a heterologous secondary challenge (Figure 3. 23, 3. 24B, 3. 25B). The previously challenged quarters of the cows that received strain FSL Z1-048 as homologous showed a peak of the bacterial concentration approximately 10^4 times lower than the peak reached following the primary challenge or the peak reached in the naive quarters in the secondary challenge (Figure 3. 24A).

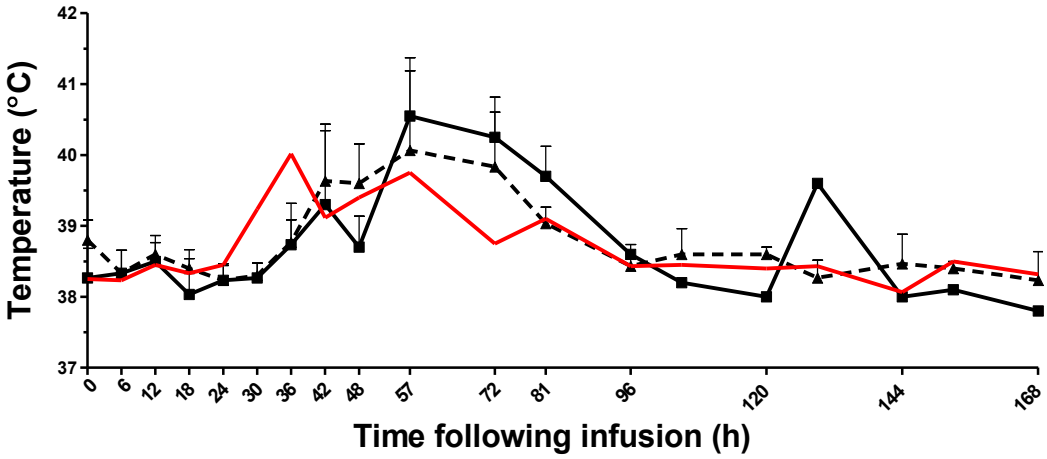

Figure 3. 23 Temperature in response to secondary challenge with *S. uberis* FSL Z1-048. Mean and, where possible, standard deviation are shown for cows that received FSL Z1-048 at secondary challenge (two quarters per cow), either as homologous strain (black dashed line, n=3) or as heterologous strain (black solid line, n=3), and in response to the primary challenge (one quarter per cow, n = 6, red solid line, see Section 2. 3. 2 for details). Two cows that received FSL Z1-048 as heterologous strain at secondary challenge, were treated with antibiotics at 48 and 81 h PI respectively and eliminated from the study at subsequent time points.

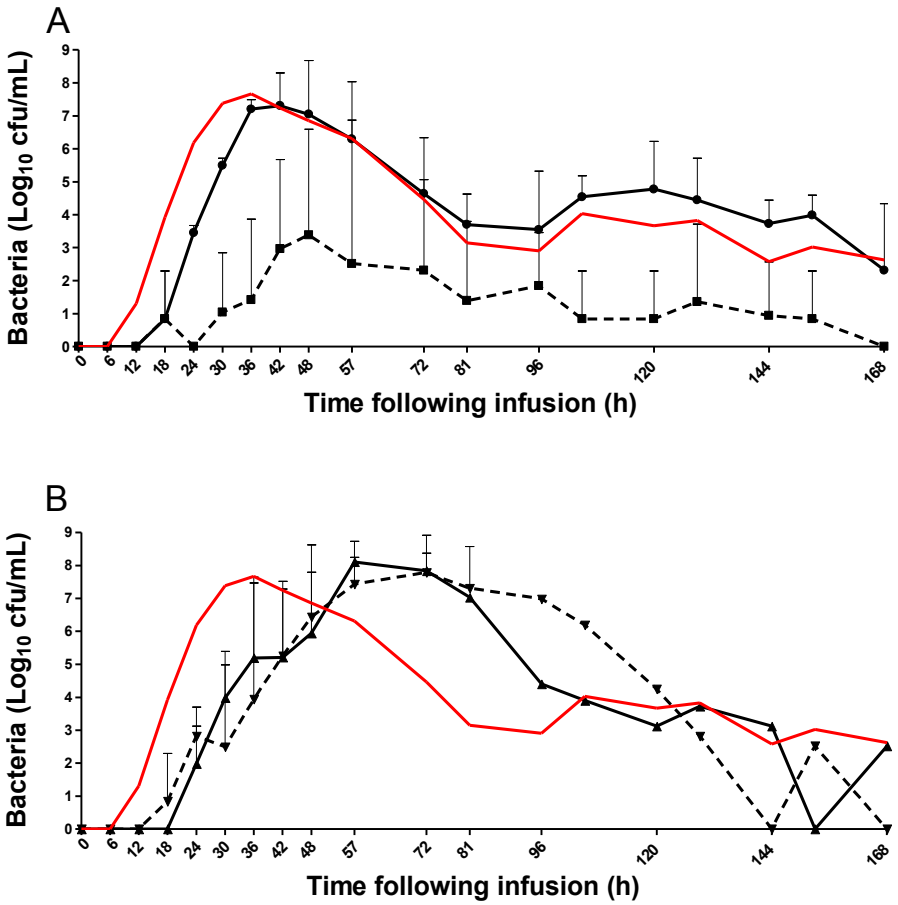

Figure 3. 24 Concentration of *S. uberis* bacteria in milk of cows after secondary challenge with strain FSL Z1-048 as homologous (A) or heterologous (B) strain. Per cow, 2 quarters were challenged, i.e. one quarter that had been challenged whit *S. uberis* before and one quarter that had been mock challenged with PBS at primary challenge of the cow. Three cows were challenged for each group. Average and, where possible, standard deviations of bacteria concentration are shown for previously challenged quarters (dashed lines) and naïve quarters (solid lines). Concentration of bacteria in quarters that received the primary challenge (n=6; one quarter per cow) is shown with the red line (see Section 2. 3. 3 for details).

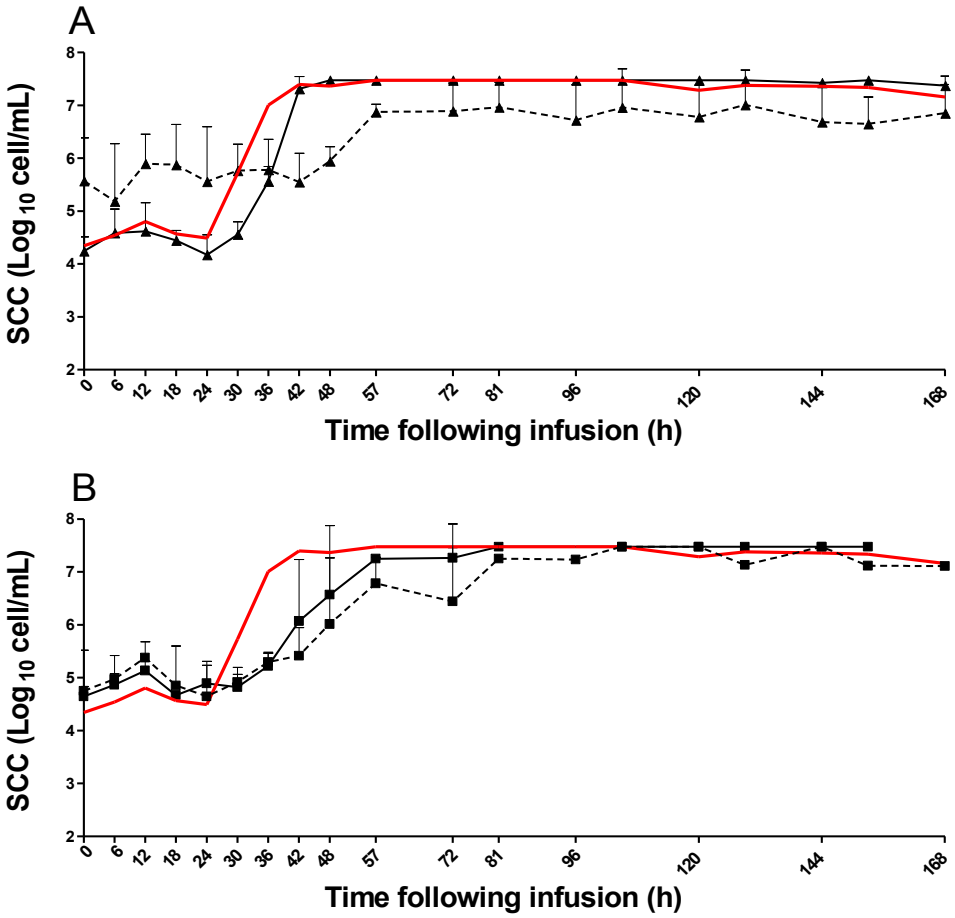

Figure 3. 25 SCC in milk of cows after secondary challenge with strain FSL Z1-048 as homologous (A) or heterologous (B). Three cows were challenged for each group. Average and standard deviations of SCC are shown for previously challenged quarters (dashed lines) and naïve quarters (solid lines). SCC in quarters that received the primary challenge (n=6, one quarter per cow) is shown with the red line (see Section 2. 3. 4 for details).

3. 4 Discussion

Putatively host adapted strain FSL Z1-048 caused mastitis in all (6/6) cows upon secondary challenge whereas none of the cows secondarily challenged with non adapted strain FSL Z1-124 developed clinical signs of mastitis. Intramammary infection in cows secondarily challenged with strain FSL Z1-048 was characterized by clinical signs, increased body temperature and reduction in milk production. *S. uberis* bacteria were isolated in 11 of the 12 quarters challenged with strain FSL Z1-048 whereas only 4 of the 11 quarters secondarily challenged with strain FSL Z1-124 became bacteriologically positive. The differences between the two strains in ability to cause intramammary infection and clinical disease are in accordance with those observed following the primary challenge (see Chapter 2). Both SCC and milk $CD3^{+}$ concentration were increased in all quarters challenged with FSL Z1-048 compared with the pre-infusion levels. As observed in the primary challenge, the host response was also characterized by production of cytokines IL-1β, TNF-α, IL-6, IL-8 IL-10 and IL-17A in the mammary gland.

Differences were observed between cows that received strain FSL Z1-048 as a homologous secondary challenge and those that received it as a heterologous secondary challenge. All the quarters that received the strain as a heterologous challenge (6/6) developed clinical mastitis, whereas only 4 out of 6 quarters that received FSL Z1-048 as homologous challenge developed clinical mastitis (e.g. no signs of inflammation and no alterations of milk were visible. Furthermore, 2 out of 3 cows challenged with FSL Z1-048 as heterologous strain had to be treated with

antibiotics before the end of the study due to severe clinical signs, whereas none of the cows challenged with FSL Z1-048 as homologous strain required antibiotic intervention.

In the present study the two quarters that did not develop clinical mastitis following challenge with FSL Z1-048 were quarters challenged with this same strain in the primary challenge. These previously challenged quarters showed an increased SCC at the time of challenge (day 0). This may explain the protective effect that was observed because high pre-challenge SCC has been described as protective factor for IMI during challenge with *S. aureus* (Schukken *et al*., 1999). Protective effect against *S. uberis* IMI mediated by high SCC was observed in the past following, intramammary infusion of IL-1β (Wedlock *et al*., 2004, Wedlock *et al*., 2008), or following implantation of an intramammary device which stimulated the influx of phagocyte cells (Nickerson *et al*., 1990) indicating that pre-existing high milk SCC can protect against IMI with *S. uberis*. However, high SCC would be unacceptable as mean to control mastitis in dairy farming because the increase in SCC would result in a deterioration of the milk quality with economical losses (Jayarao and Wolfgang, 2003).

The two previously challenged quarters that did not develop clinical mastitis also showed a lower or undetectable concentration of milk cytokines compared to that observed in naive quarters, suggesting that the inflammatory response in these quarters was lower than that observed in naive quarters and that prior infection of the quarter had attenuated the inflammatory response. The protective effect of prior

infection was limited to the affected quarter (local effect) with no evidence of protection in the adjacent quarter (no systemic effect).

Flow cytometry data suggest that the increase in SCC of previously challenged quarters secondarily challenged with the homologous strain FSL Z1-048 observed after the challenge was due to increase of PMN (Figure 3. 15) as well as $CD3^+$ population (Figure 3. 26). The increase of $CD3^+$ cells at 96 h PI was due to the coincident influx in the mammary gland of both $CD4^+$ and $CD8^+$ subsets.

In contrast, naive quarters challenged with FSL Z1-048 as homologous strain and all the quarters challenged with FSL Z1-048 as heterologous strain showed an increase of $CD4^+$ cells at 96 h PI which caused an inversion of CD4:CD8 ratio. A similar inversion of the CD4:CD8 ratio was observed in response to the primary challenge with strain FSL Z1-048 (Chapter 2). Previous studies suggest a possible role of lymphocytes in the protection given by a *S. uberis* vaccine. Vaccine studies performed in the past (Hill *et al*., 1993, Finch *et al*., 1994) showed some degree of protection against *S. uberis* IMI in animals immunized with killed *S. uberis* and that protection, when achieved, was related with a strong proliferative response of lymphocytes, either from peripheral blood or from mammary gland secretions. A recent study showed that *S. uberis* specific $CD8^+$ cells are present in mammary gland secretions (Denis *et al*., 2011) of cows previously infected with *S. uberis* as well as cows with no history of *S. uberis* infection. These memory cells were able to expand when cultured with *S. uberis* and they were able to kill this bacteria *in vitro,* suggesting a possible role in acquired immunity to *S. uberis* IMI. In our study the

increase in concentration in previously challenged quarters of $CD4^+$, $CD8^+$ and γδ subsets was observed after the secondary challenge with a homologous strain of *S. uberis* indicating an expansion of T-cell population at quarter level, suggesting a potential reactivation of a T-cell response although it has not been tested if the response is specifically raised against *S. uberis* antigens.

Host response to the secondary challenge in cows that received FSL Z1-048 as heterologous strain was largely similar to that observed in the primary challenge regardless of it was the homologous or the heterologous strain. A delayed increase of bacterial numbers in milk was observed which was reflected in a delayed host response as observed for the temperature and for the SCC. Cows used in the experiment were in mid to late lactation at primary and secondary challenge, therefore changes in immune competency due to changes in energy balance as observed in early lactating cows are unlikely to explain the delayed onset of symptoms, as most changes in immune competence occur during early lactation (Suriyasathaporn *et al*., 2000). It could be speculated that delay is due to the immune memory response to antigens shared by the two strains and the generation of cross-reactive antigen-specific immune responses. Several factors limit our ability to draw conclusions about cross-protection between strains. First, strain FSL Z1-124 largely failed to evoke a response, both at primary and at secondary challenge. If the two strains that were used had both evoked a measurable response, comparison of homologous and heterologous secondary challenge might have been more meaningful. Secondly, the number of cows and quarters per challenge group was limited to begin with and in one group (secondary challenge with FSL Z1-048 as

heterologous strain), 2 of 3 cows were lost to follow up due to severity of disease whereas in another group (secondary challenge with FSL Z1-048 as homologous strain), 2 of 3 cows failed to develop infection after challenge. This reduced the number of animals or quarters per challenge group to a single individual.

In summary, we described the host response to a secondary intramammary challenge with *S. uberis*. The study provides some indications on the immune memory that can be developed locally by the mammary gland following the infection with *S. uberis*, however the small number of the observed individual due to the complex design study and the clinical response does not allow a comparison between the groups due to the low statistical power. The observations made need to be further studied using a larger number of animals and different strains of *S. uberis*.

