An experimental challenge model in lactating dairy cows using *Streptococcus uberis* for antibiotic efficacy testing

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Abstract

The aim of this project was to develop a challenge model to test the efficacy of novel intramammary antimicrobial treatments for clinical mastitis. The use of the model, can reduces the costs of testing efficacy and accelerate the process of registration of new products. It provides controlled conditions which safeguard animal welfare.

The experimental challenge model using *Streptococcus uberis* developed in this thesis can provide the pharmaceutical industry and animal health research groups with a cost-effective method to test the efficacy of new antimicrobial products for treatment of mastitis in a safe and controlled environment. Two Cloxacillin-based antimicrobials with different formulations and treatment frequency were tested for their efficacy to cure *S. uberis* infections after infections were induced using the challenge model developed as described in the third chapter of this thesis.

The objective of the first study presented in this thesis was to choose one suitable strain from four strains of *S. uberis*, to be used in future challenge studies. Four strains were tested for their virulence and susceptibility to antibiotic therapy. A further study objective was to determine the dose (number of pathogens infused, expressed as colony forming units (CFU)) required for the tested strains to produce an acceptable proportion of clinical mastitis cases to enable future studies. The strain which accomplished the desired characteristics was then chosen and was utilised for experimental challenge in further studies (Chapters 4 and 5). The overall incidence of clinical mastitis obtained in this study at a quarter level was 54% (26/48). This study showed significant differences in the ability of different strains of *S. uberis* to cause clinical mastitis when inoculated via the intramammary route. However, only one of the four strains tested demonstrated favourable characteristics as a strain to be used in experimentally induced clinical mastitis studies.

Chapters 4 and 5 describe two challenge studies conducted using the experimental challenge model (Chapter 3) to test the efficacy of different antimicrobial drug formulations. In Chapter 4, the cure rate of one cloxacillin based product applied every 24 hr. was compared with the cure rate of a penicillin-based product applied every 12 hr. During the observation period of this investigation all challenged cows developed clinical

mastitis in at least one quarter. The incidence of clinical mastitis at the quarter level was high, with 91.25% (73/80) of challenged quarters being affected. After diagnosis of infections, the cows were randomly allocated to two treatment groups and treated accordingly. Clinical cases in which the quarter did not respond to three applications of the allocated antimicrobial product received an extended treatment of the same product. As the allocation to the extended treatment was not random, clinical and bacteriological cures were statistically evaluated for the short treatment only. Clinical cure rates for the short treatment (3 syringes) were 52.63% and 43.75% for the cloxacillin- and penicillin-based products, respectively. There was no significant difference between the treatments (P = 0.8) in their efficacy for the treatment of experimentally induced *S. uberis* clinical mastitis.

In Chapter 5, two long-acting cloxacillin containing products were compared in their efficacy to cure experimentally induced *S. uberis* infections. One commercially available product was compared with a novel long acting product (applied every 48 hr.). Out of 80 challenged quarters, 41 quarters developed clinical mastitis after inoculation (51.25%). Treatment with the novel product resulted in a total treatment success rate of 93.1% based on clinical examination, and 96.0% based on the bacteriological cure rate. Treatment with the control product resulted in total treatment success rate of 100% based on clinical and bacteriological cure rate. There was no significant difference between the products (P=0.19) in their efficacy for the treatment of experimentally induced *S. uberis* clinical mastitis.

Results in this thesis showed that experimental challenge models can be a useful tool in animal research to test the efficacy of new products in a safe and cost effective manner.

Acknowledgements

"Doing what you like is freedom,

       Liking what you do is happiness"…

            (Frank Tyger)

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List of Abbreviations

ATB: Antibiotic

BA: Blood Agar plates

BAGG: Buffered Azide Glucose Glycerol Broth

BSCC: Bulk somatic cell count

CAMP: Christie Atkins and Munch Petersen

CFU: Colony forming units

CI: Confidence interval

CLSI: Clinical Laboratory standards Institute

CM: Clinical Mastitis

CNS: *Coagulase Negative Staphylococci*

CO_2: Carbon Dioxide

DCT: Dry cow therapy

FR/RL or FL/RR: Front-Right and Rear-Left or Front-Left and Rear-Right

Hr.: Hours

IVABS: Institute of Veterinary, Animal and Biomedical Sciences

MIC: Minimum inhibition concentrations

NEB: Negative energy balance

NMC: National Mastitis Council

PBS: Phosphate buffered saline

PEB: Positive energy balance

PFGE: Pulsed-field gel electrophoresis

PMN: Polymorphonuclear cells

rm ANOVA: Repeated measure**s** analysis of variance

RR: Relative risk

SCC: Somatic cell count

SCS: somatic cell scores

S. uberis: Streptococcus uberis

Staph. aureus: Staphylococcus aureus

TS: time of sampling

CHAPTER 1

General introduction

1 General Introduction

Many diseases associated with the intensification of animal production have been demonstrated to be related to, and impact upon animal production and welfare. These diseases produce varying economic losses. Infectious diseases including parasitic diseases usually accompany intensification of animal production. Animal illnesses such as mastitis, metabolic disease, reproductive and nutritional problems affect farm profitability. Mastitis is currently the most important production animal disease impacting on the dairy industry worldwide (Hogeveen et al., 2011).

Clinical and subclinical mastitis have major economic consequences for the dairy industry. Mastitis can directly affect milk quantity and quality at any stage of lactation (Auldist and Hubble, 1998, McDougall et al., 2007). In extreme situations it may transform milk from a quality, profitable product, to a non-profitable product of poor quality. This may necessitate the farmer to treat and attempt to cure affected animals and, at some stage, try to control the illness on the farm. Mastitis can be and usually is caused by micro-organisms, particularly bacteria which invade the udder and in many cases are contagious. As such, it requires rapid detection and treatment to avoid the spread of the causal micro-organism around the herd (Hogeveen et al., 2011). As the efficacy of the drugs used to treat the mastitis-causing bacteria varies, farmers should carefully select the drugs used for treatment of clinical mastitis cases.

Experiments to determine the efficacy of products such as antibiotics and teat sprays are fundamental in mastitis research. One way to test products is through the use of challenge models. These models have been shown to be highly effective, rapid and a cost effective tool for efficacy tests (Oliver et al., 2003). An experimental challenge model is a study design that attempts to mimic a real-life scenario but with reduced variability. With regards to mastitis, one challenge model consists of the introduction of a mastitis-causing agent into the udder, resulting in the induction of a controlled mastitis infection. In the field of mastitis research, studies carried out using natural infections usually take much longer to produce results compared with challenge models, can be costly and require the use and monitoring of many more animals (Zadoks et al., 2001, Rossitto et al., 2002, Taponen et al., 2003, McDougall et al., 2007).

Mastitis knowledge can be generated using experimental challenge models (Heald, 1979, Finch et al., 1994, Brouillette et al., 2004) because the cause and consequences of the disease and the efficacy of the treatments can be studied in detail in a controlled environment. The use of challenge models has also been considered helpful when studying the pathogenicity of the agents affecting the mammary gland (Pyörälä and Syväjärvi, 1987, Bannerman et al., 2004).

Three studies designed to advance research into mastitis treatments are presented in this thesis. Since *S. uberis* is among the most common mastitis-causing agents isolated from New Zealand dairy cattle (McDougall, 1998, McDougall et al., 2007, Petrovski et al., 2011c), this pathogen was chosen for the studies documented here.

This thesis consists of seven chapters. The introduction chapter and the literature review are presented in Chapters 1 and 2. In Chapter 3 (the first field study), testing of organisms with a view to developing an experimental challenge model is described. The objective of the study was to choose one suitable strain from various strains of *S. uberis* to be used in future challenge studies. Four strains were tested for their virulence and their susceptibility to antibiotic therapy. A further study objective was to determine the dose (number of pathogens expressed as colony forming units (CFU)) required for the tested strains to produce an acceptable proportion of clinical mastitis cases when they were infused into the udder. The strain which accomplished the desired characteristics was chosen from that study and was utilised for experimental challenge in further studies (Chapters 4 and 5).

Chapters 4 and 5 describe two challenge studies conducted using the experimental challenge model (developed in Chapter 3) to test the efficacy of two new products intended for use in treatment of mastitis.

Finally, Chapter 6 presents a general discussion on the studies and their relevance to mastitis research. In the final chapter, recommendations for future research based on the findings from the current work are presented.

CHAPTER 2

Literature review

2   Literature review

2.1   The New Zealand dairy farming system

The main characteristic of the New Zealand dairy industry is the grazing system used in most farms throughout the country. Low production costs, relatively high milk prices and the seasonality of New Zealand dairy production are also characteristics of this system (Holmes, 2002). The year-round pasture grazing system for New Zealand cattle, shares some features with systems operating in South America, South Africa and some parts of Australia. Conversely, in many other countries where the dairy industry is successfully developed, cows are fed cereal grains and/or preserved pasture as the only feeds – at least for some periods of the year, or spend the winter enclosed in sheds or barns (e.g. Europe, Canada and some parts of the United States of America) due to the weather conditions.

In New Zealand, seasonality of the system is also reflected in synchronised parturition and lactation which results in a variable composition of the milk produced throughout the year, affecting the milk industry and its manufactured products (Auldist and Hubble, 1998). Milk yields and lactose concentration are reported to be highest during the postpartum period, decreasing steadily until late lactation. On the contrary, fat tends to increase as lactation proceeds (Auldist et al., 1995). The average somatic cell count (SCC) in healthy lactating cows is approximately 100,000 cells/mL of milk, rising to 1,000,000 cells/mL or more as a consequence of intramammary bacterial infections (Auldist et al., 1995, Sordillo et al., 1997, Rambeaud et al., 2003). However, high SCC and protein percentages are also seen as normal physiological findings, at the beginning and the end of lactation (Auldist et al., 1995).

As with the variability in milk constituents and SCC, the susceptibility of cows to mastitis-causing agents also varies within lactation. The periods of major susceptibility to mastitis are the peripartum and dry periods. In these periods, the immune system of the cow is suppressed by stress, by hormonal changes of advancing pregnancy or parturition and by negative energy balance (Bradley and Green, 2004, Moyes et al., 2010). This causes an increase in the susceptibility of cows to mastitis infections and a lowered response of the innate immune system (Sordillo and Streicher, 2002, Oliver et al., 2004).

In addition, environmental factors like low solar radiation, soil and air temperature and higher moisture conditions during calving season in New Zealand, have also been demonstrated to be closely related to *S. uberis* infection rates in pasture-based dairy systems (Lopez-Benavides et al., 2007).

These topics are addressed in more detail in the final discussion of this thesis.

2.2 Definition of mastitis and somatic cell counts

Mastitis is an inflammatory reaction of the mammary gland usually caused by bacterial, mycotic or algal infection. Inflammation of the mammary glands may also be caused by mechanical, thermal or chemical trauma (Zhao and Lacasse, 2008). However, the most common causes of mastitis are infections by various pathogenic agents, particularly bacteria (Zhao and Lacasse, 2008).

Clinical and subclinical mastitis are characterised by a reduction in milk yield and altered milk composition. The damage produced by mastitis agents in quarters and udders varies in severity, from a simple inflammatory reaction of the mammary epithelial tissue with a consequential rise in SCC and changes in milk composition, to alveolar cell death and consequently, losses in yields of milk constituents (Zhao and Lacasse, 2008). In severe cases, the infected quarter ceases milk production (Capuco et al., 1986, Auldist et al., 1995, Sordillo et al., 1997). Some agents (i.e. *Escherichia coli, Staphylococcus aureus* and *S. uberis*) are capable of producing gangrenous mastitis, in which, if the cow survives, the udder tissue of the quarter affected can become completely necrotic (Habuš et al., 2009).

Somatic cell counts are commonly used as an indicator of mammary health in the herd as they increase in the presence of mastitis infection (Harmon, 1994, Sordillo et al., 1997) and are easily obtained. The predominant cell types present in milk from healthy udders are macrophages, (35-79%), lymphocytes (10-24%), polymorphonuclear leucocytes (PMN; 3-26%) and dead epithelial cells (2-15%). In acute cases of clinical mastitis the SCC can reach millions of cells per millilitre and during infection, up to 90% of the cells in milk can be PMN cells (Zhao and Lacasse, 2008). Subclinical infections usually occur without clinical signs, or in other cases start with an acute and clinical infection that cannot be cleared completely and which then becomes subclinical after a few weeks. A rise in SCC can be the

only sign observed with this kind of infection (de Haas et al., 2002), thus making it more difficult to identify.

2.2.1 Mechanisms of infection and host response

Intramammary infections originate from the penetration of the infective agents (usually bacteria) through the teat canal, followed by migration and adhesion to mammary cells and bacterial proliferation (Figure 2-1). The first physical barriers of the mammary gland are the streak or teat canal, and its keratin lining which provides physical and chemical protection (Craven and Williams, 1985, Sandholm et al., 1990, Capuco et al., 1992). If the agent succeeds in defeating these first barriers, a second defence that the bacteria must overcome is the humoral and cellular defence mechanism of the immune system. Here, PMN cells (neutrophils, macrophages and natural killer cells) invade the alveolar lumen from the blood stream or from the walls of the alveoli (Figure 2-1) (Craven and Williams, 1985, Capuco et al., 1986, Sordillo et al., 1997, Sordillo and Streicher, 2002). This phase is accompanied by an increase in the SCC in milk as the cow's immune system attempts to eradicate the infection. If the micro-organisms cannot be eliminated at this stage and invasion reaches the alveolar epithelium, invasion progresses with parenchymal damage and further increases of SCC. Polymorphonuclear leucocytes in the ductular and alveolar lumen engulf bacteria (phagocytosis) and lysosomal enzymes are released in the lumen that damage alveolar cell membranes (Capuco et al., 1986). In some cases, the tissue damage can be so severe that the milk-blood barrier breaks down and extracellular fluid leaks into the milk, giving the milk a "watery" appearance that can be visually detected (Zhao and Lacasse, 2008).

The damage caused by infection can be temporary or permanent (Trinidad et al., 1990, Oliver and Calvinho, 1995). However, the economic cost produced as a consequence of decreased milk yields and quality are permanent (Auldist, 2003). That is, the wasted milk cannot be regained and in some cases, the milk quality does not return to normal.
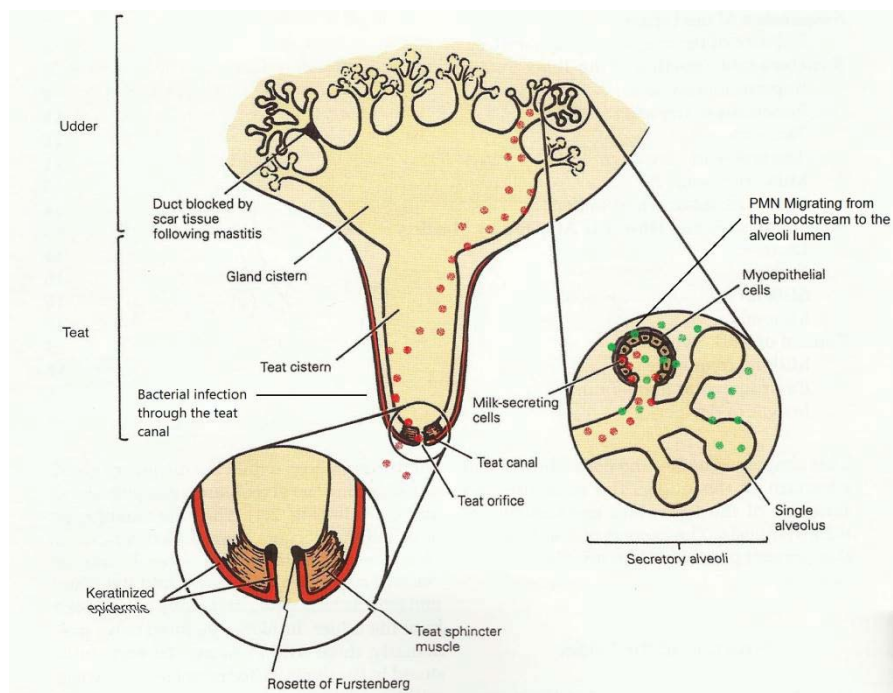
Figure 2-1: Mastitis Infection mechanisms. Adapted from Blowey and Edmondson, (2010)

2.3 Mastitis-causing bacteria

The classifications of bacteria which can infect the mammary gland can be based on:

A) The source of infection

B) The damage produced to the tissue

2.3.1 Classification of the clinical mastitis agents according to the source of infection

Based on the source of infection, agents are usually classified as "contagious" and "non-contagious", or "environmental" agents. Typical contagious agents are *Staphylococcus aureus* and *Streptococcus agalactiae*. Although not commonly found in countries other than the USA, *Mycoplasma* spp. can be also considered as a contagious agent (Fox et al., 2005). The characterisation of a mastitis-causing agent as "contagious" is based on the concept that infections are transmitted during milking and/or they are transmitted directly from cow to cow and the infected cows are the reservoir of the causal agent in the herd.

Conversely, environmental agents are for example *S. uberis, S. dysgalactiae, E. coli* and coagulase negative *Staphylococcus* spp. Such bacteria do not necessarily live in the mammary glands and they can be found in the cows' environment, in the bedding, in the soil, on the cows' skin and in faeces (Zadoks et al., 2001).

Clear-cut classification into contagious agents or environmental agents is weakening over time as there are bacteria from the former group found in the environment and bacteria from the latter group, that pass from cow to cow during milking, for example *S. uberis* and *S. dysgalactiae* (Zadoks et al., 2003), thus blurring the line between contagious and environmental agents.

2.3.2 Classification of the mastitis agents according to the damage induced to the tissue

Mastitis-causing agents can also be classified as "major" and "minor" based on the damage caused in the udder tissue. Under this classification, major agents like *Staph. aureus, S. uberis* and *E. coli*, are associated with major clinical signs, elevated SCC and rapid, marked decreases in protein, lactose, fat and milk yields. Minor pathogens such as *Corynebacterium bovis* and coagulase negative *Staphylococcus* spp. are associated with these changes in milk quality but to a minor extent (Benites et al., 2002, Coulona et al., 2002).

2.4 Major mastitis-causing agents

In clinical mastitis caused by major pathogens, histological changes produced in the tissue become evident at early stages of the microbial infection. Oedema, cell damage and PMN leucocyte infiltration, accompanied by signs of tissue repairing processes such as the increment of connective tissue and fibrosis are common findings in mastitis cases caused by major pathogens (Benites et al., 2002). However, there are some particular features that characterise the damage produced by different agents.

The major pathogen chosen for the development of the studies in this thesis was *S. uberis* and thus, it will be the focus of the literature review. Other major mastitis-causing agents such as *Staph. aureus* and *E. coli* will be described briefly.

2.4.1   *Streptococcus uberis*

Streptococcus uberis can be morphologically identified as a Gram positive, facultative anaerobic coccus. The phenotypic identification of the organism is based on its ability to hydrolyse aesculin, and ferment inulin, and its lack of growth in buffered azide glucose glycerol broth (BAGG). *Streptococcus uberis* is responsible for a high proportion (23-24%) of cases of clinical and subclinical mastitis in lactating cows in New Zealand (Table 2-1; McDougall, 1998, McDougall, 2003, McDougall et al., 2007, Petrovski et al., 2009, Petrovski et al., 2011c). Clinical *S. uberis* infections are characterised by the presence of clots in milk and swollen, painful reddened quarters. Most of the changes observed in milk, like the presence of clots and/or the changes in milk colour are produced by PMN cells. These cells can represent up to 90% of the total cells in milk during a case of clinical mastitis (Zhao and Lacasse, 2008).

In comparison with other major pathogens like *Treuperella pyogenes*, and *E. coli* the consequences of *S. uberis* infections are mild (Pyörälä and Syväjärvi, 1987). Synthesis of milk constituents is not greatly affected, but there is a substantial decrease in milk yield during clinical infection (Coulona et al., 2002). Many virulence factors have been discovered while studying the pathogenicity of *S. uberis* and some of them might explain the susceptibility of *S. uberis* to a varied range of treatments. Most of the virulence factors, which are described here, require the presence of this bacterium in the lumen of the alveoli for long periods after the infection (Thomas et al., 1994, Leigh and Lincoln, 1997, Leigh, 1999). Therefore, it has been suggested that the presence of *S. uberis* in the lumen of the mammary gland allows it to be reached easily by high doses of intramammary antibiotics, reinforcing the theory that the development of new and more effective intramammary antibiotics to control *S. uberis* could be beneficial (Hillerton and Kliem, 2002, Pyorala, 2006). On the other hand, the time spent by this bacterium in the lumen, is important for its survival (Leigh and Lincoln, 1997), as *S. uberis* can break down proteins from the milk and tissues in order to get nutrients (Mills and Thomas, 1981, Leigh and Lincoln, 1997).

The **S. uberis infection process** produces a marked inflammatory reaction in the mammary gland. Neutrophils and macrophages are recruited to the lumen in high numbers. Somatic cell count rises in *S. uberis* clinical infections and can reach in excess of 1,000,000 cells/mL (Leigh and Lincoln, 1997, Zhao and Lacasse, 2008). Despite the inflammation

observed in some cases, neither neutrophils nor macrophages are able to eliminate the infection completely (Finch et al., 1994).

In early studies investigating *S. uberis* colonisation of udder tissue, it was not possible to induce a *S. uberis* infection *in vitro*, in an intact monolayer of mammary tissue (Thomas et al., 1992). Researchers were unable to imitate the PMN reaction *in vitro*, so it was concluded that the pathogenicity of *S. uberis* and adhesion to epithelial cells in the mammary gland was a direct consequence of the damage produced by inflammatory cells, and not by the presence of *S. uberis* in the ducts (Thomas et al., 1992). The final conclusion was that the lesion of the mammary cells was produced by lysosomal enzymes and oxidative damage. Contrary to those findings, other studies have demonstrated that an invasion by *S. uberis* strains of a cell line of mammary epithelial tissue (MAC-T) intact cells does occur, and it was concluded that the capacity of *S. uberis* to attach to alveolar cells depends on microfilaments present in their capsule, and was not related to inflammation (Matthews et al., 1994a).

Some strains of *S. uberis* have demonstrated an ability to invade secretory epithelial cells, suggesting an intracellular location which could protect the bacteria from host defence mechanisms and perhaps also, from the effects of antibiotics (Falkow et al., 1992, Matthews et al., 1994b). This process of internalisation in the mammary cells (Almeida et al., 2006) occurs with some strains of *S. uberis* and results in no morphological damage to the alveolar cells (Matthews et al., 1994a).

One of the most interesting discoveries that is possibly related to the pathogenicity of *S. uberis* is the extensive **genetic variability** which exist between different strains which could involve between 15-18% of the genome (Pryor et al., 2009), even up to 40-50% (Douglas et al., 2000). The organisms' energy metabolism, the resistance to PMN engulfment, the virulence factors and the nutritional requirements, are different between strains (Pryor et al., 2009). Variability between *S. uberis* strains has also been reported in their resistance to antibiotics (Phuektes et al., 2001). For instance, while some strains from the same herds can share the same pulsed-field gel electrophoresis (PFGE) profiles and the same antibiotic susceptibility patterns, other strains with identical PFGE binding patterns originating from different herds may have different antibiotic susceptibility patterns (Phuektes et al., 2001). This high variability between *S. uberis* strains and their virulence

factors are among the main reasons why an ideal control method against *S. uberis* has not yet been developed.

A hyaluronic capsule and the production of hyaluronidase, plasminogen activating proteins, lactoferrin binding proteins and Christie Atkins and Munch Petersen (CAMP) factor are some of the putative **virulence factors** of *S. uberis* (Fontaine et al., 2002).

The **hyaluronic capsule** avoids engulfment by macrophages and neutrophils as it prevents the binding of antibodies, complement and phagocytic receptors to the bacterial cell wall and consequently, protects it against phagocytosis (Horwitz and Silverstein, 1980, Almeida and Oliver, 1993).

When **hyaluronidase** is excreted in the alveolar lumen, this enzyme breaks the anatomical barrier which exists between blood and milk, and allows phagocytic cell invasion from the blood stream in high numbers which determines the clear signs of inflammation observed in the infected quarter (Oliver et al., 1998). The inflammation induced by *S. uberis* is one of the main reasons why *S. uberis* infections are more frequently associated with clinical, rather than subclinical mastitis (Todhunter et al., 1995). However, subclinical *S. uberis* mastitis has been reported (Jayarao et al., 1999, Zadoks et al., 2003).

Plasminogen is a protein commonly present in bovine milk. A **bovine plasminogen activator** is produced by the majority of *S. uberis* strains (Leigh and Lincoln, 1997, Leigh, 1999). The molecule modifies plasminogen, and converts it into plasmin. In milk, plasmin hydrolyses casein and other proteins, thus helping *S. uberis* to obtain the essential amino acids necessary for its survival (Johnsen et al., 1999).

Streptokinase is also produced by *S. uberis* and is capable of activating plasminogen (Leigh, 1993).

Other putative virulence factors of *S. uberis* of unclear significance are the **lactoferrin binding proteins.** These proteins can bind lactoferrin to the bacterial cell wall. Lactoferrin takes part in non-specific defence mechanisms of the udder (Harmon et al., 1976, Welty et al., 1976, Smith and Oliver, 1981). Lactoferrin is a glycoprotein which binds iron to its molecule, restricting iron availability for the bacteria. Although some bacteria (Gram-negative organisms) need iron to multiply and grow, *S. uberis* does not require iron for survival, but it does appear to require lactoferrin (Fang et al., 2000). Furthermore, it has

been shown that lactoferrin enhances the adherence of *S. uberis* to the host cells (Fang et al., 2000).

Some species of *Streptococcus* (e.g. *S. agalactiae)* are capable of producing a different form of complete haemolysis when cultured near *Staphylococcus* β-haemolytic cultures (Christie et al., 1944). This property was initially only related to *S. agalactiae*. **Christie Atkins and Munch Petersen** molecules extracted from one strain of *S. agalactiae* had cross immunity with *S. uberis* CAMP molecules (Jiang et al., 1996) thereby showing that they are immunologically similar. However, not all the strains of *S. uberis* synthesise CAMP factor showing again a high genetic variability between the strains. For example, only 25% of the total *S. uberis* strains isolated from Argentinian herds were CAMP positive (Lasagno et al., 2011), whereas in New Zeland, 35.4% of the *S. uberis* isolates resulted in CAMP positive tests (Elliott et al., 1976).

2.4.1.1 *Epidemiology of S. uberis infection*

The epidemiology of *S. uberis* infection is not completely understood. Natural *S. uberis* infections combine the features of environmental and contagious agents. This allows for many strains of the same micro-organism to live together in the same herd and sometimes in the same cow, infecting different or the same quarters (Douglas et al., 2000, Phuektes et al., 2001, Zadoks et al., 2003). This phenomenon of multi-strain infection has been reported in several studies. It was postulated that this could be due to a primary infection of the quarter with an environmental strain and a subsequent infection with a different strain acquired from an infected cow during milking or *vice versa* (Zadoks et al., 2003, Pryor et al., 2009).

Even though it is believed that the clinical or subclinical cases present in a herd could be an important reservoir of *S. uberis*, the main reservoir is the environment (Oliver et al., 1998, Phuektes et al., 2001, Zadoks et al., 2003). This bacterium has been isolated from skin and udder surfaces, urogenital tract, tonsils, rectum, rumen and faeces of cows (Sharma and Packer, 1970; King, 1981; Kruze and Bramley, 1982; Zadoks et al., 2005; Lopez-Benavides et al., 2007). The presence of *S. uberis* in wet areas of paddocks has also been reported (Cullen, 1966, Lopez-Benavides et al., 2007) indicating that cows can be infected outside the milking shed. In some countries, *S. uberis* has been isolated from water, soil, plant matter, bedding material, flies and hay during the winter-housing period (Zadoks et al.,

2005). Furthermore, isolates of the same strain have been obtained from infected cows and also from the milking equipment after the infected cows were milked, demonstrating that in addition to the environmental source of infection, cow to cow transmission can occur through milking (Douglas et al., 2000, Zadoks et al., 2003, Lopez-Benavides et al., 2007, Rato et al., 2008).

