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The host-pathogen interaction of ecologically diverse coagulase-negative staphylococci in bovine mastitis, with a focus on prolactin

Kristine Piccart

Merelbeke, 2016

"*You must look at the facts, because they look at you*."

Winston Churchill, 1925

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Cover: Hans De Clercq (design), Kristine Piccart (photos)

Printing: University Press, Zelzate

This research was financed by the Special Research Fund (BOF) of Ghent University.

Project number BOF10/STA/054.

UNIVERSITEIT GENT

M-team UGent

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Dissertation submitted in the fulfillment of the requirements for the degree of Doctor in Veterinary Sciences, Faculty of Veterinary Medicine, Ghent University, May 17, 2017

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Gastheer-pathogeen interactie van ecologisch diverse coagulase-negatieve staphylokokken in boviene mastitis, met een focus op prolactine

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Proefschrift voorgedragen tot het behalen van de graad van Doctor in de Diergeneeskundige Wetenschappen aan de Faculteit Diergeneeskunde, Universiteit Gent, 17 mei 2017

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List of abbreviations

BSA	Bovine serum albumin
C5a	Complement component 5a
CFU	Colony forming units
CNS	Coagulase-negative staphylococci
cpm	Counts per minute
cDNA	Complementary DNA
gDNA	Genomic DNA
DNA	Deoxyribonucleic acid
FAO	Food and Agricultural Organization of the United Nations
GH	Growth hormone
GM-CSF	Granulocyte macrophage colony stimulating factor
H2A	Histone H2A
IgG_1	Immunoglobin G_1
IL	Interleukin
IQR	Interquartile range
IMI	Intramammary infection
LPS	Lipopolysaccharide
LSM	Least square means
MAMP	Microorganism-associated molecular patterns
MDL	Minimal detection limit
MEC	Mammary epithelial cells
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NF-κB	Nuclear factor kappa B
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PI	Post-inoculation
PL	Placental lactogen
PMN	Polymorphonuclear neutrophil leukocyte
PRL	Prolactin
PRR	Pattern recognition receptor

QMY	Quarter milk yield
qPCR	Quantitative PCR
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPS15A	Ribosomal Protein S15a
RT-PCR	Reverse transcription PCR
SCC	Somatic cell count
SD	Standard deviation
SDHA	Succinate dehydrogenase subunit A
SEM	Standard error of the mean
SV40	Simian virus 40
tDNA-PCR	Transfer RNA intergenic spacer PCR
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRIR	Total RNA isolation agent
UBC	Ubiquitine C

Chapter 1

General introduction

1.1 Bovine mastitis, a burden on the dairy industry

Cow's milk and dairy products make up a substantial part of the human diet. The Food and Agricultural Organization of the United Nations (FAO) estimates that the worldwide milk demand will increase with 1.3% over the next years due to changing global consumption patterns (FAO, 2013). The European Union currently produces around 160 million tons of milk on an annual basis (Eurostat, 2015). In contrast to the past decennia, the dairy cattle population has expanded over the past three years in Western Europe (Eurostat, 2016). Belgium counted approximately 508.000 lactating dairy cows in 2015 (FPSE, 2016). The Belgian dairy farms may be dwindling in numbers, but the herds are getting larger: the average number of dairy cows per farm has increased with almost 52% since 2000 (from 33 to 50 cows in 2012 [FPSE, 2012]). This trend towards intensification is also seen at the individual cow level. Nowadays, the average Flemish dairy cow produces around 8.500 kg of milk each year, which is an increase of more than 7% compared to 10 years ago (CRV, 2015). The higher level of milk yield is generally associated with negative health effects, such as an increased incidence of mastitis, i.e. an inflammation of the mammary gland (Simianer et al., 1991; Van Dorp et al., 1998; Koeck et al., 2014). Despite the implementation of well-known control and treatment strategies, mastitis remains to be a burden on the dairy sector. Since the abolition of the European milk quota in 2015, the average annual costs of mastitis are estimated at €240 per cow on Dutch dairy farms (van Soest et al., 2016). These high costs are not surprising, since mastitis results in milk losses, high treatment costs, higher probability of culling, etc. (Lescourret and Coulon, 1994; Halasa et al., 2007). Mastitis also affects the milk quality. Milk from inflamed udder quarters, for instance, affects curd formation and produces off-flavors during the processing of cheese (Le Marechal et al., 2011a). Locally administered intramammary antimicrobials account for the majority of antimicrobial consumption in dairy herds, either as dry-cow treatment or mastitis therapy (Stevens et al., 2016). Moreover, the use of antimicrobials in food-producing animals has been linked to the development of antimicrobial resistance (Chantziaras et al., 2014). Furthermore, there is also the issue of animal welfare, since clinical mastitis is considered to be a painful condition (Leslie and Petersson-Wolfe, 2012). In Flanders,

the mean incidence rate of clinical mastitis is estimated at 7.4 quarters cases per 10,000 cow-days at risk (Verbeke et al., 2014).

1.2 Etiology, clinical presentation and immune response

1.2.1. Etiology of bovine mastitis

Although several microorganisms can infect the mammary gland, the vast majority of bovine mastitis cases are caused by bacteria (Watts, 1988). Nearly 80% of the mastitis cases can be attributed to either staphylococci (which are routinely classified into coagulase-negative and -positive species), streptococci or *Escherichia coli* (Bradley, 2002). In general, the bacteria enter through the teat canal of the udder quarter, resulting in an intramammary infection (IMI) and subsequent inflammation (Blowey and Edmondson, 2010).

In the past, it was generally assumed that the uninfected, healthy bovine mammary gland was a sterile environment (Sheather, 1924; Perkins et al., 2009). This view can no longer be supported though, due to the growing insight into the milk microbiota. Even healthy, non-mastitic milk samples display great bacterial diversity after DNA sequencing (Kuehn et al., 2013; Oikonomou et al., 2014). The bacteria found in healthy, uninfected quarters include *Pseudomonas* spp., *Ralstonia* spp., *Psychrobacter* spp., *Faecalibacterium* spp., *Propionibacterium* spp., *Aeribacillus* spp. and unclassified *Lachnospiraceae* (Kuehn et al., 2013; Oikonomou et al., 2014). One study also found DNA of *Streptococcus* spp. and *Staphylococcus* spp. in milk samples from healthy quarters (Oikonomou et al., 2012). Some of these bacteria cannot be readily cultured during routine (aerobic) microbiological analyses, leading to culture-negative results (Kuehn et al., 2013). It has even been suggested that mastitis could be the result of a mammary dysbacteriosis, as opposed to a primary infection (Fernandez et al., 2013). The importance of the mammary microbiota is also supported by incidental reports of clinical mastitis outbreaks following a 'blitz' antimicrobial therapy (i.e. treating the entire herd or multiple infected animals with antibiotics, in this case to eradicate *Streptococcus agalactiae* IMI [Edmondson, 2010]).

2.2 Clinical presentation

Based on the clinical presentation, mastitis can be classified into two types, namely clinical and subclinical mastitis. Clinical mastitis is characterized by visual abnormalities in the milk (e.g. clots, discoloration), the udder (e.g. swelling and redness) or the animal itself (sickness). Subclinical mastitis, on the other hand, occurs when the gland is infected without any observable symptoms of inflammation. It can be detected by, for instance, measuring the somatic cell count (SCC) in milk, which increases through the influx of polymorphonuclear neutrophil leukocytes (PMN) in response to invading pathogens (Harmon, 1994). Though different cut-off values are used in practice to distinguish healthy from inflamed quarters, a threshold between 200,000 and 250,000 cells/mL at quarter level is ideal to minimize diagnostic error (Schukken et al., 2003). In practice, cow level cell counts of 250,000 cells/mL and 150,000 cells/mL are often used as a threshold for multiparous cows and heifers, respectively. The bulk milk SCC is considered an important indicator of the milk quality. As stated by the Council Directive 92/46/EEC, the geometric average bulk milk SCC over a period of three months should not exceed 400,000 cells/mL in the European Union.

Photo 1.1 shows a dairy cow with clinical mastitis in her left hind quarter, recognizable by the swelling of that particular quarter. **Photo 1.2** shows the difference between milk from a cow with subclinical (i.e. no observable signs of inflammation) and clinical mastitis (in this case watery, yellowish milk with few clots).