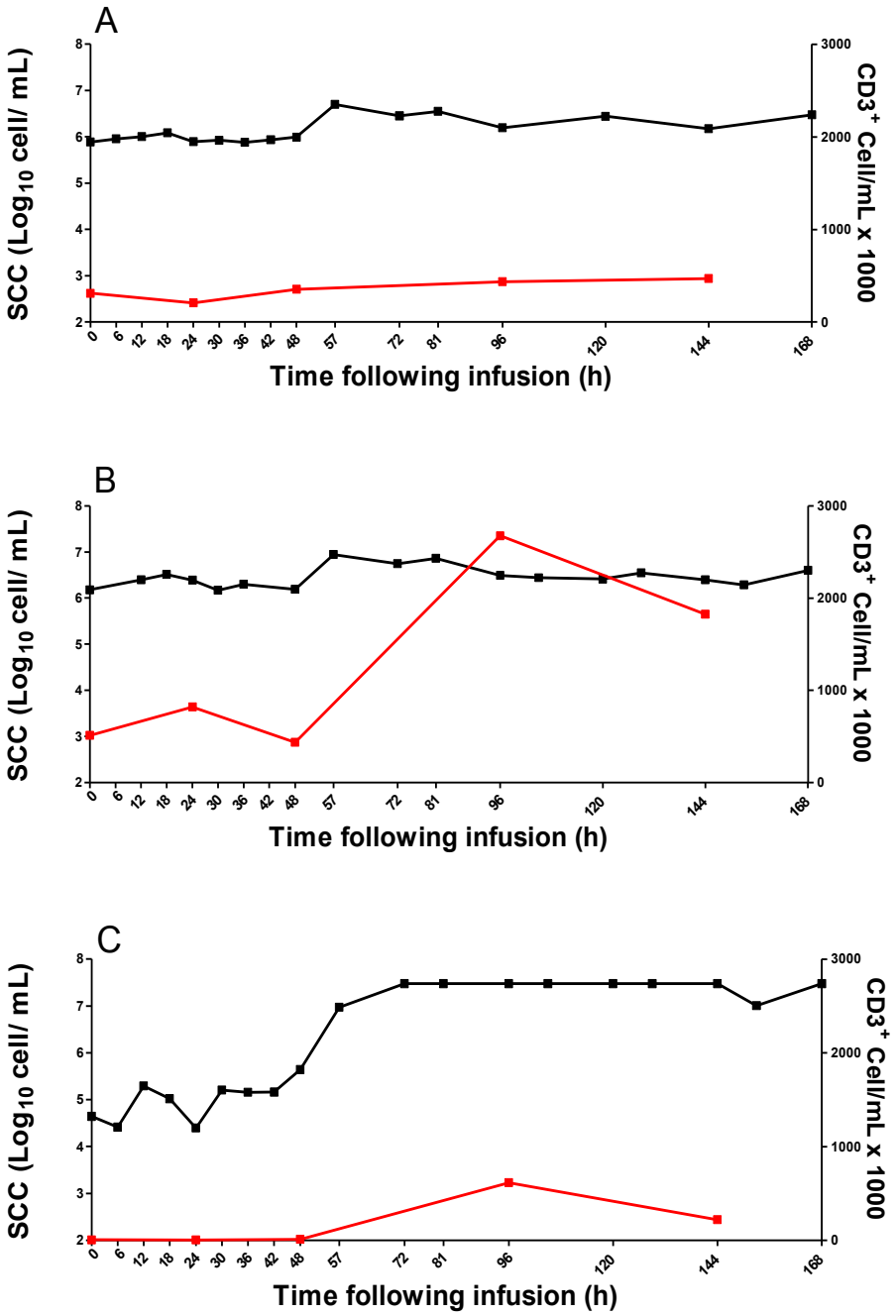

Figure 3. 26 Concentration of SCC (black lines, left Y axes) and $CD3^+$ cells (red lines, right Y axes) in milk from individuals quarters. Mammary quarters were previously challenged with strain FSL Z1-048 and received the same strain as secondary challenge.

CHAPTER 4

Adhesion and Invasion of Mammary Epithelial Cells by *Streptococcus uberis*

4. 1 Introduction

In our *in vivo* challenge experiment we observed clear differences in the pathogenicity between the putatively adapted strain FSL Z1-048 and the non adapted strain FSL Z1-124 (Chapter 2 and 3). Although able to grow in milk as demonstrated *in vitro* (Chapter 2), strain FSL Z1-124 was not able to cause mastitis in any of the cows challenged. Several mechanisms may contribute to the observed difference in the strains' ability to cause intramammary infection (IMI). Mechanisms that have been postulated to play a role in the pathogenesis of IMI include biofilm formation and bacterial adhesion and invasion. Adhesion to and internalization into epithelial cells is considered a crucial stage for the infection process in bacterial diseases because this allows the pathogen to target the appropriate tissue (Finlay and Cossart, 1997). In the context of mastitis the adherence to the mammary gland epithelia would allow the bacteria to colonize the lactating mammary gland despite the flow of milk (Leigh, 1999), as during milking non-adherent bacteria are excreted with milk. The importance of adhesion and invasion mechanisms has been shown for major mastitis pathogens such as *Staphylococcus aureus* (Lammers *et al*., 1999, Hebert *et al*., 2000) and *Escherichia coli* (Dopfer *et al*., 2000; Dogan *et al*., 2007), although all evidence is based on *in vitro* studies rather than *in viv*o studies. Invasion of the cells would also prevent the phagocytes eliminating the bacteria and protect it from antimicrobial therapy with those compounds which do not penetrate intracellularly (Zadoks, 2007).

Pathogenesis of intramammary infection due to *Streptococcus uberis* is poorly understood (Leigh, 1999; Zadoks, 2007). It has been hypothesized that adhesion to and invasion into mammary epithelial cells play a major role during the invasion of the mammary gland by *S. uberis* but very little evidence of adhesion and invasion of the mammary alveolar epithelium were found *in vivo* . Studies conducted on explant tissue obtained from bovine mammary glands showed that S. *uberis* failed to adhere to the live epithelium but showed a limited tropism for epithelial dead cells and a marked tropism for exposed connective tissue, as demonstrated by light and electronic microscopy (Thomas *et al*., 1992). However the EF20 strain used in the study was able to cause mastitis in a limited number of cows when infused intramammarily (Hill, 1988; Leigh *et al*., 1990).

Despite the fact that invasion of epithelial cells by *S. uberis* has never been observed *in vivo* or in primary cell culture, extensive work performed *in vitro* using the mammary epithelial cell line MAC-T suggests that invasion is an important mechanism in *S. uberis* mastitis. Several strains of *S. uberis* are able to adhere and invade MAC-T cells *in vitro* (Matthews *et al*., 1994). In these studies, bacteria were observed in intracellular vacuoles, and in a separate study were found to survive and possibly replicate in the epithelial cells up to 120 h after the challenge (Tamilselvam *et al*., 2006). In this second study, differences were found between the strains, with strain UT888, which was more able to cause chronic mastitis, surviving intracellularly for longer than strain UT366, which caused a more transient mastitis. This study suggests that invasion of mammary epithelial cells may be important in

the pathogenesis of IMI, by providing an intracellular reservoir in the mammary gland tissue which protect the bacteria from the host immune response.

A protein involved in the adhesion process of *S. uberis* to the epithelial cells has been identified (Fang *et al*., 1999). The protein SUAM (*Streptoccus uberis* adhesion molecule), identified initially as lactoferrin binding protein for its ability to bind lactoferrin, was found to mediate the internalization process in the mammary epithelial cells *in vitro* (Almeida *et al*., 2006). Antibodies against SUAM reduce the ability of *S. uberis* strains to adhere to and invade MAC-T cells *in vitro*, suggesting a crucial role of this molecule in the adhesion and invasion process (Almeida *et al*., 2006; Prado *et al*., 2011). The gene encoding SUAM has been identified by PCR in numerous strains of *S. uberis* suggesting that it is highly conserved (Luther *et al*., 2008). The role of the SUAM as an adhesin and the fact that it is highly conserved in *S. uberis* strains raised the interest in its potential use as target for a vaccine (Prado *et al*., 2011). After the experimental immunization with SUAM antigen cows produce specific antibodies against the molecule in serum and in colostrum, although the ability of the antibodies to reduce the adhesion and the invasion was tested and proven only *in vitro* (Prado *et al*., 2011).

Like the role of invasion and SUAM, the role of biofilm in intramammary infection has been investigated *in vitro*. Biofilm is a sessile form of bacterial growth. Bacteria growing as biofilm are described as "*a structured community formed by bacteria themselves enclosed in a self produced polymeric matrix and attached to an inert or living surface"* (Consterton *et al*., 1999). This would allow bacteria to colonize

hostile environments. In the context of bovine mastitis this would allow the bacteria to resist the host defences within the mammary gland and to resist antibiotics (Melchior *et al*., 2006). Differences in the ability to form biofilm have been found between different strains of *S. aureus* with strains isolated from the mammary gland more capable of forming biofilm *in vitro* than strains isolated from extra-mammary sources such as teat skin and milk liners (Fox *et al*., 2005). This suggests a possible role for biofilm formation in bacterial colonisation of the mammary gland. Ability to grow as biofilm has also been shown for *S. uberis* (Varihmo *et al*., 2010; Crowley *et al*., 2011; Gilchrist, 2011), where biofilm growth has been associated with the up-regulation of genes involved in adhesion and internalization of *S. uberis* to the mammary epithelial cells and sugar metabolism (Crowley *et al*., 2011). This suggests that biofilm formation may important for colonization of the mammary gland by *S. uberis*, although for several species of coagulase-negative staphylococci (CNS) no correlation has been found between the ability to form biofilm and persistence of IMI or severity of mastitis (Simojoki *et al*., 2012).

The aim of this study was to test the ability of the host-adapted and non-host adapted *S. uberis* strains from the challenge study, FSL Z1-048 and FSL Z1-124 respectively, to adhere and to invade mammary epithelial cells *in vitro*. In addition, both strains and additional representatives of putatively host-adapted and non-adapted *S. uberis* ecotypes were tested for their ability to form biofilm *in vitro*. The hypothesis to be tested was that host-adapted strains are better able to colonize the mammary gland compared to non-host adapted strains as a result of increased epithelial adherence, invasion and/or biofilm formation.

4. 2 Materials and Methods

4. 2. 1 Epithelial Cell Adhesion and Invasion Assay

4. 2. 1. 1 Tissue Culture Conditions

Immortalized bovine mammary epithelial cell line BME-UV1 cells (Zavizion *et al*., 1996) were cultured at 37 °C 5% CO_2 in 75 cm^2 tissue culture flask (Corning). BME-UV1 complete medium consisted of a mix containing 40% Ham`s F-12 nutrient mixture, 30% RPMI-1640 medium and 20% NCTC 135 medium. All the media were supplied by Gibco (Paisley ,UK). The medium was supplemented with 10% (wt/vol) heat inactivated fetal calf serum, 0.1% (wt/vol) lactose, 0.1% (wt/vol) lactalbumin hydrolysate, 1.2 mM glutathione, 10 µg/mL L-ascorbic acid, 1 µg/mL hydrocortisone, 1 µg/mL insulin, 200 U/mL penicillin and 200 U/mL streptomycin. All reagents were supplied by Sigma-Aldrich. Cells were cultured until 70 to- 80 % confluency then washed with warm PBS and treated with 1 mL of TrypLE™ Express dissociation media (Gibco) supplemented with 4 U/mL of porcine elastase (Sigma-Aldrich), incubated at 37°C until 70 to 80% of the cells were showing a shrunken morphology, medium was discarded and 1 mL of TrypLE™ Express dissociation medium per flask was added. Flasks were incubated until the cells were completely detached from the plastic surface. Complete medium was added and cells transferred to a new flask. Cells at passage 5 to 10 were transferred to 24 well tissue culture plates (Corning) at 1×10^5 cells /well and cultured 1 day in complete medium.

Eighteen hours before the assay, medium was discarded and cells were rinsed 3 times with warm PBS and antibiotic free medium was added.

4. 2. 1. 2 Bacteria Preparation

S. uberis strains FSL Z1-048 and FSL Z1-124 were grown in BHI for 18 h at 37°C under shaking (200 rpm). *Salmonella enterica* serovar Typhimurium strain 12023 and *Escherichia coli* laboratory strain TOP10 were grown in LH broth for 18 h at 37°C under shaking (200 rpm). Bacterial suspensions were washed three times in PBS (e.g. pellets resuspended in PBS after centrifugation at 4000×g for 20 min at 4°C) and resuspended in PBS. Concentrations of live bacteria were determined by viable count (See section 2. 2. 2 for details). Suspensions were diluted at target concentrations and stored at 4° C prior to use.

4. 2. 1. 3 Adhesion and Invasion Assay

The bacteria challenge preparation was added to the supernatant of the completely confluent monolayer of BME-UV1 cells. *S. uberis* strains FSL Z1-048 and FSL Z1-124 were added at multiplicity of infection (MOI) of 10 and 100 (e. i. bacteria: epithelial cells ratio). Positive and negative controls for the invasion assay were included. Invasive *Salmonella enterica* serovar Typhimurium strain 12023 (Sherry *et al*., 2011) kindly provided by Professor David GE Smith (Moredun Research Institute) was included as positive control for the invasion assay. *Escherichia coli* laboratory strain TOP10 (Invitrogen, Paisley, UK) was included as negative control for the invasion assay. Epithelial cells were challenged with approximately 1×10^6 cfu of positive or negative control per well. Cells and bacteria were co-incubated for 3 h

at 37° C and 5% CO_2. Cell culture supernatants were then discarded and wells were vigorously washed 3 times with warm PBS in order to eliminate non-adherent bacteria. Cells were washed 3 times with warm PBS and lysed by adding 500 µL per well of 0.1% vol/vol Triton X-100 (Sigma-Aldrich) diluted in PBS. Lysates were serially diluted in cold PBS and 20 µL spots of the serial dilutions were plated in triplicates on blood agar plates (E&O Laboratories). Plates were incubated overnight at 37° C, colonies were counted and the concentration of bacteria (cfu/mL) associated with the cells (adhered to the surface and internalized) was calculated for the invasion assay. Washed cells were incubated 1 h at 37° C 5% CO_2 with 1 mL per well of growth medium supplemented with 150 µg/mL gentamicin to kill the extracellular bacteria. Cells were washed 3 times with warm PBS and lysed by adding 500 µL per well of 0.1% vol/vol Triton X-100 (Sigma-Aldrich) diluted in PBS. Lysates were serially diluted in cold PBS and 20 µL spots of the serial dilutions were plated in triplicates on blood agar plates (E&O Laboratories). Plates were incubated overnight at 37° C, colonies were counted and the concentration of bacteria (cfu/mL) internalized in the cells was calculated. The number of bacteria that adhered on the surface was obtained by subtracting the bacteria internalized from the total bacteria associated with the cells.

4. 2. 2 Biofilm Assay

4. 2. 2. 1 *S. uberis* Strains

Putatively host adapted and putatively non adapted strains of *S. uberis* were selected based on epidemiological data (Lang *et al*., 2009). Moreover two of the strains: putatively adapted FSL Z1-048 and putatively non adapted FSL Z1-124 were characterized by a very different outcome when experimentally infused in the lactating mammary gland (See Chapters 2 and 3), with strain FSL Z1-048 able to cause clinical mastitis in all the cows challenged and strain FSL Z1-124 not able to cause mastitis. Strains FSL Z1-048 and FSL Z1-124 were used for the challenge study as representatives of putatively host-adapted and non-adapted strains of *S. uberis* (Lang *et al*., 2009). Both strains, as well as 4 additional strains representative of each of the two categories were used to test their ability to form biofilm. Adapted strains were isolated from multiple cows in multiple herds whereas non adapted strains were unique isolates which caused mastitis only in individual animals (Table 4. 1).

	Isolate	Country	Epidemiological context
1	FSL Z1-048	The Netherlands	Three countries, outbreak
2	QMP B5-005	USA (NY)	Three herds, multiple cows per herd
3	FSL Z3-369	France	Three countries, outbreak
4	QMP B5-143	Italy	Multiple cows in herd
5	QMP B5-157	Italy	Two herds, multiple cows in one herd
6	FSL Z1-124	The Netherlands	Unique ST
7	FSL Z3-396	France	Unique ST
8	FSL Z1-266	New Zealand	Unique ST
9	QMP B5-001	USA (NY)	Unique ST
10	QMP B5-153	Italy	Unique ST
11	20539	N/A	N/A

(Adapted from Lang *et al*., 2009)

Table 4. 1 *S. uberis* strains used for the biofilm formation assay. Isolates 1 to 5 were chosen as representative of putatively host adapted strains, isolates 6 to 10 as representative of putatively non adapted strains. Strain 20539 was included as positive control as known biofilm producer (Gilchrist, 2011).

4. 2 .2. 2 Biofilm Formation Assay

The ability to form biofilm was assessed by using the protocol described by Gilchrist (2011) with some modifications. *S. uberis* strains were cultured in brain heart infusion broth (BHI; Oxoid, Basingstoke, UK) for 16 h at 37 °C under shaking (150 rpm). BHI culture was diluted 100 fold in one of three different media preparations which have been tested: (1) BME-UV1 complete medium, (2) BME-UV1 complete medium supplemented with 0.5% wt/vol casein, and (3) RPMI-1640 chemically defined medium (CDM) supplemented with 0.5% casein) RPMI-1640 CDM was composed of RPMI-1640 (Sigma-Aldrich) supplemented with 0.8% wt/vol Lactose, 260 mg/L L-Glutamic acid, 150 mg/L Magnesium sulphate heptahydrate, 10 mg/L

Ferrous sulphate heptahydrate, 10 mg/L Manganese sulphate tetrahydrate 10 mg/L. All the reagents were supplied by Sigma-Aldrich. Two hundred µL per well of inoculated media were aliquoted into 96 well flat bottom polystyrene tissue culture plates (Corning). Plates were incubated statically at 37 °C 5% CO_2 for 24 h. Medium was discarded and wells were washed 3 times with distilled water to remove cells not adhered to the plate. Plates were air dried for 45 min then biofilm (associated with the adherent cells) were stained by adding 100 µL/well of 1% crystal violet solution (Fisher scientific, Loughborough, UK). Stain was discarded and wells washed with distilled water until excess stain was completely removed. Plates were air dried for 45 min then 200 µL/well of ethanol were added and incubated for 15 min. Optical density at 562 nm was read with a ELx808 absorbance micro plate reader (Biotek instruments, Potton, UK) to quantify the biofilm formation. For each strain 8 technical replicates (wells) were performed. For RPMI-1640 CDM supplemented with 0.5% casein, 3 biological replicates were performed whereas the biofilm formation in BME-UV1 cells growth medium and BME-UV1 cells growth medium supplemented with 0.5% casein was tested once based on results from the first round of assays (see Results).

4. 2. 3 Statistical analysis

Adhesion and invasion data expressed as concentration of bacteria that invaded cell and concentration of bacteria that adhered to the cells respectively were analysed by analysis of variance (ANOVA) using Genstat software (VSN International, Hemel Hempstead, UK). Adhesion and invasion concentrations were base-10 logarithmic transformed to ensure the data from each treatment group had an approximately

normal distribution and used as outcome variable in a two way ANOVA. Strain and MOI were used as treatment factor, interaction between strain and MOI was also evaluated. Normal distribution of the residuals was visually assessed by plotting them on charts. Post-hoc comparisons with Tukey HSD test were performed where appropriate with statistical significance declared when $P < 0.05$.