2.4.1.2   *Disease course*

Infection with *S. uberis* in a herd can be clinical or subclinical. *Streptococcus uberis* can cause 95% of subclinical cases (Jayarao et al., 1999, Zadoks et al., 2003). However, in New Zealand, it has been reported that only 23% of the *S. uberis* cases are subclinical, whereas most of the cases (between 70 and 80%) are clinical (McDougall, 1998).

2.4.1.3   *Infection periods*

Streptococcus uberis is a very common infection during the first weeks of the dry period and also the first months of lactation (McDougall et al., 2007, Petrovski et al., 2011c) when the susceptibility of the cow to mastitis-causing agents is high (Figure 2-2). This pattern appears to have been subjected to changes over time, as in the 1970s the incidence of *S. uberis* mastitis during the dry period was low (Sharma and Packer, 1970). The development of the dairy industry might be the reason for those modifications in the infection periods, as genetic selection pressure (Cai et al., 1994), cow density (stocking rate) and individual milk production have increased, producing a higher exposure and susceptibility of the udder to micro-organisms (Sordillo and Streicher, 2002).
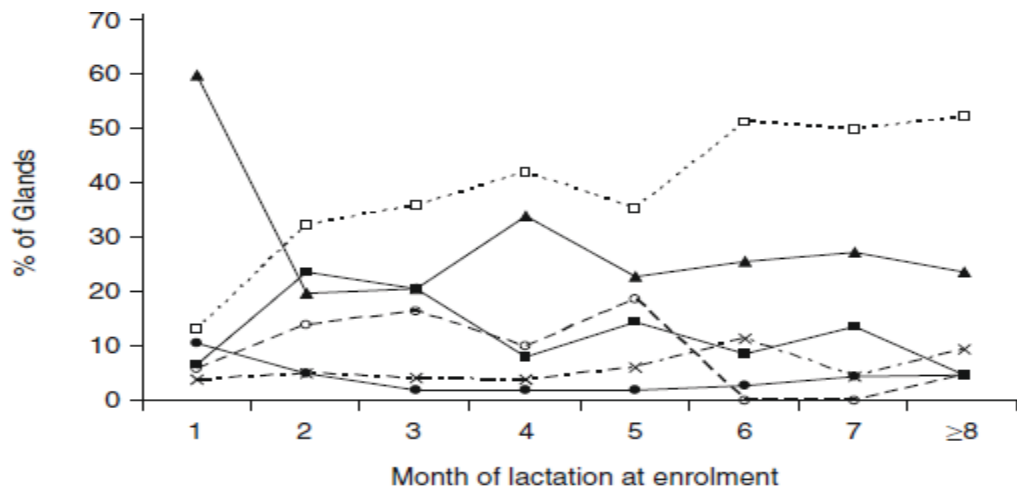
Figure 2-2 Percentage of glands diagnosed with clinical mastitis by month of lactation. (▲ = *Streptococcus uberis;* □ = *Staphylococcus aureus;* = ● *coagulase-negative staphylococci;* ○ = *Streptococcus dysgalactiae;* X = other species; ■ = two bacterial species isolated). From (McDougall et al., 2007).

2.4.1.4 Prevention of S. uberis mastitis

A large variety of control methods like antibiotics and pre-dipping solutions are available to control *S. uberis* clinical mastitis in dairy herds with variable efficacy. Management of hygiene such as general hygiene during milking, teat disinfectant after milking and avoiding muddy, dirty environments for the cows are necessary for prevention and control (Zadoks et al., 2003).

Further prevention methods such as vaccination, are currently being developed. The genetic variability of *S. uberis* is extensive in New Zealand and around the world. At least 40% genetic dissimilarity between the analysed strains has been reported (Roguinsky, 1971, Douglas et al., 2000, Lopez-Benavides et al., 2007). For this reason the development of vaccines to prevent *S. uberis* infections is challenging.

2.4.1.5 Antibiotics as a control method against S. uberis

Since the discovery of penicillin, antibiotics have been extensively used within the dairy industry for the treatment of mastitis-causing bacteria. Although *S. uberis* can be killed by antibiotics like penicillin G, amoxicillin, cloxacillin and other beta-lactam antibiotics, its ability to survive in the environment allows it to easily re-infect the cows after treatment. This opportunistic characteristic is one of the reasons why *S. uberis* persists in some herds

after treatment of the clinical cases. Another reasons for persistency of *S. uberis* in a herd could be the recurrence of clinical cases treated with antibiotics that achieve low bacteriological cure rates, which leave *S. uberis* active in the quarter, but in the subclinical form, able to be spread from cow to cow through the milking machine (Zadoks et al., 2003, Milne et al., 2005). The availability of drugs and formulations with better pharmacokinetic parameters, and higher bacteriological cure rates against *S. uberis* could be helpful in controlling this agent in dairy herds by avoiding recurrences produced by viable *S. uberis* being left in the quarter after the treatment of clinical cases.

2.4.2 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, facultative anaerobic, catalase- and coagulase-positive coccus which can produce clinical or subclinical infections. Clinical mastitis caused by *Staph. aureus* is classified as "peracute", "acute" and "chronic" (Matsunaga et al., 1993). However, the most common courses of the disease are the subclinical and chronic cases (de Haas et al., 2002).

Peracute infection is characterised by a sudden onset, severe inflammation of the quarter, agalactia, anorexia, hyperthermia and other systemic signs. Acute infections are characterised by local inflammation of the mammary gland. Chronic infections usually occur without clinical signs, or in other cases, start with an acute infection that cannot be cleared completely and become chronic after a few weeks. A rise in SCC might be the only sign observed with this kind of infection, accompanied by 'lumps' in the udder upon palpation after milking. Different patterns of increases in SCC were found to accompany subclinical *Staph. aureus* infections (de Haas et al., 2002). Somatic cell counts in natural *Staph. aureus* infections have been shown to be elevated from 100 days before a clinical episode of a *Staph. aureus* infection to 100 days following the episode, suggesting that subclinical infections with *Staph. aureus* may occur a long time before the clinical signs are observed (Harmon, 1994, Schepers et al., 1997, de Haas et al., 2002).

Histopathological changes produced by *Staph. aureus* were extensively studied in the 1970s and 1980s (cited from Zhao and Lacasse, 2008). Massive PMN infiltration and necrosis of secretory tissues are some of the characteristics of natural infections by *Staph. aureus* (Chandler et al., 1974). This bacterium also causes necrosis of the secretory tissue with its

replacement by non-secretory tissue (Heald, 1979, Nickerson and Heald, 1981). Therefore, it can lower the total life production of an infected cow.

2.4.3   *Escherichia coli*

Escherichia coli is one of the most important mastitis-causing agents in dairy cows in the USA and Europe, primarily in intensive dairy farms and it is one of the most deleterious organisms (Bradley, 2002). In severe cases, *E. coli* can produce loss in lactiferous sinus epithelia and haemorrhages, accompanied by general toxaemia and death of an infected cow. The degree of tissue damage is variable from cow to cow (Burvenich et al., 2003) but it is still considered one of the most dangerous mastitis agents worldwide, including in New Zealand where due to differences in nutrition and animal husbandry, its occurrence is rare (McDougall, 2003, Petrovski et al., 2011c).

Cow faeces can be the main source of the *E. coli* infections. However, only strains which are able to adapt to the udder conditions and survive, are able to infect the udder (Nemeth, et al., 1994).

2.5   Minor mastitis-causing agents

2.5.1   *Corynebacterium bovis*

These Gram-positive coryneform bacteria are not generally associated with clinical signs, and do not appear to alter milk composition. However, some studies have shown significant differences in SCC between quarters infected with *C. bovis* and non-infected quarters (Ngatia et al., 1991). On the other hand, some studies, did not report SCC changes (Coulona et al., 2002). Infection produced by *C. bovis* is usually related to reduced or non-use of teat disinfectant after milking (Black et al., 1972). It has been proven that it is easily killed and eliminated from farms by regular teat disinfection and by the use of dry cow therapy (DCT) (Ngatia et al., 1991). The inflammatory changes produced by *C. bovis* were similar to those found in clinical mastitis caused by major agents, but to a minor extent. The presence of fibrinocellular exudate in the teat lumen and signs of inflammation in the teat cistern are some of the most common changes reported (Ngatia et al., 1991). Despite

the known presence of this agent in New Zealand farms, its incidence has been reported as "low" in the surveys carried out in this country (McDougall, 1998, Petrovski et al., 2011c).

2.5.2 *Coagulase negative staphylococci* **(CNS)**

These are among the most common agents isolated from the mammary gland around the world. In New Zealand, 25% of the subclinical infections reported by McDougall (1998) resulted in positive cultures of CNS. Consequences of CNS infections are considered of minor importance and in rare cases can produce clinical mastitis. However, the rise in SCC and losses in milk yields have been described in many studies (e.g. Nickerson and Boddie, 1994, Chaffer et al., 1999, Taponen et al., 2006). The most common agents isolated in CNS mastitis are *Staph. epidermidis, Staph. chromogenes and Staph. haemolyticus,* (Chaffer et al., 1999). Some studies reported a protective effect for quarters by infection with CNS against major mastitis-causing agents (Nickerson and Boddie, 1994, White et al., 2001). However, other authors could not find protective effects in CNS subclinical infections (Zadoks et al., 2001). Despite all the studies which have been carried out with these bacteria, more research is needed to analyse the problems that these minor agents could produce for the dairy industry (Schukken et al., 2009).

2.6 Treatment of mastitis

The common way to treat clinical mastitis infections is by the use of antibiotics. The choice of the most successful treatment for each farm depends on many factors, such as:

1. The identification of the infectious agent and its virulence

2. Interaction of host and agent

3. The pharmacokinetics of the antibiotic chosen for treatment

4. The *in vitro* and *in vivo* susceptibility/resistance of the mastitis-causing agent to antibiotics used.

2.6.1 Knowing the infectious agents

Many infectious agents produce mastitis. More than 137 species of mastitis-causing agents have been identified (Watts, 1988). Surveys have been carried out in many countries regarding the geographical distribution of mastitis pathogens. Record keeping allows the analysis of mastitis incidence and prevalence to be studied. The European Nordic countries (Denmark, Finland, Sweden and Norway) have unique data records that are frequently and thoroughly analysed (Heringstad et al., 2000, Pitkälä et al., 2004). This intense monitoring has allowed farmers in these countries to identify and treat mastitis earlier, and treat each case more specifically within their herds, thus giving them low SCC when compared with other countries.

Since 1960, some surveys have been conducted in New Zealand, regarding mastitis-causing agents and their prevalence in different areas. However, mastitis agent identification, geographical distribution and comparisons between regions are not continuously conducted or reported. Bacterial distributions in New Zealand have undergone many changes since the beginning of the expansion of the dairy industry resulting in regional differences between farms in the same period (Table 2-1). For example, surveys done in Northland (Petrovski et al., 2009) show different incidence of the most common pathogens when compared with other work (McDougall et al., 2007, Petrovski et al., 2011b).

A possible explanation for the differences observed, is that contagious agents like *Staph. aureus* and *S. agalactiae*, have been better controlled in recent years. More recent management practices such as disinfecting the teats after milking, application of DCT and culling of chronically infected cows have been very useful tools to eliminate contagious micro-organisms from dairy herds (Williamson et al., 1995). Consequently, environmental agents like *S. uberis* are more commonly seen, even on well-managed dairy farms (Oliver et al., 2004, Petrovski et al., 2009). The variability reported in the surveys over the last few decades means that they should be considered by veterinary practitioners and mastitis consultants to enable them to remain current with these constantly changing data. However, such data/information is not always available to clinicians.

Table 2-1: Surveys carried out in New Zealand reporting the relative prevalence of mastitis agents in different regions as a percentage of all isolations.

	Streptococcus agalactiae	*Staphylococcus aureus*	*Streptococcus dysgalactiae*	*Streptococcus uberis*	*Coagulase Negative Staph*	*E. coli*
Brookbanks (1966) and Elliot et al., (1976) (North and South Island)	14-18%	27-41%	2-4%	<3%	25%	-
McDougall, (2003) (Waikato)	-	2%	3%	56%	8%	3%
McDougall et al., (2007)(North and South Island)	-	16.4%	6%	32%	5.5%	-
Petrovski et al., (2009)(Northland)	-	24%	-	23%	-	-
Petrovski et al, (2011c) (North and South Island)		23.5%	6.2%	23.6%	7.2%	3.7%

2.6.2 Interaction between host and agent

Interactions between a host and the mastitis-causing agent have already occurred when a clinical mastitis (CM) case is diagnosed and the treatment is administered. The host could be responsible for the failure of the treatment applied. Treatment failure due to host factors could be influenced by depressed individual immunity, stage of lactation or concurrent illnesses (Curtis, 1983). Occluded ducts (Figure 2-1) may also prevent a drug infused to the mammary gland from reaching the infected tissues (Sandholm et al., 1990). The intrinsic characteristics of some bacteria allow them to be protected from host immunity and/or antibiotic treatment and could also make a mastitis treatment fail. Host-agent interactions that can influence the response to mastitis treatments are for example the capacity of *S. uberis* to become intracellular (Almeida et al., 2006) and the ability of *Staph. aureus* to create granulomas or abscesses, and also to survive intracellularly in the PMN after phagocytosis (Craven and Williams, 1985).

2.6.3 Action mechanisms, pharmacokinetics and pharmacodynamics of intramammary antibiotics

In some studies, intramammary antimicrobials have been demonstrated to be a more effective way to treat mastitis than systemically administered antibiotics (Gruet et al., 2001, Hillerton and Kliem, 2002). However, in other studies, no significant differences were found between the effectiveness of intramammary and parenteral treatment by subcutaneous injection (McDougall, 1998).

Intramammary antimicrobial drugs with a good and/or limited distribution in the mammary tissue should be chosen as a first treatment measure against mastitis infection (Table 2-2). Another condition that must be considered before the election of the antimicrobial to be used for intramammary treatments is the amount of drug that is absorbed systemically. The less absorbed drugs are more likely to remain in contact with the mastitis-causing agent for longer periods. Current data on the pharmacokinetics and pharmacodynamics of antibiotics are incomplete and more research regarding efficacy and distribution of the drugs used for mastitis treatment and control should be done using infected glands rather than healthy glands. Milk pH changes, alterations in blood circulation and the disruption of the blood-milk barrier are only some examples of the physiological

changes that occur after infection which can affect the distribution of the antimicrobial substance in the udder. The challenge model developed in this thesis could be a useful tool for the development of these kinds of studies in the future.

Table 2-2 Antimicrobial drug classification grouped by its distribution in the udder after intramammary treatments (Saran and Chaffer, 2000, p. 76). Not all these drugs are available to be used for animal treatment.

Distribution in the udder	Antimicrobial Drug
Good	Erythromycin, Tylosin, Spiramycin, Tilmicosin, Lincomycin, Clindamycin, Thiamphenicol, Florphenicol, Trimethoprim, Aditroprim, Ampicillin, Amoxycillin, Hetacillin, Cephalexin, Sulfodexin, S-dimetoxin, Novobiocin, Rifampin, Rifamycin
Limited	Penicillin G (Benzyl penicillin), Cloxacillin, Oxacillin, Cephalotin, Cephoxazole, Cephalonium, Cephapirin, Cephazolin, Cephacetril, Cefuroxime, Ceftiofur, Cephoperazon, Oxytetracycline, Tetracycline, Doxicycline, Nitrofuranes
Poor	Dihydrostreptomycin, Neomycin, Kanamycin, Gentamycin, Tobramycin, Spectinomycin, Aminosidin, Polymixin B, Colisitin, Rifaximin

The majority of mastitis agents are Gram positive cocci of the *Streptococcus* and *Staphylococcus* genuses*,* naturally susceptible to β-lactam antibiotics like penicillins, which are the most common drugs used against mastitis. Most of the *Streptococcus* spp. are susceptible to penicillins (Salmon et al., 1998, Pitkälä et al., 2004, Petrovski et al., 2011b).

After the discovery of penicillin in 1928 by Fleming, it became the primary tool for attempting to control mastitis on dairy farms. The first use of penicillin for mastitis treatment is registered in the 1940s (Schofield, 1946). The action mechanism of β-lactam antibiotics is to enter the bacterial cell, and inhibit the synthesis of wall proteins. In contact with the molecule of penicillin, the bacteria are unable to maintain the external wall intact and die by osmotic lysis.

Over the years, as a consequence of the use of penicillins on dairy farms, agents like *S. agalactiae* have been disappearing. Some strains of *Staphylococcus* spp. and particularly *Staph. aureus* have developed resistance mechanisms to penicillins due to the acquired ability to synthesise β-lactamases or penicillinase enzymes. These enzymes destroy the antibiotic molecule inside the bacterial cell before it can have any effect (Sandholm et al., 1990). In recent years, due to selective pressure of antimicrobial treatments, strains resistant to antibiotic treatments have been spreading in some places (Guérin-Faublée et al., 2002). This spreading of resistant strains provides further reasons for the development of new antimicrobial drugs.

The resistance patterns are considerably different between bacteria and between different strains of the same bacterium (Salmon et al., 1998). Different resistance patterns for cloxacillin were found between strains of *S. uberis* obtained from US herds and in strains obtained from NZ and Danish herds (Salmon et al., 1998). High variability between *S. uberis* strains in their genetic expression of virulence factors and antibiotic susceptibility has also been reported (Douglas et al., 2000, Lasagno et al., 2011).

New Zealand strains of *S. uberis* have been demonstrated to be 100% susceptible to cloxacillin (Petrovski et al., 2011b). Cloxacillin sodium is a type of antibiotic classified as penicillinase-resistant penicillin. Thus, cloxacillin and other penicillinase-resistant antibiotics are considered useful in fighting certain bacterial infections such as *Staphylococcus* when other penicillins would not be effective (Mattie et al., 1973, Davis et al., 1975). Despite penicillinase-resistant antibiotics like cloxacillin being specifically developed to treat penicillin-resistant bacteria like *Staph. aureus*, the high sensitivity that *S. uberis* has shown to this antibiotic in NZ (Petrovski et al., 2011b) has demonstrated that it could be a useful drug to treat clinical mastitis on dairy farms. This is supported by the fact that prior to treatment, it is very difficult for farmers to diagnose the agent that is causing each clinical case and therefore, to choose the right drug for each case. Consequently, choosing a drug that is able to effectively treat at least the two major mastitis-causing agents (*Staph. aureus* and *S. uberis*) could be useful for treating mastitis in NZ herds (McDougall et al., 2007, Petrovski et al., 2011b).

2.6.4 Bacterial Susceptibility and Resistance

Once clinical mastitis has been correctly diagnosed, or an infection by a specific agent is suspected, a susceptibility test can be done in order to select an effective drug to treat that clinical case. The most common practice to determine the susceptibility of an agent to antibacterial drugs *in vitro* is the Kirby-Bauer sensitivity test (Langston and Davis, 1989). The test evaluates the capacity of bacteria to multiply despite the presence of an inhibitory substance such as an antibiotic. The bacterium to be tested is spread on a culture plate and small disks containing antibiotic (one disk per drug tested) are placed onto that plate. The plate is incubated for up to 24 hr. in the conditions required to allow the bacteria to grow (e.g. aerobic or CO_2-enriched environment). The results obtained in a successful test show bacterial growth on the plate and an area of inhibition around the antibiotic disk (Figure 2-3). The agent can be classified as susceptible (if there is no growth around the disk), intermediate, or resistant (if there is growth despite the presence of the antibiotic disc). The concentration of antibiotic achieved using the disk is usually approximately the concentration reached by the tested antibiotic in the tissues.
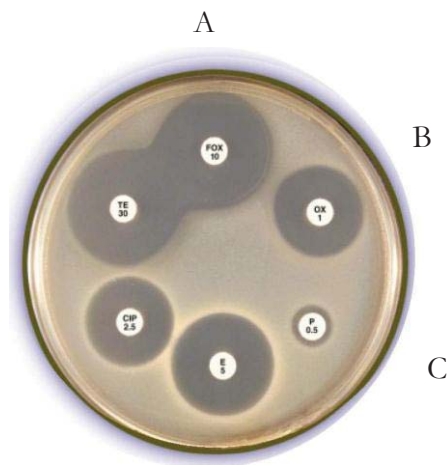
Figure 2-3 Kirby-Bauer sensitivity tests. A: Susceptible; B: Intermediate; C: Resistant

Although *in vitro* tests are a quick and inexpensive way to evaluate bacterial susceptibility (Figure 2-3), it is considered that *in vitro* testing of mastitis pathogens is not precise when comparing test results with the *in vivo* results (Sandholm et al., 1990, Constable and Morin, 2003). The behaviour of a bacterial infection *in vivo* is different to observations made on

bacteria cultured *in vitro*. The rapidity of bacterial multiplication in the enriched media of an agar petri dish is not comparable with the reproduction of the same bacteria in tissue (Sandholm et al., 1990, Constable and Morin, 2003). This implies that *in vivo* testing is necessary to obtain true results and that *in vitro* tests should be used only as preliminary tests. However, despite this, *in vitro* tests may be helpful in some specific cases. The use of *in vitro* susceptibility tests on *Staph. aureus* isolates has been recommended as a means to detect β-lactamase resistant strains (Taponen et al., 2003). If the agents are found to be resistant to an antibiotic by *in vitro* testing they will, most likely, also be resistant *in vivo*. When the agent is susceptible *in vitro*, there are no guarantees that it will also be susceptible *in vivo*. It has been demonstrated that different strains of the same agent, for example *Staph. aureus*, show different resistance patterns when tested in *in vitro* and *in vivo* (Taponen et al., 2003).

In the field of bovine mastitis, *in vitro* susceptibility tests are still very controversial, as the applicability of the results of susceptibility tests are questionable (Craven, 1987, Rossitto et al., 2002, Constable and Morin, 2003). The main reasons these tests are not completely reliable are:

1. There are no standards for minimum inhibition concentrations (MIC) validated for mastitis pathogens,

2. The pharmacokinetics and pharmacodynamics of the drugs in lactating dairy cows with mastitis are not completely understood, and

3. There are not enough field studies that validate the data from *in vitro* tests.

Current knowledge about breakpoints for mastitis-causing agents is limited (Hillerton and Kliem, 2002, Rossitto et al., 2002, Constable and Morin, 2003). The three points above support the idea that more efficacy tests are required to be carried out with infected cows.

A research technique that is used to improve current knowledge about the effectiveness of treatments against clinical mastitis in infected glands is the design and conduct of experimental challenge studies.

2.7 Experimental challenge model

An experimental challenge model is a study that uses a model of a natural system. It contains some of the risk factors which apply in the natural state, such as host defence variability, and is a simplification of on-farm or field scenarios.

Challenge models have been used to create an experimental infection of the mammary gland in various animals. In the past, mice and small ruminants have primarily been used (Brouillette and Malouin, 2005), but the udders of modern dairy cows differ markedly from those of mice and small ruminants. Genetic selection over many years to maximise individual cow milk production has increased metabolic stress to the cows and consequently has decreased their capacity to resist mastitis infections (Cai et al., 1994). Cow density (stocking rate) has also increased, producing a higher exposure of the udder to micro-organisms (Sordillo and Streicher, 2002). Therefore, new challenge models should be developed to assist the understanding of mastitis in the modern dairy cow and to aid developing new, efficacious treatments.

2.7.1 Uses of Experimental challenge models

The goal of mastitis challenge models is to cause controlled udder infections. They are considered helpful for testing the efficacy of products such as antibiotics or teat disinfectants (Foret et al., 2003, Oliver et al., 2003, Poutrel et al., 2008). Their use may also help to discover reasons for failure of treatments (Schukken et al., 1999). Challenge models provide an opportunity to realistically study the consequences of mastitis in cows, such as the effects on reproduction, the immune response of the cow and the mammary gland (Heyneman et al., 1990, Finch et al., 1994, Moyes et al., 2010), cytological changes in the udder tissues and various inflammatory changes (Heald, 1979).

The proportion of mastitis infections achieved following an artificial challenge in different studies is variable. One hundred percent incidence has been reported, following the insertion of 5mL of challenge suspension containing 800 CFU/mL of *Staph. aureus* and *S. uberis* into the mammary glands (Sladek and Rysanek, 2006, Moyes et al., 2010). Previous works carried out by Ryan (1999) and Pedersen (2003) also achieved 100% infection using strains of *S. uberis* and *S. dysgalactiae,* respectively. However, other studies using *S. uberis*

strains obtained lower incidences after challenge (between 76%-80%; Oliver et al., 2003, Rambeaud et al., 2003).

The variability in the incidence of clinical mastitis in quarters challenged with a micro-organism depends not only on factors inherent to the micro-organisms such as virulence and infective dose (CFU/mL), but also on animal and environmental factors (Burvenich et al., 2003).

Animal factors influencing mastitis occurrence include stage of lactation (Sordillo et al., 1997, Schukken et al., 1999), age (Poutrel and Lerondelle, 1978, Sordillo et al., 1997), season and nutritional status (Baumgard et al., 2006). Most of these influence the immunological responses of animals (Harmon, 1994). There are significant differences between breeds (e.g. Friesian *vs.* Jersey) in their abilities to respond to infections (Busato et al., 2000, Washburn et al., 2002).

Environmental factors reflect the surroundings of the animals. Confinement *vs.* pasture system, type of housing in confinement (free-stall, dry lots, etc.) and the type of bedding (sand, straw, etc.) have all been shown to influence the incidence of mastitis in dairy herds (Busato et al., 2000). Individual and environmental variables such as those mentioned above can be better controlled in experimental challenges than in natural conditions. Variables possibly affecting the proportion of infections achieved after bacterial challenge in the model developed in this thesis are analysed thoroughly with the objective of clarifying some unknowns around this useful tool for mastitis research.

2.7.2   Challenge Methods

An extensive list of experimental challenge models for use in studies on clinical mastitis in lactating dairy cows is available, and such models have been used for different purposes (Table 2-3). Some of the commonly used techniques are: the introduction of the agent directly into the mammary gland by infusion through the teat canal (Nickerson and Heald, 1981, Hillerton and Bramley, 1989, Heyneman et al., 1990, Hockett et al., 2000, Oliver et al., 2004, Moyes et al., 2010), dipping the teat into a challenge suspension (Nickerson and Boddie, 1994, Boddie and Nickerson, 1996, Boddie et al., 2000, Foret et al., 2003, Boddie et al., 2004, Petrovski et al., 2011a) and injecting the agent directly into the parenchyma of

the mammary gland (Heald, 1979). Each application route has a specific formula and method for preparing the challenge suspension.

Some routes are considered to have advantages over others. One of the disadvantages of dipping the teat in a challenge solution is that a higher concentration of CFU is required to produce an infection in the udder. This is in contrast to the intraparenchymal injection technique, which can produce an infection in a shorter time than the teat-dipping method, and with a minimum concentration of CFU (Heald, 1979). Heald (1979) induced *Staph. aureus* mastitis by injecting an inoculum of 5,000 CFU/0.2 mL (2.5×10^4 CFU/mL) into the udder. In some dipping experiments the concentration of the dipping challenge suspension was 5×10^7 CFU/mL (Boddie and Nickerson, 1996) – much higher than the amount of CFU used in the intraparenchymal study. Lower concentrations of the challenge suspension are advantageous from the animal welfare perspective to avoid overwhelming the hosts immune response and endangering animal's life (Mercer et al., 1974a).