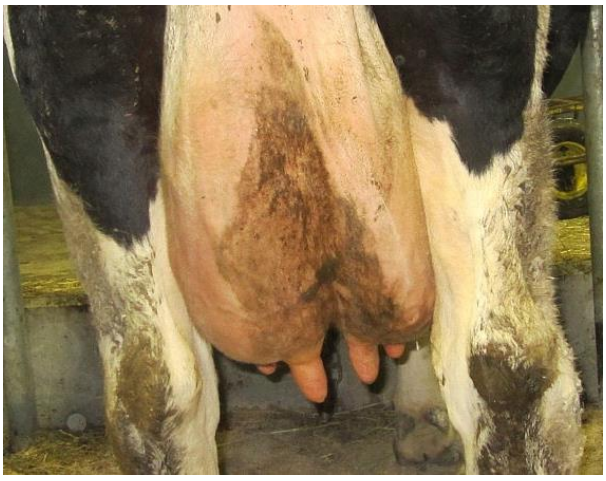

Photo 1.1 Cow with clinical mastitis in her left hind quarter.

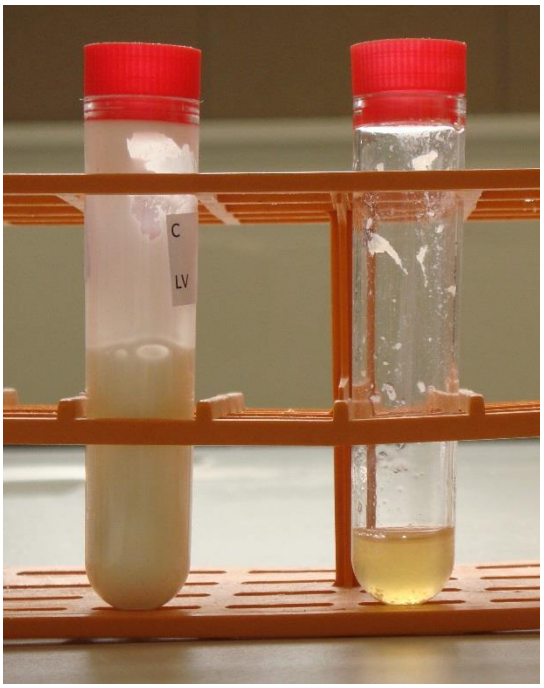

Photo 1.2 Milk samples from udder quarters

with subclinical (left) and clinical mastitis (right).

The severity of mastitis cases depends on both the involved pathogen and the immune response capacity of the cow. Host factors, such as the lactation stage, parity and genetic predisposition, have an effect on the clinical course of an IMI (Burvenich et al., 2003). During early lactation, cows suffer from an increased incidence of severe clinical mastitis due to the decreased function of leukocytes around parturition (Mehrzad et al., 2001; Vangroenweghe et al., 2005). The clinical outcome of mastitis is generally more severe in multiparous cows than in heifers (VanWerven et al., 1997; Mehrzad et al., 2002; Vangroenweghe et al., 2004a), likely due to an age-associated impairment of the immune system (Wojdak-Maksymiec et al., 2013). Managerial factors that affect the health status of the cow (e.g. nutrition, housing, …) can also have an impact on the development risk of IMI (Smith et al., 1984; Barkema et al., 1999a; Janosi et al., 2003).

In addition to the inherent and acquired host immune factors, the initial infection dose (Vangroenweghe et al., 2004b; Günther et al., 2010) and the virulence of the involved bacterial strain (Haveri et al., 2005) also affect the disease progression. Various mastitis pathogens, such as

Staphylococcus aureus, *Streptococcus uberis* or *Streptococcus dysgalactiae*, display strain-specific differences in their ability to cause disease (Higgs et al., 1980; Haveri et al., 2007; Le Marechal et al., 2011b; Tassi et al., 2013). For semantic clarity, a bacterial strain is defined in this thesis as "an isolate or a group of isolates exhibiting characteristics that set it apart from other isolates belonging to the same species" (Zadoks and Schukken, 2006).

1.2.3. Mammary gland immunity

A variety of defense mechanisms protect the mammary gland against invading pathogens (**Figure 1.1**). First, pathogens need to overcome the teat canal, which constitutes an anatomical barrier with keratin lining and sphincter muscles at the teat end (Sordillo et al., 1997). When bacteria successfully invade the mammary gland, the innate branch of the immune system comes into play. The innate immune response is activated within the first few hours, regardless of any previous exposure to the pathogen, and is carried out by professional phagocytes (e.g. macrophages, PMN, dendritic cells, etc.) and mammary epithelial cells (MEC), which are also able to recognize and respond to pathogens by producing pro-inflammatory cytokines (Lahouassa et al., 2007). Foreign, microbial components are recognized by immune cells with pattern recognition receptors (PRR), such as the transmembrane toll-like receptor proteins (TLR). So far, ten different TLR have been described in cattle (Fisher et al., 2011). The bacteria are recognized by the PRR due to highly conserved structures, referred to as microorganism-associated molecular patterns (MAMP). Lipopolysaccharides (LPS) of Gram-negative bacteria, for instance, are recognized by TLR4, whereas lipoteichoic acid of Gram-positive bacteria binds to TLR2 (Rainard and Riollet, 2006). Following the recognition of the pathogen, various inflammatory pathways are initiated, resulting in the upregulation of different cytokines and chemokines (**Table 1.1**). Virtually every cell type is capable of producing and reacting to cytokines (Dinarello, 2000). Though pro-inflammatory cytokines play a vital role in the host response, they can also be harmful to the host, depending on the quantity and the extent of their expression (Bannerman, 2009). Tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) induce a systemic host

response, resulting in fever and the production of acute phase proteins (Dinarello, 2000). Especially IMI caused by Gram-negative bacteria (e.g. *E. coli*) induce the production of TNF-α, which correlates with the clinical severity of mastitis cases (Burvenich et al., 2003; Bannerman et al., 2004). A well-described chemokine in bovine mastitis is interleukin 8 (IL-8), which binds to receptors CXC-receptor 1 (CXCR1) and CXCR2 (Lahouassa et al., 2008). Polymorphonuclear neutrophil leukocytes are drawn to the site of infection in massive numbers in response to IL-8 and other inflammatory mediators, such as complement component 5a (C5a) (Stevens et al., 2012) or leukotriene B4 (Boutet et al., 2003).

The SCC can increase dramatically within 12 hours after exposure to bacteria, reaching more than 10^6 cells/mL (Rainard and Riollet, 2006). Macrophages and lymphocytes account for the majority of the SCC in milk from uninfected quarters, but the cell type distribution shifts during inflammation, making PMN the predominant cell population (Pilla et al., 2012; Damm et al., 2017). The accumulation of PMN in the mammary gland cuts both ways though. While PMN are essential for the elimination of pathogens, they may also damage the surrounding mammary gland tissue through the release of extracellular proteolytic enzymes (such as elastase) and reactive oxygen species (Zhao and Lacasse, 2008). Consequently, their life span is limited: the PMN eventually undergo apoptosis, or programmed cell death (Paape et al., 2003). Apoptotic PMN are quickly eliminated by macrophages, while their membrane is still intact, to prevent the release of cytotoxic molecules and subsequent tissue damage (Kennedy and DeLeo, 2009). The viability of PMN is linked to their activity (Mehrzad et al., 2004).

When the innate immune system is unable to eliminate the pathogen, the acquired or adaptive immune system kicks in. The adaptive immunity, carried out by B- and T-lymphocytes, establishes an antigen-specific immunological “memory”, enabling a faster, more efficient response in case of repeated exposure to the pathogen (Sordillo et al., 2002; Iwasaki and Medzhitov, 2010). A previous encounter with a particular pathogen results, for instance, in a larger influx of milk leukocytes in case of repeated exposure (Rainard et al., 2016). The adaptive immune response can be classified into a cell-mediated and antibody component, driven by the T-lymphocytes and B-lymphocytes, respectively. Before the receptors on T-lymphocytes can recognize an antigen, it must be internalized, processed

and bound to the major histocompatibility complex (MHC) on the surface of antigen-presenting cells (such as dendritic cells, macrophages and B-lymphocytes) or non-professional antigen-presenting cells (such as epithelial cells) (Fitzpatrick et al. 1992; Quinn et al., 2015). Activated B-lymphocytes will proliferate and differentiate into antibody-producing plasma-cells, or dormant memory cells (Sordillo et al., 2002). However, Schukken et al. (2009a) propose that the mammary gland's immunological memory is limited, since the clinical response of dairy cows suffering from recurring infections is not necessarily attenuated. The mastitis pathogens in that particular study were not identified at the strain level though.