Ability to produce biofilm expressed as absorbance was analysed by one-way analysis of variance (ANOVA) using Genstat software (VSN International, Hemel Hempstead, UK). Normal distribution of the residuals was visually assessed by plotting them on charts. Least square difference (LSD) post-hoc comparison were performed with statistical significance declared when $P < 0.05$.

4. 2. 4 Genome Analysis for Sua Gene

4. 2. 4. 1 Growth Conditions and DNA Extraction

The bacterial isolates FSL Z1-048 and FSL Z1-124 were streaked onto 5% (v/v) sheep blood agar plates (E&O Laboratories) and grown aerobically at 37°C for 24 h in order to assess purity. A single colony was then selected and used to inoculate 5 mL of BHI broth (Oxoid). After overnight incubation at 37˚C in an aerobic environment, genomic DNA was extracted from cells harvested from 1 mL of BHI broth culture using an Epicentre MasterPure Gram-positive DNA purification kit according to manufacturer`s instruction (Epicentre, Madison, WI). DNA concentration was quantified using a NanoDrop 1000 (Thermo Scientific) and the product (0.5µg DNA) visually examined on a Gel Red (Cambridge Bioscience,

Cambridge, UK) stained 1% agarose gel (running conditions: 100V/cm for 1 h) in order to assess the absence of DNA shearing.

4. 2. 4 .2 Genome Sequencing and Assembly

Whole genome sequencing of strains FSL Z1-048 and FSL Z1-124 was performed using an Illumina Solexa Genome Analyzer by the Genepool sequencing core facility (University of Edinburgh, UK). *De novo* assembly of Solexa reads was performed by Genepool using VELVET 0.6.

4. 2. 4. 3 Genomic Analysis for Strain Identity Confirmation

The presence of the encoding gene for *Streptococcus uberis* adhesion molecule (*sua*) within the whole genome sequences of *S. uberis* strains FSL-Z1 O48 and FSL-Z1 124 was evaluated using BioEdit software (Hall, 1999; http://www.mb-io.ncsu.edu/bioedit /bioedit.html). A local nucleotide database file for strain FSL Z1-048 and FSL Z1-124 was created within BioEdit, thereby enabling to assess the presence of known nucleotide sequences within the strains through the Basic Local Alignment Search Tool (BLASTN). The reference nucleotide sequence of *sua* was extracted from NCBI (NCBI Accession no. DQ232760) and BLAST searched against FSL Z1-048 and FSL Z1-124 genomes using BioEdit software. The 2970 bp nucleotide sequence of *sua* was further analysed using sequence manipulation suit ORF finder (http://www.bioinformatics.org/sms2/orf_find.html Stothard, 2000) to assess the presence of stop codons.

4. 2. 4. 4 *Sua* Sequencing

In order to further confirm the nucleotide sequence of the *Sua* gene of strains FSL Z1-048 and FSL Z1-124 it was amplified by a PCR and amplicons were sequenced. The 2970 bp nucleotide sequence was amplified using the forward primer LfbpDL5 5`-GTCATTTGGTAGGAGTGGCTG-3`and the reverse primer LfbpDL6 5`-TGGTTGATATAGCACTTGGTGAC-3 (Luther *et al*., 2008) which provide the full length amplification of the 2970 bp gene. PCR was conducted in a final volume of 100 µL containing 50 µL of GoTaq green master mix (Promega, Madison, WI, USA), 50 µL of water, 300 nM of primers LfbpDL5 and LfbpDL6 and 100 ng of genomic DNA as template. The cycling profile consisted of 94°C for 2 min followed by 30 cycles of 30 s at 94°C, 30 s at 54°C, and 2 min at 68°C followed by 7 min at 68°C. Fifteen µL of the PCR product was visualized on a 1% agarose gel. The PCR products were purified using Genclean turbo kit (MP Biomedicals, Solon, OH, USA) following manufacturer`s instructions. Two 15 µL aliquots of the purified PCR products were forwarded to Eurofins Genetic Services (London, UK) for bidirectional sequencing using primers LfbpDL5 and LfbpDL6. Sequencing reads were aliged and analysed using the SeqMan program within the Lasergene 11 package (DNASTAR, WI, USA).

4. 3 Results

4. 3. 1 Adhesion and Invasion Assay

Both *S. uberis* strain FSL Z1-048 and FSL Z1-124 were able to adhere to the bovine mammary epithelial cell line BME-UV1 after 3 h of co-incubation. However strain FSL Z1-048 was approximately 1000 fold more able to adhere to the cells than strain FSL Z1-124 at MOI 10 and 100 ($P<0.01$) (Figure 4. 2). Adherence was increased at MOI 100 compared to MOI 10 approximately 15 fold for both strain FSL Z1-048 ($P < 0.01$) and strain FSL Z1-124 ($P < 0.01$) (Figure 4. 1). Both strains were able to invade epithelial cells after 3 h of incubation (Figure 4. 2). FSL Z1-124 showed higher ability to invade cells than strain FSL Z1-048. FSL Z1-124 was 37 fold and 1.7 fold more invasive than strain FSL Z1-048 at MOI 10 and MOI 100 respectively. Bacterial concentration had an effect on internalization of strain FSL Z1-048, which was 63 fold more invasive at MOI 100 compared to that observed at MOI 10 ($P< 0.01$), whereas for strain FSL Z1-124 internalization at MOI 100 was not significantly different to that observed at MOI 10 (Figure 4. 2).The positive control included in the invasion assay *S. enterica* serovar Typhimurium was able to adhere (Figure 4. 1) and to invade (Figure 4. 2) the mammary epithelial cells in all the assays. The negative control *E. coli* laboratory strain was not able to invade the cells in any assay (data not shown).

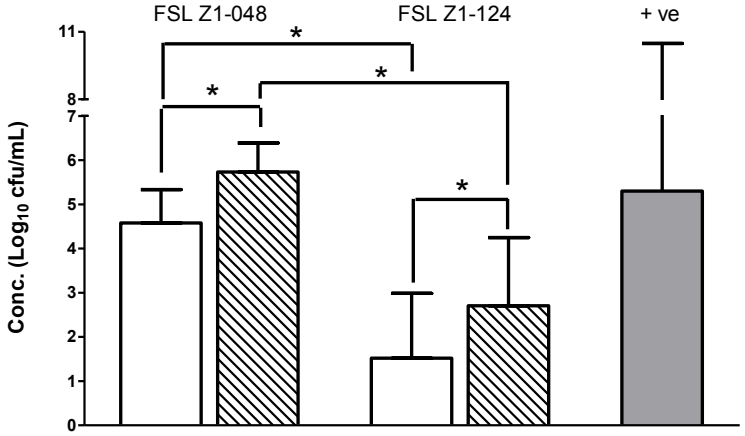

Figure 4. 1 Adhesion of *S. uberis* to epithelial cells *in vitro*. Results are expressed as concentration of bacteria recovered attached to the mammary epithelial cells BME-UV1 after 3 h of co-incubation. Cells were incubated with strain FSL Z1-048 or strain FSL Z1-124, at a multiplicity of infection (e.g. bacteria: cells) of 10 (white columns) and 100 (striped columns). The values represent the mean and the standard deviation of 3 replicates. The solid grey bar represents the number of *S. enterica* serovar Typhimurium strain 12023 bacteria used as positive control. Statistically significant differences between groups are marked with asterisks (Tukey HSD test after ANOVA, * $P < 0.05$).

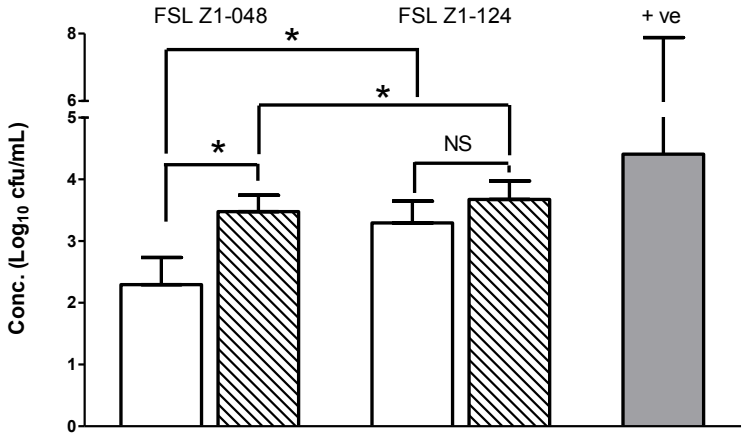

Figure 4. 2 Invasion of *S. uberis* to epithelial cells *in vitro*. Results are expressed as concentration of bacteria recovered internalized in the mammary epithelial cells BME-UV1 after 3 h of co-incubation. Cells were incubated with strain FSL Z1-048 or strain FSL Z1-124, at a multiplicity of infection of 10 (white columns) and 100 (striped columns). The values represent the mean and the standard deviation of 3 replicates. The solid grey bar represents the number of *S. enterica* serovar Thypimurium strain 12023 bacteria used as positive control. Statistically significant and non-significant differences between groups are marked with asterisks and NS respectively (Tukey HSD test after ANOVA, *$P < 0.05$).

4. 3. 2 Biofilm Assay

Ability to form biofilm was tested in 5 putatively host adapted and five putatively non adapted strains of *S. uberis*. Ability to form biofilm when grown in RPMI-1640 CDM supplemented with 0.5% casein varied amongst the strains tested (Figure 4. 3). The two strains with the highest ability to form biofilm were the putatively non adapted strains FSL Z1-124 and QMP B5-153. The ability to form biofilm of these two strains was similar to the positive control strain 20539. The host adapted strain QMP B5-005 and the non adapted FSL Z3-396 exhibited moderate biofilm formation, which was significantly lower than non adapted strains FSL Z1-124 and QMP B5-153 ($P < 0.05$), but significantly higher than all the other strains tested (FSL Z1-048, FSL Z3-369,QMP B5-143, QMP B5-157, FSL Z1-266, QMP B5-001) ($P < 0.05$). In preliminary experiments ability to form biofilm was also tested in BME-UV1 complete medium and BME-UV1 complete medium supplemented with casein, however none of the strains were able to form biofilm in these media with the exception of strain QMP B5-143 in BME-UV1 medium with casein (results not shown). Therefore, no additional replicates were performed with those media. The results show that test strains used in the mammary epithelial cell assays do not form biofilm in the culture media used in those assays, implying that adherence results will not have been affected by biofilm formation.

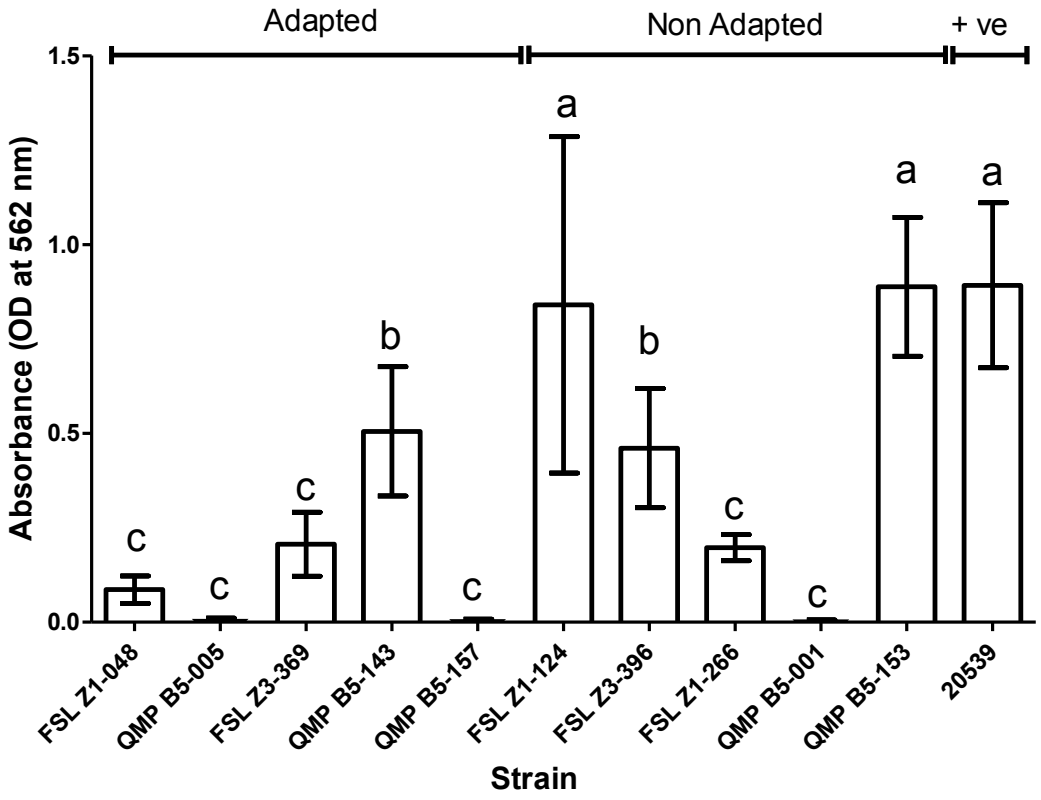

Figure 4. 3 Biofilm formation of 10 strains of *S. uberis* either putatively host adapted or non adapted. Ability to form biofilm was tested in RPMI-1640 CDM medium supplemented with 0.5% wt/vol casein. Bars represent the amount of biofilm produced after 24 h of culture as optical density and standard deviations. For each strain 3 replicates were performed, for each replicate 8 technical replicate (e.g. wells) were performed. Letters (a, b ,c) mark strains with a similar ability to produce biofilm ($P < 0.05$).