Knowledge about the pathogenicity of the agents to be used in challenge models is required prior to the development of the model. In the examples mentioned above, *S. uberis* is known as an intra-ductal mastitis agent (Leigh and Lincoln, 1997), whereas *Staph. aureus* can be found in the parenchyma (Heald, 1979, Trinidad et al., 1990). Therefore, the experimental challenge models could use different techniques for different agents – intraparenchymal for *Staph. aureus* and intramammary for *S. uberis*.

2.7.3   Advantages of experimental challenge models

Experimental challenge models can be advantageous when compared to field experiments using natural infections (Newbould, 1974, Poutrel and Lerondelle, 1978, Heald, 1979, Oliver et al., 2003). The advantages are that a smaller number of cows can be involved, results can be obtained rapidly and at lower costs. In addition, the infection can be closely monitored, and through knowing in advance the strain characteristics and it susceptibility to antibiotics, induced infection can be better controlled. At the completion of the trial, researchers are generally able to stop the infections by treatment, thus avoiding unnecessary suffering to the cows (Oliver et al., 2003).

Table 2-3 Summary of experimental challenge models carried out by different authors

Study Objective	Type of challenge	Bacteria and concentration of the challenge suspension	Proportion of successful infections in quarters	Reference
Efficacy of teat dip	Dipping	5×10^7 CFU/mL of *S. aureus* 5×10^7 CFU/mL of *Strep agalactiae*	10/40 *S. aureus* in the dip group 30/40 *S. aureus* in the control group 8/29 *S. agalactiae* in the dip group 21/29 in the control group	Boddie and Nickerson, 1996
Efficacy of teat dips (chlorine and iodine)	Dipping	5×10^7 CFU/mL of *S. aureus* 5×10^7 CFU/mL of *Strep agalactiae*	2/58 *S. aureus* in the dip group 22/58 in the control group 3 and 23 *S. agalactiae* infections in dip and control groups respectively	Boddie et al., 2000
Efficacy of teat dip against control	Dipping	5×10^7 CFU/mL and of *S. aureus Strep agalactiae* 5×10^7 CFU/mL	2.1 and 5% quarters infected with *S. aureus* and *S. agalactiae* in the dip group and 15.8 and 14.3% infections in the control groups	Boddie et al., 2004
Efficacy of two iodine dips	Dipping	5×10^7 CFU/mL of *S. aureus* and 5×10^7 CFU/mL of *Strep agalactiae*	2.2 and 6.5% quarters infected with *S. aureus* and *S. agalactiae* in the dip group and 20.2 and 21.7% infections in the control groups	Foret et al., 2003
To monitor CM incidence after challenge and to determine the role of CNS in intramammary infections	Dipping	5×10^7 CFU/ml of *S. aureus* 5×10^7 CFU/mL of *Strep agalactiae*	20.95% of CNS uninfected quarters became infected with *S. aureus* and *S. agalactiae*. 14,97% of the CNS infected quarters were infected with *S. aureus* and *S. agalactiae* after challenge	Nickerson and Boddie, 1994
Efficacy of a teat sealant with chlorhexidine	Dipping	Day 2 7.7×10^8 Day 4 5.4×10^7 Day 16 2.3×10^7	Treated quarters 7/252 (1.5%) 1.2% for the treated with a novel product. Untreated quarters 13/40 (26.8%)	Petrovski et al., 2011a

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Table 2-3 Cont.

Study Objective	Type of challenge	Bacteria and dose	Proportion of successful infections in quarters	Reference
S. uberis intramammary and subcutaneus vaccines efficacy	Intramammary and subcutaneous	300 CFU of *S. uberis*	Intramammary vaccinated: No clinical cases. All non-vaccinated quarters developed clinical mastitis	Finch et al., 1994
Inmunity response against *E. coli*	Intramammary	10^4 CFU of *E. coli*	100%	Heyneman et al., 1990
Infection following a challenge. *Peptococcus indolicus* and *Actinomyces pyogenes*	Intramammary	10^7 CFU/mL of *Peptococcus indolicus*	Mid lactation: 2/12 quarters *P. indolicus* Adv. Lactation: 8/12 quarters *Pept. ind.* Dried off: 12/12 at the dry off became infected *Actinomyces pyogenes* 9/12 lactating cows and 100% of dry cows	Hillerton and Bramley, 1989
Reproductive consequences of experimentally induced mastitis	Intramammary	600 to 1000 CFU/ml of *S. uberis*	12/19 Challenged (+)=63% 2 culled control cows displaying signs of CM	Hockett et al., 2000
Effect of bovine lactoferrin in experimental challenge	Intramammary	1500 CFU of *E. coli*	100%	Kutila et al., 2004
Efficacy of pirlimycin and usefulness of *S. uberis* in experimental model for future efficacy test	Intramammary	1000 CFU *S. uberis*	100%	Oliver et al., 2003

Table 2-3 Cont.

Study Objective	Type of challenge	Bacteria and dose	Proportion of successful infections in quarters	Reference
Determinate the effect of the NEB on the innate immune response against *S.uberis*	Intramammary	2500 CFU/ml *S.uberis*	100%	Moyes et al., 2010
Compare gene expression profile in blood PMN cells between cows in negative energy balance (NEB) cows and positive energy balance (PEB) ones all challenged with *S. uberis*	Intramammary	2500 CFU/ml *S. uberis*	100%	Moyes et al., 2009
Evaluate effectiveness of intramammary devices models	Intramammary	565 CFU/ml	8% case quarters developed mastitis and 4% of the control quarters	Nickerson et al., 1990
Determine the effect of impaired tissue of infected glands on drug excretion	Intramammary	Experiment 1: 42×10^2 Exp 2: 12×10^3 Exp 3: 30×10^5 Exp 4: 48×10^4 Exp 5: 40×10^9	Exp 1: 50% of the quarters were infected Exp 2-5: 100%	Mercer et al., 1974a
Evaluate the efficacy of danofloxacine after a single dose intravenously	Intramammary	26 CFU *E. coli*	20/23 quarters challenged developed mastitis	Poutrel et al., 2008
To study the influence of the month of lactation in mastitis incidence	Intramammary	500 CFU/ml *S. aureus*	90%	Poutrel and Lerondelle, 1978

Table 2-3 Cont.

Study Objective	Type of challenge	Bacteria and dose	Proportion of successful infections in quarters	Reference
To monitor the effects of two therapy regimens on experimental mastitis	Intramammary	1500 CFU of *E coli*	100%	Pyörälä et al., 1994
To monitor the leukocyte and cytokine dynamics during experimentally induced *S. uberis* mastitis	Intramammary	5×10^8 CFU/ml	16/20	Rambeaud et al., 2003
To evaluate the efficacy of one teat seal containing lacticin 3147 (a natural bactericide against *Streptococcus* and *Staphylococcus*	Intramammary. In the teat sinus	*S. dysgalactiae* 1.5×10^4 CFU/ml	14/33 mastitis in the infused only seal 2/35 mast in the seal+ lacticin 4 quarters not sealed	Ryan et al., 1999
To evaluate efficacy of cefaquinome against *E. coli* infections	Intramammary	400-750 CFU/ml	100%	Shpigel et al., 1997
To Develop a challenge model to study host response	Intramammary	2.1×10^6 CFU/ml *Staphylococcus chromogenes*	100%	Simojoki et al., 2009

Table 2-3 Cont.

Study Objective	Type of challenge	Bacteria and dose	Proportion of success infections in quarters	Reference
To investigate the expression of CD14 on neutrophils and macrophages to determinate their intervention in mastitis resolution	Intramammary	*S. aureus* and *S. uberis* (8x10^6 CFU/ml)	100%	Sladek and Rysanek, 2006
To show the effect of carprofen in induced *E. coli* mastitis in primiparous cows	Intramammary	1x104 CFU/ml *E. coli*	100%	Vangroenweghe et al., 2005
To elucidate early pathogenesis within the first few hours after intramammary inoculation of *S. uberis*	Intramammary	10x7 CFU/ml *S. uberis*	100%	Pedersen et al., 2003

2.7.4 Studies using natural infections

Using natural infections to test the efficacy of some products has some disadvantages in comparison with experimental challenge models. Firstly, a study carried out using natural infections could take many years (Zadoks et al., 2001, Rossitto et al., 2002, Taponen et al., 2003, McDougall et al., 2007). In order to prove the efficacy of tested products the researchers are required to include a sufficient number of clinical mastitis cases for a study to be statistically significant. Variability in the aetiology, in the duration of the infections, animal variation and differences in herd management between the farms included in the study may confound the results (Craven, 1987). Studies using natural infections are however important for efficacy tests of mastitis products. The ability of the product to treat clinical mastitis with a multi-agent infection might be proven.

Secondly, product testing in natural environments could be considered very risky, as there is a chance that the experimentally tested product may not stop (or prevent) infections. This could affect herd health, bringing major economic consequences to the farm used for testing due to lost production and potentially animal loss through death and culling. Finally, some of the field strains found on farms are naturally resistant to some drugs. Consequently, results obtained in experiments carried out to test the efficacy of any product, could be confounded by non-target field strains of bacteria, thus requiring a larger number of animals to be enrolled in a study and for these to be on multiple farms.

2.8 Summary

The aim of this Literature Review was to outline concepts about clinical mastitis infections, to clarify some details about the pathogenicity of *S. uberis* and finally to present the advantages and disadvantages of experimental challenge models as research tools.

Basic concepts on the pathogenicity of *S. uberis* in clinical mastitis have been extensively studied. However, there are still some unknown features about how different strains could affect the udder health of dairy herds.

Development of new products to treat bacterial mastitis have been pursued over the last four or five decades. Despite all the efforts to develop new products to prevent and/or treat it, the pharmaceutical industry still applies inconsistent methods to test the efficacy of

those products. In the reviewed literature, efficacy tests of antimicrobials done *in vitro* or *in vivo*, in natural or experimental conditions seem to be considered equal in statistical power, when this is unlikely to be so. The breakpoints used in antibiotic susceptibility testing of antimicrobial product efficacy for veterinary use and for mastitis-causing agents are not clearly defined.

Experimental challenge models with the objective of testing novel products like those described in the following chapters, would allow the pharmaceutical industry to carry out small, controlled, and safe experiments in consistent populations which will support the generation of key data about the efficacy of drugs and their interaction with the host response.

Chapter 3 of this thesis describes the *in vivo* testing of four strains of *S. uberis* for their virulence and susceptibility to antibiotic therapy. The objective of this study was to choose one strain of *S. uberis* for use in future challenge studies. A further study objective was to determine the dose (number of pathogens infused, expressed as colony forming units (CFU)) required for the tested strains to produce an acceptable proportion of clinical mastitis cases to enable the studies. The strain which had the desired characteristics was then chosen and utilised for experimental challenge in further studies (Chapters 4 and 5). Chapters 4 and 5 describe two challenge studies conducted using the experimental challenge model (Chapter 3) to test the efficacy of different antimicrobial drug formulations.

The studies presented in this thesis, provide the possibility to compare the efficacy of different products, to set breakpoints and guidelines for antimicrobial doses and will thus allow improvement of effectiveness in the area of udder health research in future.

CHAPTER 3

Development of a bacterial challenge model for inducing

Streptococcus uberis clinical mastitis in lactating cows

3 Development of a bacterial challenge model for inducing *Streptococcus uberis* clinical mastitis in lactating cows

3.1 Abstract

The aim of this study was to develop a challenge model to induce clinical mastitis caused by *Streptococcus uberis,* which could be used for future *in vivo* efficacy testing of antimicrobial drugs. Four strains of *S. uberis* were used and their capability to induce clinical mastitis at two different bacterial concentrations ("Low" and "High") was evaluated. Twenty-four crossbred lactating cows were enrolled in the study. The cows were randomly allocated to four groups of six cows each. Cows in each group were challenged with a different strain of *S. uberis* in two contralateral quarters (Front-Right and Rear-Left or Front-Left and Rear-Right; FR/RL or FL/RR) immediately after the morning milking. The challenge suspension used to induce clinical mastitis consisted of 4 mL of bacterial suspension at a concentration of 10^4 colony forming units (CFU)/mL ("Low" concentration) in one quarter, and 10^6 CFU/mL ("High" concentration) in the other quarter. Individual quarters were inspected for signs of clinical mastitis at every successive milking for ten consecutive days following the challenge. Milk samples for bacteriological analysis were taken from the clinically-affected quarters and from non-clinical quarters on Study Day 4.

The incidence of clinical mastitis at a quarter level was 54% (26/48). Most clinical cases in the challenged quarters were detected within 96 hours after challenge (Day 4). There were significant differences in the proportions of clinical mastitis achieved between the groups. Strain 26LB resulted in 4/6 and 2/6 cases of clinical mastitis in quarters challenged with the Low and High concentration, respectively. Strain S115 resulted in 2/6 and 5/6 cases of clinical mastitis with the Low and High concentrations respectively. Strain S418 resulted in clinical mastitis in all challenged quarters, for both High and Low concentrations and these infections did not respond well to antimicrobial treatment. Conversely, Strain S523 resulted in only one case of clinical mastitis in a quarter challenged with the Low concentration.

This study showed significant differences in the ability of different strains to cause clinical mastitis when inoculated via the intramammary route. One of the four strains tested (S115) demonstrated the most desirable characteristics for a strain to be used in experimentally induced clinical mastitis studies. Mild clinical mastitis cases were obtained in a high

proportion of quarters after challenge with this strain. A positive response after treatment and the ability to be isolated after culture were considered desirable characteristics of a strain to be used in experimental challenge models. Therefore, *S. uberis* S115 was chosen for use as a challenge organism in future studies

3.2 Introduction

Clinical mastitis is a cause of significant loss to the dairy industry worldwide. In New Zealand, the cumulative incidence of clinical mastitis in dairy herds per year is approximately 15% and *S. uberis* has been reported as the predominant mastitis-causing organism (McDougall, 1998, McDougall et al., 2007, Petrovski et al., 2009, Petrovski et al., 2011b).

The veterinary pharmaceutical industry faces increasing regulatory requirements and costs for the development of new antimicrobial drugs and novel formulations. Efficacy of such drugs is usually assessed *in vivo* with field trials, using naturally infected cows. In order to accumulate a sufficient number of observations this procedure can be costly with lengthy waits required. A successful mastitis model using experimental infections with mastitis-causing pathogens may reduce the costs associated with drug development, ultimately allowing a higher number of formulations to be tested within budget (Oliver et al., 2003).

From an animal welfare perspective, the advantages of experimental challenge models are the smaller number of cows required in order to achieve sufficient power (due to reduced variability), and a close veterinary follow up with prompt treatment of clinical infections. In fact, the infection can be closely monitored, and prior knowledge on the antimicrobial susceptibility of the challenge organism may enhance the treatment outcome when conventional treatment is required during the study. For the industry, there are advantages associated with the short time required in order to obtain the results and the possibility of testing very specific pharmacological aspects. Motivated by these advantages, the objective of this study was to induce clinical mastitis in lactating dairy cows by means of experimental challenge with four strains of *S. uberis,* and to select a strain with suitable characteristics for use in future challenge studies.

3.3 Materials and methods

3.3.1 Animal selection and husbandry

The Massey University N°1 Dairy farm (Manawatu Region, New Zealand) was selected for the study. The farm had a herd of approximately 170 autumn-calving cows. The inclusion criteria for pre-selection were: having four functional quarters, no history of mastitis in the current lactation, no anti-microbial treatments received in the 14 days prior to the commencement of the study and a somatic cell count (SCC) <250,000 cells/mL at a herd test carried out six days before challenge. Thirty-five cows complied with these pre-selection criteria. Milk samples were taken from these cows for bacteriological analysis. Eleven cows were eliminated from the study due to obtaining contaminated samples or because quarters were infected with major pathogens, leaving 24 cows which were enrolled in the study. Cows with minor pathogens isolated (e.g. coagulase negative staphylococci or *Corynebacterium* spp.) were included.

Using blocked randomisation based on milk yield at the pre-screen test, cows were allocated to one of four groups of six cows. This was done by ranking the 24 cows according to descending milk yield and placing them into six blocks of four replicates. Block 1 consisted of the cows with highest yields (11.5 to 14 L/day) and Blocks 2 to 6 consisted of cows with the following milk yields: 10 to 11.5 L/day, 9.5 L/day, 9.0 L/day, 8.5 to 9.0 L/day and 7.5 to 8.0 L/day. One replicate from each block was randomly assigned to each of the four groups (one per strain) using the random number function of Excel 2007 (Microsoft Corporation, USA).

The 24 cows were managed as a single mob and were milked twice daily in a 24 a side herringbone milking shed. The feeding regimen was pasture-based nutrition on rye grass-clover (*Lolium perenne* and *Trifolium repens*) cultivars. Water was available *ad libitum*.

3.3.2 Culturing of milk specimens

The culture procedures followed the National Mastitis Council (NMC) Guidelines (National Mastitis Council, 1999). Briefly, 10 μL of aseptically collected single-quarter milk from each cow was plated onto one quadrant of a trypticase soy agar plate supplemented with 5% defibrinated sheep blood (Fort Richard, Auckland, New Zealand). Plates were

incubated at 35 ± 2°C in an aerobic environment and bacterial growth was observed after 24 and 48 hr of incubation. Infected and contaminated quarters were identified according to the criteria recommended in the NMC guidelines.

3.3.3 Preparation of the challenge suspension

All the *S. uberis* strains used for the challenge originated from cows with clinical mastitis from the Wairarapa and Waikato regions of New Zealand. Initially, seven strains were taken at random from a collection of strains maintained in glycerol at -80ºC in the IVABS Microbiology Laboratory and were re-identified phenotypically as *S. uberis* by means of biochemical test. The *in vitro* antimicrobial susceptibility of these isolates was assessed by means of the disc diffusion method.

The isolates were cultured on 5% defibrinated blood agar (BA) plates incubated in aerobic conditions at 35 ± 2°C for 24 hr. One colony from each strain was selected from the plate and transferred onto a new BA plate and incubated as above. The bacterial growth was suspended in phosphate buffered saline (PBS) tubes (0.01 M, pH 7.3). The inoculum density was standardised using McFarland standard (Remel, Lenexa, Kansas, USA). A sterile cotton swab was dipped into each inoculum suspension and swabbed over the entire surface of 5% BA plates. Commercial filter paper disks (Oxoid, Auckland, New Zealand) containing known antibiotics in known concentrations were placed on the plate surface. All plates were incubated at 35 ± 2°C for 24 hr. The diameter of inhibition around the disk was measured and the strains characterised as susceptible or resistant to the antibiotics according to the criteria established in the Clinical and Laboratory Standards Institute, 2008 (CLSI, 2008). The breakpoint for susceptibility to any antimicrobial product is >20 mm of inhibition around the disk (National Committee for Clinical Laboratory Standards, and Watts, 1999). Three strains (26LB, S418 and S523) were selected for the study based on their antimicrobial susceptibility, which indicated high susceptibility to penicillin (40-46 mm), to ampicillin (34-46 mm) and to oxacillin (24-26 mm). The fourth strain (S115) was provided by the scientific team of Dairy NZ (Newstead, Hamilton). Antimicrobial susceptibility testing was also performed on this strain that showed similar susceptibility patterns. The selected isolates were then re-identified by culture and testing.

The preparation of the challenge suspension of *S. uberis* strains was carried out at the IVABS Microbiology Laboratory as follows. Four days before the day of the challenge, an

aliquot of each frozen strain was thawed and streaked onto 5% BA plates. The plates were incubated at 35 ± 2°C for 48 hr. in a 5-10% CO_2 enriched environment. The plates were observed for purity and one colony was suspended in PBS pH 7.3. Ten plates were flooded using 100 μL of this suspension and incubated for 48 hr as above. On the day of the challenge (Day 0), bacterial growth was harvested using sterile cotton swabs and re-suspended in PBS to a turbidity corresponding to the 0.5 McFarland standard. Such a procedure was previously shown to generate bacterial suspensions containing approximately 10^7 CFU/mL (Petrovski et al., 2011a). Following the turbidity adjustment, the working suspension was further diluted using serial ten-fold dilution in PBS to generate final bacterial suspensions containing 10^6 CFU/mL (High concentration) and 10^4 CFU/mL (Low concentration) at volumes which were sufficient to challenge the quarters with 4 mL of suspension. These final suspensions were transferred into series of sterile syringes for intramammary administration by drawing 4 mL into the syringes, so that each syringe contained an estimated of 4×10^4 or 4×10^6 CFU/mL.

Retrospective purity testing and bacterial counting was performed on the day of challenge using aliquots of the two final microbial suspensions. Briefly, 1 mL of the final microbial suspension was serially diluted ten-fold in PBS and 100 μL of dilutions 10^{-2}, 10^{-3} and 10^{-4} were spread in triplicate onto BA plates and incubated as previously described. The arithmetic means of the number of colonies that grew on those plates showing 30 to 300 bacterial colonies was used to estimate the number of CFU/4 mL of inoculum for each challenge suspension of the four challenge strains.

3.3.4 Challenge procedure

Immediately after the morning milking, each cow was experimentally challenged in two contralateral quarters (*i.e.* Front Right and Rear Left or Front Left and Rear Right) by intramammary inoculation of the High or Low bacterial concentration suspensions by full insertion of the syringe nozzle into the teat canal. Before the inoculation, the teat-ends were thoroughly cleaned using cotton swabs dampened in 70% ethanol. The entire content of each syringe (4 mL) was inoculated into the quarters. The two unchallenged quarters served as negative controls.

3.3.5 Clinical examination

Clinical examinations were conducted for ten consecutive days (Day 0-9) during each milking following the challenge. Prior to milking, the foremilk of each quarter was examined for clinical signs of mastitis in milk (*i.e.* clots, colour changes) using the blackboard strip test (Thomas, 1949). Udders and individual quarters were inspected and palpated for clinical signs of mastitis, *i.e.* heat, swelling, redness and/or pain. A clinical score ranging from 0 to 5 was determined for each quarter based on the observed signs, according to Table 3-1.

Table 3-1 Clinical scoring criteria applied for the challenged quarters during the examination period. Adapted from Petrovski et al., (2011a).

Score	Description
0	No evidence of irritation, pain and/or redness or swelling of the quarter.
1	No or virtually no evidence of irritation, pain, redness or very slight swelling of the quarter.
2	Evidence of irritation and/or pain of a minor intensity, slight redness and/or swelling, likely to originate from residual milk in the quarter.
3	Evidence of irritation and/or pain of a moderate intensity or moderate redness, swelling and/or heat of the quarter. Secretion contains small clots or flecks.
4	Evidence of irritation and/or pain of a severe intensity, severe redness and/or severe swelling and heat of the quarter. The secretion contains large clots or flecks.
5	Evidence of severe irritation or pain, severe swelling, redness and/or heat, associated with generally sick animal. The secretion contains large clots or flecks.

Quarters were considered to have clinical mastitis if clots or flecks were present in the foremilk, or if the clinical score was equal to or greater than 3 (Table 3-1).

Quarters diagnosed with mastitis were immediately treated with a penicillin-based intramammary product (Lactapen G, Bomac Laboratories, Ltd., Auckland, New Zealand) three or six times as necessary for recovery. Systemic penicillin-based antibiotics were used only for quarters with high clinical score ≥ 4 (Penethaject, Bomac Laboratories Ltd. Auckland New Zealand). All the clinical cases were cured by the end of the study.

3.3.6   Collection of Milk Specimens

As each clinical case was diagnosed in a quarter, milk specimens were aseptically collected in duplicate. These specimens were then submitted for bacteriological analysis. In order to estimate the number of subclinical infections, milk specimens were also collected from the unaffected challenged quarters on the morning of Day 4. The duplicate specimens were cultured if the first sample was contaminated. Composite milk specimens were collected using in-line Waikato Milk System samplers each morning for SCC determination (performed by Milk Test NZ, Te Rapa, Hamilton).

3.3.7   Statistical analysis

Records of the number of clinical mastitis cases, bacteriological culture results and clinical scores were analysed at the quarter level, whilst the SCC values were obtained and analysed at the cow level.

The occurrence of clinical mastitis was compared using the relative risk (RR) of clinical mastitis, its 95% confidence interval (CI), and its probability under the null hypothesis H_0: RR=1 (logistic regression models could not be fit due to unbalanced designs). In order to account for the two different challenge concentrations (High and Low), the counts of clinical mastitis were stratified, and the Mantel Haenszel RR and Wald Test for homogeneity of strata were calculated. These analyses were undertaken in Excel, using Episheet 2004 (Rothman, 2002). Time from the challenge to the diagnosis of the clinical mastitis was compared between the strains by Kaplan-Mayer survival analysis using the function provided in XL-Stats package (XL-Stats 2012).

Clinical udder scores (per quarter) were compared between and within each strain, among the quarters challenged with High and Low bacterial concentrations, using the Wilcoxon non-parametric test (XL-Stats 2012).

Somatic cell counts were not normally distributed and were therefore transformed into somatic cell scores (SCS), calculated as SCS= $\log_2$ (SCC/1000). The differences between the SCS in the four strains were analysed by means of a repeated measures analysis of variance (rm ANOVA) using the MIXED PROCEDURE of SAS (version 9.3 SAS Institute Inc., Cary, NC, USA), with a model that included the fixed effect of strain and time of sampling (TS), the interaction of strain and TS, and a random effect to account for the variability

within each cow. The geometric means and standard errors of the SCC for each treatment group and day of measurement were calculated as they are more representative of the real mean than the least square means.

3.4 Results

3.4.1 Clinical mastitis and bacteriological results

Out of 48 quarters challenged with the four different strains, 26 quarters (54%) were diagnosed with clinical mastitis. The occurrence of clinical mastitis differed between the strains ($P<0.05$; Table 3-2). Clinical mastitis occurrence with S523 was the lowest, with only one case of clinical mastitis in one of the quarters challenged with the Low concentration and no cases in the quarters challenged with the High concentration. Strain 26LB resulted in four and two cases of clinical mastitis in quarters challenged with the Low and High concentrations respectively. Strain S115 resulted in two and five cases of clinical mastitis with Low and High concentration, respectively. All the quarters challenged with S418 showed signs of clinical mastitis. The proportion of clinical mastitis induced among quarters challenged with S115 was statistically higher ($P<0.05$) than both: the proportion of mastitis in the unchallenged quarters and the proportion in quarters challenged with S523 (Table 3-2). For quarters challenged with S523, the proportion of clinical mastitis was not significantly different from that in the unchallenged quarters, and there was no significant difference in the proportion of clinical mastitis between the quarters challenged with either strain 26LB or S115 (Table 3-2). However, the proportion of clinical mastitis in quarters challenged with S418 was significantly greater than in those challenged with the other three strains ($P<0.05$) suggesting a naturally higher capability of this strain to cause clinical mastitis in lactating cows.