The dairy cow's immune system reacts differently to IMI caused by epidemiologically and ecologically varying species (Schukken et al., 2011; Günther et al., 2016). Typically, Gram-negative mastitis pathogens (such as *E. coli* or *Klebsiella* spp.) evoke a more drastic host response than Gram-positive bacteria, given the LPS in their cell walls (Schukken et al., 2011). However, the host response does not only depend on the involved pathogen, but also on various cow factors (Burvenich et al., 2003), there has been much debate on the "most desirable" type of response in dairy cattle following IMI (Benjamin et al., 2015). In case of experimental *E. coli* mastitis, an early mobilization of PMN leads to lower bacterial growth and faster clearance of the infection (Vandeputte-Van Messom et al.; 1993, Mehrzad et al., 2005). Some authors even consider very low pre-challenge quarter milk SCC levels (< 20.000 cells/mL) a risk factor for establishing IMI (Schukken et al., 1999, Wellnitz et al., 2010). Another study investigated the potential of the bacterial molecules LPS for enhancing leukocyte recruitment during an experimental *S. aureus* challenge (Kauf et al., 2007). Although LPS increased the SCC levels in the *S. aureus*-infected quarters, it did not facilitate the bacterial clearance.

Table 1.1. A non-exhaustive summary of cytokines involved in bovine mastitis (1/2).

Cytokine	Primarily produced by	Functions	Pathogen-specific response
IL-1	Macrophages, lymphocytes, epithelial cells, etc.	The IL-1 family consists of 11 cytokines, including IL-1α and IL-1β. IL-1 induces local and systemic effects, such as fever and the synthesis of acute phase proteins.	Mainly Gram-negative mastitis pathogens induce a temporal IL-1β response (ranging between 0.3 – 8 ng/mL). The response varies greatly between individual cows.
IL-2	T-helper cells (Th1)	IL-2 stimulates the clonal expansion of T-lymphocytes, activates cytotoxic T-cells and natural killer (NK) cells, and the proliferation of B-lymphocytes.	IL-2 occurs in both healthy and infected mammary glands. The transcription of IL-2 decreases during *S. aureus* infection.
IL-4	T-helper cells (Th2)	IL-4 is a so-called anti-inflammatory cytokine that promotes the differentiation of T-cells into Th2 cells. It is the primary cytokine expressed by mononuclear cells in the post-partum period.	Little is currently known about the involvement of IL-4 in bovine mastitis.
IL-6	PMN, macrophages, lymphocytes, epithelial cells, etc.	IL-6 has both pro- and anti-inflammatory properties. It promotes the synthesis of hepatic APP. It has been suggested that IL-6 enables the shift from PMN to monocytes during inflammation.	Although IL-6 is found in milk from mammary quarters infected with Gram-negative and -positive bacteria, the transcription is minimal in case of *S. aureus* infections.
IL-8	MEC, PMN, lymphocytes, etc.	IL-8 is a chemotactic cytokine (chemokine) that attracts PMN to the site of infection, and enhances their activity.	IL-8 increases within 20h during Gram-negative IMI (ranging between 100 – 1000 pg/mL). IL-8 transcription is diminished or absent in *S. aureus* IMI.

Table 1.1. A non-exhaustive summary of cytokines involved in bovine mastitis (2/2).

Cytokine	Primarily produced by	Functions	Pathogen-specific response
IL-10	Th2 cells, B cells, eosinophils, mast cells, etc.	IL-10 is an anti-inflammatory that inhibits the production of pro-inflammatory cytokines in PMN, and impairs the Th1 response.	Various mastitis pathogens evoke an IL-10 response, although the expression is not always seen in *S. aureus* IMI. The production of IL-10 is preceded by an increase in TNF-α.
IL-12	Monocytes, macrophages, dendritic cells	IL-12 links the innate to the adaptive immune system: it promotes the differentiation of T-cells into Th1-cells, and stimulates the production of IFN- γ.	Similar elevations in IL-12 are found in *E. coli* and *S. aureus* infections. The increase in IL-12 coincides with the elevated IFN- γ levels.
IL-17	T-helper cells (Th17)	The IL-17 cytokine family contains 6 members, including IL-17A. IL-17A stimulates the inflammatory response of MEC, and attracts PMN and macrophages to the site of infection.	IL-17A is expressed in mammary tissue infected with *E. coli*, *S. aureus* or *S. uberis*. Intramammary infusion of IL-17A in *E. coli* IMI is associated with lower bacterial numbers, increased PMN recruitment and lower IL-10 levels.
IFN-γ	Monocytes, lymphocytes	IFN-γ stimulates the activity of PMN and macrophages, and upregulates the expression of the major histocompatibility complex (MHC) class I molecules, enabling pathogen recognition by T-cells.	IFN-γ transcription occurs in both Gram-negative and –positive mastitis cases, but the highest concentrations are found in persistent IMI.
TNF-α	Macrophages, lymphocytes, PMN, epithelial cells, etc.	TNF-α induces systemic effects, such as fever and the production of APP, and weakens the blood-milk barrier. TNF-α is also associated with shock, tissue damage and organ failure.	The TNF-α level increases in the blood and milk in a dose-responsive manner during Gram-negative IMI. The TNF-α response is absent in S. aureus IMI.

References: Sordillo and Streicher, 2002; Waller, 2002; Alluwaimi et al., 2003; Alluwaimi, 2004; Rainard and Riollet, 2006; Dinarello, 2007; Bannerman, 2009; Günther et al., 2011; Porcherie et al., 2016