4. 3. 3 Sua Gene

The Sua gene was identified in the whole genome sequences of both strain FSL Z1-048 and strain FSL Z1-124. Identity of the *sua* sequence of strain FSL Z1-048 with *sua* of *S. uberis* strain UT888 (NCBI, National Center for Biotechnology Information, Bethesda, MD, USA, GenBank, Accession no. DQ232760.1) was 99% (2955/2971), with one base pair gap in position 700 (5`→3`) of the submitted sequence.(Figure 4. 5). For strain FSL Z1-124 identity with strain UT888 was 99% (2939/2971) and no gaps were present. The presence of *sua* was confirmed by amplification by PCR in both strains. Sequencing of the PCR products confirmed the genome sequence of the two strains including the deletion of a single base from *sua* in FSL Z1-048. Analysis of open reading frames (ORF) in the full length 2971 bp Sua gene showed that strain FSL Z1-124 has an ORF on the direct strand extending from base 94 to base 2811 translating in a protein of 905 amino acids in length (Figure 5. 5), which is the full length of the SUAM protein described (Almeida *et al*., 2006) with 98% (884/905) identity with strain UT888 SUAM protein. The nucleotide base missing in *sua* of strain FSL Z1-048 resulted in an ORF extending from base 94 to base 744 which would be translated in a protein of 216 amino acids in length, resulting in a truncated SUAM protein (Figure 4. 5).

```
UT888          GTCATTTGGTAGGAGTGGCTGTTTTTGCTGAAAATGCTAAAGAAGAACGTGAACAGATGG 60
FSLZ1-048      ************************************************************ 60
FSLZ1-124      ************************************************************ 60

UT888          CATATAAATCATTGCTTAAAGTTTCTGAAATAGATGTCAAGAACAATAAAGTCGTCGTTG 120
FSLZ1-048      ************************************************************ 120
FSLZ1-124      ************************************************************ 120

UT888          AAGTTGGGAATATTTTTAACGATATATAATGTATGGAGAGAAAAAGGGAATATTATGGAA 180
FSLZ1-048      ************************************************************ 180
FSLZ1-124      ******************T*****G**********************T********** 180

UT888          CTCGAAAACACAAAATCTAATCAGATTAAAACAACACTTGCTTTAACGTCAACACTCGCA 240
FSLZ1-048      T*********************************************************** 240
FSLZ1-124      T*********************************************************** 240

UT888          CTTCTTGGAACTGGTGTTGGTATGGGACATACCGTTAATGCGGATGACATGACAACTGCT 300
FSLZ1-048      ************************************************************ 300
FSLZ1-124      ***************************************************A******** 300

UT888          GATCAATCACCTAAATTACAAGGTGAAGAAGCAACATTGGCGCCTACAAACATTGAAGAT 360
FSLZ1-048      ************************************************************ 360
FSLZ1-124      ****************************************A******************* 360

UT888          ACTAAAGCAGCCATTGATACTAAAACAGCTACATTAGCAGAACAAACCGATGCTCTTAAT 420
FSLZ1-048      ******************T*****************************G*********** 420
FSLZ1-124      ****G******************************************************* 420

UT888          ACTGTAAATGAGACAATCACAAGCACAAATGAAGAATTAGCTACTTTAGAAGGAGGCTTA 480
FSLZ1-048      ************************************************************ 480
FSLZ1-124      ************************************************************ 480

UT888          GCTGATAAAGAAACAGCAGTTGCAGATGCTGAAAAAACATTGGAGTCTGTTTCAAATGCC 540
FSLZ1-048      ************************************************************ 540
FSLZ1-124      ************************************************************ 540

UT888          TCAGAAGAAGAATTTAATCAATTAGCAGAACAAAATAAAGCTGACTTAGCTAAAACTCAA 600
FSLZ1-048      ************************************************************ 600
FSLZ1-124      ****************************************C******************* 600

UT888          GAGGAGCTAAAACTTGCTGAAGCAACAAAAGAAGAAGTTGCAACACAGGTATTGACACAA 660
FSLZ1-048      ************************************************************ 660
FSLZ1-124      ************************************************************ 660

                                                       ⬇
UT888          TCTGACGAGGTAACAGCTGCAGCTAATGAAGCTAAAAAAATGGCTGAAAAAGTTGCACAA 720
FSLZ1-048      ****************************************-******************* 719
FSLZ1-124      ************************************************************ 720

UT888          GCAGAGACAAAAGTTTCAGACTTGACGAAAATGGTCAATCAACCAGAAGCAATAACAGCT 780
FSLZ1-048      ************************************************************ 779
FSLZ1-124      ****************T*****************A********************** 780

UT888          CAAGTTGAAATAGAACAAAACAATGTCAAAATCATTTCGGAAGATTTAGCAAAAGCCAAA 840
FSLZ1-048      ************************************************************ 839
FSLZ1-124      ************************************************************ 840

UT888          ACTGATTTAGTTGCTGTAACAGATAATACAAAAACACAATTAGCAAATGATTTAGCGACT 900
FSLZ1-048      ************************************************************ 899
FSLZ1-124      ************************************************************ 900
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UT888          GCTCAATCTAGCTTAAGTGCCAAACAAAATGAATTAGCTAAAGTACAGTCACAAACAAGT 960
FSLZ1-048      ************************************************************ 959
FSLZ1-124      ************************************************************ 960

UT888          AATGTCGCAGTGAATGTTATGGGTGCTAATAAAATGGTTGCTCCAACTAATTACCCAATT 1020
FSLZ1-048      ************************************************************ 1019
FSLZ1-124      ************************************************************ 1020

UT888          AATGAAATCAAAAAATTAATGTCAAGTGGTTACATTGGGACACAATCTTATCTAAATACA 1080
FSLZ1-048      ************************************************************ 1079
FSLZ1-124      ************************************************************ 1080

UT888          TTCTATGCTTTAAAAGATCAACTGGTTTCTAAAGCAGAAGTTGGGGCATACTTAAATCAT 1140
FSLZ1-048      ************************************************************ 1139
FSLZ1-124      **************************C*********A********************** 1140

UT888          TACGTTGATATCGCAAGTGACTTAAACCGTATCGTTAACCCAGATAACTTATCAGTTGAG 1200
FSLZ1-048      ************************************************************ 1199
FSLZ1-124      ************************************************************ 1200

UT888          GTTCAAAATGAATTGGCTGTATTTGCAGCAACATTGATTAATTCTGTTCGTCAGCAATTT 1260
FSLZ1-048      ********************************************************A******* 1259
FSLZ1-124      ************************************************************ 1260

UT888          GGTCTTTCTGCAGTCGAAGTGACGCAAGGTGCTCAAGAGTTTGCTCGCACTTTGACTCAA 1320
FSLZ1-048      ************************************************************G* 1319
FSLZ1-124      ************************A*********************************** 1320

UT888          AACTATAAAGCAACACATGGAAACACTGTTCCTTTCTTTAATTACAATCAACCTGGCAAG 1380
FSLZ1-048      **********T************************************************* 1379
FSLZ1-124      ************************************************************ 1380

UT888          AATGGTCATATAGGCATTGGTCCACACGATAGAACAATTATCGAACAAGCAGCTACAAGT 1440
FSLZ1-048      ************************************************************ 1439
FSLZ1-124      **********C************************************************* 1440

UT888          GTTGGCTTAAAAGCTAATGATGATAACATGTATGAAAACATCGGATTCTTTGATGATGTT 1500
FSLZ1-048      ***************************T******************************** 1499
FSLZ1-124      ************************************************************ 1500

UT888          CATACTGTTAATGGTATCAAACGTAGTATTTATAACAGTATTAAGTACATGCTGTTTACA 1560
FSLZ1-048      ************************************************************ 1559
FSLZ1-124      ************************************C*********************** 1560

UT888          GACCTCACCTATGGAAATACATTTGGACATACGGTTAACTTGTTGCGTTCTGATAAAACA 1620
FSLZ1-048      ***T******************************************************** 1619
FSLZ1-124      ***T******************************************************** 1620

UT888          AACCCAAGTGCTCCGGTCTATTTAGGAGTTTCAACAGAAACTGTTGGTGGTTTAAATACC 1680
FSLZ1-048      ************************************************************ 1679
FSLZ1-124      ****************T******************************************* 1680

UT888          CACTATGTTATCTTCCCGGCAAGCAATATTGTAAATGCCAGCCAGTTCAGCAAACAAGTG 1740
FSLZ1-048      *******************************************A*************** 1739
FSLZ1-124      **************************C*********A********A*************** 1740
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UT888          GTTTCAGGTCCATTAACAACAGTTGATAACAGTGCTAAAATTAGCACTCTTCAAGCAAGT 1800
FSLZ1-048      ************************************************************ 1799
FSLZ1-124      ************************************************************ 1800


UT888          ATTGCTTCTGTTGAGTCTAAAATTCAAACCTTACAAAAACGTATTGCAAATATTTCTTCA 1860
FSLZ1-048      ***A******************************************************** 1859
FSLZ1-124      ************************************************************ 1860


UT888          GAAGCACTAGTTATCTCTGCACAGAGAAAAGTAGATGGTTTAGCTGCAAAACTTCAAAAA 1920
FSLZ1-048      ************G*********************************************** 1919
FSLZ1-124      ************************************************G********** 1920


UT888          GCTGAATCTAACGTTGAAAAAGCAAAAGCTCAACTTCAACAGTTAAAAGATTCAAAAGAA 1980
FSLZ1-048      *********************************************C************** 1979
FSLZ1-124      *********************************************C************** 1980


UT888          GATTTACATAAACAACTTGCTTTTGCCCTTTCAACTCGTAAGGATTTAAAAGGTCAACTT 2040
FSLZ1-048      ************************T*********************************** 2039
FSLZ1-124      ************************************************************ 2040


UT888          GACGAATCGCTTGTTCACCTAAATCAGTCTAAAATTCTTTTTCATAGCTTAGAAGAAAAA 2100
FSLZ1-048      ****************************************A******************* 2099
FSLZ1-124      ****************************************A******************* 2100


UT888          CAAAGTCAAGTGGCAAGTCAAATTAACGTCTTGACATTGAAGAAGGCACAACTTGAAAAA 2160
FSLZ1-048      ************************************************************ 2159
FSLZ1-124      *********************************************A************** 2160


UT888          GAACTAGCCTTTAACTCTCATCCAAATCGTGAAAAAGTTGCAAAAGAAAAAGTTGAAGAG 2220
FSLZ1-048      ************************************************************ 2219
FSLZ1-124      ************************************************************ 2220


UT888          GCTCAAAAAGCATTAACAGAAACCTTATCTCAAATTAAAACTAAAAAAGCTATCTTAAAT 2280
FSLZ1-048      ************************************************************ 2279
FSLZ1-124      ************************************************************ 2280


UT888          GATTTAACACAAGAAAAAGCAAAATTGACGTCAGCAATCACAACAACTGAACAACAAATT 2340
FSLZ1-048      ************************************************************ 2339
FSLZ1-124      ************************************************************ 2340


UT888          GTTTTGTTGAAGAATCATTTAGCAAATCAAGTGGCGAATGCTCCAAAAATCAGCAGTATT 2400
FSLZ1-048      ************************************************************ 2399
FSLZ1-124      ************************************************A*********** 2400


UT888          GTCCAAAGATCAGAAAACAATGGAGTAAGACCTGATGTTTCTGATACAAGAGAGAAGGCA 2460
FSLZ1-048      ********************A*************************************** 2459
FSLZ1-124      **********T**********A********G***************************** 2460


UT888          GTAGATACTGCTCAAGAAGCGACAATTCTTGCTCAAGCAGAAACAATGGCTGAAGAAGTC 2520
FSLZ1-048      ************************************************************ 2519
FSLZ1-124      *********************************************G*****A******** 2520


UT888          ATTACAAATTCTGCAAAAGCCATTGTTGCAAATGCTCAAAATGTTGCACAAGAGATTATG 2580
FSLZ1-048      ************************************************************ 2579
FSLZ1-124      ************************A*********************************** 2580
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UT888           AAAGTAGCACCTGAAGTAACACCTGATCAAGGAGTTGTTGCAAAAGTTGCAGATAATATT 2640
FSLZ1-048       ************************************************************ 2639
FSLZ1-124       ************************************************************ 2640

UT888           AAGAAAAATAATGCCCCAGCAAGTAAATCATATGGTGCAAGTTCATCAACTGTAGGAAAT 2700
FSLZ1-048       ************************************************G*********** 2699
FSLZ1-124       ************************************************************ 2700

UT888           GCTACTTCTTCACGAGATGAAAGTACAAAACGTGCTTTAAGAGCAGGAATTGTTATGCTG 2760
FSLZ1-048       ************************************************************ 2759
FSLZ1-124       ************************************************************ 2760

UT888           GCAGCAGCAGGACTTACTGGTTACAAACTCAGAAGAGATGGCAAAAAATAAGAAAATCAA 2820
FSLZ1-048       ************************************************************ 2819
FSLZ1-124       ************************************************************ 2820

UT888           AGGAAAAATTGATTGACAGAAAGTACCGTCTATGTTACTATAGTAGACGGTACTTTTTAC 2880
FSLZ1-048       ************************************************************ 2879
FSLZ1-124       ************************************************************ 2880

UT888           TTTTGGTCTCTCAAAAGTGTACAGAGACGTGCTGACAATTGTTGCAAAAGTACACACAGA 2940
FSLZ1-048       ************************************************************ 2939
FSLZ1-124       ************************************************************ 2940

UT888           TATAGGCTGTCACCAAGTGCTATATCAACCA 2971
FSLZ1-048       ******************************* 2970
FSLZ1-124       ******************************* 2971
```

Figure. 4 Alignment of DNA sequence (5`→3`) of *sua* of *S. uberis* strains FSL Z1-048 and FSL Z1-124 obtained from whole genome sequencing and confirmed by amplification and sequencing of the specific fragment with *sua* of strain UT888 (Almeida *et al*., 2006). Deletion of one base in s*ua* of strain FSL Z1-048 is marked by an arrow.

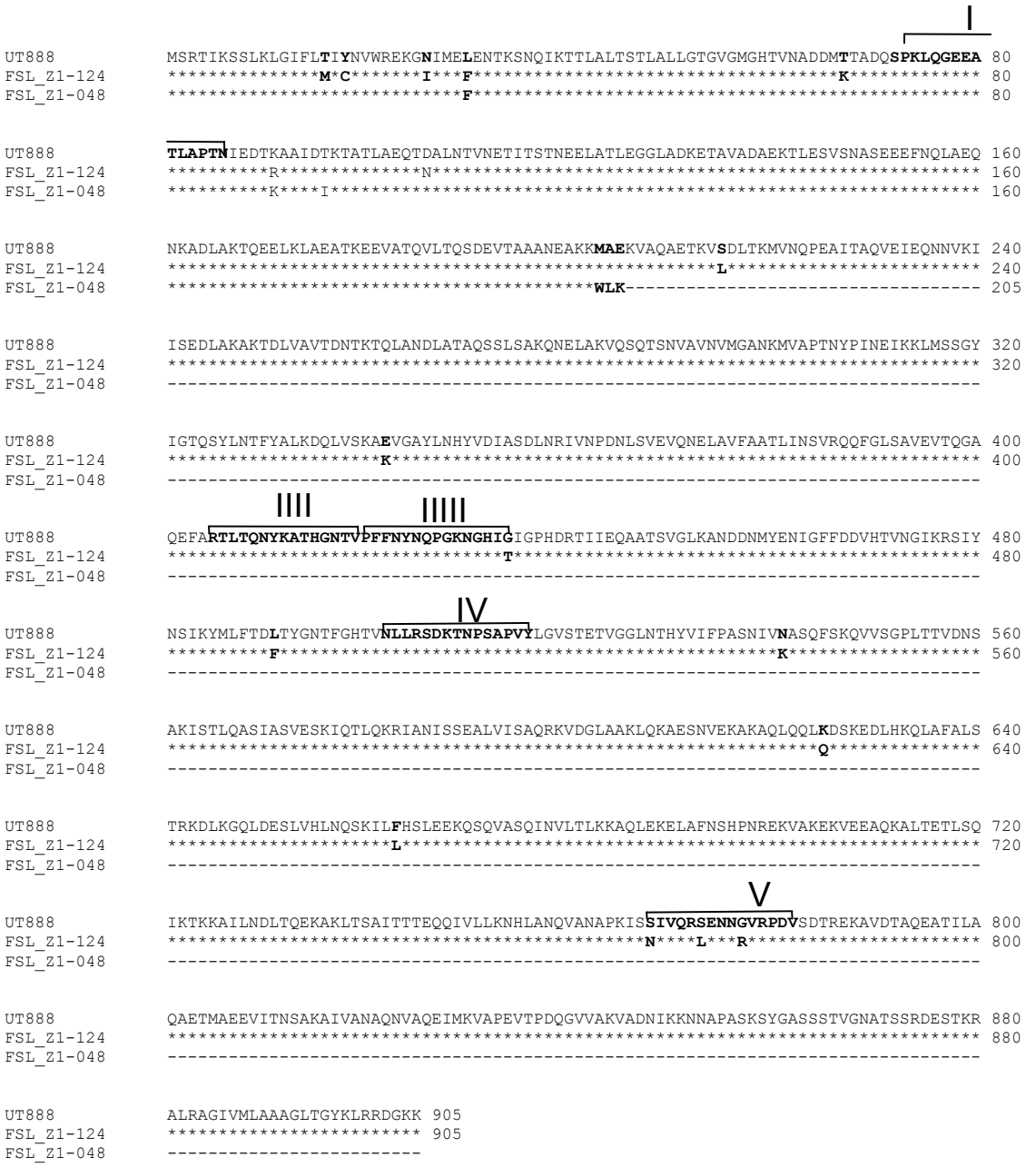

Figure 4. 4 Alignment of amino-acid translation of *sua* sequence of strains FSL Z1-124, and FSL Z1-048 with strain UT888 (Almeida *et al*., 2006). The five functional domains, identified in strain UT888 (I to V), are marked. The predicted protein of strain FSL Z1-048 resulted truncated compared to the protein identified in strain UT888 and the predicted protein of strain FSL Z1-124.

4.4 Discussion

Adhesion and invasion of epithelial cells of the mammary gland has been suggested as crucial step in development of intramammary infection of *S. uberis* (Matthews *et al*., 1994; Tamilselvam *et al*., 2006) and other mastitis pathogens such as *S. aureus* (Hebert *et al*., 2000) and *E. coli* (Dopfer *et al*., 2001; Dogan *et al*., 2007). In the present study we tested the ability of two strains of *S. uberis* that elicit a different clinical response to adhere and to internalize into bovine mammary epithelial cells *in vitro*. Strain FSL Z1-048 and FSL Z1-124 both possessed the ability to grow in fresh milk yet only strain FSL Z1-048 caused IMI and clinical signs in all challenged animals (Chapter 2). Adhesion to the epithelium of the mammary gland in the early stage of the infection could allow the bacteria to remain in the mammary gland without being eliminated during the milking and eventually cause mastitis. Both strains were able to adhere to the epithelial cells after 3 h of co-incubation however at MOI 10 strain FSL Z1-048 was approximately 1,000 fold more able to adhere than strain FSL Z1-124. These data suggest that adhesion may play a role in the higher virulence of strain FSL Z1-048 observed *in vivo.*

Both strains were also able to invade mammary epithelial cells, in this case strain FSL Z1-124 was found intracellularly 37 fold more than strain FSL Z1-048 at MOI 10. The ability of strain FSL Z1-124 to invade the epithelium did not alter with MOI, whereas ability to invade of strain FSL Z1-048 was greater with a MOI 100 compared to 10, indicating that internalization for strain FSL Z1-048, is concentration dependent. After infusion of this strain in the lactating mammary gland

we observed an increase in concentration of bacteria recovered in milk of approximately 1,000 fold between 12 and 36 h PI (see Chapter 2, Figure 2. 4A). The increase in bacterial concentration could increase the ability of *S. uberis* to invade the mammary epithelium.

Internalization in the mammary epithelial cells would protect the pathogen from the activity of the phagocytes present in the mammary gland such as PMN and macrophages. It could also provide protection against the vast majority of the antibiotics currently used in the therapy of the IMI (Zadoks, 2007). However, data obtained *in vitro* should be evaluated carefully since invasion of mammary gland cells by *S. uberis* has never been observed *in vivo* to date (Thomas *et al*., 1992; Pedersen *et al*., 2003). In the past adhesion and invasion of *S. uberis* has been studied extensively *in vitro* using the immortalized mammary epithelial cell MAC-T (Matthews *et al*., 1994; Tamilselvam *et al*., 2006). The immortalized cell line BME-UV1 used in the present study was used to study the internalization process of CNS species involved in bovine mastitis (Hyvonen *et al*., 2009). Although a direct comparison of the two cell lines as *in vitro* mastitis model has never been carried out, it could be speculated that the cell line BME-UV1 retaining many morphological and physiological characteristics of the secretory mammary epithelium such as the ability to form a monolayer capable of maintaining apical polarization and cell junction integrity (Quesnell *et al*., 1996), could represent a reasonable model of bovine mammary epithelial cells.