Table 3-2 Comparison between the strains in the ability to cause clinical mastitis RR: relative risk; CI: Confidence Interval; HC: high bacterial concentration; LC: low bacterial concentration; UCH: unchallenged quarters; ∞: infinity. *: P<0.05; **: P<0.001; MHRR Mantel Haenszel relative risk; nc: not calculable

Challenge concentration	Comparison		RR (95%CI)	P-value	Wald Test for homogeneity of strata
	26LB	**115**			
ALL QUARTERS	6/12	7/12	0.8571(0.4086-1.7980)	0.688	
H C	2/6	5/6	0.5 (0.1411- 1.7719)	0.269	
L C	4/6	2/6	1.33 (0.5004-3.5526)	0.575	
MHRR			0.8571 (0.4004-1.8348)	0.694	0.2241
	26LB	**418**			
ALL QUARTERS	6/12	12/12	0.5(0.2840-0.8804)	0.006*	
H C	2/6	6/6	0.33(0.1075- 1.0335)	0.019*	
L C	4/6	6/6	0.66(0.3786- 1.1739)	0.138	
MHRR			0.800(0.5362-1.1936)	0.269	0.2689
	26LB	**S523**			
ALL QUARTERS	6/12	1/12	6(0.8452-42.5960)	0.028*	
H C	2/6	0/6	∞	0.138	
L C	4/6	1/6	4(0.6125- 26.1239)	0.093	
MHRR			0.4000(0.1101- 1.4534)	0.147	nc
	26LB	**UCH**			
ALL QUARTERS	6/12	0/12	∞		
H C	2/6	0	∞		
L C	4/6	0	∞		
MHRR			∞		
	115	**418**			
ALL QUARTERS	7/12	12/12	0.5833(0.3616-0.9410)	0.013*	
H C	5/6	6/6	0.666(0.3786- 1.1739)	0.138	
L C	2/6	6/6	0.55(0.2246- 1.1130)	0.055	
MHRR			1.111(0.7769- 1.5892)	0.575	nc
	115	**523**			
ALL QUARTERS	7/12	1/12	7(1.0094-48,5423)	0.011*	
H C	5/6	0/6	∞	0.019*	
L C	2/6	1/6	3(0.4226)	0.241	
MHRR			1(0.3753- 2.6645)	1.000	nc
	115	**UCH**			
ALL QUARTERS	7/12	0/12	∞		
H C	5/6		∞		
L C	2/6		∞		
MHRR			∞		
	S418	**523**			
ALL QUARTERS	12/12	1/12	12(1.8374-78.37)	<0.001**	
H C	6/6	0/6	∞	0.009*	
L C	6/6	1/6	6(1.0025-35.9089)	0.005*	
MHRR			0.8571(0.6334-1.1598)	0.310	nc
	S418	**UCH**			
ALL QUARTERS	12/12	0/12	∞		
H C	6/6		∞		
L C	6/6		∞		
MHRR			∞		
	523	**UCH**			
ALL QUARTERS	1/12	0/12	∞		
H C	0/6		∞		
L C	1/6		∞		
MHRR			∞		

Based on bacteriological culture, *S. uberis* was found as the causative organism in 19 (73%) of the clinical cases. Seven cases of clinical mastitis yielded no growth. Only 16% of clinical cases (1/6) in quarters challenged with Strain 26LB were bacteriologically positive to *S. uberis* whereas the number of positive results after culture in the other strains was similar to the number of clinical cases induced (Table 3-3). Eighty five per cent (6/7) of clinical cases observed after challenge with S115 were positive to *S. uberis* on culture.

All milk samples collected from the challenged quarters for which no clinical mastitis was diagnosed, had bacteriologically negative cultures.

Table 3-3 Number of cases of clinical mastitis (CM) obtained after challenge stratified by strain and concentration; and the number of samples from which *Streptococcus uberis* was isolated after culture of the samples from CM cases. UCH= unchallenged quarters.

Strain	Concentration	No. CM/quarters challenged	No. Cultures Positives/Total CM cultured	Percentage of total positive cultures
26LB	High	2/6 (33%)	0/2	16%
26LB	Low	4/6 (66%)	1/4	
UCH		0/12		
Total CM/ total challenged		6/12 (50%)		
S115	High	5/6 (83%)	4/5	86%
S115	Low	2/6 (33%)	2/2	
UCH		0/12		
Total CM/ total challenged		7/12 (58.33%)		
S418	High	6/6 (100%)	6/6	100%
S418	Low	6/6 (100%)	6/6	
UCH		0/12		
Total CM/ total challenged		12/12 (100%)		
S523	High	0/6	0/0	100%
S523	Low	1/6 (16%)	1/1	
UCH		0/12		
Total CM/ total challenged		1/12 (8.3%)		
TOTAL		**26 (54.2%)**	**19**	**73%**

All the cases of clinical mastitis occurred between the second and eighth milking after challenge. The Kaplan Meier survival analysis (Figure 3-1) indicated a significant difference in the time of onset of clinical mastitis between the four strains (Log rank, Wilcoxon and

Tarone Ware P<0.001). The survival distribution functions indicated that strain S418 induced clinical mastitis more quickly than the other strains. The unchallenged quarters remained unaffected for the duration of the study.
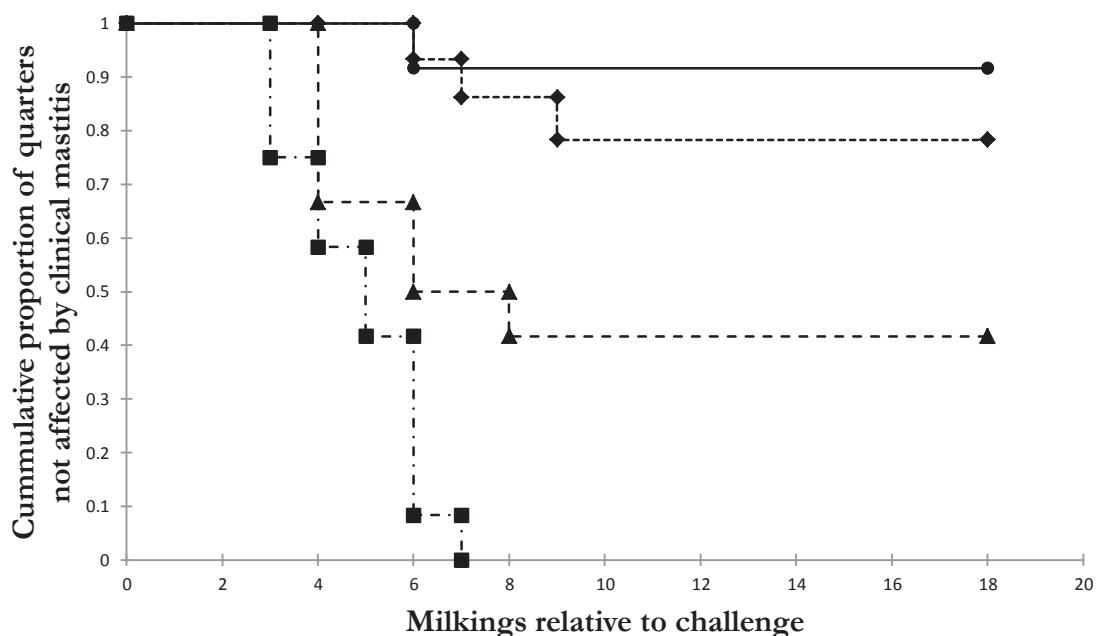
Figure 3-1 Kaplan-Meier survival analysis showing the cumulative per cent of quarters not affected by clinical mastitis over time post-challenge. The X axis value of 0 indicates the time of challenge. -●- S523; -♦- 26LB; -▲- S115; -■- S418.

3.4.2   Clinical Udder Scores

The results of the Wilcoxon test indicated significant differences in the clinical scores between strains S418 and S523 for the High concentrations (Figure 3-2; P<0.001). The differences between S418 and 26LB, S115 and 26LB, 115 and 418, and 115 and 523 were also significant (Figure 3-2; P<0.05). However, no significant differences were found between the clinical scores of 26LB and S523 (P>0.05) in the High concentration. The same results were obtained with the Low concentration, with significant differences (Figure 3-2; P<0.001) between all pairs of strains, except 26LB and S115.

 An increasing trend in the clinical scores was observed two days post challenge in all the challenged quarters, independently of the challenge concentration (Figure 3-3). Clinical scores were low for the duration of the study in the unchallenged quarters, and also in

quarters infected with S523, for which there was no evident difference between challenged and unchallenged quarters (Figure 3-3).

a)
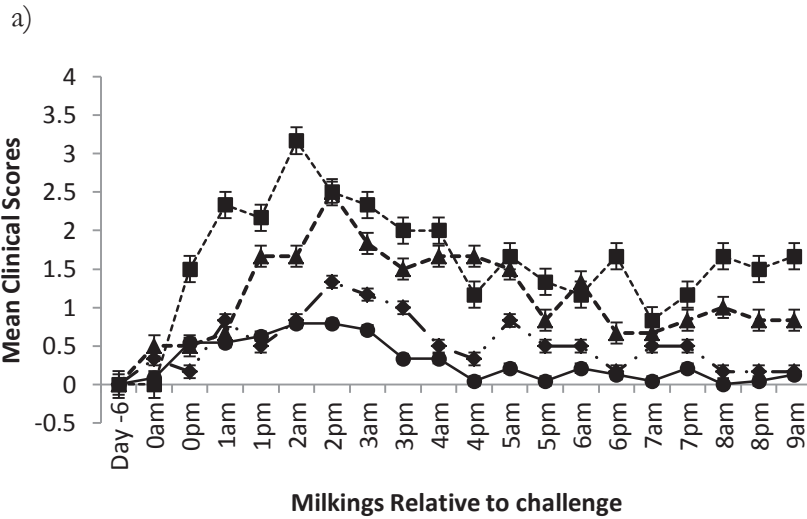
b)
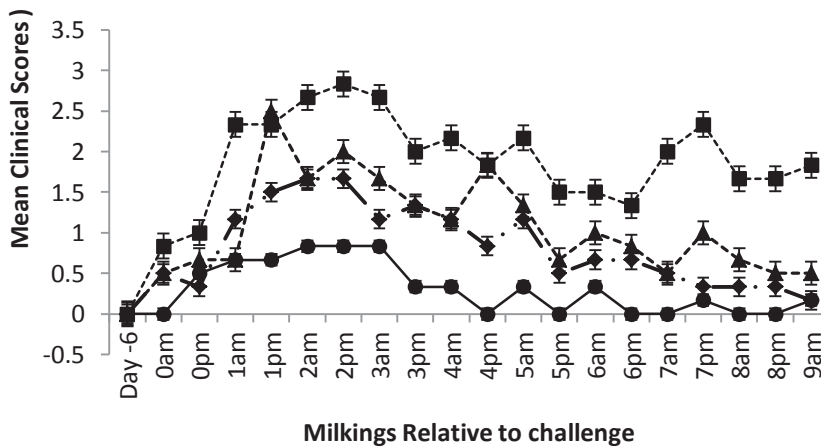
Figure 3-2 Mean clinical scores of challenged quarters. a) High concentration ($1\text{x}10^{6}$ CFU/mL) and b) Low concentration ($1\text{x}10^{4}$ CFU/mL) -▲-S115; -■- S418; -●- S523; -♦- 26LB.

a) S115

b) S418

c) S523

d) 26LB
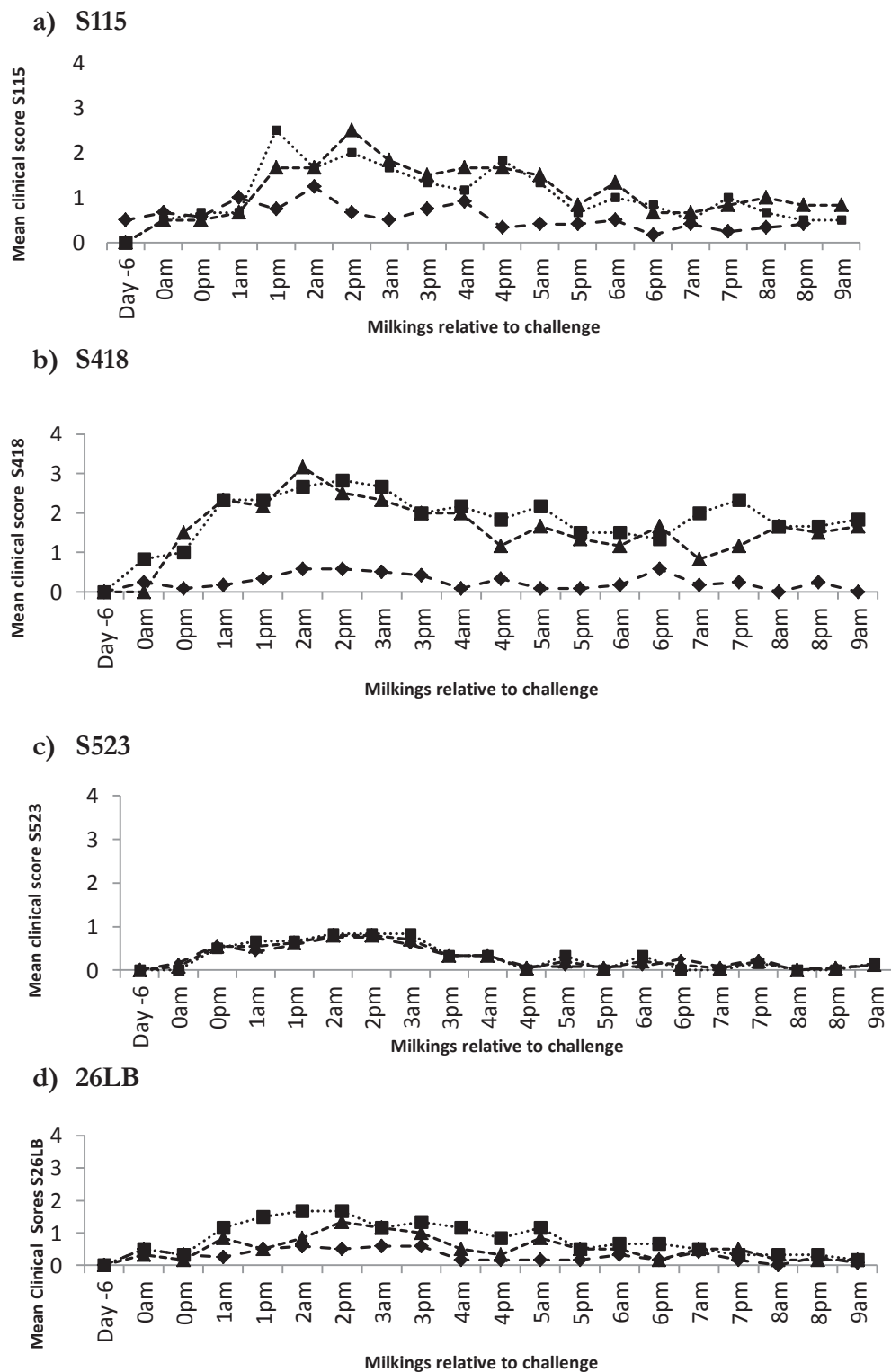
Figure 3-3 Mean Clinical Score of the challenged quarters by bacterial concentration (High, Low) and for the unchallenged quarters, by milking. Figure 3-3 a) Strain S115. Figure 3-3 b) Strain S418. Figure 3-3 c) Strain S523. Figure 3-3 d) Strain 26LB. -▲- quarters challenged with the high concentration (1×10^6 CFU/mL); -■- quarters challenged with the low concentration (1×10^4 CFU/mL); -♦- UC unchallenged quarters.

3.4.3 Somatic cell count results and milk yields

Mean SCC and milk yields were analysed from milk samples taken as a pool from the four quarters from Day -3 before the challenge day (Day 0) and during the eight days following the day of the challenge. A pooled sample from the four quarters was used due to budgetary constraints.

The mean milk yield declined 12 hours after challenge in all the cows, concomitantly with the appearance of an inflammatory response to the bacterial challenge (increased clinical scores) and an increase in SCC (Table 3-4). Since the goal of the study was not to analyse the effect of mastitis on milk yield, these results are not reported.

Somatic cell count increased the first day after challenge in all groups and remained high until the end of the study (Figure 3-4). The rise in SCC was less evident in the cows challenged with S523.
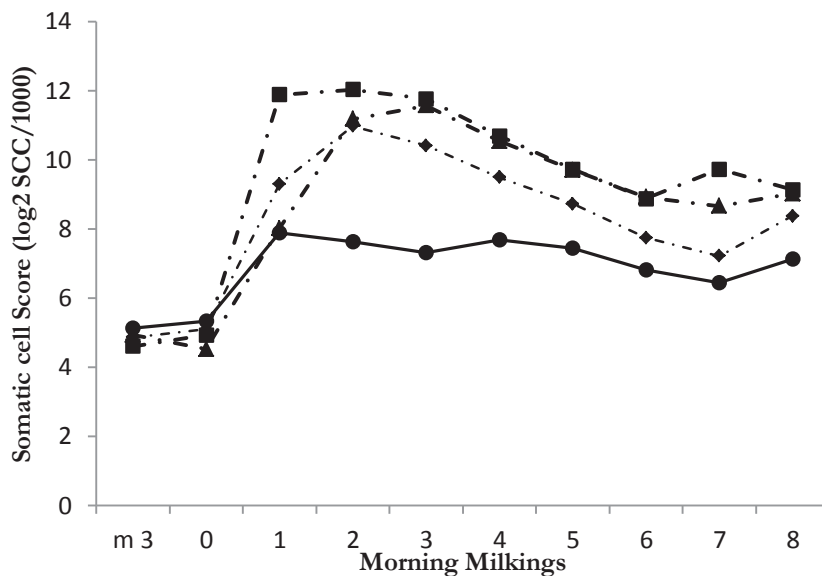
Figure 3-4 Mean somatic cell score (SCS) of the milk specimens taken every morning from three days before the challenge (m3) to Day 8 after challenge. -▲-S115; -■- S418; -●- S523; -♦- 26LB.

Table 3-4 Geometric mean of the SCC (95% CI) per strain, from Day 0 to Day 8. Milk samples were collected from a pool of challenged and non-challenged quarters at morning milkings only

Strain	Day 0	95%CI	Day 1	95%CI	Day 2	95%CI	Day 3	95%CI
26LB	34,138	(13,022-89,494)	628,557	(280,576-1,408,117)	2,009,192	(896,866-4,501,069)	1,360,103	(453,463-4,079,453)
S115	22,916	(7,636-68,772)	259,916	(116,021-582,273)	2,304,279	(1,028,587-5,162,134)	3,027,686	(1,154,135-7,942,646)
S418	30,358	(12,463-68,769)	3,753,966	(11,580-79,585)	4,178,009	(1,675,700-8,409,778	3,469,712	(1,864,985-9,359,735)
S523	40,080	(16,748-95,917)	236,878	(105,738-530,663)	197,622	(88,215-442,720)	158,749	(60,580-415,999)

Strain	Day 4	95%CI	Day 5	95%CI	Day 6	95%CI	Day 7	95%CI	Day 8	95%CI
26LB	718,876	(320,893-1,610,454)	420,890	(187,877-942,894)	213,442	(95,276-478,161)	148,663	(66,360-333040)	331,393	(147,928-742,400)
S115	1,484,746	(662,762-3,326,183)	831,977	(371,379-1,863,827)	481,503	(214,934-1,078,681)	404,109	(168,700-968,011)	519,687	(231,979-1,164,223)
S418	1,643,664	(1,156,813-10,406,952)	843,767	(733,701-3,682,199)	468,109	(376,641-1,890,238)	845,113	(208,955-1,048,675)	559,153	(249,595-1,252,636)
S523	203,954	(91,0.41-456,906)	173,549	(77,469-388,791)	112,291	(50,125-251,559)	86,259	(38,504-193,240)	139,411	(62,230-312,314)

3.5 Discussion

This study assessed the ability of four strains of *S. uberis* to induce clinical mastitis in healthy quarters after intramammary inoculation of two bacterial concentrations of a challenge suspension. The amount of clinical mastitis induced by strain S115 was higher than for strains 26LB and S523, and lower than for S418. Most cases of clinical mastitis caused by S115 responded to treatment, whereas clinical cases induced by other strains (e. g. S418), did not, suggesting that S115 is responsive to treatment with beta-lactams *in vivo*. In order to maximise curability during trials, the *in vitro* antimicrobial susceptibility of strains used in experimental challenge studies should be known in advance (Schukken and Deluyker, 1995). This is particularly relevant to animal welfare concerns, and bacteriological and clinical cures are necessary in order to ensure the return of the animals to the milk production cycle (Schukken and Deluyker, 1995).

In marked contrast to the characteristics of S115, challenge with S418 resulted in the highest proportion of clinical mastitis (100%), which was also the highest in severity, with cases persisting even after extended antimicrobial treatments. The SCC also remained high for several days after challenge. As previously stated, these characteristics of high disease severity and low curability are not desirable characteristics of a challenge bacterium to be used in experimental infection studies. Therefore, this strain was not selected for further studies.

In the present study, the overall incidence of mastitis was 54.2% and a large variability between the four different strains was observed. The proportion of clinical mastitis was relatively low compared with other studies carried out with different strains of *S. uberis*. Using the *S. uberis* strain UT888, Oliver et al., (2003) and Rambeaud et al. (2003) reported approximately 80% clinical mastitis incidence after challenge. Higher mastitis proportions (between 90 and 100%) have also been reported (Hillerton and Bramley, 1989, Pryor et al., 2009). In accordance with these results, subsequent experimental infection studies carried out with S115 achieved an infection proportion of 91% using a dose of 1.6×10^{6}/mL CFU (Chapter 4). In the study carried out by Pryor et al. (2009) five different strains of *S. uberis* were used, in which all five strains were inserted into the same quarter simultaneously. Only one strain however was isolated from the majority of the quarters challenged. The strains were also evaluated separately for their capability to cause clinical mastitis, but in contrast to

the findings of the present study, no differences were observed between the proportions of infection caused by the different strains when inoculated separately. The authors suggested that the survival mechanisms of *S. uberis* in the mammary gland might be multifactorial and highly related to individual strain virulence factors (Pryor et al., 2009). This suggestion is in accordance with the results of the present study where the incidence of mastitis differed between the strains even though all the strains were exposed to similar conditions in the mammary gland (flushing effect of milking, host immune response, etc.). It is therefore possible that there are strain-related virulence mechanisms responsible for the differences achieved in various studies.

The proportion of infected quarters achieved by the strains in the present study was expected to be variable. The Mantel Haenszel relative risk analysis did not show differences between the different strains, perhaps due to the small number of animals used. However, when comparing the aggregate RRs and clinical effects, there were significant differences between the pairs of strains S115-S418, S115-S523, 26LB-S418, 26LB-S523 and S418-S523. The capability of strain S523 to induce clinical mastitis in this study was lower than the other strains. Strain S523 induced only one case of clinical mastitis, the SCC rise was relatively moderate and clinical udder scores were lower in comparison to the other strains used here. These results also agree with results of another study where it was suggested that the capability of different strains to infect and persist in the udder could be significantly different (Pryor et al., 2009). Strains are genetically different and consequently their virulence factors are expected to vary. Genetic variability between strains of *S. uberis* can be between 40 and 50% (Douglas et al., 2000). The presence or absence of genetically coded capsule, plasmin activator and *S. uberis* adherence molecule (SUAM) are characteristics that can determine the ability of strains to colonise the mammary gland (Hill, 1988, Leigh and Lincoln, 1997, Almeida et al., 2006).

Infective dose also appears to affect the appearance of clinical mastitis signs. In this study, infective doses of 4 mL of suspension containing $1x10^6$ CFU/mL and $1x10^4$ CFU/mL were used. The peak of clinical mastitis was observed between 72 and 96 hours after challenge with no significant differences between High or Low concentrations of the challenge suspension. Rambeaud et al. (2003) observed the peak of clinical cases 144 hours after challenge when using infective doses of 6,650 CFU/mL for Jersey cows and 10,500 CFU/mL for Holstein cows, markedly lower concentrations than the ones used in the

present study. In another study 1,500 CFU/mL of the challenge suspension were administered into the mammary gland, and obtained the highest number of cases on the seventh day (or 168 hours) after challenge (Oliver et al., 2003). From this data, it appears that the higher the infective dose, the faster the signs of mastitis appear, but the highest incidence is not necessarily obtained.

According to Leigh (1999), mastitis incidence after challenge could be related more to strain virulence than to infective dose. Rambeaud et al. (2003) proposed that some strains take longer than others to establish in the mammary gland. Strain UT888 was used in studies by Rambeaud et al. (2003) and Oliver et al. (2003) with different infective doses, and both studies obtained a similar incidence although at different times after challenge, thus supporting the theory that it is the infective dose and not the strain that determines the time it takes the bacteria to establish within the mammary gland.

Another important factor that can interfere in colonisation of the udder by mastitis agents is the immune response of the host (Sordillo and Streicher, 2002, Moyes et al., 2009). Significant differences between the time of onset of clinical mastitis after challenge were found between Holstein and Jersey cows (Oliver et al., 2003). Both groups were challenged with the same strain of *S. uberis* at the same dose; however Holstein cows developed clinical mastitis earlier than Jerseys, although more Jerseys were infected (69% in Holstein cows *vs.* 86.5% in Jerseys cows). The opposite was found in another study which reported a higher incidence of mastitis in Holsteins compared with Jerseys cows (Bannerman et al., 2004). Washburn et al., (2002) and Rambeaud et. al*.,* (2003), however, found no significant difference between breeds. Unfortunately in the present study, all the cows were cross-breeds therefore such differences could not be investigated.

In order to demonstrate that the mastitis-causing agent inoculated in the study was responsible for the clinical mastitis cases, it was important to isolate the bacterium after culture of the milk specimens. In this study, Strain 26LB presented a low number of positive isolates after culture. Even though the bacterium was expected to be present in the mammary gland, it was not isolated. The milk culturing technique used in this study requires only 10 μL to be cultured on the agar plate. Consequently, it is common to find approximately 10% of the samples negative for culture due to the small amount of milk used for culture (Milne et al., 2005). The particular behaviour of strain 26LB in this study in terms of the low percentage of successful isolation after culture suggests that individual virulence

factors could exist within this strain making it difficult to isolate from clinical cases. This was considered a disadvantage of this strain in comparison with the others. The isolation of the agent after infection in experimental challenge studies is considered of high importance to avoid or at least to document "field strains" interfering in the results. This finding further reinforces the use of S115 for future research, as 6 out of 7 milk samples from clinical mastitis cases were successfully isolated and identified as *S. uberis* after culture.

3.6 Conclusions

Based on its moderate ability to produce clinical mastitis in lactating cows, its susceptibility to antibiotics, isolation after culture and the moderate severity of the clinical signs (that did not produce any long term consequences for the infected cows), the *S. uberis* strain S115 was selected for use in further experiments using challenge models.

3.7 Acknowledgment

CHAPTER 4

Efficacy of a cloxacillin based intramammary product in treating experimentally induced *Streptococcus uberis* clinical mastitis in lactating cows

4 Efficacy of a cloxacillin based intramammary product in treating experimentally induced *Streptococcus uberis* clinical mastitis in lactating cows

4.1 Abstract

The aim of this study was to test the efficacy of a novel cloxacillin-based antimicrobial product in treating experimentally induced *Streptococcus uberis* clinical mastitis in lactating cows and compare this product with a currently marketed penicillin based antimicrobial. Forty cows were experimentally challenged in two quarters by inoculating a known strain (S115) of *S. uberis* into the quarters. Out of the eighty quarters challenged, 73 quarters (91.25%) developed clinical mastitis and were treated for three consecutive treatments as soon as clinical signs appeared. Cows were randomly allocated to two treatment groups upon presentation with clinical signs. One group of 31 cows was treated with a novel cloxacillin-based product. One syringe was administered into the infected quarter every 24 hr. for three consecutive days following the manufacturer`s instructions. A positive control group of 9 cows was treated with a penicillin-based product, with one syringe administered every 12 hours for three consecutive milkings as per label instructions. Clinical cases in which the quarter did not responded to three applications (short treatment) of the allocated product received an extended treatment of the same product (n= 26). As this allocation to the extended treatment was not random, clinical and bacteriological cures were statistically analysed and compared between treatment groups for the short treatment only.

From the 31 cows allocated to the Cloxacillin group, 91.93% quarters developed clinical mastitis (57/62). Five quarters in this group did not develop mastitis (8%). Seventy per cent of the clinically infected quarters were treated with a short treatment course (40/57). Seventeen quarters did not show clinical improvement after short treatment (30%) and required extended Cloxacillin treatment. From the 40 quarters that received the short treatment, ten quarters showed recurrences three to four days after the short treatment with Cloxacillin finished, and were considered to be treatment failures.

From the nine cows allocated to the penicillin treatment group, 89% of quarters developed clinical mastitis during the observation period (16/18). Two quarters did not develop

mastitis in the penicillin treatment group. Seven of the clinical mastitis quarters (44%) responded to the short penicillin treatment course. All but one of the nine quarters which remained infected after the short course, were cured after extended treatment. The quarter that did not respond to the treatment received additional antimicrobial treatment until full recovery and was considered treatment failure for the extended treatment.