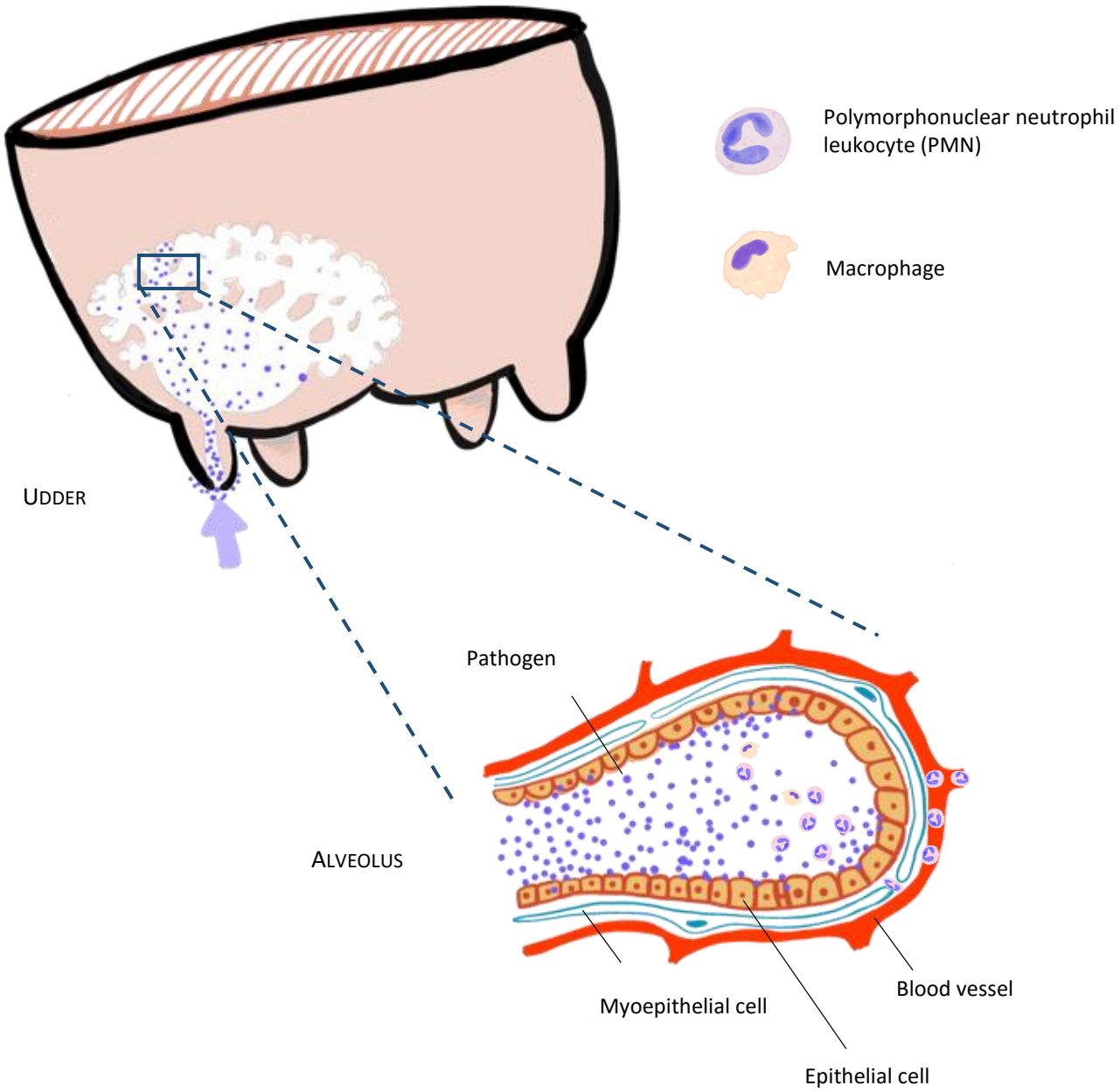

Figure 1.1. Simplified representation of an intramammary infection. Pathogens enter the mammary gland through the teat canal and colonize the mammary epithelial cells (arrow). Once the pathogen is recognized by macrophages and other immune cells, the host response is triggered. Neutrophils, or PMN, travel in massive numbers from the blood stream to the site of infection to kill the invading pathogens. Apoptotic PMN are then eliminated by macrophages to minimize tissue damage

1.3 Coagulase-negative staphylococci

1.3.1. A complex, heterogeneous group

Coagulase-negative staphylococci (CNS) are named for their inability to coagulate rabbit plasma *in vitro*. In bovine medicine, CNS is often used as a collective term for non-aureus staphylococci, although this is not entirely accurate. For example, *Staphylococcus intermedius*, a staphylococcal species found in bovine milk, is in fact coagulase-positive (Roberson et al., 1996), whereas *Staphylococcus hyicus*, *Staphylococcus agnetis* and *Staphylococcus chromogenes* have a variable coagulase activity (Taponen et al., 2012; dos Santos et al., 2016). Previously, CNS were often considered to be one large homogeneous group of bacteria (Reneau, 1986; Hogan et al., 1987). Novel molecular identification and typing techniques, however, have made it easier to study CNS on species level (Da Silva Santos et al., 2008; Park et al., 2011; Supré et al., 2011; Piessens et al., 2011; Braem et al., 2012). So far, more than 50 staphylococcal species have been identified and described (Parte, 2014). Over 20 CNS species have been found in bovine milk, but only 5 species are isolated on a routine basis: *S. chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus xylosus*, *Staphylococcus haemolyticus* and *Staphylococcus simulans* (Vanderhaeghen et al., 2014).

1.3.2. Epidemiology and ecology

Coagulase-negative staphylococci are frequently isolated from cows with subclinical mastitis (Barkema et al., 1999b; Pitkälä et al., 2004; Sampimon et al., 2009; Piepers et al., 2011; Rall et al., 2014), especially in dairy heifers (Tenhagen et al., 2006; Fox, 2009; Sampimon et al., 2009). According to Vanderhaeghen et al. (2015), CNS can be divided into two categories based on their epidemiology, namely “contagious” and “opportunistic CNS”. Whereas contagious mastitis pathogens are typically transmitted from cow to cow through a vector (such as the milking equipment or the milker’s hands) (Zadoks et al., 2011), opportunistic mastitis bacteria originate from different sources, do not spread between cows and only

cause an IMI "under conditions favouring colonisation of the udder" (Vanderhaeghen et al., 2015). In terms of their ecology (i.e. habitat), CNS are usually divided into "host-adapted" and "environmental" species (Piessens et al., 2011; De Visscher et al., 2014; Fry et al., 2014). Across multiple studies, *S. fleurettii* is regularly found in the environment of the dairy farm or milking parlor (Piessens et al., 2011; De Visscher et al., 2014). The species rarely occurs in the milk of cows (De Visscher et al., 2016). In the (occasional) occurrences that *S. fleurettii* is associated with an IMI, the infection is of a transient nature (Supré et al., 2011). At the other end of the ecological spectrum are the host-adapted CNS species and strains, which are characterized by their ability to colonize or invade the host. *Staphylococcus chromogenes* is by all means a prime example of host-adapted species. For one, *S. chromogenes* is the most frequently isolated CNS species in milk, especially in heifers (Aarestrup and Jensen, 1997; Taponen et al., 2006, Adkins and Middleton, 2016). It has been suggested by some authors that *S. chromogenes* is part of the normal skin microbiome of cattle (White et al., 1989; Taponen et al., 2008; Taponen and Pyörälä, 2009), even though the species is not always present on the skin of the teats (Braem et al., 2013; De Visscher et al., 2014). Although *S. chromogenes* can be effectively and swiftly neutralized by macrophages (Åvall-Jääskeläinen et al., 2013), the species is known to invade and replicate in bovine MEC (Hyvönen et al., 2009; Souza et al., 2016). It is not uncommon for *S. chromogenes* to cause persistent IMI lasting an entire lactation (Taponen et al., 2007; Piessens et al., 2011; Mørk et al., 2012). Ultimately though, the question remains: why do some CNS species thrive in the mammary gland, as opposed to other body sites or the surrounding environment?

1.3.3. Intramammary infections with CNS

Since the clinical impact of CNS IMI is relatively limited, they are referred to as "minor pathogens" (Djabri et al., 2002; Taponen et al., 2006). An IMI with CNS usually manifests itself as subclinical or mild clinical mastitis (Waller et al., 2011). In general, the SCC increase caused by CNS is small compared to the response seen in IMI with major mastitis pathogens (Sampimon et al., 2010). Some CNS species, such as *S. chromogenes* and *S. simulans*, provoke a larger SCC increase than others though (Fry et al.,

2014, De Visscher et al., 2016a). *Staphylococcus chromogenes* can even evoke an increase in SCC similar to the response seen in *S. aureus* IMI (Supré et al., 2011).

All in all, not much is known about the interaction between bovine-associated CNS and their host. Gram-positive bacteria generally induce a weaker pro-inflammatory cytokine response than Gram-negative mastitis pathogens (Riollet et al., 2000; Bannerman et al., 2004; Günther et al., 2016). Previous challenge studies show that *S. epidermidis* is able to induce the production of IL-1β, IL-8 and TNF-α in cows and ewes (Winter et al., 2003; Simojoki et al., 2011). This is in stark contrast to experimental infections with *S. aureus*, where IL-8 and TNF-α are generally not detected in milk (Riollet et al., 2000; Bannerman et al., 2004). The IL-1β response seen in an *S. epidermidis* or *S. simulans* challenge is similar to *S. aureus* IMI (Simojoki et al., 2011), but can vary greatly between individual animals (Winter et al., 2003; Bannerman, 2009).