Despite its ability to invade the epithelial cells *in vitro* strain FSL Z1-124 was not able to cause mastitis in any of the mammary glands challenged (Chapter 2). After the infusion in the mammary gland strain FSL Z1-124 was recovered at a maximal concentration of approximately 10^5 lower than that observed for strain FSL Z1-048, and only at sporadic time points up to 168 h after intramammary infusion (see Chapter 2, Figure 2. 4). These observations may suggest that invasion can happen *in vivo* but is not a crucial step in the development of intramammary infection. It is possible that strain FSL Z1-124, after entering into the mammary gland, invades epithelial cells rapidly where it does not multiply, whereas strain FSL Z1-048, being only attached to epithelial cells, can multiply using milk as source of nutrients.

Mammary epithelial cells are able to recognize mastitis pathogens and initiate the host immune response through the synthesis and the release of chemo attractants such as IL-8 and pro inflammatory cytokines such as IL-1β and IL-6 (Rainard and Riollet, 2006). IL-8 is chemoattractant expressed by mammary epithelial cells in response to contact with major mastitis pathogens such as *S. aureus*, *E. coli* (Lahouassa *et al*., 2007) and *S. uberis* (Swanson *et al*., 2009; Wellnitz *et al*., 2012) and the expression of IL-8 is dependent on concentration of the pathogen (Günther *et al*., 2010; Wellnitz *et al*., 2012). Pro- inflammatory cytokines were absent in the milk of the mammary gland challenged with strain FSL Z1-124 except for very low levels of IL-8. Interestingly, IL-8 was only detected in quarters in which the bacteria were recovered, suggesting IL-8 production may have be a result of an interaction between the bacteria and the mammary epithelium in a similar manner to that seen *in vitro*,

although the concentration reached by the cytokine *in vivo* following challenge with strain FSL Z1-124 was not sufficient to evoke other detectable effects.

In addition to the findings in this study, internalization of *S. uberis* has previously been observed *in vitro* following challenge of MAC-T cells, an immortalized bovine mammary epithelial cell line (Almeida *et al*., 2006; Tamilselvam *et al*., 2006). In MAC-T challenge studies internalization *in vitro* was shown to be partially mediated by *Streptococcus uberis* adhesion molecule (SUAM), with deletion of *sua* reducing the ability of *S. uberis* to adhere and internalize to the mammary epithelial cells(Kerro Dego *et al*., 2011). The gene encoding SUAM (*sua*) has been shown to be conserved in strains of *S. uberis* from different geographical locations (Luther *et al*., 2007). Analysis of genome sequence of the two challenge isolates showed the presence of *sua* encoding for the SUAM in both strain FSL Z1-048 and FSL Z1-124. Further analysis revealed that sua gene in strain FSL Z1-124 would code for a protein of 905 amino acids in length as described by Luther *et al*. (2008) whereas *sua* in strain FSL Z1-048 is predicted to code for a truncated protein of 216 amino acids in length. Four out of 5 predicted epitopes thought to be involved in the process of adhesion and adhesion mediated by SUAM are located between 342 and 719 amino acid residue (Almeida *et al*., 2011). It is therefore unlikely that SUAM produced by strain FSL Z1-048 is completely functional as an adhesin.

Recent studies suggest that SUAM is not the only adhesin involved in the adhesion and invasion process of the mammary epithelial cells. Deletion of *sua* reduced but did not eliminate completely the ability of *S. uberis* to adhere to and to invade MAC-

T cells *in vitro* (Chen *et al*., 2011), moreover production of random mutants revealed that other adhesins could be involved in the adhesion and invasion process (Kerro Dego *et al*., 2011). Therefore, adherence of strain FSL Z1-048 to the mammary epithelium is likely to be mediated by other, as yet unidentified, SUAM independent mechanisms.

In addition to the challenge strains FSL Z1-048 and FSL Z1-124, 4 putatively adapted and 4 putatively non adapted strains of *S. uberis* selected based on epidemiological and genetic characteristics were tested for the ability to form biofilm *in vitro*. The non adapted strain FSL Z1-124 showed a higher ability to form biofilm compared to strain FSL Z1-048. Our results however suggest that ability to form biofilm does not play a role in the pathogenesis of IMI caused by *S. uberis*. In addition no clear association between host adaptation and ability to form biofilm was observed among other strains tested. These observations need to be considered carefully because biofilm formation greatly depends on culture conditions (Gilchrist, 2011), as exemplified by the lack of biofilm formation in BME-UV1 medium compared to RPMI CDM. Therefore, different culture conditions should be tested to confirm these results.

In conclusion adhesion to the mammary epithelial cells may at least partially explain the differences observed in the pathogenicity of the strains FSL Z1-048 and FSL Z1-124 suggesting that the adhesion to the mammary epithelial cells play a role in the pathogenesis of the IMI caused by this *S. uberis* strain.

CHAPTER 5

Activity of Bovine Phagocytes Against

Streptococcus uberis

5. 1 Introduction

In our challenge experiments (Chapter 1 and 2) we observed differences in the ability of two strains of *S. uberis* to cause mastitis when infused in the lactating mammary gland. The putatively host adapted strain FSL Z1-048 caused clinical mastitis in all the lactating cows challenged whereas the putatively non adapted strain FSL Z1-124 was not able to cause mastitis in any of the quarters challenged. *S. uberis* bacteria were recovered in milk from 4 cows challenged with strain FSL Z1-124 at sporadic time points (Chapter 3, Figure 3. 1 A and B). Concentration reached by strain FSL Z1-124 was also 10^5 fold lower than the maximal concentration reached by strain FSL Z1-048. In contrast FSL Z1-048 was recovered in all the quarters challenged within 18 h PI (post infusion). Since both the strains were able to grow in milk samples obtained from the challenged quarters pre infusion to a similar degree (Chapter 2, Figure 2. 2), we hypothesize that strain FSL Z1-124 is less able to invade the mammary gland at early stages of the infection. This may be explained, at least partially, by a the higher ability of strain FSL Z1-048 to adhere to the mammary epithelial cells as shown in Chapter 4. In addition, differences might be explained by a different ability to resist to the host defense of the mammary gland, specifically to the cellular defenses represented by phagocytes such as macrophage and PMN. In the present experiment we tested the ability of bovine macrophages and PMN to kill *S. uberis* strains FSL Z1-048 and FSL Z1-124.

Although the proportion of the total mammary leukocyte population reported varies depending on the study, macrophages represent a major cell population in the healthy

lactating mammary gland (Schwarz *et al*., 2011). Macrophage are phagocytic cells that represent the first line of defense against pathogens in the mammary gland (Rainard and Riollet, 2006; Sordillo and Streicher, 2002). Their function is to phagocytose and kill pathogens by the production of nitrogen reactive species such as nitric oxide. Moreover, mammary gland macrophages are involved in the initiation of the immune response following the recognition of pathogens via pattern recognition receptors by the production of proinflammatory cytokines such as TNF-α and IL-1β (Rainard and Riollet, 2006).

The role of macrophages in IMI caused by *S. uberis* is not entirely clear. After intramammary challenge with *S. uberis* strain 0140J, microscopic analysis demonstrated bacteria were within the cytoplasm of mammary macrophages, suggesting they may play a role in clearance of *S. uberis* from the mammary gland (Thomas *et al*., 1994). However subsequent studies conducted *in vitro* showed that ability to kill the pathogenic strain 0140J was different between macrophages isolated from the lactating mammary gland (e.g. from milk) and macrophages isolated from the dry mammary gland secretions or blood monocytes (Denis *et al*., 2006). In fact, macrophages from dry cow secretions and blood monocytes were able to kill *S. uberis*, whereas macrophages isolated from lactating mammary gland were able to ingest but not to kill the bacteria. These same milk macrophages were, however, able to kill *S. aureus*, suggesting that cells were not damaged during the isolation procedure and that the failure of milk macrophages to kill *S. uberis* in this study was due to a specific ability of *S. uberis* to resist to macrophage bactericidal activity (Denis *et al*., 2006).

Polymorphonuclear leukocytes (PMN) are the first cell population recruited in the mammary gland following a bacterial infection (Rainard and Riollet, 2006). Migration of the cells from the blood to the mammary gland is mediated by pro inflammatory cytokines such as TNF-α, IL-1β and IL-8 (Paape *et al*., 2003). The role of PMN is to ingest and kill the bacteria at the site of the infection. PMN are crucial in response to the intramammary infection caused by major mastitis pathogens such as *Escherichia coli* and *Staphylococcus aureus*. It was observed that cows with a better recruitment of PMN after the intramammary challenge with *E. coli* showed less severe clinical signs (Shuster *et al*., 1996). Similar findings were observed in cows with chronic sub-clinical *S. aureus* mastitis where the induced depletion of circulating PMN resulted in a gangrenous mastitis (Schalm *et al*., 1976). Both the studies suggest that PMN, even if not able to clear completely the infection, limit the number of bacteria thus reducing the symptoms during an IMI.

The role of PMN in IMI caused by *S. uberis* is less clear. Differences in the ability of PMN to kill different strains of *S. uberis* have been observed *in vitro*. Similar to our study, a difference in the ability of *S. uberis* strains to cause clinical mastitis was observed by Hill (1988b) and Leigh (1990). In their studies strains 0140J and EF20 were shown to differ in their ability to resist to PMN bactericidal activity, with O140J being more resistant to PMN killing than EF20 *in vitro*. This may explain the difference observed in clinical outcome in the challenge studies conducted with these two strains Hill (1988b), with strain 0140J able to cause mastitis in the vast majority of the cows challenged and EF20 causing mastitis only in a small proportion of animals, and suggests that resistance to PMN killing is important determinant of

strain pathogenicity. Additional studies also suggest that different strains of *S. uberis* have different abilities to resist PMN. A separate challenge study conducted with strain 0140J (Bannerman *et al*., 2004) and a study conducted with strain UT888 (Rambeaud *et al*., 2003) showed that despite the massive influx of PMN in the mammary gland after the infusion, PMN were not able to control the infection, consistent with the idea that some strains are resistant to PMN killing. In contrast, a challenge study conducted with strain U103 (Pedersen *et al*., 2003) showed that influx of PMN reduced the number of bacteria after an initial peak suggesting that PMN are able to control the intramammary infection by this *S. uberis* strain.

We observed a similar result in our challenge experiment with an influx of PMN in the mammary gland following the infection with strain FSL Z1-048 being coincident with a decrease in concentration of bacteria after a peak, which led to the clearance of the infection by 14 days in 5 of 6 animals (Chapter 2, Figure 2. 4). In our *in vivo* challenge experiments we also observed an increase of IL-17A in milk of 4 quarters challenged with strain FSL Z1-048. This increase coincided with a drop in milk bacterial concentration and cows cleared the infection by 14 days PI (Chapter 2, Figure 2. 10), suggesting a possible role of IL-17 in resolution of the IMI. IL-17, produced mainly by Th17 CD4 cells, is considered an amplifier of the acute inflammatory response by the innate immune system (Murphy, 2012). This function in considered important in controlling extracellular infection (Curtis and Way, 2009). Recent studies showed that IL-17 simulates the phagocytosis ability of human macrophages (Silverpil *et al*., 2006) and the bactericidal activity of PMN from mice and humans (Lu *et al*., 2008). Interferon-γ is a cytokine produced by Th1

lymphocytes (Ellis and Beaman, 2004), in the context of bovine mastitis, it has been shown that IFN-γ is capable of stimulating neutrophil killing activity against the mastitis pathogens *S. aureus* and *E. coli* (Fox *et al*., 1990), but not the killing activity of mammary gland macrophages (Quiroga *et al*., 1993) suggesting that IFN-γ can play a role in the response to the IMI. Although IFN-γ was not elevated in response to challenge with strain FSL Z1-048 in our challenge experiment (Chapter 2, Figure 2. 9) it has been observed in response to the strain 0140J (Bannerman *et al*., 2004) suggesting that it may be important in the response to other strains of *S. uberis*.

The objective of the present experiment is to test the ability of bovine macrophages and PMN to kill the *S. uberis* challenge strains FSL Z1-048 and FSL Z1-124 and the ability of IL-17A to stimulate the bactericidal activity of bovine macrophage and PMN toward *S. uberis*. Moreover ability of IFN-γ to stimulate the bactericidal activity of PMN toward *S. uberis* is tested.

5. 2 Materials and Methods

5. 2. 1 Strains

FSL Z1-048 and FSL Z1-124 were chosen respectively as representative of putatively host adapted and putatively non adapted strains of *S. uberis* for the macrophage killing assay. See section (Chapter 2 and 4 for the details).

5. 2. 2 Monocyte Derived Macrophage Killing Assay

The ability of bovine monocyte derived macrophage to kill *S. uberis* strain FSL Z1-048 and FSL Z1-124 was tested. Cells were obtained from 3 Holstein non-lactating heifers. Age was comprised between 9 and 12 months). The experiment was conducted at Moredun Research Institute (Penicuick, UK) with approval of the Institute's Experiments and Ethical Review Committee in accordance with the Animals (Scientific Procedures) Act 1986. Approximately 100 mL of blood were collected from the jugular vein and mixed immediately with an equal volume of Alsever`s solution as anticoagulant (D-glucose 113.76 m*M*, sodium chloride 71.87 m*M*, sodium citrate dihydrate 27.20 m*M*, citric acid 2.86 m*M* in water). The mix of blood and anticoagulant was layered on phicoll plus (GE healthcare, Amersham, UK) at a ratio of 2:1 and the PBMC layer was separated by centrifuging at 900 × *g* for 30 min at 4°C. The PBMC layer was pipetted off and transferred to a new falcon tube and washed 3 times in complete medium (RPMI-1640 supplemented with 10% vol/vol heat inactivated fetal calf serum, 200 U/mL penicillin, 200 U/mL streptomycin, 1% vol/vol glutamine (Sigma-Aldrich, Dorset, UK). Cells were finally resuspended in up to 2 mL buffer, then labelled with mouse anti-human CD14

microbeads (Miltenyi, Bisley, UK) and isolated by positive selection on LS magnetic column (Miltenyi-Biotech) following manufacturer`s instructions. Viable cells were counted by trypan blue dye exclusion and seeded in complete medium at 1×10^6 cells/well in flat bottom 96 wells polystyrene tissue culture plates (Corning, NY, USA). Cells were cultured for 6 days at 37°C, 5% CO_2. Medium was changed every second day. 24 h before the assay, complete medium was replaced with complete medium without antimicrobials and where appropriate supplemented with 10 ng/mL of recombinant bovine IL-17A (Kingfisher Biotech, Saint Paul, MN, USA). To conduct the assays, 100 µL of supernatant were removed and replaced with 100 µL of medium containing *S. uberis* bacteria strain FSL Z1-048 or FSL Z1-124 pre-incubated for 20 min at 37°C with heat inactivated adult bovine serum (Gibco) as source of opsonins. Based on preliminary experiments bacteria were diluted to have a multiplicity of infection (MOI) of 5 (i.e. 5 bacteria per cell). Bacteria and cells were co-incubated for 2 h at 37°C, 5% CO_2 before 50 µL of 0.1% vol/vol tryton X-100 (Sigma-Aldrich) diluted in PBS were added in order to lyse the cells. Cell lysates were 10-fold serially diluted in cold PBS and 20 µL spots were plated in triplicates on blood agar plates (E&O Laboratories, Bonnybridge, UK). Cfu were counted where possible for the dilution factor presenting 5 to 50 colonies per spot and concentration was calculated. For each treatment (strain, IL-17A stimulation) 3 technical replicates (i.e. wells) were performed. The entire experiment was repeated in a 2-week period, collecting each time blood from the three animals. In preliminary experiments purity of macrophage culture after the magnetic bead separation was assessed by flow cytometry. Briefly, 10 µL of anti CD14 antibody (clone VPM65, mouse IgG1 isotype, Moredun Research Institute) were added to 1

mL of cell suspension in complete medium containing 1×10^6 cells and incubated for 30 min at 4°C. Cells were washed 3 times by centrifugation at 1500 × g for 5 min at 4°C. 100 µL of goat anti mouse IgG Alexa 488 conjugated secondary antibody diluted 1:2000 were added and incubated for 30 min at 4°C. Cells were acquired with a 2 laser Cyan flow cytometer (Beckman Coulter, High Wycombe, UK). Data were collected for a minimum of 10,000 cells for each cell preparation sample, and cells were gated based on forward and side scatter to exclude cellular debris from the analysis. Data were analysed using FlowJo software (Tree Star). The percentage of CD14 positive cells was calculated. Cytocentrifuge preparations of selected cell samples after 7 days of culture were prepared using a Shandon Cytospin 4 cytocentrifuge (Thermo Electron Corporation, Milford, MA, USA) and stained using a REASTAIN Quick-Diff Kit (Reagena Toivala, Finland) for microscopic examination.