Clinical cure rates for the short treatment (3 syringes) were 52.63% (30/57) and 43.75% (7/16) for the cloxacillin and penicillin-based treatment groups, respectively. There was no significant difference between the treatments (P = 0.8). Both treatments were found to be similar in their efficacy for the treatment of experimentally induced *S. uberis* clinical mastitis.

4.2 Introduction

In recent years, the prevalence of environmental micro-organisms has increased in most of the dairy countries around the world (Oliver et al., 2003). *Streptococcus uberis* has become one of the most important mastitis-causing agents isolated from not only New Zealand dairy farms but also worldwide (McDougall, 1998, Oliver et al., 2003, McDougall et al., 2007, Petrovski et al., 2011). It is primarily considered an environmental agent, although it can be transmitted from cow to cow (Zadoks et al., 2005). Its high genetic variability, virulence factors and its ubiquity in the environment of the cow, have made this agent difficult to eliminate from herds (Oliver et al., 2003). Currently, there are several control methods under development, such as the development and use of new beta-lactam products, which attempt to limit *S. uberis* infections on the farms and reduce economic losses to the dairy industry.

Cloxacillin sodium is a beta-lactam antibiotic classified as penicillinase-resistant penicillin. Cloxacillin and other penicillinase-resistant antibiotics are considered useful for treating certain bacterial infections such as *Staphylococcus sp.* infections when other penicillin products appear to fail (Mattie et al., 1973, Davis et al., 1975). In addition, cloxacillin has shown 100% effectiveness in eliminating *S. uberis* from *in vitro* tests carried out with New Zealand strains (Petrovski et al., 2011b). For this reason, it was the drug chosen for efficacy testing against clinical mastitis artificially induced with the *S. uberis* strain S115.

The primary objective of this study was to determine the efficacy of a cloxacillin-based product for treating experimentally induced *S. uberis* mastitis infections and compare it with the efficacy of a penicillin-based antimicrobial that is already in the market. Clinical and microbiological cure rates were evaluated after treatment with this product. The experimental challenge model used in the present study was also evaluated to determine its capacity to induce clinical mastitis in future efficacy tests.

4.3 Materials and methods

4.3.1 Animal selection and husbandry

Eighty crossbred lactating cows (Friesian x Jersey) from a herd of approximately 460 spring-calving cows from Massey University N°4 Dairy farm (Manawatu Region, New Zealand) were enrolled in the pre-selection phase of the study which was conducted in November 2011.

Average days in milk (DIM) was 71±17 DIM for the cows included in the study and they were 5±1.7 years old. The inclusion criteria for pre-selection were: Healthy cows with four functional quarters, no history of mastitis in the current lactation, no anti-microbial treatments given within 14 days of study commencement, SCC <250,000 cells/mL and negative milk cultures for major pathogens. Cows with minor pathogens (e.g. coagulase negative staphylococci or *Corynebacterium* spp.) were however included.

Cows were managed as a single mob separated from the main milking herd and milked twice a day in a fifty-bale rotary milking parlour. Cows were identified by permanent, uniquely numbered neck collars. The feeding regimen was pasture-based with rye grass and clover cultivars (*Lolium perenne* and *Trifolium repens)*, and water was available *ad libitum*.

Cows enrolled in the study were randomly allocated to be challenged in either the Front Right/Rear Left (FR/RL) or Front Left/Rear Right (FL/RR) contralateral quarters (20 cows for each). For allocation to challenge groups and randomisation cows were ranked according their average milk yields in the 7 days before challenge (range 14.3–27 L per cow per day). Cows were blocked in pairs and each consecutive pair of cows in this ranked list received a random block number using the random number function of Excel 2007

(Microsoft Corporation, USA). Two different challenge distributions (e.g. FR/RL or FL/RR) were assigned randomly to each block. Once the cows presented with clinical mastitis they were randomly allocated to treatment at a ratio of 3:1 (3 cloxacillin-based product to 1 penicillin-based product). Cows with a second infected quarter which manifested after the first, received the same product as the first infected quarter.

4.3.2   Culturing of milk samples

Milk specimens were collected from the pre-selected cows using an aseptic technique. These specimens were cultured at the Microbiology Laboratory at the Institute of Veterinary, Animal and Biomedical Sciences (IVABS), Massey University, Palmerston North. The culture procedure followed the National Mastitis Council Guidelines (National Mastitis Council, 1999). Briefly, an aliquot of 10 μL from each quarter was plated on one quadrant of a trypticase soy agar plate supplemented with 5% defibrinated sheep blood (Fort Richard, Auckland, New Zealand). Plates were incubated at 35 $\pm$ 2°C in an aerobic environment and bacterial growth was observed after 24 and 48 hr of incubation. Infected and contaminated quarters were identified and cows infected with major mastitis-causing agents were excluded from the experiment. Forty cows that met the selection criteria were enrolled in this study.

4.3.3   Challenge strain

The *S. uberis* strain (S115) used for the challenge was provided by the scientific team of Dairy NZ (Newstead, Hamilton), originated from a clinical case from Wairarapa region, and was stored in the IVABS Microbiology Department Laboratory, Massey University. The strain was phenotypically identified as *S. uberis* by means of biochemical test. Its capacity to cause the development of clinical mastitis was shown in a previous study (Chapter 3).

Preparation of the *S. uberis* challenge suspension was carried out at the IVABS Microbiology Laboratory. Four days before the challenge, an aliquot of the frozen challenge strain was thawed and streaked onto 5% blood agar (BA) plates (Fort Richard, Auckland, New Zealand). The plates were incubated at 35 $\pm$ 2°C for 48 hr. in the presence

of 5-10% CO_2 enriched environment. The colony-growth of bacteria was observed for purity, then harvested and suspended in phosphate buffered saline (PBS) (0.01 M, pH 7.3). From this suspension, 100 μL were used to flood 10 BA plates and these were incubated for 48 hr. as above.

On the day of the challenge (Day 0), *S. uberis* bacteria were harvested from the BA plates using cotton swabs soaked in sterile PBS. The swab was then rinsed in 5-10 mL of sterile PBS into a sterile tube. In the tube, the suspension was visually adjusted for turbidity corresponding to a McFarland turbidity standard of 0.5 (Remel, Lenexa, Kansas, USA) using sterile PBS. Such a procedure was expected to generate a bacterial suspension containing approximately 10^7 CFU/mL (Petrovski et al., 2011a). Following the adjustment of turbidity, the working suspension was further diluted using serial dilution in PBS at one ten-fold dilution, generating a final microbial suspension of approximately 10^6 CFU/mL. This final suspension was transferred into series of sterile syringes for intramammary administration by drawing 4 mL of the suspension into the syringe. Once the syringe was filled, the nozzle cover was applied. These ready-to-use syringes were then stored in portable chilled boxes with ice packs for transportation to the farm.

On the day of challenge, 1 mL of the final microbial suspension was diluted ten-fold in PBS. The serial ten-fold dilutions were carried out until the suspension was estimated to be at 10^{-1}, 10^{-2} and 10^{-4} CFU, and 100 μL was spread in triplicate on 5% BA. Plates were incubated as previously described. The arithmetic means of the number of CFU that grew with approximate 30 to 300 CFU on the plates was used to estimate the number of CFU/4mL of inoculum for the challenge suspension. This retrospective purity test estimated the final microbial suspension to be 1.64×10^6 CFU/mL.

4.3.4 Challenge procedure

Immediately after milking was completed, each cow was experimentally challenged in two contralateral quarters (*i.e.* FR/RL or FL/RR). Before inoculation, the teat-ends were thoroughly cleaned using alcohol-dampened cotton swabs. Challenge suspension was inoculated into the quarters by full insertion of the nozzle through the teat canal. The quarter was then massaged in an upward direction to ensure the pathogen was dispersed as far up into the quarter as possible. The entire content of one syringe was administered to

each of the quarters to be challenged. The two non-inoculated quarters served as negative controls.

4.3.5 Clinical examination

Clinical examinations were conducted at each milking following challenge for 13 consecutive days (Study Day 0-12) for signs of clinical mastitis (clots, colour changes in the milk, etc.). The foremilk of each quarter was examined prior to milking using the blackboard strip test (Thomas, 1949). Individual quarters of the udders were inspected after milking and palpated for clinical signs consistent with mastitis (i.e. heat, swelling, redness and painful quarter/s). Table 4-1 shows the udder clinical scoring system used for the udder examinations.

Table 4-1 Clinical scoring criteria applied for the challenged quarters during the examination period. Adapted from Petrovski et al., (2011a).

Score	Description
0	No evidence of irritation, pain and/or redness or swelling of the quarter.
1	No or virtually no evidence of irritation, pain, redness or very slight swelling of the quarter.
2	Evidence of irritation and/or pain of a minor intensity, slight redness and/or swelling, likely to originate from residual milk in the quarter.
3	Evidence of irritation and/or pain of a moderate intensity or moderate redness, swelling and/or heat of the quarter. Secretion contains small clots or flecks.
4	Evidence of irritation and/or pain of a severe intensity, severe redness and/or severe swelling and heat of the quarter. The secretion contains large clots or flecks.
5	Evidence of severe irritation or pain, severe swelling, redness and/or heat, associated with generally sick animal. The secretion contains large clots or flecks.

Mammary glands were considered to have clinical mastitis when the clinical examination score was equal to or higher than three, or when the foremilk secretion presented with clots and/or flecks.

4.3.6   Sample Collection

Once signs of mastitis were detected by palpation or foremilk appearance, milk samples were collected in duplicate using an aseptic collection technique. Cows were then milked as per normal practice. On the afternoon of Study Day 4 aseptic milk samples were collected from all challenged quarters that had not yet shown clinical signs of mastitis. These samples were submitted for microbiological culture and were used to indicate subclinical mastitis when there was bacterial growth after culturing.

4.3.7   Clinical mastitis treatment

Quarters with clinical mastitis were treated with a cloxacillin-based product every 24 hr. or a penicillin-based product every 12 hr. for three consecutive administrations. The treatment chosen for each cow depended on their allocation to treatment group. Clinical cure was determined when there was no swelling in the quarter (udder clinical score of 1 or 0) and no flecks/clots were observed in the foremilk. A quarter was defined as microbiologically cured when the results of the laboratory cultures done five to six days after the last treatment were negative.

 If no clinical improvement was observed after the third administration, the result was recorded as a treatment failure and an extended treatment continued immediately for a further three syringes. Cows that were not cured with extended treatments received penethamate hydrodide (Penethaject, Bayer Animal Health Laboratories Ltd., Auckland, New Zealand) and ketoprofen (Ketofen™ Merial Animal Health Ltd.) systemically, until full recovery. Clinical cases in which clinical improvement was observed after three administrations, but where the quarters showed recurrence of the clinical signs after three to four days beyond conclusion of the first treatment, were considered to be treatment failures.

Clinical and microbiological cure were assessed 5 days after the last treatment of the initial treatment course (3 syringes = short treatment) and 5 days after the extended treatment (6 Syringes) (Figure 4-1)

Cloxacillin   Penicillin

57 Clinical quarters   16 Clinical quarters

40 Short   17 Extended   7 Short   9 Extended

Figure 4-1 Diagram of the allocation to treatment for cloxacillin and penicillin groups

4.3.8 Statistical analysis

The number of clinical mastitis cases, bacteriological culture results and clinical scores were analysed at the quarter level, whilst the SCC values were analysed at the cow level. The proportions of clinical and bacteriological cures were compared between the treatment groups using the two tailed Fisher's exact test using Excel, Episheet 2004 (Rothman, 2002).

Somatic cell count were not normally distributed and were transformed into somatic cell scores (SCS), calculated as SCS= $\log_2$ (SCC/1000). The differences in SCS between the two treatments were analysed by means of repeated measurements analysis of variance (rmANOVA) using the MIXED PROCEDURE of SAS (version 9.3 SAS Institute Inc., Cary, NC, USA) with a 5% significance level (P<0.05). The model included treatment, time of sampling (TS), and the interaction between treatment and TS as a fixed effect, and cow as a random effect to account for the within cow variability. As SCC was collected in the mornings only, another analysis was performed with the same model but including time to the first SCC as a variable. This variable showed no significance and was dropped from the statistical model. The geometric means and standard errors of SCC for each treatment group, and day of measurement were also calculated as they are more representative of the real mean than the least square means.

4.4 Results

4.4.1 Clinical cure

During the observation period, all challenged cows developed clinical mastitis in at least one quarter. The incidence of clinical mastitis at the quarter level was high, with 91.25% (73/80) of challenged quarters affected. Only one unchallenged quarter showed signs of clinical mastitis and was not included in the analysis.

Thirty-one cows were allocated to the Cloxacillin group. From these 62 quarters, 92% (57/62) were observed with clinical mastitis and treated. Only 8% (5/62) of the quarters in this group did not develop mastitis.

From the clinically infected quarters in the Cloxacillin group, 70% (40/57) appeared to be clinically cured after the third treatment. Thirty per cent of the quarters (17/57) showed clinical signs of infection even after the third treatment (palpation scores >3 and/or clots in milk). These quarters were recorded as treatment failure for the short course and received the extended course of treatment (6 syringes; Figure 4-2). Between three to five days after the third treatment, ten cows that had appeared to be clinically cured after the short course of treatment presented signs of recurrence (palpation scores >3 and/or clots in milk). These cows were included in the treatment failure group, leaving a total of 52.63% (30/57) clinically cured quarters after the short treatment with cloxacillin (Table 4-2).

From the nine cows allocated to the penicillin treatment group, 89% (16/18) of the challenged quarters developed clinical mastitis during the observation period (Figure 4-2). Two out of eighteen quarters (11%) did not develop clinical mastitis.

Forty-four per cent (7/16) responded to the short penicillin treatment course and 56% (9/16) remained infected and therefore received an extended treatment (6 syringes). Eight out of the nine quarters that received extended treatment with penicillin, were clinically cured after treatment (Figure 4-2). No recurrences were observed in this group. There was no significant difference between the clinical cure rates of the two treatment groups for the short treatment course (Fisher's two tailed exact P=0.8; Table 4-2).
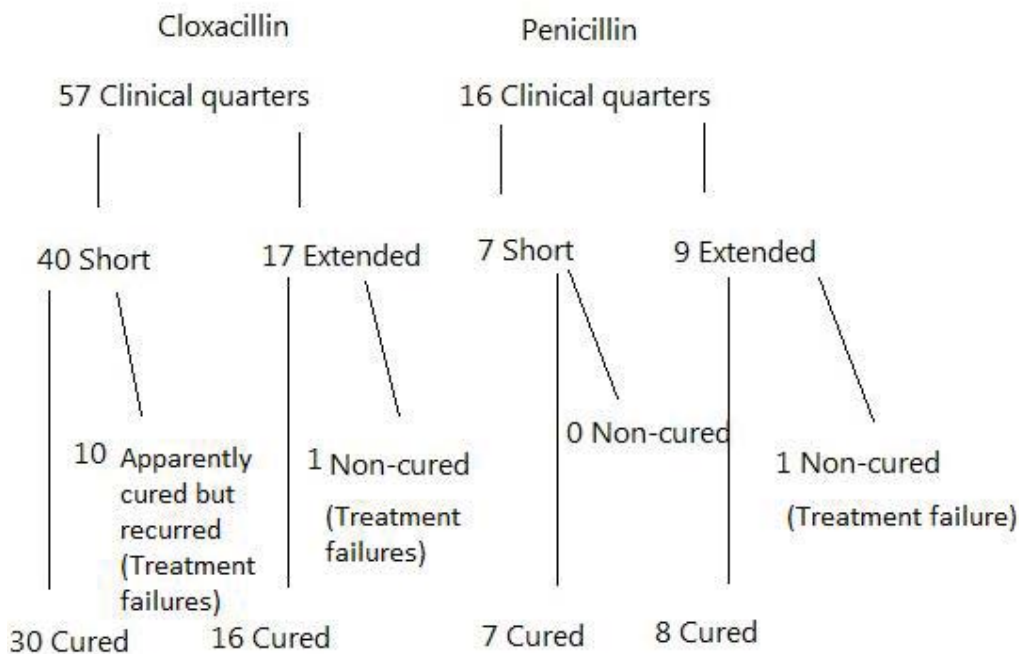
Figure 4-2 Diagram of the treatment group allocation and results after treatment

Extended Cloxacillin treatment of the quarters resulted in 94% of the infected quarters being cured (16/17) (Table 4-2). The proportion cured by extended treatment in the Penicillin group was not as high as in the Cloxacillin group, only 89% (8/9) of the quarters responded to the extended treatment. No statistical analysis was carried out on the extended treatment groups as the allocation to treatment was not random.

Table 4-2 Clinical cure rates for short, and extended treatments in percentages. [a] Derived from the Fischer Exact test two-tailed analysis

	Cloxacillin	Penicillin	Total	P-values [a]
Cure Short treatment	52.63% (30/57)	43.75% (7/16)	50.68% (37/73)	0.8
Cure Extended treatment	94.12% (16/17)	88.89% (8/9)	92.30% (24/26)	
TOTAL	**80.70% (46/57)**	**93.75%(15/16)**	**83.56% (61/73)**	**0.8**

4.4.2   Bacteriological cure

By the end of the study 80 milk samples had been cultured, 91.25% (73/80) of which were from clinical cases. Each treated quarter was sampled 5 days after the administration of the last antimicrobial treatment. Eight non-clinical samples were cultured on Study Day 4 for determination of subclinical infections. One of these quarters later developed clinical mastitis and was subsequently included in the analysis for clinical cases, leaving 7 quarters that were not clinical for the duration of the study (Table 4-4). Two of those samples were positive for *S. uberis* on that day, but by Study Day 13 had negative culture results indicating "self-cure". Five samples were negative on Study Day 4. On Study Day 13 one sample was contaminated, and the remaining 6 samples were negative for any pathogens (Table 4-3).

Table 4-3 Culture results from the non-clinical cases in Day 4 and 13 of the study

	Study Day 4	Study Day 13
Positive	2	0
Negative	5	6
Contaminated	0	1
Total Non-Clinical	7	7

Bacteriological cure was determined when no growth was observed after 48 hr. incubation in the samples taken from infected quarters 5 days after the last treatment.

From the 73 clinical cases, 89.0% had a positive culture prior to treatment and 11.0% had a negative culture despite showing clinical signs (changes in appearance of milk or high clinical scores). These animals were treated and included in the analysis based on clinical signs (Table 4-4).

A single sample still had a positive culture after treatment in the Cloxacillin group and received further treatment. The remaining samples were either contaminated 6.8% (5/73) or the culture was not performed 6.8% (5/73).

Post-treatment culture results showed an overall bacteriological cure (negative cultures) of 84.9%. In the Cloxacillin group, 85.9% of the quarters presented bacteriological cure and

75% in the Penicillin group (Table 4-4). The difference in bacteriological cures between the treatments was not statistically different (P=0.83).

Table 4-4 Culture results of the samples, taken before and after treatment (short or extended treatments are shown together). Pre-T: Pre-treatment sample; Post-T: Post-Treatment sample. Fischer Exact test two tailed analysis (P=0.83).

	Cloxacillin		Penicillin		Total
	Pre-T	Post-T	Pre-T	Post-T	
Negative Culture	12.2%(7/57)	85.9%(49/57)	12.5% (2/16)	75% (12/16)	
Positive Culture	50	1	14	0	65/73 (89%)
Contaminated	0	4	0	1	5/73 (6.8%)
Not cultured	0	2	0	3	5/73 (6.8%)

4.4.3 Somatic Cell Count

Somatic cell scores (SCS= log2 SCC/1000) were analysed at the cow level from a 4 quarters composite milk sample to compare treatment groups.

In the cloxacillin and penicillin group, 31 and 9 cows were analysed respectively (Figure 4-3). To evaluate the effect of treatment on the SCC, the data was standardised to compare SCS on the day after the clinical mastitis was diagnosed and treated (Day 1). The main reasons for the rises in the SCC observed were recurrences of clinical mastitis after treatment failures or second quarters showing clinical signs after the first quarter was diagnosed (Figure 4-3).

A significant difference (P<0.001) was found between the capability of the treatments to lower the SCC (Figure 4-3). By Day 9 after treatment, both treatments achieved normal physiological SCC levels (≤250,000 SCC/mL). The time taken for SCC to return to normal physiological levels was 9 and 6 days for the cloxacillin and penicillin-based products, respectively.

The geometric mean of the SCC of the cows at the first day after treatment for the cloxacillin group was 1,004,109 cells/mL (range 620,915-1,623,788), whereas in the penicillin group SCC was 662,419 cells/mL (range 274,029-1,601,282 cells/mL). This difference between the treatments was significant during the period analysed (P<0.001; Table 4-5).
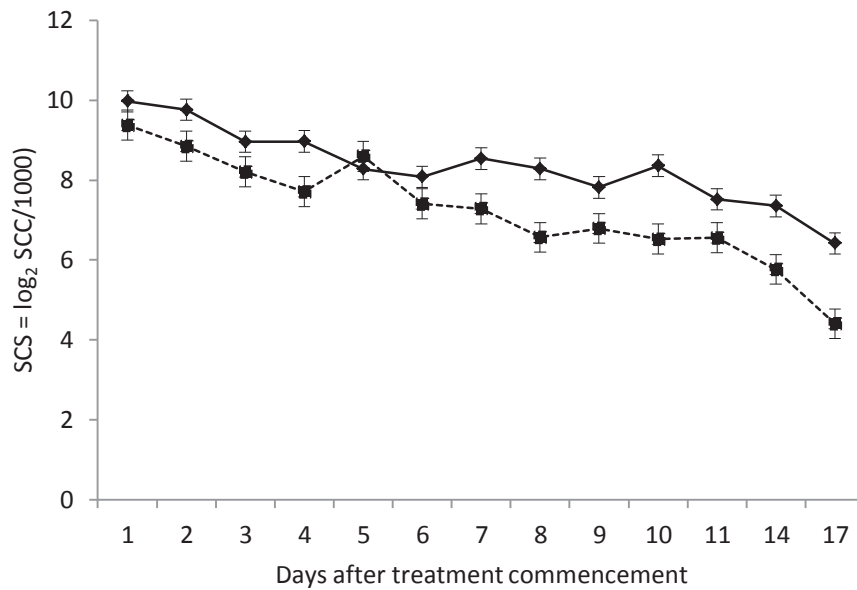
Figure 4-3 Patterns of Somatic Cell Scores (SCS= $\log_2$ SCC/1000) for cows since the first day after treatment. -♦- Cloxacillin; -■- Penicillin.

Table 4-5 Somatic cell scores, Standard errors (SE), geometric means and 95% confidence intervals for the somatic cell count (SCC) for cows treated with Cloxacllin and Penicillin group from 1 milking after positive diagnosis with clinical mastitis. n: number of cows with clinical mastitis (CM) by day after diagnosis of the clinical case

Milking post diagnosis	Cloxacillin					Penicillin				
	SCS	SE	SCC	95% CI	*n*	SCS	SE	SCC	95% CI	*n*
1	9.9717	0.3538	1,004,109	620,915- 1,623,788	31	9.3716	0.6497	662,419	274,029- 1,601,282	9
2	9.7613	0.3538	867,849	536,655- 1,403,436	31	8.8484	0.6497	460,929	190,677- 1,114,215	9
3	8.9579	0.3578	497,275	305,835- 808,547	31	8.2054	0.6497	295,170	122,105- 713,520	9
4	8.9665	0.3824	500,248	297,551- 841,024	31	7.7055	0.6497	208,731	86,347- 504,570	9
5	8.2797	0.3822	310,769	184,898- 522,327	31	8.5946	0.7495	386,574	139,641- 1,070,166	9
6	8.0833	0.4167	271,216	153,976- 477,723	31	7.402	0.7496	169,131	61,086- 468,276	9
7	8.538	0.3879	371,701	219,445- 629,596	31	7.281	0.8036	155,525	52,198- 463,381	9
8	8.2831	0.3538	311,503	192,625- 503,744	31	6.5668	0.6497	94,799	39,216- 229,160	9
9	7.818	0.3538	225,659	139,541- 364,922.	30	6.7904	0.6497	110,691	45,790- 267,577	9
10	8.3597	0.3716	328,489	198,275- 544,216	29	6.5224	0.6497	91,926	38,027- 222,214	9
11	7.5196	0.4016	183,495	106,334- 316,647	23	6.5574	0.8775	94,183	28,591- 310,252	9
14	7.3538	0.4353	163,574	90,548- 295,494	17	5.7657	0.8782	54,406	16,500- 179,392	6
17	6.4154	0.6756	85,355	34,088- 213,719	10	4.4054	1.6203	21,191	2,345- 191,498	4

4.5 Discussion

This study used an experimental challenge model with *S. uberis* to test the efficacy of two treatments. The results showed no significant differences in the efficacy between the two treatments tested. The study aimed to analyse the proportion of clinical and bacteriological cures achieved by one cloxacillin- and one penicillin-based treatment. Clinical cure rates after short treatments (three syringes per treatment) resulted in relatively low cure rates (53% and 44% for cloxacillin and penicillin-based products, respectively). The cure rates for extended treatments (six syringes) increased efficacy in both treatments groups (94.1% and 88.9% for the cloxacillin- and penicillin-based antibiotics, respectively).

In accordance with these results, Milne et al. (2005) showed that 51% of the clinical *S. uberis*-infected quarters failed to respond to conventional treatment regime (three syringes). However, unlike the current experiment, only 55% responded to extended treatments. Most conventional antibiotic treatments consist of three syringes applied every 12 or 24 hr. depending on the drug and formulation. These types of treatment have usually achieved clinical cure rates between 60-70% (Oliver et al., 2003), although bacteriological cure has been demonstrated to range between 60 and 80% (Hillerton et al., 1995, Pyörälä and Pyörälä, 1997, McDougall, 1998). After extended treatments, the present study showed higher bacteriological cure rates than the results obtained in these other studies. Bacteriological cure rates for extended treatments in the present study were 86% and 75% for the cloxacillin- and penicillin-based products respectively, indicating that extended treatments may increase bacteriological cure rates when compared to shorter treatments. These results were similar to those obtained after the extended treatment with pirlymicin which resulted in 85% of bacteriological cure after a 5-day treatment, as compared with a 2-day treatment which achieved a 67% cure rates (Oliver et al., 2003).

In recent years there has been an increasing tendency to seek more efficacious treatments for mastitis. Mistreatment of some mastitis infections has led to the development of resistant bacterial strains (Pitkälä et al., 2004). The objective of extended treatment therapies is to avoid persistent infections and in the long term, to decrease the use of antibiotics in dairy cattle. Despite that, some consider that is not economically justifiable to keep milk out of supply for the duration required for extended therapies (Swinkels et al.,

2005). However, increasing clinical and bacteriological cure, even by the use of extended treatments, decreases the risk of recurrences of clinical mastitis (Milne et al., 2005). Therefore, over the duration of lactation, the amount of antimicrobials used and milk discarded could be reduced in the long term. Several authors have agreed that extended treatments for clinical mastitis are economically more beneficial when compared with shorter ones when looking long term (Owens et al., 1997, Sol et al., 2000, Gillespie et al., 2002, Oliver et al., 2003).