Although it is assumed that most CNS infections resolve without antibiotic treatment (Taponen and Pyörälä, 2009), certain species (i.e. *S. chromogenes*, *S. simulans*, *S. epidermidis* and others) can cause persistent IMI lasting over several months (Taponen et al., 2007, Thorberg et al., 2009; Piessens et al., 2011; Fry et al., 2014). A number of virulence factors have been identified in bovine-associated CNS species, but the pathogenic potential of CNS is still not fully elucidated. Some CNS species may have virulence factors similar to *S. aureus*, such as the ability to form a biofilm (Simojoki et al., 2012), whereas other do not (Taponen and Pyörälä, 2009). In order to evade the host response and cause a long-lasting IMI, *S. aureus* attaches itself to the surface of MEC and invades them (Cifrian et al., 1994; Almeida et al., 1996). This strategy of invasion is also seen in mastitis-causing CNS, although to a lesser extent (Hyvönen et al., 2009). Similar to *S. aureus*, certain CNS species and strains are also able to adhere to and replicate in MEC (Hyvönen et al., 2009), and resist phagocytosis by macrophages (Åvall-Jääskeläinen et al., 2013). However, the potential for adhesion and internalization varies between species (Almeida and Oliver, 2001) and even within species (Souza et al., 2016). A *S. chromogenes* strain recovered from a chronically infected quarter showed higher adhesion and internalization values than another *S. chromogenes* strain originating from the teat skin (more specifically, the teat apex

[Souza et al., 2016]). The indication that some strains of *S. chromogenes* might be better suited to invade and colonize the mammary gland than others sheds new light on the heterogeneity of CNS, even within species.

1.3.4. Effect of CNS IMI on milk yield: a paradox?

Milk production losses associated with mastitis are either due to the bacterial infection itself, or to inflammatory response following the infection (Detilleux et al., 2015). Evidently, not all mastitis pathogens elicit the same degree of milk loss in dairy cattle (Gröhn et al., 2004). Over the past years, the effect of CNS IMI on milk production has received a great deal of attention (**Table 1.2**). Yet, there is no definitive consensus on the impact of CNS IMI on milk yield. Some research indicates that the milk yield decreases in response to a CNS IMI (Timms and Schultz, 1987; Gröhn et al., 2004), while others have observed barely any effect at all (Pearson et al., 2013; Tomazi et al., 2015). Interestingly though, some studies even mention a positive effect on milk yield (Schukken et al., 2009b; Piepers et al., 2010). There are multiple possible explanations for this counterintuitive finding. For one, high-yielding dairy cows might inherently be more prone to CNS infections. Gröhn et al. (2004), for instance, remarked that multiparous cows with clinical CNS mastitis produced significantly more milk (between 2.3 and 2.7 kg/day) one month before diagnosis than their non-infected herd mates. This effect was not seen in primiparous cows. According to Piepers et al. (2013), the association between milk yield and CNS IMI is only partially confounded by the genetic potential for milk production, as it did not fully account for the observed difference in milk yield. Another hypothesis for the higher milk yield in CNS-infected dairy cattle relies on the lactation hormone prolactin (PRL), which can also act as an immunomodulatory factor (see below). Other research suggests that pre-existing CNS IMI or even CNS teat apex colonization can have a protective effect against new infection with other (major) mastitis pathogens, effectively lowering the risk of developing clinical mastitis and subsequent milk loss (Rainard and Poutrel, 1988, Matthews et al., 1991, Nickerson and Boddie, 1994). The protective

mechanism of CNS infections might be attributed to bacterial competition between CNS and other pathogens in the same niche (Hibbing et al., 2010), the stimulation of the innate immune system (i.e. an increased SCC [Schukken et al., 1999]), or the production of bacteriocins and other antibacterial substances (Braem et al., 2014). Only a handful of studies have focused on the species-specific effect of CNS regarding their protecting outcome. De Vliegher et al. (2003) demonstrated that certain *S. chromogenes* isolates, originating from the teat apex of primiparous cows, are able to inhibit the *in vitro* growth of Gram-positive mastitis pathogens. In mice, the colonization of the mammary gland by *S. epidermidis* attenuates the clinical response to a later challenge with *S. aureus* or *E. coli* (Anderson, 1978).

Still, the majority of the aforementioned studies do not account for potential differences between or within CNS species, which might contribute to the overall conflicting results. Also, the protective effect of CNS is more pronounced in experimental challenge studies where major pathogens are infused directly into the mammary gland, compared to natural infections with mastitis pathogens (Reyher et al., 2012).

Table 1.2. An overview of longitudinal field studies on the effect of intramammary infections with coagulase-negative staphylococci (CNS) on the milk yield.

Authors	Animals	CNS ID[1]	Effect on milk yield	Effect size compared to uninfected animals
Timm & Schutlz (1987)	Dairy cows	Bacterial culture	↓	- 2.9 kg/d
Gröhn et al. (2004)	Dairy heifers	Bacterial culture	↓	- 3.2 to - 1.0 kg/d
Leitner et al. (2004)	Sheep & goats	/	↓	- 0.64 to 0 kg/d
Gröhn et al. (2004)	Dairy cows	Bacterial culture	→	-
Pearson et al. (2013)	Dairy cows	Bacterial culture	→	-
Tomazi et al. (2015)	Dairy cows	PCR-RFLP	→	-
Koop et al. (2010)	Goats	Bacterial culture	→	-
Koop et al. (2012)	Goats	tDNA-PCR	*Staphylococcus caprae:* ↑	/
Schukken et al. (2009)	Dairy cows	Bacterial culture	↑	+ 0.45 kg/d (SD[2] ± 0.12)
Piepers et al. (2010)	Dairy cows	Bacterial culture	↑	+ 2.95 kg/d (SEM[3] ± 0.98)
Piepers et al. (2013)	Dairy cows	Bacterial culture	↑	+ 2.0 kg/d

[1] Identification of CNS at group level with bacterial culture, or at species level using restriction fragment length polymorphism (PCR-RFLP) or transfer RNA intergenic spacer PCR (tDNA-PCR). [2] Standard deviation. [3] Standard error of the mean.

1.4 Prolactin and udder health

1.4.1. Lactation hormone

Prolactin, thought to be present in all vertebrates (Malven, 1993), is involved in a plethora of biological mechanisms. Over 300 distinct actions have been attributed to this versatile hormone (Goffin et al., 2002). Riddle et al. (1933) were the first to extract and identify the hormone from the anterior lobe of the pituitary gland. They proposed the name 'prolactin', seeing that the compound was able to promote mammary growth and milk secretion in mammals. Even though PRL is historically well-known for its role in lactation and mammary gland development, it is also involved in various behavioral mechanisms, reproduction and immunoregulation (Freeman et al., 2000). The broad function of PRL is evidenced by the vast number of tissues equipped with PRL receptors (Bole-Feysot et al., 1998). Bovine pituitary-derived PRL is a polypeptide consisting of 199 amino acids weighing approximately 23 kDa (Wallis, 1974). The hormone is primarily – though not exclusively- produced by the lactotrophs in the anterior pituitary gland (Freeman et al., 2000). Other production sites include (among others) the ovaries, the uterus, various regions of the brain, the skin, the spleen, the thymus, the tonsils and the lymph nodes (**Figure 1.2**). Mammary tissue is also capable of synthetizing PRL (Leprovost et al., 1994). Mammary-produced PRL controls the proliferation and differentiation of MEC through an autocrine or paracrine mechanism (Naylor et al., 2003; Chen et al., 2012). Bovine PRL also limits the transfer of immunoglobulins of the maternal circulation into colostrum, by reducing the expression of the IgG_1-receptor on mammary tissue (Barrington et al., 2001).