5. 2. 3 PMN Killing Assay

To test the ability of bovine PMN to kill *S. uberis* strains FSL Z1-048 and FSL Z1-124, PMN were isolated from blood of 4 mid lactating Holstein cows (parity 2 to 5). The experiment was conducted at Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University (Ithaca, NY, USA) using animals held by the Cornell Teaching and Research Facility (Ithaca, NY, USA). Approximately 20 mL of blood were collected from the jugular vein in vacutainer vials containing heparin as anti-coagulant. Blood was processed within 1 h of collection diluting it with an equal volume of PBS, the mixture was then layered on Ficoll-Paque plus (GE Healthcare, Pittsburgh, PA, USA) and centrifuged at for

30 min at 900×g 4°C. The granulocyte layer was transferred to a fresh 50 mL falcon tube. Residual erythrocytes were lysed by adding 5 mL of lysis solution (0.8% wt/vol NH_4Cl, 0.1 m*M* EDTA in water) pre warmed at 37°C. Cell suspension was gently mixed until the erythrocytes were completely lysed. 20 mL of PBS were added, then cells were washed twice by spinning at 300 × g for 10 min at 4°C and finally resuspended in RPMI-1640 (Gibco) supplemented with 10% heat inactivated FCS and 4 m*M* glutamine. Viable cells were microscopically counted based on trypan exclusion staining. Cells were seeded in 96-well polystyrene cell culture plate (Corning) at 5×10^5 cells per well in a total volume of 200 µL. Cells were stimulated with bovine recombinant cytokines IFN-γ (AbD Serotec, Kidlington, UK) or IL-17A (Kingfisher) at 10, 100, 1000 ng/mL for 2 h at 37°C, 5% CO_2. After pre-incubation for 20 min at 37°C with heat inactivated adult bovine serum (Gibco) as source of opsonins, *S. uberis* strains FSL Z1-048 and FSL Z1-124 were added to wells at MOI of 1 with PMN. Bacteria and PMN were co-incubated for 90 min at 37°C, 5% CO_2. Fifty µL of 0.1% vol/vol tryton X-100 (Sigma-Aldrich) diluted in PBS were added in order to lyse the cells. Cell lysates were 10 fold serially diluted in cold PBS and 20 µL spots were plated in triplicates on blood agar plates. Cfu were counted where possible for the dilution factor presenting 5 to 50 colonies per spot and concentration was calculated. In preliminary experiments the purity of cell population after isolation of PMN was tested with flow cytometry analysis. Cells were stained with antibodies against anti CD11b (Monoclonal FITC conjugated, Mouse IgG2b, VMRD) and anti CD14 (Monoclonal APC conjugated Mouse IgG1, VMRD) antigens using a protocol described by Schwarz *et al*. (2011). PMN from 4 animals were tested separately, for each treatment (strain, cytokine) 3 technical replicates

were performed. An average of the 3 technical replicates was used as input for the statistical analysis. All procedures were approved by the Cornell Institutional Animal Care and Use Committee

5. 2. 4 Statistical Analysis

Percentage survival was calculated as the percentage of the viable bacteria compared to viable bacteria in wells containing the medium only and no host cells. Percentage survival was used as input for a two-way analysis of variance (ANOVA) using Genstat software (VSN International, Hemel Hempstead, UK). Strain and cytokine stimulation were used as treatment factor, interaction between strain and cytokine was also evaluated. A blocking structure with cells from individual animals was used. Charts were visually checked to assess the normal distribution of the residuals. Post-hoc comparisons with Tukey HSD test and LSD test were performed where appropriate with statistical significance declared when $P < 0.05$.

5. 3 Results

5. 3. 1 Macrophage Killing Assay

The ability of macrophages derived from blood monocytes to kill *S. uberis* strains FSL Z1-048 and FSL Z1-124 was tested. Flow cytometry on preliminary experiments showed that approximately 90% of the cells after the purification from blood were $CD14^+$ (Figure 5. 1). After 7 days of culture approximately 95% of the cells at microscopic examination were showing the morphology of differentiated macrophage.

Monocyte derived macrophages were able to kill strain FSL Z1-124. The proportion of viable bacteria present after 2 h of co-incubation with host cells was 57.5 ± 21.8% of the bacteria incubated in the medium only (Figure 5. 2) indicating that blood derived macrophage were able to kill this *S. uberis* strain. In contrast, macrophages failed to kill strain FSL Z1-048. Bacteria recovered after 2 h were 123.8 ± 33.2% of the bacteria incubated in the medium only, suggesting a replication in presence of macrophages (Figure 5. 2).

Stimulation of the macrophages for 24 h with 10 ng/mL of bovine recombinant IL-17A significantly increased their ability to kill the strain FSL Z1-048 ($P < 0.05$, Figure 5. 2) whereas stimulation with IL-17A did not significantly enhance the ability of macrophages to kill strain FSL Z1-124 (Figure 5. 2)

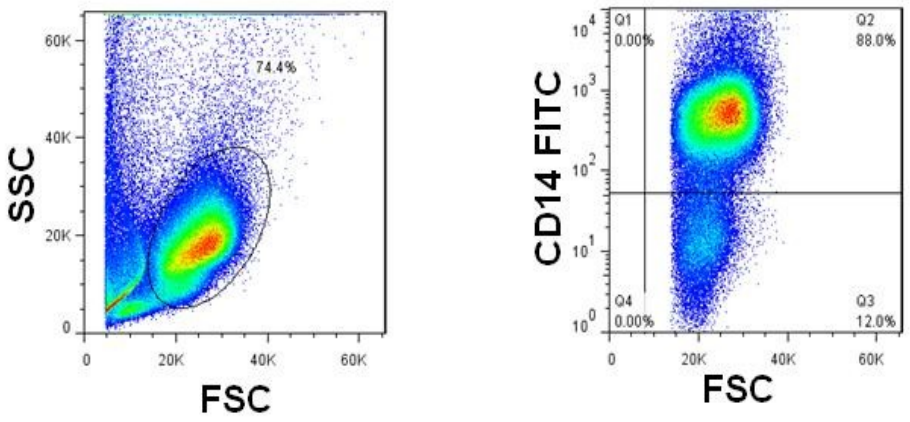

Figure 5. 1 Example of flow cytometric analysis of cells isolated from blood using CD14 beads. Cells were gated based on side and forward scatter (left figure) to exclude cell debris. Monocytes were identified as $CD14^{+}$ (FITC) cells (Q2 quadrant).

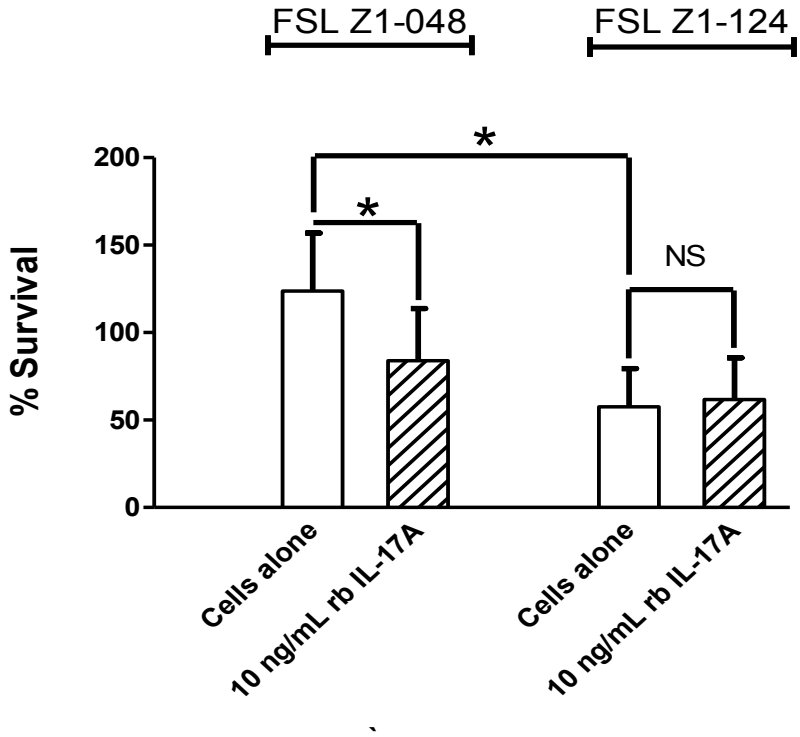

Figure 5. 2 Killing of *S. uberis* strains FSL Z1-048 and FSL Z1-124 by bovine monocyte derived macrophages. Data represent the percentage ± standard deviation of bacteria survived after 2 h of co-incubation with macrophages compared with bacteria in medium alone. Cells were stimulated for 24 h with 10 ng/mL of rbIL-17A (striped bars) or left untreated (empty bars). Cells were isolated from blood of 3 animals and the experiment was repeated twice.
Statistically significant differences (LSD test, $P < 0.05$) are marked by asterisks.

5. 3. 2 PMN Killing Assay

Flow cytometry analysis showed that approximately 75% of the cells after the PMN isolation process were $CD11b^+$ $CD14^-$ thus likely to be PMN (Figure 5. 3). In fact, CD14 antigen is expressed by monocyte but not on PMN (Schwarz *et al*., 2011). This proportion was confirmed by microscopic observation of morphology of cells present in the cell preparations

PMN cells isolated from bovine blood were able to kill both *S. uberis* strain FSL Z1-048 and FSL Z1-124 (Figure 5. 4A and B). Bactericidal ability of PMN alone was significantly different between the two strains ($P < 0.05$), with higher bactericidal activity observed with strain FSL Z1-048. Percentage survival for strain FSL Z1-048 was 32.9 ± 10.2 (Figure 5. 4A) whereas for strain FSL Z1-124 it was 55.8 ± 18.8 (Figure 5. 4B).

Stimulation with recombinant cytokines IL-17A or IFN-γ increased the killing ability of PMN, however only at certain concentrations (Figure 5. 4A and B). 10 ng/mL of IL-17A increased the killing ability toward strain FSL Z1-048 and toward strain FSL Z1-124 compared to PMN cultures without IL-17A, with the mean percentage survival of bacteria being 20.4 ± 4.3 and 43.8 ± 18.9 % for strains FSL Z1-048 and FSL Z1-124, respectively. IL-17A at 100 ng/mL increased the killing ability of PMN for strain FSL Z1-048 only, with mean percentage survival being 28.4 ± 8.1 (Figure 5. 4B). IL-17A at 1000ng/mL had no effect on the killing ability of PMN for either strain.

10 ng/mL of IFN-γ increased the killing ability of PMN towards both strain FSL Z1-048 and FSL Z1-124, with mean percentage survival being 24.3 ± 10.2 and 45.8 ± 17.8 for strains FSL Z1-048 and FSL Z1-124, respectively. IFN-γ at 100 ng/mL increased the killing ability of PMN for strain FSL Z1-124 only (mean percentage survival 39.2 ± 15.2, Figure 5. 4B), whereas IFN-γ at 1,000 ng/mL increased the killing ability of PMN for strain FSL Z1-048 only (mean percentage survival 22.1 ± 7.2, Figure 5. 4A).

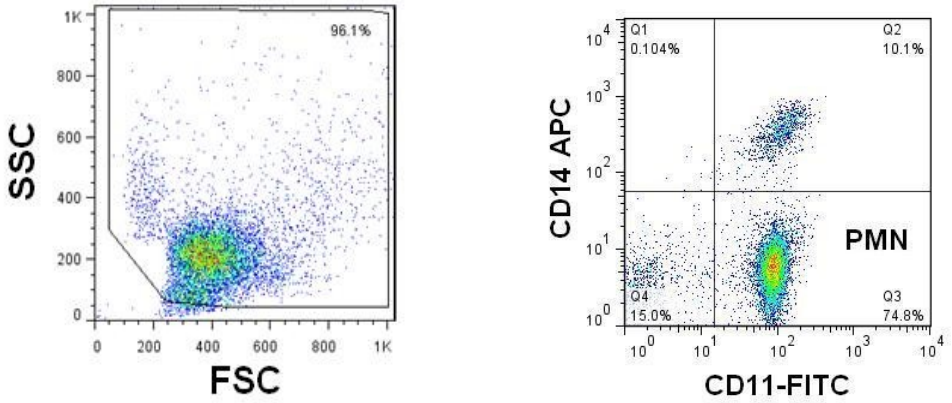

Figure 5. 3 Example of flow cytometric analysis of PMN isolated from blood. Cells were gated based on side and forward scatter (left figure) to exclude cell debris. PMN were identified as $CD11b^{+}$ (FITC) $CD14^{-}$ (APC) cells (Quadrant Q3)

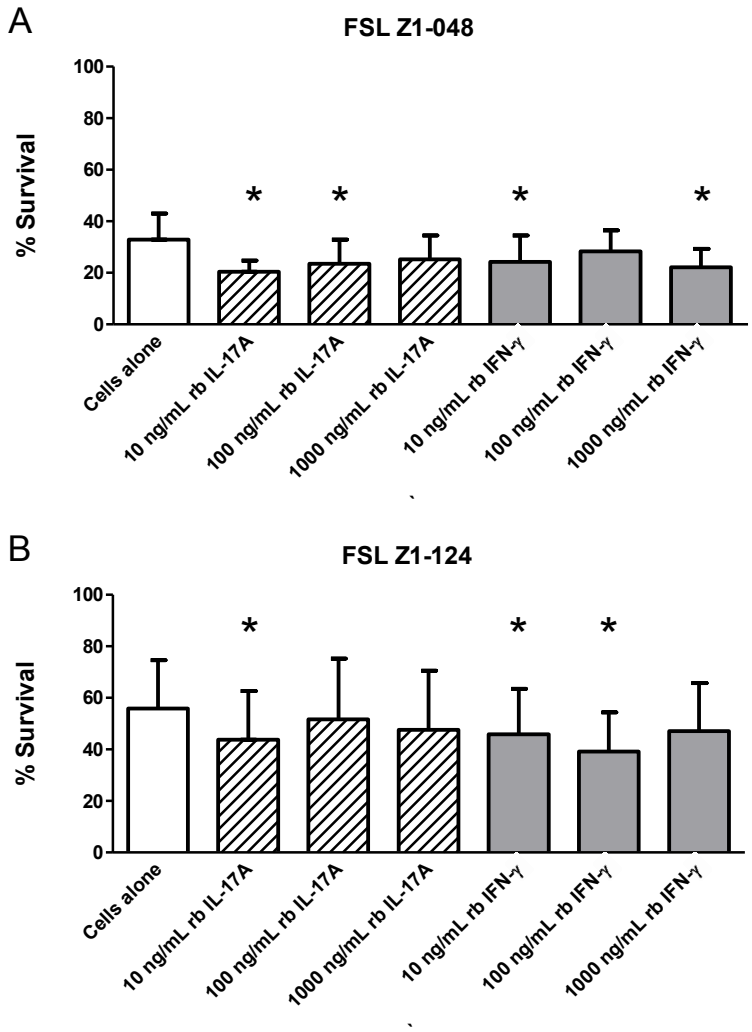

Figure 5. 4 Killing ability of PMN isolated from bovine blood. Cells isolated from blood were co-incubated with *S. uberis* strain FSL Z1-048 (A) or strain FSL Z1-124 (B). Host cells were also stimulated with recombinant cytokines IL-17A (striped bars) or IFN-γ (grey bars) at different concentrations. Means and standard deviations of percentage survival (i.e. bacteria that survived after co-incubation compared with bacteria cultured in medium only) of 4 animals for strain FSL Z1-048 and 3 animals for strain FSL Z1-124. Means significantly different (LSD test, $P < 0.05$) from cell alone were marked by asterisks. "Cells alone" refers to the combination of bacteria and host cells without addition of cytokines.

5. 4 Discussion

In this study we tested the ability of bovine macrophages and PMN to kill two different strains of *S. uberis in vitro.* These strains were chosen as representative of putatively adapted and putatively non adapted strains of *S. uberis* and elicit a different host response when infused in the lactating mammary gland (Chapter 2). In our challenge study we observed an increase in the bacterial count in the milk of cows challenged with strain FSL Z1-048 within 12 and 18 h (Table 2. 3; Fig 2. 4 A and B) whereas strain FSL Z1-124 was isolated only at sporadic time points in a limited number of cows (Fig 2. 4 A and B). This suggests that the differences between the two strains occur at an early stage after the infusion of the bacteria in the teat cistern.

Bovine macrophages were able to kill the putatively non adapted strain FSL Z1-124 *in vitro* whereas they were not able to kill the adapted strain FSL Z1-048. This suggests that differences in the pathogenicity observed *in vivo* in our challenge study (See Chapter 2 and 3) could be, at least partially, explained by the different ability of the macrophages to kill *S. uberis* at the early stages of the infection. *In vivo*, resident macrophages may be able to kill strain FSL Z1-124 and clear the bacteria before infection becomes established. Conversely, strain FSL Z1-048 would not be cleared by macrophages and thus bacteria would be able to replicate within the mammary gland. Macrophages are one of the most represented cell populations in the healthy lactating mammary gland, and are thought to be the first defence in case of infection (Raianrd and Riollet, 2006; Sordillo and Streicher 2002). Due to the technical

problems encountered in the isolation of the milk macrophages resulting in an insufficient number of cells available for the assay in this experiment, we used macrophages derived from blood monocytes. In other studies macrophages isolated from dry mammary gland secretions (Grant *et al*., 1997), milk (Denis *et al*., 2006) or blood monocytes (Denis *et al*., 2006) were used. The killing ability of monocyte derived macrophages in the present study for strain FSL Z1-048 is comparable to that reported for the pathogenic strain 0140J following incubation with milk macrophages (Denis *et al*., 2006). This suggests that ability to resist to macrophage killing may play a role in the pathogenicity of this strain. Although a comparison between the present study and the study conducted by Denis and co-workers needs to be carefully evaluated due to the diverse origin and isolation technique of the macrophages used.