In the present work, 5-6 days after the last treatment, samples were taken to evaluate bacteriological cure rates and to determine treatment success or failure. Cows were considered cured when there was no growth in the post-treatment cultured samples. This is considered one limitation to the current work as *S. uberis* bacteriological cure rates determined before 21 days following the last treatment can lead to confounding results due to bacterial persistency that cannot be detected as a consequence of residual inhibitory substances (Milne et al., 2005). Budget limitations and the study design determined the absence of a 21 days post-treatment culture. However, there are other parameters that have been evaluated in the present study to determine bacteriological cure after treatment. It has been demonstrated that there is a correlation between lower SCC and a negative bacterial culture results (Pyörälä and Pyörälä, 1997, Milne et al., 2005). In other words, a decrease in SCC is directly correlated with lower concentrations of bacteria in the udder (Pyörälä and Pyörälä, 1997, Schukken et al., 2003). Despite that correlation, SCC can remain high during some period after the elimination of mastitis-causing bacteria (Deluyker et al., 1993). As for any process of recovery from inflammation, the quarters take some time to return to physiological levels of SCC. Depending on the severity of the clinical case and the mastitis-causing agent, some quarters never recover (Heald, 1979). The individual SCC per cow in the present study was evaluated up to 17 days after treatment and the data showed a decreasing trend, with a return of normal physiological parameters 9 days after the diagnosis and treatment of the clinical case in both treatment groups, which is consistent with the high bacteriological cure rates obtained after treatment.

Despite the high bacteriological cures obtained in this study, the cloxacillin-based product was not as effective as the penicillin-based treatment at returning the SCC to levels considered to be physiologically healthy levels (200,000-250,000 cells/mL; Dohoo and Leslie, 1991). However, the time taken by cloxacillin and penicillin products to lower the SCC in this study are in accordance with other studies where the SCC took from ten days

to four weeks to return to normal physiological levels after the elimination of the infectious agent (Deluyker et al., 1993, Pyörälä and Pyörälä, 1997). This suggests that the efficacy of both products in lowering the SCC is still within the normal period for recovery despite the significant difference found between treatments.

As *S. uberis* was isolated after culture of the two clinical cases observed on Study Day 10, they were considered late cases induced by the same causal bacteria. It was postulated that those cases were produced by a field strain of *S. uberis* rather than S115. No genetic analysis was carried out on the isolates to confirm the presence of strain S115 in those or any of the clinical cases presented in this study due to limitations in the budget and because it was not considered essential, as the main objective of this study was to demonstrate the efficacy of the antimicrobial products.

Cloxacillin has been demonstrated to be effective against the most common mastitis-causing agents found on dairy farms (Davis et al., 1975) and *in vitro* studies carried out in New Zealand demonstrated that cloxacillin is highly effective against pathogens such as *S. uberis* and *Streptococcus dysgalactiae* (Petrovski et al., 2011b). For some antimicrobials, the efficacy found in *in vivo* and *in vitro* studies differ significantly (Craven, 1987). Clinical-multi-strain field trials are usually used to test new products. However, large scale field studies are considered expensive by the animal pharmaceutical industry (Schukken and Deluyker, 1995). The results obtained in this small scale study reporting the effectiveness of antimicrobials *in vivo* could be considered valuable as a predictor of the results that could be obtained in multi-strain field studies with the same product. The results of small scale studies can be obtained in a shorter period of time, they can generate statistically significant data and the animals enrolled can be monitored daily to avoid animal welfare concerns.

4.6 Conclusion

Cloxacillin resulted in similar cure rates to a penicillin-based product in a short treatment regimen. These two products can be considered equivalent in their efficacy, as the difference in the clinical and bacteriological cure rates were not statistically significant. Cure rates for both treatments increased after extending treatments from three to six applications per quarter. These results suggest that higher cure rates after extended treatments should be considered despite milk being withheld from the bulk vat for a longer period.

4.7 Acknowledgement

CHAPTER 5

Clinical and bacteriological response to treatment with long acting cloxacillin-based products of experimentally induced *Streptococcus uberis* clinical mastitis in lactating cows

5 Clinical and bacteriological response to treatment with long acting cloxacillin-based products of experimentally induced *Streptococcus uberis* clinical mastitis in lactating cows

5.1 Abstract:

Forty cows were experimentally challenged in two contralateral quarters to induce clinical mastitis by inoculating a known strain of *Streptococcus uberis* (S115) in a dose of 8×10^5 colony forming units (CFU/mL). The cows enrolled in the study were randomly allocated into two treatment groups as clinical signs were detected. After inoculation, 41 quarters developed clinical mastitis and were treated as soon as the signs of mastitis appeared. One group of 29 quarters (23 cows) was treated with a novel long-acting intramammary Cloxacillin-based product with a dose regimen of one syringe every 48 hr. for three administrations. A positive control group consisting of 12 quarters (8 cows) was treated with a different long acting cloxacillin-based product every 48 hr. following the label instructions. Clinical and bacteriological cures were evaluated in both groups after treatment.

Treatment with the novel product resulted in a treatment success of 93.1% based on clinical examination, and 96.0% based on bacterial cure. Treatment with the control product resulted in total treatment success of 100% based on clinical and bacteriological cure. There was no significant difference between the products (P=0.19).

5.2 Introduction:

Clinical mastitis is still a high cost, production-related disease for the dairy industry. Management changes such as hygiene and culling of chronically infected cows on farms, and the development of more effective products to treat mastitis agents are conducive to reducing bulk somatic cell count (BSCC) and subclinical mastitis on dairy farms. However, the annual cumulative incidence of clinical mastitis in New Zealand is still approximately 15% for most farms (McDougall, 1998, McDougall et al., 2007, Petrovski et al., 2011c).

Mastitis-causing agents have been changing in their relative prevalence. In recent years, the incidence of environmental micro-organisms has increased in most of the dairy farms around the world (Oliver et al., 2003). In the last few decades, *S. uberis* has become one of the most important mastitis-causing agents isolated from New Zealand dairy farms (McDougall, 1998, McDougall et al., 2007, Petrovski et al., 2009). Its characteristics as an environmental and contagious agent its high genetic variability and virulence factors have made this agent difficult to eliminate from farms (Oliver et al., 2003). In order to control infections caused by these bacteria on farms, or at least, to diminish the economic losses that they are producing in the dairy industry, enhanced control methods, like the development of more effective antimicrobial products, need to be implemented.

Cloxacillin sodium is an antibiotic belonging to the penicillinase-resistant penicillins group. This and other penicillinase-resistant antibiotics are considered convenient in combating certain bacterial infections such as *Staphylococcus spp.*, when other penicillins would not work (Mattie et al., 1973, Davis et al., 1975). Cloxacillin is a semi-synthetic drug derived from penicillin. Its mechanism of action is to inhibit the trans-peptidase enzyme in the wall of bacteria (Wise Jr and Park, 1965). This enzyme gives rigidity to the peptidoglycan of the bacteria. In contact with the molecule of penicillin, the bacteria are unable to maintain an intact external wall and die by osmotic lysis. To be effective, these antimicrobial substances must be in contact with the bacteria over the minimum inhibitory concentration (MIC) for sufficient periods. Bacteria must also be actively multiplying and synthesising the external wall at the time the antimicrobial is acting. This action mechanism is considered "time dependant". The pharmaceutical industry has created long acting cloxacillin-based intramammary products which can maintain their MIC for long periods without the necessity of repeating the doses every 8 or 12 hours. Consequently, labour and milking time on the farm can be reduced. Furthermore, from *in vitro* tests conducted with New Zealand and USA strains, cloxacillin has been shown to have 100% effectiveness in eliminating *S. uberis* (Petrovski et al., 2011b). For these reasons this long acting product was chosen for this efficacy study.

The primary objective of this study was to determine the efficacy of this novel long-acting cloxacillin-based product (Clox 48) in treating experimentally induced *S. uberis* mastitis infections in cows in active lactation and compare it with another long acting antimicrobial used as a control product. Clinical and bacteriological cure rates after treatment with these products were evaluated and compared.

5.3 Materials and methods:

5.3.1 Animal selection and husbandry

Eighty crossbreed lactating cows (Friesian x Jersey) from Massey University Nº4 Dairy Farm (Manawatu Region, New Zealand) were pre-selected to be enrolled in this efficacy study from a herd of approximately 460 spring-calving cows. This study was conducted in March 2012. The inclusion criteria for the pre-selection were: Healthy cows with four functional quarters, no history of mastitis in the current lactation, no anti-microbial treatments given within 14 days of study commencement, somatic cell count (SCC) <250,000 cells/mL and negative milk cultures for major pathogens. Cows with minor pathogens (e.g. coagulase negative staphylococci or *Corynebacterium* spp) were included. Average days in milk (DIM) for the cows included in the study were 184.3 (±19.0). Cows enrolled in the study were 4.3±1.3 years old.

Enrolled cows were managed separately from the main herd as a single mob and milked twice daily in a fifty-bale rotary shed. Cows were identified by permanent uniquely numbered neck collars. The feeding regimen was pasture-based nutrition with rye grass-clover cultivars (*Lolium perenne* and *Trifolium repens*) and water was available *ad libitum*.

Cows were randomly allocated to challenge in either their Front Right/Rear Left (FR/RL) or Front Left/Rear Right (FL/RR) contra lateral quarters (20 cows in each). A list of the cows was prepared based on 7 day average milk yields. The range for milk yield was between 10.2 and 25.0 L per cow per day. Cows were ranked by production and blocked into pairs. Each pair of cows received a block random number (Microsoft Excel 2010) which corresponded to challenge in either FR/RL or FL/RR. Once the cows showed clinical signs of mastitis, they were allocated to treatment at a ratio of 3:1 (Clox 48: Control). Twenty nine quarter**s** (23 cows) were allocated to the Clox 48 group and 12 (8 cows) to the Control group. After treatment, once the milk withdrawal period finished, the cows were returned to the main herd and checked periodically until Study Day 28.

5.3.2 Culturing of milk samples

Milk specimens from the eighty cows were collected from each quarter in duplicate using aseptic technique and cultured at the Institute of Veterinary, Animal and Biomedical Sciences (IVABS), Microbiology Laboratory, Massey University, Palmerston North, New Zealand. The culture procedure followed the National Mastitis Council Guidelines (National Mastitis Council, 1999). Briefly, an aliquot of 10 μ*L* from each quarter was plated into one quadrant of a trypticase soy agar plate supplemented with 5% defibrinated sheep blood (Fort Richard, Auckland, New Zealand). Plates were incubated at 35±2°C in an aerobic environment and bacterial growth was observed at 24 and 48 hr after incubation. Infected and contaminated quarters were identified and the infected cows were excluded from the study.

5.3.3 Challenge strain

The *S. uberis* strain used for the challenge S115 was obtained from the IVABS Microbiology Section Laboratory, Massey University. This strain was previously donated by the scientific team of Dairy NZ (Newstead, Hamilton). The strain was phenotypically identified as 99.9% *S. uberis*, by means of biochemical tests. Its ability to cause clinical mastitis was shown in previous studies (Chapter 3 and 4).

Preparation of *S. uberis* challenge suspension was carried out at the IVABS Microbiology Laboratory. Four days prior to the challenge, an aliquot of the frozen challenge strain was thawed in a phosphate buffer saline (PBS; 0.01 M, pH 7.3) streaked onto 5% blood agar (BA) plates (Fort Richard, Auckland, New Zealand). The plates were incubated at 35 ± 2°C for 48 hr in the presence of 5±2% CO_2. The colony-growth of bacteria was observed for purity, harvested and suspended in PBS. Ten plates were flooded with 100 μ*L* of this suspension and incubated for 48 hr as above.

On the day of challenge, *S. uberis* bacteria were harvested from the BA plates using sterile cotton swabs soaked in sterile PBS. The swabs were then rinsed in 5 to 10 mL of sterile PBS into a sterile tube. In the tube, the suspension was adjusted for turbidity corresponding to an approximate McFarland turbidity standard of 0.5 (Remel, Lenexa,

Kansas, USA) using sterile PBS. Such a procedure was expected to generate a bacterial suspension containing approximately 10^7 CFU/mL (Petrovski et al., 2011a). Following the adjustment of turbidity, the working suspension was further diluted ten-fold using serial dilution in PBS at one ten-fold dilution. This generated the final microbial suspension of 10^5 CFU. This final suspension was transferred into series of sterile syringes for intramammary administration by drawing 4 mL of the suspension into the syringe. Once the syringe was filled, the nozzle cover was applied. These ready-to-use syringes were then stored in a portable chilly box with ice pack for transportation to the farm.

On the day of challenge, 1 mL of the final microbial suspension was diluted ten-fold in PBS. The serial ten-fold dilutions were carried out until the suspension was estimated to be at 10^{-1}, 10^{-2} and 10^{-4} CFU, and 100 μL was spread in triplicate on 5% BA. Plates were incubated as previously described. The arithmetic means of the number of CFU that grew with approximately 30 to 300 CFU on the plates was used to estimate the number of CFU/4mL of inoculum for each dose of challenge suspension. This retrospective purity test estimated the final microbial suspension to be 8.3 x10^5 CFU/mL.

5.3.4 Challenge procedure

Immediately after milking had finished, each cow was experimentally challenged in two contralateral quarters (*i.e.* FR/RL or FL/RR). Before inoculation, the teat ends were thoroughly cleaned using alcohol-moistened cotton swabs. Challenge suspension was inoculated into the teats by full insertion of the nozzle through the teat canal. The quarter was then massaged in an upward direction to ensure the pathogen was distributed as far up into the quarter as possible. The entire content of one syringe was administered to each of the challenged quarters. The two non-inoculated quarters served as negative controls.

5.3.5 Clinical examination

Clinical examinations were conducted at each milking following the challenge for 19 consecutive days (Study Day 18). The foremilk of each cow was evaluated prior to milking using the blackboard strip test (Thomas, 1949) looking for signs of clinical mastitis (clots, colour changes in the milk, etc.). Individual quarters of the udders were also inspected after milking and palpated for clinical signs associated with mastitis (heat, swelling, redness and

painful quarter/s). Table 5-1 shows the udder scoring system used for the udder examinations.

Table 5-1 Clinical scoring criteria applied for the challenged quarters during the examination period. Adapted from (Petrovski et al., 2011a)

Score	Description
0	No evidence of irritation, pain and/or redness or swelling of the quarter.
1	No or virtually no evidence of irritation, pain, redness or very slight swelling of the quarter.
2	Evidence of irritation and/or pain of a minor intensity, slight redness and/or swelling, likely to originate from residual milk in the quarter.
3	Evidence of irritation and/or pain of a moderate intensity or moderate redness, swelling and/or heat of the quarter. Secretion contains small clots or flecks.
4	Evidence of irritation and/or pain of a severe intensity, severe redness and/or severe swelling and heat of the quarter. The secretion contains large clots or flecks.
5	Evidence of severe irritation or pain, severe swelling, redness and/or heat, associated with generally sick animal. The secretion contains large clots or flecks.

Mammary glands were considered to have clinical mastitis when the clinical score was equal to or higher than three, or when the foremilk secretion presented with clots or flecks.

5.3.6   Sample Collection

Once signs of mastitis were detected by palpation results or foremilk appearance, milk samples were collected in duplicate using an aseptic collection technique and cows were milked as per normal practice. These samples were cultured in the IVABS Microbiology Laboratory following the culture procedure of the National Mastitis Council Guidelines (National Mastitis Council, 1999). The second sample was cultured only when the first sample was contaminated.

On the afternoon of Study Day 4 aseptic milk samples were collected from all challenged quarters that had not yet shown clinical signs of mastitis. These samples were submitted for microbiological culture and were used as an indication of subclinical mastitis when there was bacterial growth after culture.

5.3.7 Culturing

The culturing process was as follows: An aliquot of 10 μL was plated into a trypticase soy agar plate supplemented with 5% defibrinated sheep blood. Plates were incubated at $35\pm2°C$ and bacterial growth was observed and recorded after 24 and 48 hr of incubation. Positive samples were re-cultured to obtain isolated colonies for further biochemical analysis.

5.3.8 Clinical mastitis treatment

Quarters with clinical mastitis (presence of flecks/clots or high clinical examination score) were treated with a cloxacillin-based product (Clox 48) every 48 hr. or the positive Control product every 48 hr. following the manufacturer's instructions. The treatment chosen for each cow depended on their allocation to treatment group. Cows with a second infected quarter which manifested later, received the same product as for the first infected quarter.

Any animal requiring further action after a failed treatment received penethamate hydrodide (Penethaject, Bayer Animal Health Laboratories Ltd., Auckland New Zealand) and ketoprofen (Ketofen™ Merial Animal Health Ltd.) systemically. Clinical and microbiological cure were assessed after treatment. Clinical cure was determined when no swelling was apparent in the quarter, no clots were found in milk, and the udder score returned to ≤1 or 0. A quarter was defined as microbiologically cured when the results of the laboratory cultures conducted 5 days after the last treatment were negative.

5.3.9 Statistical analysis

Records of the number of clinical mastitis cases, bacteriological culture results and clinical scores were analysed at the quarter level, whilst the SCC values were analysed at the cow level. The proportions of clinical and bacteriological cure were compared between the treatment groups using the two tailed Fisher's exact test using Excel, Episheet 2004 (Rothman, 2002).

Somatic cell counts were not normally distributed and were transformed into somatic cell scores (SCS), calculated as SCS= $\log_2$ (SCC/1000). The differences in SCS between the two treatments were analysed by means of a repeated measurements analysis of variance (rmANOVA) using the MIXED PROCEDURE of SAS (version 9.3 SAS Institute Inc.,

Cary, NC, USA) with a 5% significance level. The model included treatment and time of sampling (TS), and the interaction between treatment and TS as a fixed effect, and cow as a random effect to account for the within cow variability. The geometric means and standard errors of SCC for each treatment group and day of measurement were also calculated as they are more representative of the real mean than the least square means.

5.4 Results

5.4.1 Clinical cure

From the forty cows challenged, 52.5% of the cows developed clinical mastitis in one quarter (21/40), 25% of cows developed clinical signs in two quarters (10/40), and 22.5% of cows did not develop mastitis (9/40).

From the total of the 80 quarters challenged in the study with *S. uberis* strain S115, 51.25% (41/80) of the challenged quarters developed clinical mastitis. The percentage of quarters that did not display clinical mastitis was 43.75% (35/80). A further (4/80) challenged quarters (5%) were diagnosed with clinical mastitis by palpation at the time of the clinical examination but were removed from the analysis due to the absence of clots in milk and/or an increase in the SCC. In addition, the cultures also did not reveal the presence of *S. uberis* or any other mastitis-causing agent. These four quarters are not included in the analysis. Two non-challenged quarters from two different cows displayed clinical mastitis but were removed from the analysis since the strain infecting those quarter was unknown.

Out of the 41 clinical cases, 29 quarters were allocated to the Clox 48 group, of those 29 quarters, 27 (93.1%) were clinically cured after treatment (Table 5-2). Two quarters did not cure after treatment and were treated with a non-steroidal anti-inflammatory and a penicillin-based systemic product containing penethamate hydrodide (Penethaject, Bayer Animal Health Laboratories Ltd., Auckland, New Zealand) and ketoprofen (Ketofen™ Merial Animal Health Ltd.) systemically. One of those two quarters required nine consecutive applications of the penicillin-based product to achieve cure, and the other quarter was cured clinically after three applications of the same treatment. Both quarters were regarded as treatment failures.

Eight cows were allocated to the Control group, 12 quarters were treated with the Control product. All the quarters (100%) were judged to be clinically cured after treatment (Table 5-2). There was no significant difference in cure rates between the treatment groups (two tailed Fisher's exact test P=0.19).

Table 5-2 Quarter level clinical cure rates according to treatment groups

Treatment	Total Number of Clinical Cases	Number of quarters cured (%)
Cloxacillin 48	29	27 (93.1%)
Control	12	12 (100%)
TOTAL	41	39 (95.1%)

5.4.2 Bacteriological cure

Pre-treatment milk samples from challenged quarters were taken when clinical mastitis was identified before the antimicrobial treatment was applied. Milk samples from 43 quarters affected with clinical mastitis were cultured. Forty-one of these quarters were the challenged quarters. As previously stated, two of these quarters were unchallenged and were not considered in further analysis. Eighty per cent (33/41) of cultured milk samples were confirmed to be infected with *S. uberis* from the milk sample taken before treatment. Five samples (12.2%) were bacteriologically negative (no bacterial growth) and three samples yielded different infectious agents (one Coagulase-negative staphylococci and two *Corynebacterium bovis*). As there were only three non-*S. uberis* cases, which were distributed in the two groups, these cases were included in the statistical analysis.

Five days after treatment, from the 29 quarters treated with Clox 48, only two quarters were considered treatment failures for the clinical analysis. After the bacteriological culture, one quarter was positive for *S. uberis*. This cow had to be treated for 9 consecutive days with a systemic penicillin-based product and non-steroidal anti-inflammatories due to severe mastitis (as previously explained). The other clinically failure quarter was negative after culture. From the 12 quarters that were treated with the control product, none were positive for *S. uberis* 5 days after the last treatment. The percentage of bacteriological cure

was very similar in the two groups, with 96.5% (28/29 quarters) in the Clox 48 group and 100% for the control group (two tailed Fisher exact test P=1).

5.4.3 Somatic Cell Count

Somatic cell scores (SCS= log2 SCC/1000) were analysed at the cow level from composite samples from the four quarters comparing treatment groups (Figure 5-1). A composite sample from the four quarters was used, following the study design in the protocol.

In the Clox 48 group 23 cows were analysed and 8 in the control group. To evaluate the effect of treatment on SCC, the data was standardised to compare SCS on the Day after the clinical mastitis was diagnosed and treated.

A significant difference (P<0.001) was found between the capability of both treatments to lower the SCC (Figure 5-1). By the 6th day after treatment, both treatments achieved physiological SCC levels (lower than 250,000 SCC/mL).
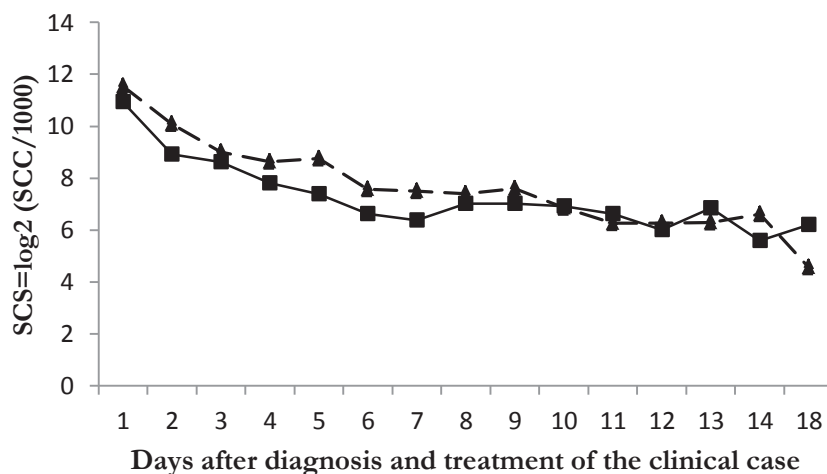
Figure 5-1 Average Somatic Cell Scores (SCS= $\log_2$ SCC/1000) for cows from 1 day to 18 days after treatment with Cloxacillin 48 (-▲-) or Control (-■-). The P-value for the interaction of treatment and day was P<0.001.

The geometric mean of the SCC of the cows in the first day after treatment for the Clox 48 group was 2,970,932 cells/mL (1,864,302-4,734,446) whereas in the control group SCC= 1,976,593 cells/mL (912,424-4,281,914 cells/mL). This difference between the treatments was significant during the period analysed (P<0.001).

Table 5-3 Somatic cell scores, Standard Error (SE), geometric means and 95% confidence intervals for the somatic cell count (SCC) for cows treated with Cloxacllin 48 and Control group from 1 milking after positive diagnosis with clinical mastitis. n: Number of cows with CM per day after diagnosis of the clinical case

Milking post diagnosis	Clox 48					Control				
	SCS	**SE**	**SCC**	**95% CI**	***n***	**SCS**	**SE**	**SCC**	**95% CI**	***n***
1	11.5367	0.343	2,970,932	1,864,302- 4,734,446	23	10.9488	0.569	1,976,593	912,424- 4,281,914	8
2	10.0844	0.287	1,085,693	735,142- 1,603,401	23	8.9231	0.4919	485,423	248,823- 947,002	8
3	9.0056	0.3484	513,991	320,179- 825,122	23	8.6235	0.569	394,396	182,059- 854,384	8
4	8.6432	0.3605	399,818	244,997- 652,476	23	7.8183	0.569	225,706	104,189- 488,949	8
5	8.7564	0.3483	432,453	269,424- 694,133	23	7.4012	0.569	169,038	78,030- 366,188	7
6	7.585	0.3542	192,005	118,666- 310,668	23	6.628	0.6303	98,907	42,009- 232,871	7
7	7.512	0.3604	182,531	111,865- 297,838	22	6.3853	0.7322	83,592	30,914- 226,037	7
8	7.404	0.3673	169,366	102,828- 278,959	21	7.0264	0.6303	130,364	55,369- 306,935	7
9	7.6082	0.3917	195,118	114,600- 332,205	19	7.0313	0.6727	130,807	52,448- 326,240	7
10	6.8544	0.402	115,712	67,018- 199,787	16	6.9241	0.6303	121,440	51,579- 285,924	7
11	6.2813	0.402	77,779	45,048- 134,291	15	6.6289	0.6728	98,969	39,676- 246,867	6
12	6.2769	0.4573	77,542	41,660- 144,328	8	6.0193	0.673	64,862	25,996- 161,835	2
13	6.2965	0.5295	78,602	38,284- 161,380	4	6.8593	0.9756	116,106	30,848- 436,996	2
14	6.6121	0.6767	97,823	39,010- 245,305	2	5.6016	0.9756	48,557	12,901- 182,756	1
18	4.5605	0.9344	23,596	6,630- 83,977	1	6.2125	1.3318	74,156	12,144- 452,831	1

5.5 Discussion

The primary objective of this study was to determine the efficacy of a novel long-acting cloxacillin-based product and compare its efficacy with another long-acting cloxacillin-based antimicrobial used as a positive control. No significant differences were found between the rates of clinical cure obtained after three applications every 48 hr. of Clox 48 (the novel product) and the control product. Both treatments demonstrated high efficacy after the treatment of experimentally induced clinical mastitis by *S. uberis*. The overall clinical cure rate obtained was 95.1% (39/41).

There was no significant difference in the bacteriological cure rates for the Clox 48 group (96.3%) and the control group (100%; P=1). These results are higher than the results obtained in previous studies where 63% and 83% of the cows treated resulted in bacteriologically negative cultures after treatment with long acting cloxacillin (sodium based) every 48 hours for three applications (Brander et al., 1964, Dodds et al., 1969).

Despite the high bacteriological cure rates obtained in this study for both treatments, there was a significant difference found between the novel and the control product in their capability to return the SCC to physiologically normal levels (200,000–250,000 cells/mL; Dohoo and Leslie, 1991). The time taken for Clox 48 and the control product to lower the SCC in this study, were shorter than the time reported in other studies, where the SCC took from ten days to four weeks return to physiologically normal levels after the elimination of the infectious agent (Deluyker et al., 1993, Pyörälä and Pyörälä, 1997). This suggests that the efficacy of both products in lowering the SCC is still within the normal time of recovery despite the significant difference found between treatments.

Cloxacillin is a drug from the group of beta-lactams. Since the discovery of penicillin and the use of beta-lactams for intramammary mastitis treatments in the 1940's, many studies which have analysed the efficacy of these drugs concluded that the time of action of the antibiotic is more important than the dose itself (Sanderson, 1966). This is true if the MIC is reached (Little and Plastridge, 1946). Consequently, penicillin and all the synthetic products derived from it, like cloxacillin, are called "time dependant antibiotics". Further investigations have demonstrated that different vehicles in combination with penicillin gave the antibiotic more stability and longer action periods in the udder, thus reducing doses per treatment, costs and milking times (Zweig, 1949, Mercer et al., 1974b). Oil with aluminium

stearate, castor oil, peanut oil and beeswax are substances that have been mixed with penicillin with the objective of extending the effective period of the product in the udder (Zweig, 1949, Mercer et al., 1974b). Cloxacillin has also been used as a benzatinic salt in long acting dry cow treatments with high subclinical mastitis cure rates after treatment (Cummins and McCaskey, 1987).