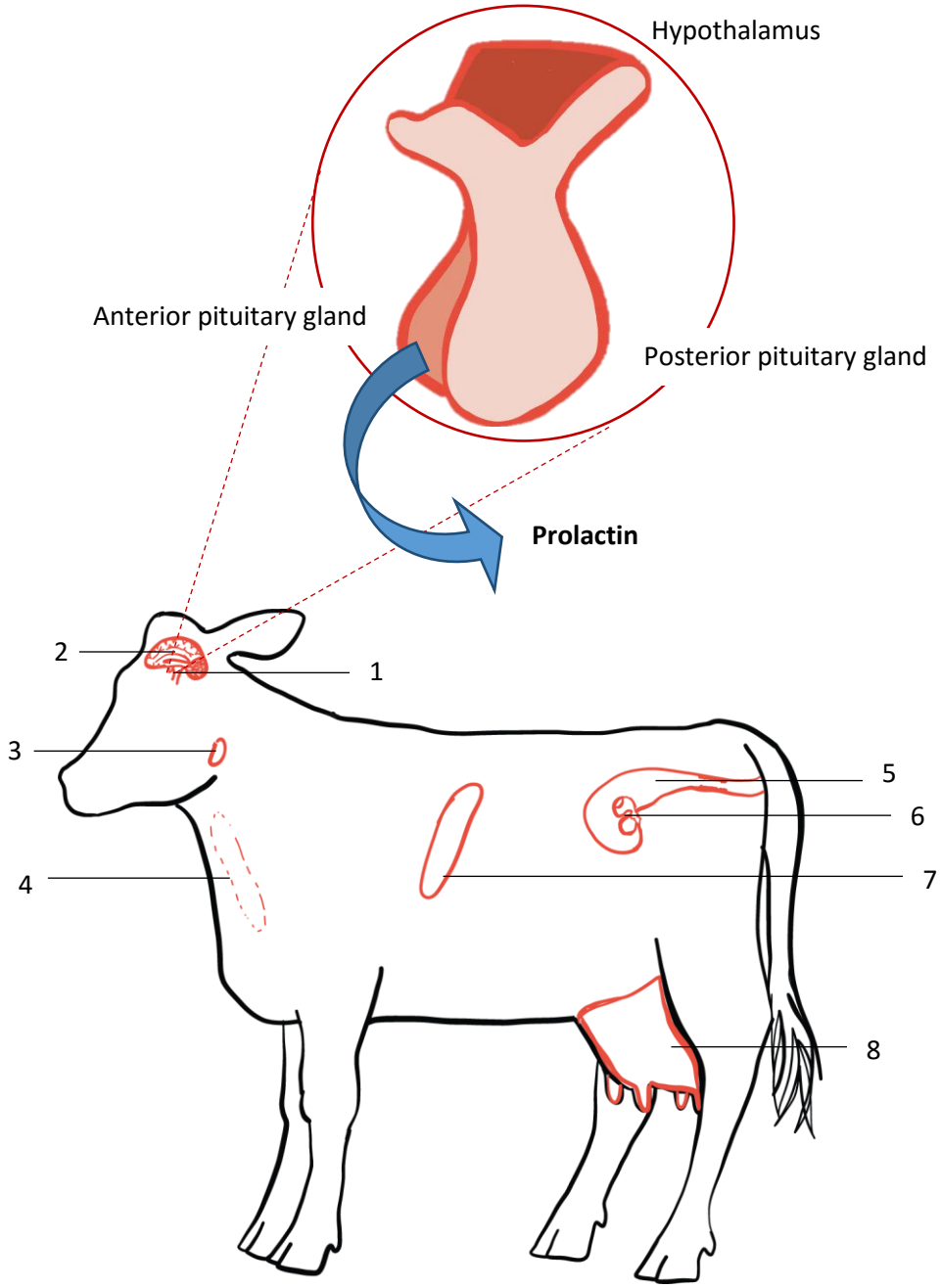

Figure 1.2. The anterior pituitary gland (1) is the primary site of prolactin synthesis. Extra-pituitary sites of prolactin production in mammals include (but are not limited to) various regions of the brain (2), the tonsils (3), the thymus (4), the myometrium (5), the ovaries (6), the spleen, (7) the mammary gland (8) and the skin. (Adapted from Marano and Ben-Jonathan, 2014)

Nowadays, it is generally accepted that PRL is pivotal for the initiation and maintenance of lactation in ruminants (Lacasse et al., 2015), although this was debated for many years. Early experiments failed to demonstrate an effect on milk yield when suppressing the PRL release with the dopamine agonist bromocriptine (Karg et al., 1972; Hart, 1973; Smith et al., 1974). Therefore, it was assumed that PRL was not a galactopoetic hormone in cattle. However, newer studies show that the milk production significantly drops when dairy cows receive a long-term quinagolide treatment (Lacasse et al., 2011), which is a more selective and effective dopamine receptor agonist (Barlier and Jaquet, 2006). The milk yield can also be increased by administering domperidone, a dopamine antagonist which increases the circulating PRL level (Lacasse and Ollier, 2015). The contradictory findings between the older and newer studies might be explained by the use of different compounds (bromocriptine versus quinagolide) or duration of treatments (varying between 2 days and nine weeks).

In general, serum PRL lies between 10 and 60 ng/mL in adult dairy cows (Koprowski et al., 1972; Malven, 1977; Fulkerson et al., 1980; Marcek and Swanson, 1984), but it can be affected by many factors, such as the ambient temperature (Wetteman and Tucker, 1974), the exposure to light (Dahl et al., 2000), or the milking process (Johke, 1970). Stress also has a high impact on circulating PRL levels (Karg and Schams, 1974). García-Ispierto et al. (2009) observed that dairy cows with intermediate to high blood cortisol levels (> 3ng/mL) had higher levels of circulating PRL.

Approximately one week before calving, the serum PRL concentration starts to rise (80 – 110 ng/mL), peaking one day before parturition (230 – 285 ng/mL) (Edgerton and Hafs, 1973; Ingalls et al., 1973). The milk PRL level is at its highest (369 ± 56 ng/mL) immediately after parturition (Malven, 1977). Besides PRL, there are many other hormones involved in the development of the mammary gland and the secretion of milk, such as oxytocin, estrogen, progesterone and various metabolic hormones (including glucocorticoids, growth hormone, etc.) (Neville et al., 2002). The mammary gland can function as a self-regulating endocrine organ, largely independent from systemic influences (Wilde and Peaker, 1990; Weaver and Hernandez, 2016). Nevertheless, the biological significance of autocrine PRL has not been studied extensively in cattle.

1.4.2. Immunological aspects

In addition to its role in lactation, PRL also acts as an immunomodulatory factor. Hypophysectomized rats, for instance, display signs of immunosuppression (more specifically normochromic normocytic anemia, leucopenia and thrombocytopenia), which can be reversed by administering PRL (Berczi and Nagy, 1981; Nagy and Berczi, 1991). Other research shows that PRL is able to inhibit the glucocorticoid-induced apoptosis of T-lymphocytes (Krishnan et al., 2003), stimulate the production of reactive oxygen species in macrophages (Edwards et al., 1987) and induces *TLR2* expression on the membrane of bovine MEC (Medina-Estrada et al., 2015). When challenging peripheral immune cells in the presence of PRL, the production of pro-inflammatory cytokines (such as TNF-α and interleukin 12 [IL-12]] increases while the production of the anti-inflammatory cytokine IL-10 decreases (Brand et al., 2004). The opposite occurs as well: the expression of the PRL-receptor is upregulated in rat fibroblasts in the presence of pro-inflammatory cytokines, making the cells more responsive to PRL (Corbacho et al., 2003).

Previous research indicates that PRL might also play a role in the udder health of dairy cattle. A PRL surge occurs around the time of calving (Convey, 1974), coinciding with the phenomenon of periparturient immunosuppression (Drackley, 1999). The risk for developing an IMI is very high after parturition (Burton and Erskine, 2003), combined with an increased severity of clinical mastitis cases (Burvenich et al., 2003). It has previously been hypothesized that PRL counteracts the immunosuppression caused by glucocorticoid hormones (Dorshkind and Horseman, 2000; Matalka, 2003; Fomicheva et al., 2004). Although the blood PRL concentration does not differ between healthy cows and cows experiencing a clinical (Hockett et al., 2000; Vanselow et al., 2006) or chronic subclinical mastitis (Boutet et al., 2007), a positive correlation exists between the SCC of infected udder quarters and the milk PRL concentration (Boutet et al., 2007). Nuclear factor κB (NF-κB), the transcription factor for the genes encoding numerous cytokines involved in the pathogenesis of mastitis (Boulanger et al., 2003), is activated by PRL in a dose-dependent manner, resulting in the up-regulation of IL-1β, IL-6, IL-8, TNF-α and macrophage colony stimulating factor (GM-CSF) in MEC (Boutet et al., 2007). However,

PRL can also elicit anti-inflammatory responses in MEC. Adding PRL to a mammary cell line infected with *S. aureus* even promotes the internalization of the mastitis pathogen (Gutierrez-Barroso et al., 2008), hereby enabling a persistent IMI (Hebert et al., 2000). Furthermore, *S. aureus* has the ability to inhibit the PRL-driven activation of NF-κB (Lara-Zarate et al., 2011), which suppresses the host's innate immune response. All in all, the role of bovine PRL as a potential cytokine in the mammary gland defense system is not yet well defined.