Bovine PMN were able to kill both strain FSL Z1-048 and FSL Z1-124. Differences between strains were observed. Surprisingly, the pathogenic strain FSL Z1-048 was killed more easily than the non-pathogenic strain FSL Z1-124. This results is in contrast with another study where ability to resist to PMN killing was related with the pathogenicity of the strain when infused it the lactating mammary gland (Hill, 1988b; Leigh *et al*., 1990). PMN are present in relatively low number in the healthy mammary gland and they are recruited in large number from the blood following an inflammatory stimulus (Paape *et al*., 2003). Our data suggests that ability of *S. uberis* strains FSL Z1-048 and FSL Z1-124 to invade at an early stage the mammary gland and cause mastitis is not related with the different ability of the two strains to resist to the PMN killing. PMN may, however, play a role in the resolution of the mastitis caused by *S. uberis* strain FSL Z1-048. In fact, in our study we observed a reduction

in the number of bacteria in milk in quarters challenged with strain FSL Z1-048 between 36 and 96 h PI of approximately 10,000 fold (Chapter 2, Figure 2. 4A). The first reduction in bacterial count was coincident with the massive influx of PMN (Figure 2. 6), and ability of PMN to kill S.uberis strain FSL Z1-048 *in vitro* supports this hypothesis. The role of PMN in the resolution of *S. uberis* appears to be highly variable between studies. PMN were not able to reduce the bacterial number following the IMI with *S. uberis* strain 0140J (Bannerman *et al*., 2004) despite the massive infiltration into the mammary gland, whereas for strain UT888 a decrease in the bacterial count was observed only 120 h following the increase of SCC (Rambeaud *et al*., 2003). In another challenge study with strain U103, a reduction of bacteria number was coincident with the influx of PMN (Pedersen *et al*., 2003). Comparison of these studies is difficult due to different challenge model used, however they suggest, together with our observations that ability to resist to PMN killing may vary among *S. uberis* strains and for certain strains such as our challenge strain FSL Z1-048 may play an important role in clearance of the IMI..

The first reduction in the bacterial count in cows challenged with strain FSL Z1-048 was also coincident in four animals with the production of IL-17A in the mammary gland (Chapter 2, Figure 2. 10), suggesting that IL-17A may play a role in resolution of infection. For this reason, the effects of IL-17A on macrophage and PMN function were assessed.

Stimulation of bovine PMN with rbIL-17A increased the killing ability against both strain FSL Z1-048 and FSL Z1-124, at least for low concentrations of IL-17A.

Studies conducted on PMN from humans and mice showed that similar concentrations of IL-17A increase the *in vitro* killing ability of human neutrophils against *Streptococcus pneumomiae* (Lu *et al*., 2008), which was attributed due to the enhancement of phagocytic activity. In our challenge experiment the maximal average concentration of IL-17A in milk was approximately 20 fold lower than the lowest concentration of IL-17A used in the *in vitro* experiment. The concentration in milk however may not be representative of the concentration in the mammary gland tissue or in the alveoli due to the dilution effect. These observations together suggest that IL-17 plays a role in the response to IMI caused by *S. uberis* by increasing the killing ability of PMN in the mammary gland.

We observed an increased *in vitro* bactericidal activity of bovine macrophage against *S. uberis* strain FSL Z1-048 following stimulation with IL-17A. Although macrophages are the least represented cell population in the milk after an inflammatory response (Koess and Hamann, 2008; Schwarz *et al*., 2011a; Schwarz *et al*., 2011b), these data suggest that IL-17 can stimulate mammary gland macrophages which may contribute to the clearance of *S. uberis* infection. A previous study showed that stimulation of mouse bronchoalveolar macrophages or human monocyte-derived macrophages stimulated with IL-17 increased their phagocytosis ability (Silverpil *et al*., 2006). These data further support the hypothesis that IL-17 plays an important role in clearance of extracellular bacteria. A recent study shown that IL-17A plays a role in the clearance of intracellular infection of macrophage with the *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) The authors investigated the mechanisms of action of IL-17A on murine macrophages finding

that IL-17A promotes the clearance of BCG internalized by macrophages by stimulating the expression of inducible nitric oxide synthase (iNOS) and production of nitric oxide (NO) (Ling *et al*., 2013). Similarly to what we observed in the present study for the augmented killing activity of PMN induced by IL-17A the response to the stimulation with this cytokine was found to be dose dependant. Without stimulation of IL17A macrophages are not capable to kill strain FSL-048 this suggests that the presence of IL-17A is necessary also *in vivo* for bacterial killing of this strain by macrophages. This suggests that the clearance of this strain observed in Chapter 2 was correlated with the appearance of this cytokine in the mammary gland. It can be speculated that activated macrophages can contribute with PMN to the clearance of IMI caused by strain FSL Z1-048.

We also observed an increased bactericidal ability of PMN following stimulation with recombinant IFN-γ. IFN-γ is a potent inducer of PMN function such as phagocytosis, production of reactive oxygen species and production of nitric oxide (Ellis and Beaman, 2004). It has also been found to increase the ability of neutrophils from the bovine mammary gland to phagocytise and kill *S. aureus* (Sordillo and Babiuk, 1991). However in our challenge study we detect IFN-γ in the mammary gland of only one cow challenged with strain FSL Z1-048 suggesting that the majority of the cows challenged did not respond to the infection with production of IFN-γ. Thus, the relevance of this mechanism in the resolution of IMI caused by FSL Z1-O48 remains unclear.

In summary, we demonstrate that ability of *S. uberis* to resist to macrophage killing may play an important role in the early pathogenesis of IMI determining the outcome of infection. We also demonstrate that bovine PMN are able to kill a pathogenic strain of *S. uberis*,. This can contribute to the clearance of the infection caused by this strain , possibly with the involvement of IL-17 .

CHAPTER 6

General Discussion

6. 1 General Discussion

The aims of the present study were to test the hypothesis that putatively host adapted strains of *S. uberis* and non adapted strains yield a different host response when infused in the bovine mammary gland. Moreover we tested the hypothesis that an immune memory exists in the mammary gland by a secondary challenge, and that immune memory if present, is strain specific and systemic. Based on the challenge results and literature we investigated *in vitro* some possible mechanisms that could explain the differences observed between the two strains in *vivo*.

We demonstrated in Chapter 2 that the putatively adapted strain FSL Z1-048 elicits a clinical and immune response different than the non-adapted strain FSL Z1-124. Surprisingly the intramammary infection with the putatively adapted strain FSL Z1-048 elicited a clinical mastitis that self cured rather than a persistent sub-clinical mastitis, as observed in the field studies that led to its isolation and characterisation (Zadoks *et al*., 2003). Different criteria for defining host adaptation in *S. uberis* mastitis have been formulated in the past, in addition to the theory proposed by Zadoks (2007). The host adaptation could be considered as the ability to cause IMI in all, or the vast majority, of the cows challenged, as described in early studies that identified strains of *S. uberis* with different abilities to cause IMI (Hill, 1988a). Considering this criteria strain FSL Z1-048 could be defined as host adapted as it was able to cause IMI in all the animals challenged. The infectious dose may be critical for determining clinical outcome following IMI, however, a low challenge dose (200 cfu) of FSL Z1-048 was used in these studies which was comparable to the

dose used in models with strain 0140J (Hill, 1988b; Bannermann *et al*., 2004) which infected all animals challenged and also strain EF20 which failed to infect the vast majority of the quarters challenged (Hill, 1988b). In a separate *S. uberis* challenge experiment approximately 10000 cfu of strain UT888 failed to infect some of the quarters (Rambeaud *et al*., 2003), indicating that even at higher doses some strains of *S. uberis* have limited capacity to infect the bovine mammary gland. The cows challenged with strain FSL Z1-048 excreted *S. uberis* with milk until the end of the study (14 days) with a concentration that reached a peak of 10^8 cfu/mL, and this level of shedding could provide the opportunity for the bacteria to spread amongst the cows as described during the outbreaks caused by this strain. Cow to cow transmissions could then occur at milking via the milking equipment, as described during an outbreak caused by strain FSL Z1-048 (Zadoks *et al*., 2003), or indirectly by contamination of the herd environment.

The differences between the two strains seem to occur at early stages after the entry of the bacteria in the mammary gland. Several mechanisms described by the literature which could explain the differences observed were investigated *in vitro.* These include ability to grow in milk, epithelial adhesion and invasion, resistance to killing by phagocytes and ability to form biofilm.

Ability to grow in milk has been suggested as a virulence factor in mastitis caused by *S. uberis* (Leigh, 1999) and *E. coli* (Kornalijnslijper *et al*., 2003), because a rapid growth would enable bacteria to replicate within the mammary gland at a rate exceeding that by which they are cleared via milking and by the activity of the

phagocytes such as macrophage and PMN. Both the strains used in the present study, although with a different rate, grew well in the milk from the cows used in the challenge experiment. This is in contrast to what has been observed for *E. coli* where faster growth rates in milk were correlated with clinical mastitis (Kornalijnslijper *et al*., 2003). In the studies performed in this thesis, growth of *S. uberis* did not differ between the individual animals suggesting that growth in milk of *S. uberis* is a characteristic of the strains rather than the composition of milk of individual animals. In the challenged experiment cows were milked 9 h after the challenge and milk samples were negative for all the cows challenged with either strain FSL Z1-048 and FSL Z1-124, suggesting that concentration of the bacteria in milk *in vivo* depends on other factors in addition to the mere ability to grow in milk. Other factors are more likely to explain the differences observed in the outcome of the intramammary challenge such as the ability to adhere and invade the mammary epithelial cells and the resistance to the killing of the phagocytes.

Adhesion and invasion is considered an important step in the process of colonization of the mammary gland by *S. uberis* (Tamilselvam *et al*., 2006).The ability of the two challenge strains to adhere and to invade the mammary epithelial cells was investigated *in vitro.* The putatively host adapted strain FSL Z1-048 was approximately 1,000 fold more able to adhere to the mammary epithelial cells after 3 h of co-incubation compared to the non-adapted strain FSL Z1-124. This suggests that the ability to adhere to the epithelial cells could, at least in part, explain the differences observed *in vivo* between the two strains. Adherence to the epithelium would in fact allow the bacteria to resist clearance from the mammary gland as a

result of milk flow during milking. In our study we tested the ability to adhere at 3 h, however, this is a relatively short time period and it would be important to further investigate the adhesion at later time points. This would allow a comparison between the results obtained *in vitro* and the *in vivo* situation. In fact, cows are normally milked with a 12 h interval. It could be hypothesised that during the milking process bacteria that are not attached to the host cells are excreted with milking and only those attached to the host cells and the bacteria in the residual milk are left in the mammary gland after milking.

Both strains were able invade the epithelial cells *in vitro,* but the non adapted strain FSL Z1-124 was more invasive than strain FSL Z1-048. Strain FSL Z1-124 was isolated in a limited number of animals challenged. From these observations it could be hypothesized that once infused in the mammary gland, strain FSL Z1-124 invades the epithelial cells (as demonstrated *in vitro* in the present study, Chapter 4) and is able to survive in the mammary epithelial cells (Tamilselvam *et al*., 2006) but does not multiply significantly intracellularly. Moreover, remaining extracellular FSL Z1-124 bacteria would either be removed by milk flow or killed efficiently by resident macrophage of the mammary gland, as suggested by *in vitro* macrophage killing data (Chapter 5) resulting in clearance of the majority of the infection at early stages. In contrast, strain FSL Z1-048 adheres to the epithelial cells and grows in the alveoli at a rate that exceeds the elimination rate with milking and the elimination by the phagocytic cells present in the milk.

Analysis of the genome, further validated by amplification and sequencing of the target gene, showed that *sua* of strain FSL Z1-048 has a one base deletion which would translate in a truncated form of *Streptococcus uberis* adhesion molecule (SUAM). SUAM has been found to mediate the adhesion and invasion of mammary epithelial cells by *S. uberis in vitro* (Almeida *et al*., 2006). *Sua ,* the gene encoding for this protein, has been detected in many strains of *S. uberis* by PCR amplification (Luther *et al*., 2008), however the presence of mutations which could affect the function of the protein as described for strain FSL Z1-048 has never been evaluated. The presence of strains which do not synthesize SUAM might reduce the efficacy of a vaccine against that elicits antibodies against that molecule.

The potential absence of functional SUAM in strain FSL Z1-048 suggests that adhesion and invasion of the epithelial cells observed *in vitro* for this strain is likely to be mediated by other mechanisms. The presence of other mechanisms of adhesion and invasion are also suggested by a study showing that deletion of the gene encoding SUAM does not abrogate completely the ability to adhere and invade the mammary epithelial cells *in vitro* (Kerro Dego *et al*., 2011).

Recent studies highlighted the importance of Sortase A anchored proteins in *S. uberis*. Sortase A (SrtA) is an enzyme encoded by SrtA gene involved in the anchoring of proteins on the cell wall after synthesis. A limited number of Srt A anchored proteins have been identified by genomic and proteomic analysis. The secretion of SrtA substrates seems to be greatly dependent on experimental growth conditions (Egan *et al*., 2010). A recent paper showed that *S. uberis* mutants lacking

SrtA are less pathogenic than the isogenic wild type strain (Leigh *et al*., 2010). The function of SrtA anchored proteins in *S. uberis* is not known. Authors, comparing the sequence of these proteins with other streptococci species, suggest that two of the SrtA anchored proteins identified in strain 0140J are homologues of proteins known to be involved in adhesion and invasion process in other *streptococci* species. For instance sub1095 is homologue of Collagen like protein SclB of *Streptococcus pyogenes*, which is involved in the adhesion to fibroblasts. Another collagen like protein found in *S. pyogenes*, Sub1145, is involved in the process of adhesion and invasion of epithelial cells. Sub1145 is found to be homologous to a protein found in group B Streptococci involved in the invasion of human epithelial cells. It could be hypothesized that lack of SrtA impairs the anchoring of proteins involved in the adhesion to host cells. Although out of the scope of this study presence of SrtA was confirmed in the genome of the two *S. uberis* challenge strains. It is therefore unlikely that the low pathogenicity of strain FSL Z1-124 is due to the lack of this enzyme.

Much of the research conducted in the past on adhesion and invasion of *S. uberis* has focused on SUAM. However our findings that FSL Z1-048 is able to adhere to the mammary epithelial cells even if not encoding for a functional form of SUAM, and recent literature data on SrtA anchored proteins suggest that other adhesins might play a role in adhesion and invasion of mammary epithelial cells by *S. uberis*.

As in previous studies, the *in vitro* adhesion and invasion data in this thesis were obtained using immortalized cell lines. In this study BME-UV1 cells were used. This cell line retains physiological functions of the mammary epithelium such as tight

junctions (Quesnall *et al*., 2006). However, since adhesion of *S. uberis* to primary epithelial mammary cells is considerably lower than that observed using MAC-T cell line (Ditcham *et al* 1996; Lammers *et al*., 2001) and little or no adhesion is observed *in vivo* with *S. uberis* (Thomas *et al*., 1994), the use of immortalized cell lines such as BME-UV1 and MAC-T cells for studying adhesion and invasion may not be optimal. It would therefore be important to further investigate the adhesion and invasion ability of *S. uberis* using a system as close as possible to the *in vivo* situation. For instance, this could be performed *in vitro* using primary epithelial cell cultures (Lammers *et al*., 2001) which retain more of the *in vivo* characteristics. Interaction with the cells and localization in the mammary gland of *S. uberis* bacteria could also be investigated *in vivo* using various techniques such as immunohistochemistry, immunofluorescence or fluorescent *in situ* hybridization (FISH). FISH has been used for detection of *S. uberis* bacteria in sheep mammary gland tissue (Addis *et al*., 2013), and although the technique needs further improvement to augment the specificity, it could be used in cattle samples. Tissue samples could be obtained from the mammary gland of animals with a natural case or an experimentally induced *S. uberis* mastitis at *post mortem* or from living animals by mammary gland biopsy as described for *E. coli* (Dogan *et al*., 2006), and co-localisation of bacteria with epithelial or other cells determined. At the end of the secondary challenge experiment (Chapter 3), tissue samples of the mammary gland were collected for further analysis. Although out of the scope of this study, preliminary results of gram stained tissue samples showed clusters of bacteria associated with alveolar mammary epithelial cells in those quarters that were secondary challenged with strain FSL Z1-048.