Cloxacillin in sodium salt, combined with a proper excipient has demonstrated a long duration above the MIC in the bovine udder (Brander et al., 1964, Dodds et al., 1969). This has driven the animal pharmaceutical industry to create long-acting products -such as the one presented in this study- which are equally as effective as other products applied more frequently and in addition, are adapted to the treatment requirements for lactating cows. Some disadvantages of the use of these products might be the longer milk withholding period and a higher probability of missing a treatment because the applications are not on consecutive days (Craven, 1987). On the other hand, milking times can be reduced and lesions in the teat ends caused by the frequent use of intramammary treatments could also be reduced.

During the last 30 years, the advantages of shorter intramammary treatments for clinical mastitis cases has been discussed and presented as a tool to reduce costs of mastitis problems (Craven, 1987, Swinkels et al., 2005). However, longer treatments are associated with a clear reduction in recurrences after treatments, and higher bacteriological cure rates that will, in the long term reduce the use of antibiotic on dairy farms and the long term costs of mastitis treatment (Chapter 4; Owens et al., 1997, Milne et al., 2005).

5.6 Conclusion

It is concluded that there was no significant difference in the cure rates after treatment of induced clinical mastitis produced by *S. uberis* with Clox 48 and the control product.

5.7 Acknowledgment

CHAPTER 6

General Discussion

6   General Discussion

Clinical and subclinical mastitis are among the most costly diseases for dairy production (Milne et al., 2005, Denis et al., 2009, Cha et al., 2011, Hogeveen et al., 2011, Steeneveld et al., 2011) and *S. uberis* is one of the most prevalent mastitis-causing agents in lactating cows in New Zealand (McDougall, 1998, McDougall, 2003, McDougall et al., 2007, Petrovski et al., 2009, Petrovski et al., 2011c). Although the consequences of clinical mastitis caused by *S. uberis* are considered mild (Pyörälä and Syväjärvi, 1987), the opportunistic nature of this environmentally widespread agent make it difficult to eradicate from farms (Zadoks et al., 2001, Zadoks et al., 2003, Zadoks et al., 2005, McDougall et al., 2007). Due to a physiological reduction in the immune response and the ubiquitous characteristic of this bacteria, the first weeks of the dry period and the first stages of lactation are high risk periods for intramammary infections with mastitis-causing agents such as *S. uberis* (Kashiwazaki, 1984, Craven and Williams, 1985, Kehrli et al., 1989, Sordillo et al., 1997, Sordillo and Streicher, 2002, McDougall, 2003, Oliver et al., 2003, Oliver et al., 2004, McDougall et al., 2007, Moyes et al., 2010, Lopez-Benavides, 2007). *Streptococcus uberis* often causes clinical mastitis that requires treatment intervention. Currently, the increasing incidence of *S. uberis* on farms could be interpreted as a sign of low efficacy of the treatments applied against *S. uberis* infections or of some resistance mechanisms of *S. uberis* to the current therapies (Oliver et al., 2003). Therefore, improving the effectiveness of antimicrobial drug use in these periods could enhance the control of *S. uberis* mastitis. In addition, many authors believe that the main reason for the increasing incidence of *S. uberis* in dairy herds is that contagious agents like *Staph. aureus* and *S. agalactiae*, have been better controlled in recent years. Consequently, environmental agents like *S. uberis* are more commonly seen, even on well-managed dairy farms (Oliver et al., 2004, Petrovski et al., 2009).

One of the aims of this project was to develop an experimental challenge model using *S. uberis*, which could aid research into clinical mastitis treatment and lead to the development of more efficacious treatments. An experimental challenge model with *S. uberis* was developed in the first study (Chapter 3), and was used later to test the efficacy of two antimicrobial drugs (Chapter 4 and 5). In the study presented in Chapter 3, four

strains of *S. uberis* were tested for their ability to induce clinical mastitis in four groups of lactating cows. Two concentrations of the challenge suspension (High and Low) were used for the challenge. The proportion of clinical mastitis and the clinical scores were compared between the strains and between concentrations. The presence of clinical signs was assessed by observation of the foremilk for changes, such as the presence of clots and/or colour changes, and by manual palpation and observation of the quarters using a scoring scale ranging from 0 to 5.

There were significant differences in the incidence of clinical mastitis between the groups. Strain 26LB resulted in 4/6 and 2/6 cases of clinical mastitis in quarters challenged with the Low and High concentration, respectively. Strain S115 resulted in 2/6 and 5/6 cases of clinical mastitis for Low and High concentration and strain S418 resulted in clinical mastitis in all challenged quarters for both concentrations, and these infections were difficult to cure after treatment. Conversely, Strain S523 resulted in only one case of clinical mastitis in a quarter challenged with the Low concentration.

Ultimately, Strain S115 was selected for use in the following challenge studies, for a number of reasons. First, the clinical presentation of the mastitis caused by this strain was mild in comparison with the other strains. In addition, although the affected quarters presented only mild inflammation (scores ≤3), there were clots in milk and a rise in SCC was observed, allowing the assessment of clinical improvement. In fact, the mastitis cases were responsive to treatment and the clinical signs improved shortly after treatment. From an animal welfare perspective, curability is an important requisite and strains used as a challenge to induce clinical or subclinical mastitis must not induce a severe, incurable mastitis or cause excessive suffering to the cow (Mercer et al., 1974b, Oliver et al., 2003). S115 matched this requisite also.

Lastly, S115 showed a high isolation proportion from the experimentally infected quarters, as compared for instance, to strain 26LB, which induced a high proportion of clinical mastitis but was isolated from only one out of six clinical cases. Conversely, S418 was successfully isolated from all the clinical cases, but because of its low curability after treatment, was discarded as a candidate for future studies. Strain S523 induced a single clinical mastitis case and therefore, was discarded due to its low capability of producing clinical cases. This variability in the ability of the strains to cause clinical mastitis and

variability in the isolation of organisms is consistent with previous studies (Hogan et al., 1989, McDougall, 2003).

This experimental challenge model was subsequently used in two drug efficacy studies (Chapters 4 and 5), where the efficacy of two cloxacillin-based products for the treatment of experimental *S. uberis* mastitis was assessed. In Chapter 4, the efficacy of a new cloxacillin-based intramammary product administered once daily with a 3-treatment regime, was compared with a registered, twice-a-day penicillin-based product with a similar number of treatments. The results indicated that the two products had similar efficacy against *S. uberis* experimental infections using S115, and that extended treatments enhance clinical cure rates after treatment.

In Chapter 5, the efficacy of a new long-acting cloxacillin-based product was compared with an existing long-acting intramammary product containing the same active ingredient and assumed similar posology (cloxacillin administered every 48 hours). The efficacy of the novel product did not differ significantly from the product which was used as a positive control.

Interestingly, a statistically significant difference in the incidence of clinical mastitis following the experimental challenge with strain S115 was found between Chapters 4 and 5 (Chapter 4, clinical mastitis proportion 73/80 = 91.25%; Chapter 5, clinical mastitis proportion 41/80 = 50%, Table 6-1).

Table 6-1 Concentration of the challenge suspension (CFU: colony-forming units) , DIM (Days in Milk) and proportion of clinical mastitis observed in Chapters 4 and 5 (quarters showing clinical mastitis signs/total quarters challenged).

	Chapter 4	**Chapter 5**
Concentration of the challenge suspension	1.6×10^6 CFU/mL	8.3×10^5 CFU/mL
Mean DIM (± Standard error)	71.0(±17.0)	184.3 (±19.0)
Proportion of clinical mastitis	73/80 = 91.2%	41/80= 51.2%

The difference in the proportion of clinical mastitis observed between the two trials which used the same strain on the same farm, indicates that environmental and/or individual factors may influence the susceptibility of the cow to experimental intramammary challenge. As described previously in Chapter 2, the incidence of mastitis on dairy herds depends on factors related to the infecting microorganism (e.g. virulence and infectivity), environmental factors which determine its survival in the environment (e.g. temperature and humidity) and host factors such as immunity, concurrent diseases, DIM and cow age.

The trials described in Chapters 4 and 5, were conducted in similar environments (similar nutrition, milking shed, milking routine etc.). However, variations were observed in the time of the year when the studies were carried out and therefore, the stage of lactation. The study in Chapter 4 was carried out in October 2011, using spring-calving cows that were an average of 71 (± 17) DIM. The study in Chapter 5 was conducted in March 2012, using cows with an average of 184 (± 19) DIM. In Chapter 3, the challenged cows were approximately 180 DIM. It is plausible that the difference in stage of lactation between the studies accounted for some of the difference in clinical mastitis proportions observed between studies in Chapters 4 and 5. However, there was also a two-fold difference in the infectious dose administered between the trials, which could also account for some of the variability in response to the challenge (Table 6-1).

Cow age and stage of lactation are among the most studied and reported factors affecting the incidence of clinical mastitis in herds (Poutrel, 1982, Schukken et al., 1999). Erb and Martin (1978) did not find significant differences in mastitis incidence between old and young stock, but other authors (Poutrel, 1982, Mellado and Reyes, 1994, Oltenacu and Ekesbo, 1994) have reported a higher risk for older cows getting clinical mastitis. Possible reasons for the "age effect" are a higher prevalence of teat skin lesions, wider and longer teat canals seen in older animals which mean a shorter distance between the teat orifice and the ground (Poutrel, 1982). Given that the average age of the cows in the studies presented in this thesis was similar for both studies (5 years ± 1.7 and 4.5 years ± 1.3 for Chapters 4 and 5, respectively), age can be discounted as a reason for observed difference between mastitis proportions in the two studies.

Increased incidence of mastitis after parturition and in early stages of lactation has been previously reported (Kashiwazaki, 1984, Craven and Williams, 1985, Kehrli et al., 1989, Sordillo et al., 1997, Sordillo and Streicher, 2002, Oliver et al., 2003, Oliver et al., 2004,

McDougall et al., 2007, Moyes et al., 2010), suggesting low mammary immunity around this period. Kehrli (1989) described a depletion of neutrophil activity after parturition as compared with the pre-calving period. Cows in negative energetic balance (NEB) have been shown to have altered expression of genes involved in the immune response, suggesting that cows in early lactation may be unable to mount an adequate immune response against invading pathogens (Moyes et al., 2010). Given that a NEB is considered normal physiological condition after parturition (Baumgard et al., 2006), it could be deduced that cows in early lactation are physiologically more susceptible to mastitis.

Despite the difference in infection proportions observed between Chapters 4 and 5 (91.25% and 51.25%, respectively), in both studies the infections could be eradicated by antimicrobial treatment and no long-lasting damage to the udder was observed, similar to the pilot study presented in Chapter 3.

As stated above, the two-fold differences found in the concentration of the challenge suspensions between Chapters 4 and 5 could also have had an effect on the incidence of clinical mastitis induced. Some authors have reported differences in clinical mastitis incidence obtained following inoculation with varying bacterial doses of *Staph. aureus* (Mercer et al., 1974b, Heald, 1979). However, as can be seen in Chapter 3, the difference in the incidence of infection between High and Low concentration of S115 did not differ significantly, although there was a hundred-fold difference between the two challenge concentrations of (1×10^6 CFU/mL *vs*. 1×10^4 CFU/mL). However, the lack of significant difference between the High and Low concentrations could also be due to the small number of observations (six quarters per strain per concentration). The challenge suspensions were obtained using visual assessment of the turbidity of the bacterial suspension, and this method does not allow a precise prediction of the number of CFU/mL in the challenge suspension used for the challenge. Future studies could benefit from a more precise estimation of the bacterial concentration used, for example using a spectrophotometer.

Significant difference in the mean SCC was found between the studies presented in Chapters 4 and 5. In Chapter 4, the mean SCC was lower than in Chapter 5. In accordance with these results, Green et al., (2004) speculated that the higher incidence of clinical mastitis post-partum could be a reflection of a lower response and slower recruitment of PMNs. Although lower SCC numbers at the peak of lactation (42 days post-partum) are

considered normal and are consistent with the dilution theory (high milk volume = lower concentration of SCC), it could be argued that the low SCC usually found after calving and during the peak of lactation is a direct consequence of a lower immune response of the cow against mastitis agents, rather than the mere consequence of the dilution of the cells in a greater volume of milk (Green et al., 2004).

Results in this thesis show that experimental challenge models can be useful in the animal pharmaceutical industry to test the efficacy of new products in a safe and cost effective manner. In addition, experimental challenge models like that developed in this thesis could also have additional scientific value through allowing researchers to observe and make conclusions regarding the host response against infections, clinical behaviour of different strains of mastitis-causing agents and further characteristics of the infectious agent utilised for the challenge.

6.1 Limitations of the studies

Chapter 3 was a pilot study carried out with a modest number of cows and a lower statistical power than the other reported studies (Schukken et al., 1999, Oliver et al., 2003). Nevertheless, the study met the objective and allowed the identification of one suitable strain for the subsequent clinical trials.

Another limitation of the studies presented here was the lack of a genetic typing of the strains prior to or following the challenge. Therefore, although it is likely that most strains isolated from the clinical cases were the same strain as was used for the challenge (due to the close proximity of the two events), this could not be corroborated due to a limited budget allocated to the studies and because the studies were designed to provide clinical cases in order to test antimicrobial products, strain typing was not considered necessary in this case.

Treatment groups in Chapters 4 and 5 were not balanced for number of cows. Consequently, only a result showing significant differences between the treatments could justify such as low number of individuals tested in the positive control group. However, for reasons of animal welfare and costs, it was not possible to carry out this type of study with a higher number of animals.

6.2 Recommendations for future research

The three studies presented in this thesis allowed the development of a robust challenge model that is currently in use to test the efficacy of novel treatments against *S. uberis* mastitis. The use of the model reduces the costs of efficacy testing and also accelerates the process of registration of new products. It provides controlled, safe conditions which safeguard the welfare of animals.

In order to carry out this type of study, it is essential to know the susceptibility of the strain to antimicrobials, to estimate the incidence of infection that could be obtained after the challenge, and to know the severity of the infection induced. Developing a successful challenge model is therefore an 'art', and an iterative process. For instance, the different proportions of mastitis observed in the studies presented in this thesis, highlights the difficulty in estimating the number of animals needed for a trial based on a pre-trial power analysis, as the assumed infection incidence may not prove correct. Indeed, despite the controlled conditions and the use of the same strain at similar infection doses, on the same farm, it has been very difficult to predict with accuracy the infection incidence before the studies. Nevertheless, the variability between the infection proportions obtained is not excessive and the results presented in this thesis provide solid ground for future trials, and will lead to minimising the number of cows necessary to efficiently perform efficacy studies. Further studies should take into account the causes of variability described in this thesis to calculate the number of cows that should be involved in the study (e.g. age and stage of lactation). Repeatability of the study in different stages of lactation and using a variable number of cows could provide more accurate conclusions.

The risk of producing long-term and/or acute infections when infecting a cow with a mastitis-causing agent always exists. However, it has been demonstrated in these studies that prompt and specific treatment, can protect the health of the cow and of the whole herd. This suggests that a controlled artificial infection may be possible without major animal welfare concerns. As occurs with natural infections, the infection incidences obtained after experimental challenge are dose and strain-dependant and this is true for most of the mastitis-causing agents, whereas, in experimental challenge models those variables are better known and can be better controlled.

There are more than 135 mastitis-causing agents affecting dairy cattle (Watts et al., 1988). Major ones like *S. uberis* and *Staph. aureus* are the most prevalent in New Zealand. There are no mastitis-causing agents which are "safer" than others when they are used to induce clinical mastitis in udders, but researchers are responsible for conducting experiments, whilst taking into account animal health and welfare.

Future experimental challenge models using different mastitis-causing agents and/or multi-strain challenges could be developed, opening the gate to efficacy testing of new antimicrobials and the development of different control methods against other bacteria. The close monitoring and the prompt treatment, the availability of data about the strain that is going to be used for the challenge (e.g. antibiotic susceptibility tests done in advance), could enhance the welfare of the cows involved in this type of study. Continued research by animal pharmacological industries and scientist is essential to the dairy industry, not only in New Zealand, but also worldwide.

List of References

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Almeida, R. A., Luther, D. A., Park, H.-M. & Oliver, S. P. 2006. Identification, isolation, and partial characterization of a novel *Streptococcus uberis* adhesion molecule (SUAM). *Veterinary Microbiology,* 115, 183-191.

Almeida, R. A. & Oliver, S. P. 1993. Antiphagocytic effect of the capsule of *Streptococcus uberis*. *Journal of Veterinary Medicine, Series B,* 40, 707-714.

Auldist, M. (ed.) 2003. *Milk quality and udder health. Effect on processing characteristics*

Auldist, M. J., Coats, S., Rogers, G. L. & McDowell, G. H. 1995. Changes in the composition of milk from healthy and mastitic dairy cows during the lactation cycle. *Animal Production Science,* 35, 427-436.

Auldist, M. J. & Hubble, I. B. 1998. Effects of mastitis on raw milk and dairy products. *Australian Journal of Dairy Technology,* 53, 28-36.

Bannerman, D. D., Paape, M. J., Goff, J. P., Kimura, K., Lippolis, J. D. & Hope, J. C. 2004. Innate immune response to intramammary infection with *Serratia marcescens* and *Streptococcus uberis*. *Veterinary Research,* 35, 681-700.

Baumgard, L. H., Odens, L. J., Kay, J. K., Rhoads, R. P., VanBaale, M. J. & Collier, R. J. 2006. Does negative energy balance (NEBAL) limit milk synthesis in early lactation. *Proc. Southwest Nutr. Conf.*

Benites, N. R., Guerra, J. L., Melville, P. A. & Costa, E. O. 2002. Aetiology and histopathology of bovine mastitis of espontaneous occurrence. *Journal of Veterinary Medicine, Series B,* 49, 366-370.

Black, R. T., Marshall, R. T. & Bourland, C. T. 1972. Locus of mammary gland infections of *Corynebacterium bovis*. *Journal of Dairy Science,* 55, 413-416.

Blowey, R. W. & Edmondson, P. 2010. *Mastitis control in dairy herds,* Wallingford, UK Cambridge, MA, CABI.

Boddie, R. L. & Nickerson, S. C. 1996. Efficacy of teat dips containing a hypochlorous acid germicide against experimental challenge with *Staphylococcus aureus* and *Streptococcus agalactiae*. *Journal of Dairy Science,* 79, 1683-1688.

Boddie, R. L., Nickerson, S. C. & Adkinson, R. W. 2000. Efficacies of chlorine dioxide and iodophor teat dips during experimental challenge with *Staphylococcus aureus* and *Streptococcus agalactiae*. *Journal of Dairy Science,* 83, 2975-2979.

Boddie, R. L., Owens, W. E., Foret, C. J. & Janowicz, P. 2004. Efficacy of a 0.1% Iodine teat dip against *Staphylococcus aureus* and *Streptococcus agalactiae* during experimental challenge. *Journal of Dairy Science,* 87, 3089-3091.

Bradley, A. J. 2002. Bovine Mastitis: An evolving disease. *The Veterinary Journal,* 164, 116-128.

Bradley, A. J. & Green, M. J. 2004. The importance of the nonlactating period in the epidemiology of intramammary infection and strategies for prevention. *The Veterinary clinics of North America. Food animal practice,* 20, 547.

Brander, G. C., Jones, A., Neave, F. K., Dodd, F. H., Kingwill, R. G. & Westgarth, D. R. 1964. Antibiotics in slow and quick release bases. *Veterinary Record,* 76, 329.

Brookbanks, E. O. 1966. A report on surveys of the incidence of mastitis infection in New Zealand dairy herds. *New Zealand Veterinary Journal,* 14, 62-70.

Brouillette, E., Grondin, G., Lefebvre, C., Talbot, B. G. & Malouin, F. 2004. Mouse mastitis model of infection for antimicrobial compound efficacy studies against intracellular and extracellular forms of *Staphylococcus aureus*. *Veterinary Microbiology,* 101, 253-262.

Brouillette, E. & Malouin, F. 2005. The pathogenesis and control of *Staphylococcus aureus*-induced mastitis: study models in the mouse. *Microbes and Infection,* 7, 560-568.

Burvenich, C., Van Merris, V., Mehrzad, J., Diez-Fraile, A. & Duchateau, L. 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. *Veterinary Research,* 34, 521-564.

Busato, A., Trachsel, P., Schällibaum, M. & Blum, J. W. 2000. Udder health and risk factors for subclinical mastitis in organic dairy farms in Switzerland. *Preventive Veterinary Medicine,* 44, 205-220.

Cai, T. Q., Weston, P. G., Lund, L. A., Brodie, B., McKenna, D. J. & Wagner, W. C. 1994. Association between neutrophil functions and periparturient disorders in cows. *American Journal of Veterinary Research,* 55, 934- 943.

Capuco, A. V., Bright, S. A., Pankey, J. W., Wood, D. L., Miller, R. H. & Bitman, J. 1992. Increased susceptibility to intramammary infection following removal of teat canal keratin. *Journal of Dairy Science,* 75, 2126-2130.

Capuco, A. V., Paape, M. J. & Nickerson, S. C. 1986. In vitro study of polymorphonuclear leukocyte damage to mammary tissues of lactating cows. *American Journal of Veterinary Research,* 47, 663-8.

Cha, E., Bar, D., Hertl, J. A., Tauer, L. W., Bennett, G., González, R. N., Schukken, Y. H., Welcome, F. L. & Gröhn, Y. T. 2011. The cost and management of different types of clinical mastitis in dairy cows estimated by dynamic programming. *Journal of Dairy Science,* 94, 4476-4487.

Chaffer, M., Leitner, G., Winkler, M., Glickman, A., Krifucks, O., Ezra, E. & Saran, A. 1999. Coagulase-negative Staphylococci and mammary gland infections in cows. *Journal of Veterinary Medicine, Series B,* 46, 707-712.

Chandler, R. L., Reid, I. M., Harrison, R. & France, B. R. 1974. Ultrastructural, morphometric and associated observations on experimental mastitis in cattle. *Journal of Comparative Pathology,* 84, 517-531.

Christie, R., Atkins, N. & Munch-Petersen, E. 1944. A note on a lytic phenomenon shown by group B streptococci. *Australian Journal of Experimental Biology & Medical Science* 22, 197-200.

Constable, P. D. & Morin, D. E. 2003. Treatment of clinical mastitis: Using antimicrobial susceptibility profiles for treatment decisions. *Veterinary Clinics of North America: Food Animal Practice,* 19, 139-155.

Coulona, J.-B., Gasquib, P., Barnouin, J., Ollier, A., Pradel, P. & Pomiès, D. 2002. Effect of mastitis and related-germ on milk yield and composition during naturally-occurring udder infections in dairy cows. *Animal Research,* 51, 383-393.

Craven, N. 1987. Efficacy and financial value of antibiotic treatment of bovine clinical mastitis during lactation : A review. *British Veterinary Journal,* 143, 410-422.

Craven, N. & Williams, M. R. 1985. Defences of the bovine mammary gland against infection and prospects for their enhancement. *Veterinary Immunology and Immunopathology,* 10, 71-127.

Cullen, G. A. 1966. The ecology of *Streptococcus uberis. British Veterinary Journal* 122, 333-339.

Cummins, K. A. & McCaskey, T. A. 1987. Multiple infusions of cloxacillin for treatment of mastitis during the dry period. *Journal of Dairy Science,* 70, 2658-2665.

Curtis, S. E. 1983. *Environmental management in animal agriculture*, Iowa State University Press.

Davis, W. T., Maplesden, D. C., Natzke, R. P. & Philpot, W. N. 1975. Sodium cloxacillin for treatment of mastitis in lactating cows. *Journal of Dairy Science,* 58, 1822-1827.

de Haas, Y., Barkema, H. W. & Veerkamp, R. F. 2002. The effect of pathogen-specific clinical mastitis on the lactation curve for somatic cell count. *Journal of Dairy Science,* 85, 1314-1323.

Deluyker, H. A., Gay, J. M. & Weaver, L. D. 1993. Interrelationships of somatic cell count, mastitis, and milk yield in a low somatic cell count herd. *Journal of Dairy Science,* 76, 3445-3452.

Denis, M., Wedlock, D. N., Lacy-Hulbert, S. J., Hillerton, J. E. & Buddle, B. M. 2009. Vaccines against bovine mastitis in the New Zealand context: What is the best way forward? *New Zealand Veterinary Journal,* 57, 132-140.

Dodds, J. S., Nelson, F. C., Meads, E. B. & Hébert, C. 1969. Cloxacillin mastitis ointment in the treatment of bovine mastitis. *The Canadian Veterinary Journal,* 10, 188.

Dohoo, I. R. & Leslie, K. E. 1991. Evaluation of changes in somatic cell counts as indicators of new intramammary infections. *Preventive Veterinary Medicine,* 10, 225-237.

Douglas, V. L., Fenwick, S. G., Pfeiffer, D. U., Williamson, N. B. & Holmes, C. W. 2000. Genomic typing of *Streptococcus uberis* isolates from cases of mastitis, in New Zealand dairy cows, using pulsed-field gel electrophoresis. *Veterinary Microbiology,* 75, 27-41.

Elliott, R. E. W., Tattersfield, J. G. & Brookbanks, E. O. 1976. New Zealand national mastitis survey: 1965–6. *New Zealand Veterinary Journal,* 24, 80-84.

Erb, H. N. & Martin, S. W. 1978. Age, breed and seasonal patterns in the occurrence of ten dairy cow diseases: a case control study. *Canadian Journal of Comparative Medicine,* 42, 1.

Falkow, S., Isberg, R. R. & Portnoy, D. A. 1992. The interaction of bacteria with mammalian cells. *Annual Review of Cell Biology,* 8, 333-363.

Fang, W., Almeida, R. A. & Oliver, S. P. 2000. Effects of lactoferrin and milk on adherence of *Streptococcus uberis* to bovine mammary epithelial cells. *American Journal of Veterinary Research,* 61, 275-279.

Finch, J. M., Hill, A. W., Field, T. R. & Leigh, J. A. 1994. Local vaccination with killed *Streptococcus uberis* protects the bovine mammary gland against experimental intramammary challenge with the homologous strain. *Infection and Immunity,* 62, 3599-3603.

Fontaine, M. C., Perez-Casal, J., Song, X. M., Shelford, J., Willson, P. J. & Potter, A. A. 2002. Immunisation of dairy cattle with recombinant *Streptococcus uberis* GapC or a chimeric CAMP antigen confers protection against heterologous bacterial challenge. *Vaccine,* 20, 2278-2286.

Foret, C. J., Owens, W. E., Boddie, R. L. & Janowicz, P. 2003. Efficacy of two iodine teat dips during experimental challenge with *Staphylococcus aureus* and *Streptococcus agalactiae*. *Journal of Dairy Science,* 86, 3783-3786.

Fox, L. K., Kirk, J. H. & B ritten, A. 2005. *Mycoplasma* Mastitis: A review of transmission and control. *Journal of Veterinary Medicine Series B,* 52, 153-160.

Gillespie, B. E., Moorehead, H., Lunn, P., Dowlen, H., Johnson, D., Lamar, K., Lewis, M., Ivey, S., Hallberg, J. & Chester, S. 2002. Efficacy of extended pirlimycin hydrochloride therapy for treatment of environmental *Streptococcus spp* and *Staphylococcus aureus* intramammary infections in lactating dairy cows. *Vet Ther,* 3, 373-80.

Green, M. J., Green, L. E., Schukken, Y. H., Bradley, A. J., Peeler, E. J., Barkema, H. W., de Haas, Y., Collis, V. J. & Medley, G. F. 2004. Somatic cell count distributions during lactation predict clinical mastitis. *Journal of Dairy Science,* 87, 1256-1264.

Gruet, P., Maincent, P., Berthelot, X. & Kaltsatos, V. 2001. Bovine mastitis and intramammary drug delivery: Review and perspectives. *Advanced Drug Delivery Reviews,* 50, 245-259.