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Chapter 2

Aims of the thesis

Coagulase-negative staphylococci are a broad, heterogeneous group of bacteria that occupy a wide range of habitats. Certain CNS species, such as *S. chromogenes*, are routinely found in bovine milk, whereas other species, e.g. *S. fleurettii*, mostly occur in the environment of dairy cattle. A number of studies indicate that CNS-infected dairy cattle produce more milk than their non-infected herd mates, although the involved bacteria were never identified at the species or strain level. All in all, current knowledge on the host-pathogen interaction of CNS in dairy cattle is limited, and the mechanism behind the reported elevated milk yield in CNS-infected cattle remains unexplained at present.

The general aim of this thesis is to broaden the current knowledge on the interaction between primiparous cows and CNS. This work focuses on two representative species from both the environmental and the host-associated habitat: S. fleurettii (Photo 2.1.A) and S. chromogenes, respectively. Staphylococcus fleurettii was selected for this research, because the species epitomizes the group of so-called environmental CNS. Staphylococcus chromogenes, on the other hand, is both part of the normal microflora, and the most prevalent species in CNS mastitis. Therefore, two different strains will be considered in this work; one isolated from the left hind quarter of a multiparous cow with a persistent IMI lasting more than 300 days (Photo 2.1.B), and another from the teat apex of a heifer, which is able to inhibit the growth of mastitis pathogens under laboratory conditions (Photo 2.1.C). In light of the apparent increased milk yield in CNS-infected cows, this thesis will also examine the role of PRL in CNS mastitis, as a potential autocrine lactation hormone and immunomodulatory factor. Heifers in mid-lactation are used as a study model, since the prevalence of CNS IMI appears to be higher in primiparous cows than in multiparous cows. We specifically selected heifers in mid-lactation, to circumvent the periparturient immunosuppression seen in dairy cattle.

A B C

Photo 2.1. The three strains used in this thesis plated on esculin-blood agar: *Staphylococcus fleurettii* (A), *Staphylococcus chromogenes* from a persistent intramammary infection (B) and *S. chromogenes* from the teat apex of a heifer (C).

The following specific research questions were addressed:

- Do host-associated CNS species (i.e. *S. chromogenes*) evoke a different local host response in the mammary glands of primiparous cows than environmental CNS species (i.e. *S. fleurettii*)? (**Chapter 3**).
- Do different strains of *S. chromogenes* from varying habitats (isolated from either extra- or intramammary sites) evoke a different local host response in the mammary glands of primiparous cows? (**Chapter 3**)
- Does the milk PRL concentration increase in response to an experimental infection with *S. chromogenes* or *S. fleurettii*? (**Chapter 4.1**)
- Can the local *PRL* expression in the mammary gland be increased by an experimental infection with *S. chromogenes* or *S. fleurettii*? (**Chapter 4.2**)

Chapter 3

Local host response following an intramammary challenge with *Staphylococcus fleurettii* and different strains of *Staphylococcus chromogenes* in dairy heifers

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Veterinary Research, 2016, 47:5

3.1 Abstract

Coagulase-negative staphylococci (CNS) are a common cause of subclinical mastitis in dairy cattle. The CNS inhabit various ecological habitats, ranging between the environment and the host. In order to obtain a better insight into the host response, an experimental infection was carried out in eight healthy heifers in mid-lactation with three different CNS strains: a *Staphylococcus fleurettii* strain originating from sawdust bedding, an intramammary *Staphylococcus chromogenes* strain originating from a persistent intramammary infection (*S. chromogenes* IM) and a *S. chromogenes* strain isolated from a heifer's teat apex (*S. chromogenes* TA). Each heifer was inoculated in the mammary gland with 1.0 x 10^6 colony forming units of each bacterial strain (one strain per udder quarter), whereas the remaining quarter was infused with phosphate-buffered saline. Overall, the CNS evoked a mild local host response. The somatic cell count increased in all *S. fleurettii*-inoculated quarters, although the strain was eliminated within 12h. The two *S. chromogenes* strains were shed in larger numbers for a longer period. Bacterial and somatic cell counts, as well as neutrophil responses, were higher after inoculation with *S. chromogenes* IM than with *S. chromogenes* TA. In conclusion, these results suggest that *S. chromogenes* might be better adapted to the mammary gland than *S. fleurettii*. Furthermore, not all *S. chromogenes* strains induce the same local host response.

Key words: coagulase-negative staphylococci, dairy heifer, mastitis, host response

3.2 Introduction

Coagulase-negative staphylococci are the principal cause of subclinical mastitis in dairy cattle (Pyörälä and Taponen, 2009), especially in primiparous cows (Sampimon et al., 2009a). The impact of CNS on the udder health of dairy cattle has gained more attention in the past decade. The CNS were initially reported as one large, uniform group of bacteria (Hogan et al., 1987; Nickerson and Boddie, 1994). However, thanks to recent advances in molecular identification techniques, individual CNS species have become easier to identify and study (Supré et al., 2009; Braem et al., 2011). Over 20 different CNS species have been isolated from bovine milk (Vanderhaeghen et al., 2014). Nonetheless, the bovine-associated CNS cover a wide range of ecological habitats, varying from essentially environmental species to host-adapted species (De Visscher et al., 2014). Some CNS species rarely occur in bovine milk, but rather thrive in the environment of the cow and the barn (e.g. air, sawdust, bedding, and floors) (Piessens et al., 2011). These so-called environmental CNS species include, among others, *Staphylococcus equorum* and *Staphylococcus fleurettii* (De Visscher et al., 2014). On the other end of the spectrum are the so-called host-adapted CNS species, specialized in survival in the udder and on the cow. *Staphylococcus chromogenes* is considered such a species (Fry et al., 2014), since it is the predominant CNS species found in milk (Waller et al.,2011; De Visscher et al., 2014). Furthermore, *S. chromogenes* is also present on the teat apex (De Vliegher et al., 2003; De Visscher et al., 2016), streak canal, and other extra-mammary body sites (Taponen et al., 2008). *Staphylococcus chromogenes* is one of the main CNS species involved in IMI (Sampimon et al., 2009; Park et al., 2011). In general, *S. chromogenes* causes a minor to moderate increase in the milk somatic cell count (SCC) (Tomazi et al., 2015), although one study noted a rise in SCC comparable to that of *S. aureus* infections (Supré et al., 2011). On the other hand, teat apex colonization with *S. chromogenes* has also been associated with a lower quarter milk SCC in early lactating heifers (De Vliegher et al., 2003), whereas some strains can even inhibit the growth of other mastitis pathogens *in vitro* (De Vliegher et al., 2004).

Epidemiological data suggest that not all CNS species exhibit the same degree of pathogenicity (Supré et al., 2011), but little is known about the different host responses caused by an IMI with representatives of the supposed environmental or host-adapted species (or strains). Therefore, the first objective of the research was to examine the host response and bacterial shedding following an experimental intramammary inoculation in heifers with one distinctive host-adapted CNS species and another typical environmental one (*S. chromogenes* versus *S. fleurettii*). The second objective was to evaluate whether the elicited host response and bacterial shedding differs between strains belonging to the same species, in this case *S. chromogenes*.

3.3 Materials and Methods

The study is in compliance with the European Directive 2010/63/EU, and was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University (EC2012/73).

Animals

The experiment was performed at the research dairy farm of Ghent University (Biocentrum Agri-Vet, Melle, Belgium). Eight clinically healthy Holstein-Friesian heifers in mid-lactation (78 – 278 days in milk) were selected. Heifers with a previous history of clinical mastitis or persistent high SCC (>150,000 cells/mL) on Dairy Herd Improvement records were not included. To increase the likelihood that all quarters were free from IMI, the animals received 15 days before the start of the experiment 3 daily intramuscular injections of 10 g penethamate hydroiodide (Mamyzin, Boehringer Ingelheim GmbH) combined with an intramammary treatment of 200 mg cephalexin and 100,000 I.U. kanamycin (Ubrolexin, Boehringer Ingelheim GmbH) in each quarter for 2 days. The heifers were moved to a separate tie-stall barn 48h prior to inoculation, and kept there until the end of the experiment (i.e. 78h after inoculation). The heifers were milked twice a day, at 08:00h and 20:00h. After milking, the teats

were dipped with an iodine-based barrier dip (Io-Shield, Ecolab, Northwich, UK) and before sampling, the teats were cleansed with a lactic acid based foam product (Oxy-Foam D, Ecolab, Northwich, UK).