Although not able to induce mastitis in our challenge experiments, strain FSL Z1-124 was isolated from a clinical case of mastitis affecting a heifer at parturition (Zadoks, personal communication) suggesting that is not a completely a-virulent strain. It could be hypothesised that this strain and other non adapted strains can cause the intramammary infection only when the host defences are impaired as for other environmental pathogens. This can happen frequently during the peripartum period and early lactation. The negative energy balance in the early lactation can cause hyperketonemia which impairs the udder defence against pathogens (reviewed by Suriyasathaporn *et al*., 2000). Ketones can reduce the bactericidal activity of bovine macrophage *in vitro* by reducing the production of oxidative species and by reducing their phagocytosis activity (Cerone *et al*., 2007). Considering our *in vitro* observations which demonstrated efficient killing of FSL Z1-124 by monocyte-derived macrophages (Chapter 4), it could be speculated that during the peripartum period macrophages resident in the mammary which normally kill non adapted *S. uberis* strains such as FSL Z1-124 are unable to kill the bacteria allowing them to replicate. Mastitis at parturition can be also caused by pathogens that infect the mammary gland during the dry period and persist until the calving without signs of mastitis, as suggested for *E. coli* (Quesnell *et al*., 2012). Authors suggest that the inflammatory response in the mammary gland is suppressed during the late gestation due to an IL-10 mediated Th2 type response, which allows bacteria to survive intracellularly in the epithelial cells. At parturition, a shift toward a Th1 type response causes the appearance of clinical signs. Given the ability of *S. uberis* to infect dry cows (Zadoks, 2007) and to invade the epithelial cells as suggested by both

the literature (Almeida and Oliver, 2006) and by the studies in this thesis (Chapter 4), it is possible that this mechanism could play a role in IMI caused by *S. uberi*s. This could be investigated in challenge studies of dry cows or periparturient cows.

S. uberis is a highly heterogeneous bacterial species (Leigh, 1999, Zadoks 2007). It would therefore be important to extend the observations made *in vitro* for the two strains FSL Z1-048 and FSL Z1-124 to other strains. Several putatively host adapted and non-adapted strains have been collected and are available for further experiments. In addition to the criteria that have been used to define host adaptation characteristics in this study, other criteria can be used to select strains. For instance strains can be selected based on the host species (i.e. ovine and bovine stains, Gilchrist *et al*., 2013). It would be possible to repeat the *in vitro* assays conducted in the present study to determine if the differences observed between the two *S. uberis* strains are common amongst other host adapted and non adapted strains. This would be important in order to avoid approaches in mastitis management and/or vaccine development based on data obtained from a limited number of strains which could target antigens or mechanisms present in only a limited number of *S. uberis* strains.

The primary challenge with strain FSL Z1-048 showed that clearance of the infection could be due to the influx of PMN which cause the initial reduction of the bacterial concentration and to the influx in the mammary gland of T-cells at later stages. A recent study showed that bovine $CD8^+$ T cells are able to directly kill *S. uberis in vitro* (Denis *et al*., 2011), providing a possible explanation for our observations. The efficacy of PMN in controlling *S. uberis* is debated since some strains induced a

massive influx of PMN which is not able to clear the infection (Rambeaud *et al*., 2003; Bannerman *et al*., 2004), whereas in another study the influx of PMN was concomitant with a drop in the bacterial concentration (Pedersen *et al*., 2003). The differences observed could be due to the different strains used in these models. Following our observation made *in vivo* that the influx of PMN coincides with the initial drop in bacterial concentration (described in Chapter 2), we tested the ability of bovine PMN to kill the challenge strains *in vitro.* Bovine PMN were able to kill approximately 70% of FSL Z1-048 bacteria following co-incubation for 90 min, indicating that the influx of PMN could have been at least partly responsible for the clearance of bacteria from the mammary gland.

To further study the observations made in this thesis, mathematical modelling could be used. This study provides several parameters that could be used to generate a mathematical model which describes the intramammary challenge. Mathematical models have been used to study various aspects of intramammary infection in cattle. For example, a predator prey model was developed to study the role of neutrophils in response to *Staphylococcus aureus* (Detilleaux *et al*, 2004), using a simple model considering bacterial growth and PMN killing rate. Another group (White *et al*., 2009) developed a model with the aim of describing the outcome of intramammary infection (e.g. transient or persistent) with *E. coli*, taking in account pro-inflammatory and anti-inflammatory cytokine responses. The present study provides several parameters obtained *in vivo* and *in vitro* that could be used to construct a mathematical model to further study the role of single components of the host response. These data include: growth rate *in vitro*, growth *in vivo*, PMN and

macrophage killing, adhesion to and invasion of mammary epithelial cells *in vitro*, concentration of cytokines *in vivo*. For instance, a simple model could be constructed using the data obtained *in vitro* on PMN killing and the effect of IL-17A on bactericidal activity of these cells to elucidate the role of PMN, IL-17 and T-cells in the clearance of *S. uberis* infection, determining for example if the presence of IL-17 is necessary to clear the infection as observed in our challenge study in 4/6 cows.

Recent data highlights the importance of the early immune events during the intramammary infection (Schukkent *et al*., 2011, Wellnitz and Bruckmeier 2012). One area that could be investigated to explain the differences between host adapted and non adapted *S. uberis* strains is the immune response of the mammary epithelial cells following the interaction with *S. uberis*. A recent transcriptomic study showed that epithelial cells along with infiltrated immune cells play an important role in the response to *S. uberis* IMI (deGreeff *et al*., 2013). Moreover, mammary epithelial cells respond differently *in vitro* to different strains of *S. uberis*. The two strains chosen present also a different clinical response in the live animal (Wellnitz *et al*, 2013). The authors suggest that a stronger immune response of the epithelial cells in response to one of the two strains tested leads to the production of pro-inflammatory cytokines such as IL-8 and IL-1β which start the inflammatory response that ultimately clears that strain *in vivo*. Similar results were obtained in a microarray study showing that primary epithelial cells isolated from animals with high susceptibility to mastitis had a lower immune response to either *E. coli* and *S. aureus* than cows with low susceptibility to mastitis (Brand *et al*., 2011). In our challenge study the concentration of pro-inflammatory cytokines in milk from cows challenged

with non-pathogenic strain FSL Z1-124 was undetectable except for IL-8 at limited time points. However, the ELISA tests used in these studies is not able to detect very small changes in cytokines measured due to a lack of sensitivity. Therefore, the possibility that strain FSL Z1-124 is recognized earlier by the innate immune system cannot be ruled out and further studies would be needed to determine if any immune response is generated. The inflammatory response of mammary epithelial cells to the strains used in the present study could be further studied *in vivo* or *in vitro*. Tissue samples from the mammary gland could be collected from experimentally challenged animals and used for microarray analysis (Swanson *et al*., 2009, de Greaff *et al*., 2013) and IHC could be used to identify leukocyte migration into the mammary gland. The use of *in vitro* mammary gland primary cell cultures or immortalized cell lines would allow a more accurate dissection of the response due to the use of a single cell type. Tissue slices have been used for lung infection models (Liberati *et al*., 2013). It may be possible to develop a similar technique for the mammary gland. The main advantage of using tissue slices compared to the tissue sample is that it is possible to exclude the inflammatory cells migrating in the tissue in response to the stimuli. Expression of genes of interest in these *in vitro* systems could be measured with quantitative RT-PCR or microarray analysis, whereas production of the protein product could be quantified by ELISA.

Production of cytokines and other inflammatory mediators could also be investigated with proteome analysis. A recent study measured the production of several inflammatory mediators in the mammary gland in response to *S. uberis* mastitis (Bislev *et al*., 2012a). In this study the peptides were extracted from the tissue,

however it is also possible to perform proteomic analysis on cell cultures and milk. A database is also available providing data for proteomic analysis of bovine mammary gland peptides (Bislev *et al*., 2012a). Proteome targeted analysis is capable of quantification of peptides present in very low concentration.

Along with management measures such as mastitis control plans, vaccination is considered an important tool in the reduction of IMI in dairy cattle (Denis *et al*., 2009, Bharathan and Mullarky, 2011). However, the development of an effective vaccine against mastitis pathogens, and particularly against *S. uberis*, has proven difficult (Leigh 1999, Denis *et al*., 2011). It is still a matter of debate if the mammary gland is able to mount an efficient immune response against the most common mastitis pathogens after an immunization. Studies conducted on natural cases of mastitis showed no protection in cows following an IMI to a subsequent case of IMI (Schukken *et al*., 2009, Schukken *et al*., 2013). This however could have be due to different strains involved in the subsequent infections. Experiments conducted using immunogens such as tuberculin (Nonnecke *et al*., 1986) and ovalbumin (Rainard *et al*., 2013) showed that the mammary gland presents an immunological memory as defined by an inflammatory response in the animals immunized a response following the re-challenge with the same immunogen used. Milk lymphocytes are however less responsive than blood lymphocytes (Nonnecke and Kehrli, 1985; Nonnecke *et al*., 1986) and this could explain the lack of protection to the IMI.

In our study we showed that previous IMI with *S. uberis* does not induce complete protection against a subsequent *S. uberis* infection. However a degree of protection,

with a reduction of bacterial concentration and a reduction of inflammatory cytokine responses, was observed in quarters primary challenged with strain FSL Z1-048 and secondary challenged with the same (homologous) strain. Similar findings with partial protection were reported by vaccines studies conducted using killed (Finch *et al*., 1994) or live (Finch *et al*., 1997) *S. uberis*.

A limitation of the present study was represented by the high SCC in quarters primary challenged with strain FSL Z1-048 at the time of the secondary challenge. High SCC is protective against IMI (Schukken *et al*., 1999) although unacceptable as mean of control in dairy farming as it reduces the quality of milk. The presence of high SCC at challenge did not allow to determine if the protective response observed was due to the PMN infiltration itself as a result of the primary challenge, or to an immunological adaptive memory response. The flow cytometry data, however, suggested that a memory immune response, characterised by a more rapid expansion of T cell populations (CD4, CD8 and γδ) within the milk, was present after the secondary challenge. A protective response characterised by lymphocyte influx into the mammary gland has been observed before following *S. uberis* vaccination experiments (Finch *et al*., 1994; Finch *et al*., 1997). This suggests that the increased influx of mammary lymphocyte populations following secondary challenge may play a role in protection.

Lymphocyte responses are considered crucial in the development of vaccine against mastitis pathogens (Denis, 2009; Barathan and Mullarky, 2011). Although antibody responses can be induced in the mammary gland they are not thought to be

protective: In fact the presence of antibodies against *S. uberis* do not increase the killing activity of neutrophils. (Leigh and Field, 1994). It is therefore important to further study the cell mediated immune response to *S. uberis* IMI. The secondary challenge experiment described in Chapter 3 could be repeated using strain FSL Z1-048 strain as primary and secondary challenge strain. Given the low pathogenicity of strain FSL Z1-124 other secondary heterologous strains might be selected to be tested as secondary challenge stains to test the strain specificity of the immune response.

To allow the SCC to return to the physiological level (i.e. < 200,000 cells/mL) and avoid difficulties in interpreting the mechanism(s) of protection following secondary challenge, a longer rest period could be given to the challenged animals prior to the secondary challenge. It would be important to increase the frequency of sampling for flow cytometry analysis to allow a more precise study of the timing of the influx of lymphocytes in the mammary gland. To further study the role of cell mediated immune response, T-cells could be isolated from the blood and from the milk of the challenge quarters. The isolation could be achieved using antibody coated magnetic beads as described by Denis *et al*., (2011). Once isolated cells can be cultured and used for a range of experiments, such as re stimulation with *S. uberis* antigens to determine if *S. uberis* specific T cells are present if so, characterise the responding cell types.

IL-17A is the prototypic member of a family of cytokines which act as effectors of the Th17 response (Curtis and Way, 2009). Its function is to potentiate the innate

immune mechanisms, such as the recruitment functional enhancement of phagocytes and production of antimicrobial compounds (Pappu *et al*., 2011). The Th17 response is considered particular important in response to extracellular pathogens such as bacteria (Curtis and Way, 2009) and there is growing evidence that IL-17 is important in the context of the immune response to IMI (Bharathan and Mullarky, 2011; Rainard *et al*., 2013). IL -17A was detected in milk of 4 cows following the primary challenge with strain FSL Z1-048. This, to our knowledge, is the first description of IL-17A at protein level in response to an intramammary infection in cattle. The challenge data also suggest that IL-17A is involved in the clearance of the intramammary infection. In fact, the increase in concentration of IL-17A in milk coincided with the decrease of the bacterial concentration.

The effect of IL-17A toward phagocytes present in the mammary gland during the intramammary infection with *S. uberis* was further investigated *in vitro* showing an increased killing ability of bovine PMN stimulated with recombinant IL-17A toward strain FSL Z1-048. Moreover the stimulation of bovine macrophages with recombinant IL-17A augmented the killing activity of these cells against strain FSL Z1-048. These data together suggest that IL-17A plays a role in the clearance of the IMI of *S. uberis* by stimulating the killing activity of phagocytes. Since the vast majority of the cells present in the milk after the challenge were PMN this potentiating activity of IL-17 on phagocyte function would be potentially important *in vivo*.

Recent studies conducted *in vitro* (Bougarn *et al*., 2011) and *in vivo* (Rainard *et al*., 2013) have shown that IL-17 up-regulates the production of peptides by mammary

epithelial cells with anti-bacterial functions such as calgranulin A, calgranulin B, Lingual antimicrobial peptide (LAP) and Tracheal antimicrobial peptide (TAP), as well as production of inducible. Nitric oxide synthases (iNOS) (Bougran *et al*., 2011). These compounds may contribute to the response observed against the intramammary infection with *S. uberis*.

IL-17 is mainly produced by Th17 $CD4^+$ lymphocytes (Curtis and Way, 2009), and in our challenge study we observed the increase of IL-17A in milk coincident with the influx of $CD4^+$ cells in the mammary gland. This suggests that $CD4^+$ T cells were the source of IL-17, although these observation needs to be carefully considered due to the limited number of sampling points for the flow cytometry analysis. A recent study (Rainard *et al*., 2013) showed that during mastitis caused by intramammary challenge with Ovalbumin, IL-17 is produced in the mammary gland, further supporting a role for IL-17 in bovine mastitis. The source of IL-17 in response to the intramammary infection with *S. uberis* could be further investigated using the challenge model that we developed. T cells could be isolated from the milk following the challenge with strain FSL Z1-048 and intracellular labelling for IL-17 by flow cytometry could be used to determine if T-cells are the indeed source of this cytokine at protein level.

Results of the present study further support the hypothesis that Th17 response plays a central role in the context of the mammary gland immunity, as illustrated in Figure 6. 1. In support of this hypothesis it has been found that protective immune memory induced by vaccine against a number of extracellular pathogens such as

Streptococcus pneumoniae (Lu *et al*., 2008; Moffit *et al*., 2011), *Klebsiella pneumoniae* (Chen *et al*., 2011) and *Bordetella pertussis* (Warfel and Merkel., 2013) is mediated by CD4 Th17 cells. The development of a vaccine toward *S. uberis* and other mastitis pathogens that stimulates a Th17 response is therefore an attractive option (Bharathan and Mullarky, 2011).

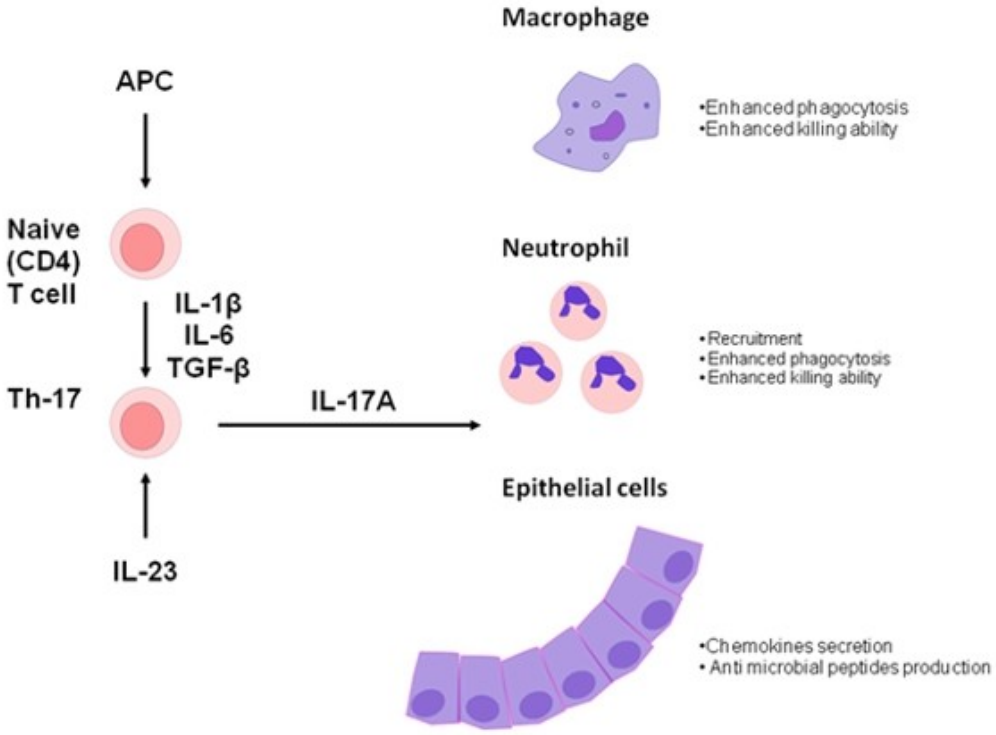

Figure 6. 1 Effects of IL-17A on phagocytes and epithelial cells.

6. 2 Conclusions

In conclusion the present studies demonstrate that host adapted and non adapted strains of *S. uberis* elicit different clinical and immune responses after experimental challenge of lactating dairy cows. These differences may be due to different interactions of the two strains with mammary gland macrophages and the mammary epithelial cells but do not appear to be associated with their ability to grow in milk. These observations need further investigation to determine the mechanisms involved and to extend these conclusions apply to other strains. PMN, lymphocyte responses, and in particular the IL-17 response appear to be involved in the reduction of the bacterial load observed *in vivo* leading to the clearance of the IMI caused by the adapted strain, and are worthy of further investigation.

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