Guérin-Faublée, V., Tardy, F., Bouveron, C. & Carret, G. 2002. Antimicrobial susceptibility of *Streptococcus* species isolated from clinical mastitis in dairy cows. *International Journal of Antimicrobial Agents,* 19, 219-226.

Habuš, J., Buić, V., Zobel, R., Milas, Z., Starešina, V., Barbić, L., Štritof, Z., Stevanović, V. & Turk, N. Outbreak of gangrenous mastitis in cows. European Buiatrics Forum, 2009.

Harmon, R. J. 1994. Physiology of mastitis and factors affecting somatic cell counts. *Journal of Dairy Science,* 77, 2103-2112.

Harmon, R. J., Schanbacher, F. L., Ferguson, L. C. & Smith, K. L. 1976. Changes in lactoferrin, immunoglobulin G, bovine serum albumin, and alpha-lactalbumin during acute experimental and natural coliform mastitis in cows. *Infection and Immunity,* 13, 533-542.

Heald, C. W. 1979. Morphometric study of experimentally induced *Staphylococcus bovis* mastitis in the cow. *American Journal of Veterinary Research,* 40, 1294-1298.

Heringstad, B., Klemetsdal, G. & Ruane, J. 2000. Selection for mastitis resistance in dairy cattle: A review with focus on the situation in the Nordic countries. *Livestock Production Science,* 64, 95-106.

Heyneman, R., Burvenich, C. & Vercauteren, R. 1990. Interaction between the respiratory burst activity of neutrophil leukocytes and experimentally induced *Escherichia coli* mastitis in cows. *Journal of Dairy Science,* 73, 985-994.

Hill, A. W. 1988. Protective effect of previous intramammary infection with *Streptococcus uberis* against subsequent clinical mastitis in the cow. *Research in veterinary science,* 44, 386.

Hillerton, J. E. & Bramley, A. J. 1989. Infection following challenge of the lactating and dry udder of dairy cows with *Actinomyces pyogenes* and *Peptostreptococcus indolicus*. *British Veterinary Journal,* 145, 148-158.

Hillerton, J. E., Bramley, A. J., Staker, R. T. & McKinnon, C. H. 1995. Patterns of intramammary infection and clinical mastitis over a 5 year period in a closely monitored herd applying mastitis control measures. *Journal of Dairy Research,* 62, 39-50.

Hillerton, J. E. & Kliem, K. E. 2002. Effective treatment of *Streptococcus uberis* clinical mastitis to minimize the use of antibiotics. *Journal of Dairy Science,* 85, 1009-1014.

Hockett, M. E., Hopkins, F. M., Lewis, M. J., Saxton, A. M., Dowlen, H. H., Oliver, S. P. & Schrick, F. N. 2000. Endocrine profiles of dairy cows following experimentally induced clinical mastitis during early lactation. *Animal Reproduction Science,* 58, 241-251.

Hogan, J. S., Smith, K. L., Hoblet, K. H., Schoenberger, P. S., Todhunter, D. A., Hueston, W. D., Pritchard, D. E., Bowman, G. L., Heider, L. E., Brockett, B. L. & Conrad, H. R. 1989. Field survey of clinical mastitis in low somatic cell count herds. *Journal of Dairy Science,* 72, 1547-1556.

Hogeveen, H., Huijps, K. & Lam, T. 2011. Economic aspects of mastitis: New developments. *New Zealand Veterinary Journal,* 59, 16-23.

Holmes, C. W. 2002. Milk production from pasture. Palmerston North, N.Z.: Massey University.

Horwitz, M. A. & Silverstein, S. C. 1980. Influence of the *Escherichia coli* capsule on complement fixation and on phagocytosis and killing by human phagocytes. *The Journal of Clinical Investigation,* 65, 82-94.

Jayarao, B. M., Gillespie, B. E., Lewis, M. J., Dowlen, H. H. & Oliver, S. P. 1999. Epidemiology of *Streptococcus uberis* Intramammary Infections in a Dairy Herd. *Journal of Veterinary Medicine, Series B,* 46, 433-442.

Jiang, M., Babiuk, L. A. & Potter, A. A. 1996. Cloning, sequencing and expression of the CAMP factor gene of *Streptococcus uberis*. *Microbial Pathogenesis,* 20, 297-307.

Johnsen, L. B., Poulsen, K., Kilian, M. & Petersen, T. E. 1999. Purification and Cloning of a Streptokinase from *Streptococcus uberis*. *Infection and Immunity,* 67, 1072-1078.

Kashiwazaki, Y. 1984. Lymphocyte activities in dairy cows with special reference to outbreak of mastitis in pre-and postpartus. *Japanese Journal of Veterinary Research,* 32, 101-101.

Kehrli, M. E., Nonnecke, B. J. & Roth, J. A. 1989. Alterations in bovine neutrophil function during the periparturient period. *American Journal of Veterinary Research,* 50, 207.

King, J. S. 1981. *Streptococcus uberis:* A review of its role as a causative organism of bovine mastitis. I. Characteristics of the organism. *The British veterinary journal,* 137, 36-52.

Kruze, J. & Bramley, A. J. 1982. Sources of *Streptococcus uberis* in the dairy herd. *Journal of Dairy Research,* 49, 375-379.

Kutila, T., Suojala, L., Lehtolainen, T., Saloniemi, H., Kaartinen, L., Tähti, M., Seppälä, K. & Pyörälä, S. 2004. The efficacy of bovine lactoferrin in the treatment of cows with experimentally induced *Escherichia coli* mastitis. *Journal of Veterinary Pharmacology and Therapeutics,* 27, 197-202.

Langston, V. C. & Davis, L. E. 1989. Factors to consider in the selection of antimicrobial drugs and therapy. *The Compendium on continuing education for the practicing veterinarian,* 11 p. 355-359.

Lasagno, M. C., Reinoso, E. B., Dieser, S. A., Calvinho, L. F., Buzzola, F., Vissio, C., Bogni, C. I. & Odierno, L. M. 2011. Phenotypic and genotypic characterization of *Streptococcus uberis* isolated from bovine subclinical mastitis in Argentinean dairy farms. 212-217.

Leigh, J. A. 1993. Activation of bovine plasminogen by *Streptococcus uberis*. *FEMS Microbiology Letters,* 114, 67-71.

Leigh, J. A. 1999. *Streptococcus uberis*: A Permanent Barrier to the Control of Bovine Mastitis? *The Veterinary Journal,* 157, 225-238.

Leigh, J. A. & Lincoln, R. A. 1997. *Streptococcus uberis* acquires plasmin activity following growth in the presence of bovine plasminogen through the action of its specific plasminogen activator. *FEMS microbiology letters,* 154, 123-129.

Little, R. B. & Plastridge, W. N. 1946. *Bovine mastitis: A symposium*, McGraw-Hill Book Company, Incorporated.

Lopez-Benavides, M. G., Williamson, J. H., Pullinger, G. D., Lacy-Hulbert, S. J., Cursons, R. T. & Leigh, J. A. 2007. Field observations on the variation of *Streptococcus uberis* populations in a pasture-based dairy farm. *Journal of Dairy Science,* 90, 5558-5566.

Matsunaga, T., Kamata, S., Kakiichi, N. & Uchida, K. 1993. Characteristics of *Staphylococcus aureus* isolated from peracute, acute and chronic bovine mastitis. *The Journal of veterinary medical science/the Japanese Society of Veterinary Science,* 55, 297.

Matthews, K. R., Almeida, R. A. & Oliver, S. P. 1994a. Bovine mammary epithelial cell invasion by *Streptococcus uberis*. *Infection and Immunity,* 62, 5641-5646.

Matthews, K. R., Jayarao, B. M., Guidry, A. J., Erbe, E. F., Wergin, W. P. & Oliver, S. P. 1994b. Encapsulation of *Streptococcus uberis:* Influence of storage and cultural conditions. *Veterinary Microbiology,* 39, 361-367.

Mattie, H., Goslings, W. R. O. & Noach, E. L. 1973. Cloxacillin and nafcillin: serum binding and its relationship to antibacterial effect in mice. *The Journal of Infectious Diseases,* 128, 170-177.

McDougall, S. 1998. Efficacy of two antibiotic treatments in curing clinical and subclinical mastitis in lactating dairy cows. *New Zealand Veterinary Journal,* 46, 226-232.

McDougall, S. 2003. Intramammary treatment of clinical mastitis of dairy cows with a combination of lincomycin and neomycin, or penicillin and dihydrostreptomycin. *New Zealand Veterinary Journal,* 51, 111-116.

McDougall, S., Arthur, D., Bryan, M., Vermunt, J. & Weir, A. 2007. Clinical and bacteriological response to treatment of clinical mastitis with one of three intramammary antibiotics. *New Zealand Veterinary Journal,* 55, 161-170.

Mellado, M. & Reyes, C. 1994. Associations between periparturient disorders and reproductive efficiency in Holstein cows in northern Mexico. *Preventive Veterinary Medicine,* 19, 203-212.

Mercer, H. D., Geleta, J. & Carter, G. 1974a. Absorption and excretion of penicillin G from the mastitic bovine udder. *Journal of the American Veterinary Medical Association,* 164, 613.

Mercer, H. D., Geleta, J., Porteous, L. A. & Condon, R. J. 1974b. Excretion of penicillin G and dihydrostreptomycin from quarters of cows with experimentally induced staphylococcal mastitis. *American Journal of Veterinary Research,* 35, 1191.

Mills, O. E. & Thomas, T. D. 1981. Nitrogen sources for growth of lactic streptococci in milk. *New Zealand Journal of Dairy Science & Technology* 16, 43-55.

Milne, M. H., Biggs, A. M., Barrett, D. C., Young, F. J., Doherty, S., Innocent, G. T. & Fitzpatrick, J. L. 2005. Treatment of persistent intramammary infections with *Streptococcus uberis* in dairy cows. *Veterinary record,* 157, 245-250.

Moyes, K. M., Drackley, J. K., Morin, D. E. & Loor, J. J. 2010. Greater expression of TLR2, TLR4, and IL6 due to negative energy balance is associated with lower expression of HLA-DRA and HLA-A in bovine blood neutrophils after intramammary mastitis challenge with *Streptococcus uberis*. *Functional & integrative genomics,* 10, 53-61.

Moyes, K. M., Drackley, J. K., Salak-Johnson, J. L., Morin, D. E., Hope, J. C. & Loor, J. J. 2009. Dietary-induced negative energy balance has minimal effects on innate immunity during a *Streptococcus uberis* mastitis challenge in dairy cows during midlactation. *Journal of Dairy Science,* 92, 4301-4316.

National Committee for Clinical Laboratory Standards. & Watts, J. L. 1999. *Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals : Approved standard,* Wayne, Pa., NCCLS.

National Mastitis Council 1999. Laboratory Handbook on Bovine Mastitis. *In:* Hogan (ed.) *Laboratory Handbook on Bovine Mastitis B2 - Laboratory Handbook on Bovine Mastitis.* Madison WI, USA: National Mastitis Council.

Nemeth, J. Muckle, C. A. & Gyles, C. L. 1994. In vitro comparison of bovine mastitis and fecal *Escherichia coli* isolates. *Veterinary Microbiology,* 40, 231-238.

Newbould, F. 1974. Antibiotic treatment of experimental Staphylococcus aureus infections of the bovine mammary gland. *Canadian Journal of Comparative Medicine,* 38, 411.

Ngatia, T. A., Jensen, N. E. & Berg, B. B. 1991. Changes in the bovine udder quarters naturally infected by *Corynebacterium bovis*. *British Veterinary Journal,* 147, 463-468.

Nickerson, S. C. & Boddie, R. L. 1994. Effect of naturally occurring coagulase-negative staphylococcal infections on experimental challenge with major mastitis pathogens. *Journal of Dairy Science,* 77, 2526-2536.

Nickerson, S. C., Boddie, R. L., Owens, W. E. & Watts, J. L. 1990. Effects of novel intramammary device models on incidence of mastitis after experimental challenge. *Journal of Dairy Science,* 73, 2774-2784.

Nickerson, S. C. & Heald, C. W. 1981. Histopathologic response of the bovine mammary gland to experimentally induced *Staphylococcus aureus* infection. *American Journal of Veterinary Research,* 42, 1351-1355.

Oitenacu, P. A. & Ekesbo, I. 1994. Epidemiological study of clinical mastitis in dairy cattle. *Veterinary Research,* 25, 208-212.

Oliver, S. P., Almeida, R. A. & Calvinho, L. F. 1998. Virulence factors of *Streptococcus uberis* isolated from cows with mastitis. *Journal of Veterinary Medicine Series B,* 45, 461.

Oliver, S. P., Almeida, R. A., Gillespie, B. E., Ivey, S. J., Moorehead, H., Lunn, P., Dowlen, H. H., Johnson, D. L. & Lamar, K. C. 2003. Efficacy of extended pirlimycin therapy for treatment of experimentally induced *Streptococcus uberis* intramammary infections in lactating dairy cattle. *Veterinary Therapeutics,* 4, 299-308.

Oliver, S. P. & Calvinho, L. F. 1995. Influence of inflammation on mammary gland metabolism and milk composition. *Journal of Animal Science,* 73, 18-33.

Oliver, S. P., Gillespie, B. E., Headrick, S. J., Moorehead, H., Lunn, P., Dowlen, H. H., Johnson, D. L., Lamar, K. C., Chester, S. T. & Moseley, W. M. 2004. Efficacy of extended ceftiofur intramammary therapy for treatment of subclinical mastitis in lactating dairy cows. *Journal of Dairy Science,* 87, 2393-2400.

Owens, W. E., Ray, C. H., Watts, J. L. & Yancey, R. J. 1997. Comparison of success of antibiotic therapy during lactation and results of antimicrobial susceptibility tests for bovine mastitis. *Journal of Dairy Science,* 80, 313-317.

Pedersen, L. H., Aalbæk, B., Røntved, C. M., Ingvartsen, K. L., Sorensen, N. S., Heegaard, P. M. H. & Jensen, H. E. 2003. Early pathogenesis and inflammatory response in experimental bovine mastitis due to *Streptococcus uberis*. *Journal of Comparative Pathology,* 128, 156-164.

Petrovski, K. R., Caicedo-Caldas, A., Williamson, N. B., Lopez-Villalobos, N., Grinberg, A., Parkinson, T. J. & Tucker, I. G. 2011a. Efficacy of a novel internal dry period teat sealant containing 0.5% chlorhexidine against experimental challenge with *Streptococcus uberis* in dairy cattle. *Journal of Dairy Science,* 94, 3366-3375.

Petrovski, K. R., Heuer, C., Parkinson, T. J. & Williamson, N. B. 2009. The incidence and aetiology of clinical bovine mastitis on 14 farms in Northland, New Zealand. *New Zealand Veterinary Journal,* 57, 109-115.

Petrovski, K. R., Laven, R. A. & Lopez-Villalobos, N. 2011b. A descriptive analysis of the antimicrobial susceptibility of mastitis-causing bacteria isolated from samples submitted to commercial diagnostic laboratories in New Zealand (2003–2006). *New Zealand Veterinary Journal,* 59, 59-66.

Petrovski, K. R., Williamson, N. B., Lopez-Villalobos, N., Parkinson, T. J. & Tucker, I. G. 2011c. Culture results from milk samples submitted to veterinary diagnostic

laboratories from August 2003 to December 2006 in New Zealand. *New Zealand Veterinary Journal,* 59, 317-322.

Phuektes, P., Mansell, P. D., Dyson, R. S., Hooper, N. D., Dick, J. S. & Browning, G. F. 2001. Molecular epidemiology of *Streptococcus uberis* isolates from dairy cows with mastitis. *Journal of Clinical Microbiology,* 39, 1460-1466.

Pitkälä, A., Haveri, M., Pyörälä, S., Myllys, V. & Honkanen-Buzalski, T. 2004. Bovine mastitis in Finland 2001—prevalence, distribution of bacteria, and antimicrobial resistance. *Journal of Dairy Science,* 87, 2433-2441.

Poutrel, B. 1982. Susceptibility to mastitis: A review of factors related to the cow. *Annales de Recherches Veterinaires* 13, 85-99.

Poutrel, B. & Lerondelle, C. 1978. Induced staphylococcal infections in the bovine mammary gland. *Annales de Recherches Veterinaires* 39, 29-35.

Poutrel, B., Stegemann, M. R., Roy, O., Pothier, F., Tilt, N. & Payne-Johnson, M. 2008. Evaluation of the efficacy of systemic danofloxacin in the treatment of induced acute *Escherichia coli* bovine mastitis. *Journal of Dairy Research,* 75, 310-318

Pryor, S. M., Cursons, R. T., Williamson, J. H. & Lacy-Hulbert, S. J. 2009. Experimentally induced intramammary infection with multiple strains of *Streptococcus uberis. Journal of Dairy Science,* 92, 5467-5475.

Pyorala, S. 2006. Treatment of clinical mastitis: Local and/or systemic? Short or long? *In:* Navetat, H. & Schelcher, F. (eds.) *24th World Buiatrics Congress, Nice, France, 15-19 October, 2006.* Paris, France: World Association for Buiatrics.

Pyörälä, S., Kaartinen, L., Käck, H. & Rainio, V. 1994. Efficacy of two therapy regimens for treatment of experimentally induced *Escherichia coli* mastitis in cows. *Journal of Dairy Science,* 77, 453-461.

Pyörälä, S. & Pyörälä, E. 1997. Accuracy of methods using somatic cell count and n-acetyl-β-d-glucosaminidase activity in milk to assess the bacteriological cure of bovine clinical mastitis. *Journal of Dairy Science,* 80, 2820-2825.

Pyörälä, S. & Syväjärvi, J. 1987. Bovine acute mastitis part i. Clinical aspects and parameters of inflammation in mastitis caused by different pathogens. *Journal of Veterinary Medicine, Series B,* 34, 573-584.

Rambeaud, M., Almeida, R., Pighetti, G. & Oliver, S. 2003. Dynamics of leukocytes and cytokines during experimentally induced *Streptococcus uberis* mastitis. *Veterinary Immunology and Immunopathology,* 96, 193-205.

Rato, M. G., Bexiga, R., Nunes, S. F., Cavaco, L. M., Vilela, C. L. & Santos-Sanches, I. 2008. Molecular epidemiology and population structure of bovine *Streptococcus uberis*. *Journal of Dairy Science,* 91, 4542-4551.

Roguinsky, M. 1971. Biochemical and serological characters of *Streptococcus uberis*. . *Ann. Inst. Pasteur (Paris),* 120, 154-63.

Rossitto, P. V., Ruiz, L., Kikuchi, Y., Glenn, K., Luiz, K., Watts, J. L. & Cullor, J. S. 2002. Antibiotic susceptibility patterns for environmental streptococci isolated from bovine mastitis in central california dairies. *Journal of Dairy Science,* 85, 132-138.

Rothman, K. J. 2002. Epidemiology: An introduction Oxford University Press. *Inc., New York.*

Ryan, M. P., Flynn, J., Hill, C., Ross, R. P. & Meaney, W. J. 1999. The natural food grade inhibitor, lacticin 3147, reduced the incidence of mastitis after experimental challenge with *Streptococcus dysgalactiae* in nonlactating dairy cows. *Journal of Dairy Science,* 82, 2625-2631.

Salmon, S. A., Watts, J. L., Aarestrup, F. M., Pankey, J. W. & Yancey Jr, R. J. 1998. Minimum inhibitory concentrations for selected antimicrobial agents against organisms isolated from the mammary glands of dairy heifers in New Zealand and Denmark. *Journal of Dairy Science,* 81, 570-578.

Sanderson, C. J. 1966. The treatment of mastitis with intramammary infusions. *Australian Veterinary Journal,* 42, 47-53.

Sandholm, M., Kaartinen, L. & Pyorala, S. 1990. Bovine mastitis-why does antibiotic therapy not always work? An overview. *Journal of Veterinary Pharmacology and Therapeutics,* 13, 248-260.

Saran, A. & Chaffer, M. 2000. Mastitis y calidad de leche. Page 76. Inter-Médica Buenos Aires.

Schepers, A. J., Lam, T., Schukken, Y. H., Wilmink, J. B. M. & Hanekamp, W. J. A. 1997. Estimation of variance components for somatic cell counts to determine thresholds for uninfected quarters. *Journal of Dairy Science,* 80, 1833-1840.

Schofield, F. W. 1946. Penicillin in the treatment of mastitis. *Canadian Journal of Comparative Medicine and Veterinary Science,* 10, 63.

Schukken, Y. H. & Deluyker, H. A. 1995. Design of field trials for the evaluation of antibacterial products for therapy of bovine clinical mastitis. *Journal of Veterinary Pharmacology and Therapeutics,* 18, 274-283.

Schukken, Y. H., González, R. N., Tikofsky, L. L., Schulte, H. F., Santisteban, C. G., Welcome, F. L., Bennett, G. J., Zurakowski, M. J. & Zadoks, R. N. 2009. CNS mastitis: Nothing to worry about? *Veterinary Microbiology,* 134, 9-14.

Schukken, Y. H., Leslie, K. E., Barnum, D. A., Mallard, B. A., Lumsden, J. H., Dick, P. C., Vessie, G. H. & Kehrli, M. E. 1999. Experimental *Staphylococcus aureus* intramammary challenge in late lactation dairy cows: quarter and cow effects determining the probability of infection *Journal of Dairy Science,* 82, 2393-2401.

Schukken, Y. H., Wilson, D. J., Welcome, F., Garrison-Tikofsky, L. & Gonzalez, R. N. 2003. Monitoring udder health and milk quality using somatic cell counts. *Veterinary Research,* 34, 579-596.

Sharma, R. M. & Packer, R. A. 1970. Occurrence and ecologic features of *Streptococcus uberis* in the dairy cow. *American Journal of Veterinary Research,* 31, 1197-202.

Shpigel, N. Y., Levin, D., Winkler, M., Saran, A., Ziv, G. & Böttner, A. 1997. Efficacy of cefquinome for treatment of cows with mastitis experimentally induced using *Escherichia coli. Journal of Dairy Science,* 80, 318-323.

Simojoki, H., Orro, T., Taponen, S. & Pyörälä, S. 2009. Host response in bovine mastitis experimentally induced with *Staphylococcus chromogenes. Veterinary Microbiology,* 134, 95-99.

Sladek, Z. & Rysanek, D. 2006. The role of CD14 during resolution of experimentally induced *Staphylococcus aureus* and *Streptococcus uberis* mastitis. *Comparative Immunology, Microbiology and Infectious Diseases,* 29, 243-262.

Smith, K. L. & Oliver, S. P. 1981. Lactoferrin: A component of nonspecific defense of the involuting bovine mammary gland. *Advances in Experimental Medicine and Biology,* 137, 535.

Sol, J., Sampimon, O. C., Barkema, H. W. & Schukken, Y. H. 2000. Factors associated with cure after therapy of clinical mastitis caused by *Staphylococcus aureus. Journal of Dairy Science,* 83, 278-284.

Sordillo, L. M., Shafer-Weaver, K. & DeRosa, D. 1997. Immunobiology of the mammary gland. *Journal of Dairy Science,* 80, 1851-1865.

Sordillo, L. M. & Streicher, K. L. 2002. Mammary gland immunity and mastitis susceptibility. *Journal of Mammary Gland Biology and Neoplasia,* 7, 135-146.

Steeneveld, W., van Werven, T., Barkema, H. W. & Hogeveen, H. 2011. Cow-specific treatment of clinical mastitis: An economic approach. *Journal of Dairy Science,* 94, 174-188.

Swinkels, J. M., Hogeveen, H. & Zadoks, R. N. 2005. A partial budget model to estimate economic benefits of lactational treatment of subclinical *Staphylococcus aureus* mastitis. *Journal of Dairy Science,* 88, 4273-4287.

Taponen, S., Jantunen, A., Pyorala, E. & Pyorala, S. 2003. Efficacy of targeted 5-day combined parenteral and intramammary treatment of clinical mastitis caused by penicillin-susceptible or penicillin-resistant *Staphylococcus aureus*. *Acta Veterinaria Scandinavica,* 44, 53 - 62.

Taponen, S., Simojoki, H., Haveri, M., Larsen, H. D. & Pyörälä, S. 2006. Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. *Veterinary Microbiology,* 115, 199-207.

Thomas, C. A. 1949. *Strip cup - US Patent Number 2467949* [Online]. United States Patent and Trademark Office. Available: http://www.google.com/patents/US2467949.

Thomas, L. H., Haider, W., Hill, A. W. & Cook, R. 1994. Pathologic findings of experimentally induced *Streptococcus uberis* infection in the mammary gland of cows. *American Journal of Veterinary Research,* 55, 1723.

Thomas, L. H., Leigh, J. A., Bland, A. P. & Cook, R. S. 1992. Adherence and colonization by bacterial pathogens in explant cultures of bovine mammary tissue. *Veterinary Research Communications,* 16, 87-96.

Todhunter, D. A., Smith, K. L. & Hogan, J. S. 1995. Environmental streptococcal intramammary infections of the bovine mammary gland. *Journal of Dairy Science,* 78, 2366-2374.

Trinidad, P., Nickerson, S. & Alley, T. 1990. Prevalence of intramammary infection and teat canal colonization in unbred and primigravid dairy heifers1. *Journal of Dairy Science,* 73, 107-114.

Vangroenweghe, F., Duchateau, L., Boutet, P., Lekeux, P., Rainard, P., Paape, M. J. & Burvenich, C. 2005. Effect of carprofen treatment following experimentally induced *Escherichia coli* mastitis in primiparous cows. *Journal of Dairy Science,* 88, 2361-2376.

Washburn, S. P., Silvia, W. J., Brown, C. H., McDaniel, B. T. & McAllister, A. J. 2002. Trends in reproductive performance in southeastern Holstein and Jersey DHI herds. *Journal of Dairy Science,* 85, 244-251.

Watts, J. L. 1988. Etiological agents of bovine mastitis. *Veterinary Microbiology,* 16, 41-66.

Welty, F. K., Larry Smith, K. & Schanbacher, F. L. 1976. Lactoferrin concentration during involution of the bovine mammary gland. *Journal of Dairy Science,* 59, 224-231.

White, L., Schukken, Y. H., Lam, T., Medley, G. & Chappell, M. J. 2001. A multispecies model for the transmission and control of mastitis in dairy cows. *Epidemiology and Infection,* 127, 567-576.

Williamson, J. H., Woolford, M. W. & Day, A. M. 1995. The prophylactic effect of a dry-cow antibiotic against *Streptococcus uberis*. *New Zealand Veterinary Journal,* 43, 228-234.

Wise Jr, E. M. & Park, J. T. 1965. Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. *Proceedings of the National Academy of Sciences of the United States of America,* 54, 75.

Zadoks, R. N., Allore, H. G., Barkema, H. W., Sampimon, O. C., Wellenberg, G. J., Gröhn, Y. T. & Schukken, Y. H. 2001. Cow-and Quarter-Level Risk Factors for *Streptococcus uberis* and *Staphylococcus aureus* Mastitis. *Journal of Dairy Science,* 84, 2649-2663.

Zadoks, R. N., Gillespie, B. E., Barkema, H. W., Sampimon, O. C., Oliver, S. P. & Schukken, Y. H. 2003. Clinical, epidemiological and molecular characteristics of *Streptococcus uberis* infections in dairy herds. *Epidemiology and infection,* 130, 335-349.

Zadoks, R. N., Tikofsky, L. L. & Boor, K. J. 2005. Ribotyping of *Streptococcus uberis* from a dairy's environment, bovine feces and milk. *Veterinary Microbiology,* 109, 257-265.

Zhao, X. & Lacasse, P. 2008. Mammary tissue damage during bovine mastitis: Causes and control. *Journal of animal science,* 86, 57-65.

Zweig, J. 1949. Procaine penicillin G in the bovine udder. *Veterinary Record,* 61, 811.