Study design

All heifers were challenged following a split-udder design (Piccart et al., 2015; Verbeke et al., 2015). Three quarters of each heifer were simultaneously inoculated with 1.0×10^6 colony forming units (CFU) of the bacterial strains (one strain per udder quarter) in 5 mL sterile phosphate-buffered saline (PBS) using a sterile polyvinyl chloride catheter of 18 cm. The remaining quarter was inoculated in the same manner with 5 mL sterile PBS (Thermo Scientific, Waltham, USA) and served as a control. All inocula were directly infused into the gland cistern. To ensure a balanced distribution between the quarter positions, the inocula were allocated to the quarters using restricted randomization. The heifers were examined clinically and their rectal temperature was registered at each sampling.

Inocula

Three different CNS strains were used in this experimental challenge (**Table 3.1**). Two field strains of *S. chromogenes* were used; one isolated from the teat apex of a heifer with no signs of mastitis (*S. chromogenes* TA) (De Vliegher et al., 2004) and the other from the left hind quarter of a multiparous cow with a persistent, intramammary infection lasting over 300 days (*S. chromogenes* IM) (Piessens et al., 2011). Although no genotypic strain typing was performed, there were notable phenotypic differences between both *S. chromogenes* isolates (**Table 3.1**). For instance, the bacterial inhibitory capacities of the present strains were tested against a field isolate of *S. aureus* in a previously described modified cross-streaking culture (De Vliegher et al., 2004). *Staphylococcus chromogenes* TA is able to inhibit the growth of *S. aureus*, whereas *S. chromogenes IM* is not. Both strains elicit a different immune response in mice (Breyne et al., 2015) and interact differently with MEC (Souza et al., 2016). The third CNS strain used in this study was *S. fleurettii* (Piessens et al., 2011b). The strains were initially stored at -80°C (Microbank, Pro-Lab Diagnostics). A growth curve was set up for each strain by

incubating one colony in brain-heart infusion broth at 37°C. The bacteria were collected during the late logarithmic growth phase. The bacteria were washed 3 times in sterile PBS by centrifugation at 4,000 x *g* for 10 minutes. The pellet was resuspended in PBS with 15% (v/v) glycerol and stored at -80°C. To confirm the viable bacterial count in the stock solution, serial dilutions were plated on tryptic soy agar (TSA, Oxoid, Basingstoke, UK). An infection dose of 1.0 x 10^6 CFU was selected based on the results of a preliminary challenge trial to induce subclinical mastitis rather than clinical mastitis (Verbeke et al., 2015b).

Table 3.1 The characteristics of the coagulase-negative staphylococcal isolates used in this study.

Strain	Origin	Colony characteristics				Author
		Color	Shape	Consistency	*In vitro* growth inhibition of *S. aureus*	
S. fleurettii	Sawdust	Grey	Round	Creamy	No	Piessens et al. (2011)
S. chromogenes (teat apex; TA)	Heifer's teat apex	Beige	Round	Creamy	Yes	De Vliegher et al. (2004)
S. chromogenes (intramammary; IM)	Intramammary infection in a cow lasting > 300 days	Orange	Round	Creamy	No	Piessens et al. (2011)

Milk samples

Collection. Milk samples were collected aseptically from the CNS-challenged and control quarters in duplicate 24h before inoculation (b.i.) and at 0, 4, 6, 9, 12, 18, 24, 28, 32, 36, 48, 54, 60, 72, and 78h post-inoculation (p.i.) for microbiology, SCC and cytokine measurements. The apoptosis and necrosis of polymorphonuclear neutrophil leukocytes (PMN) was determined 24h b.i. and at 0, 6, 12, 18, 24, 48 and 72h p.i.. Additional milk samples were collected after the actual experiment, at 144 and 216h p.i., to evaluate the progression of bacterial shedding and quarter milk SCC. Milk samples were kept on ice

during transportation to the laboratory of Mastitis and Milk Quality Research Lab (Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium).

Microbiology and somatic cell counts. The SCC was determined by a DeLaval Cell counter (DeLaval, Tumba, Sweden). The milk samples (10 µL) were plated in duplicate on aesculin-blood and MacConkey agar (Oxoid, Basingstoke, UK) according to the guidelines of the National Mastitis Council (Hogan et al., 1999). The plates were incubated at 37°C in aerobic conditions, and examined after 24 and 48h. Additionally, serial dilutions of the milk were plated in duplicate on TSA for counting the CFU. Every morphologically dissimilar colony type on TSA was collected, and subjected to Gram staining, together with catalase, DNAse and tube coagulase testing (i.e. the routine identification methods for CNS). One colony of each morphologically dissimilar Gram-positive, catalase-positive and coagulase-negative isolate was stored at -80°C (Microbank, Pro-Lab Diagnostics, Richmond Hill, Canada) and subjected to transfer RNA-intergenic spacer PCR (tDNA-PCR) for further identification at the species level (Supré et al., 2009). Only if the isolates could not be identified using tDNA-PCR, *rpoB* sequencing was carried out (Mollet et al., 1997).

Apoptosis and necrosis of milk PMN. The apoptosis and necrosis of the milk PMN, considered an indirect indicator of their impaired functionality (Mehrzad et al., 2004), were determined 24h b.i. and at 0, 6, 12, 18, 24, 48 and 72h p.i. by means of flow cytometry (Piepers et al., 2009). This dual staining technique with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) differentiates the (early) apoptotic (FITC+/PI-) and necrotic (FITC+/PI+) PMN from the intact, viable cells (FITC-/PI-). The raw data were acquired and analyzed with FACSDiva Software (BD Biosciences, San Jose, USA).

Cytokine measurement. The cytokines interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), together with the chemokine interleukin 8 (IL-8) were measured. First, the fresh milk samples were centrifuged at 16,000 x g for 30 minutes at 4°C (Centrifuge 5418R, Eppendorf, Hamburg Germany). The

fat-depleted whey fraction was stored at -80°C. The concentration of IL-1β, IL-8 and TNF-α was determined by sandwich ELISA. A commercially available kit was used to measure IL-8 (DY208, R&D Systems, Minneapolis, USA) and IL-1β (ESS0027, Thermo Scientific, Waltham, USA) according to the manufacturer's instructions. The TNF-α measurement was based on another study (Simojoki et al., 2011) (MCA2334, PBP005 and MCA2335B, AbD Serotech, Oxford, UK).

3.4 Statistical analysis

The entire data of one quarter were not included in the final analysis due to an elevated SCC at the moment of inoculation. The data of another quarter were also omitted from the analysis due to a naturally occurring IMI with *Staphylococcus epidermidis*, but only 12h after the inoculation. Linear mixed regression analysis was used to model the relationship between the inoculum (categorical variable: control, *S. fleurettii*, *S. chromogenes* IM and TA), the time of sampling (continuous variable: from 4h until 78h p.i.), the quadratic term of time of sampling (continuous variable) and the different outcome variables (bacterial count, SCC, % apoptotic milk PMN, and % necrotic milk PMN) (PROC MIXED, SAS 9.4, SAS Institute Inc.). The bacterial count was $\log_{10}$-transformed, whereas the % apoptotic PMN, % necrotic PMN, and SCC/µL underwent a natural logarithmic transformation to obtain a normalized distribution. Heifer and quarter were incorporated as random effects in every model to account for the correlated nature of the data. Compound symmetry was selected as a covariance pattern to correct for the clustering of multiple samplings per quarter. To emphasize the response after challenge, measurements prior to inoculation were not included in the analysis. The interaction between inoculum and time of sampling was tested each time, and kept in the model when significant. The significance level was set at $P \leq 0.05$. For pairwise comparisons between the different bacterial strains and control, a Bonferroni adjustment was used.

3.5 Results

Clinical parameters

None of the inoculated quarters showed physical signs of clinical mastitis during the trial. A short period of fever was observed in three heifers though (> 39.5°C and < 40.6°C) between 9h and 12h p.i..

Somatic cell count

The milk SCC was significantly higher in the quarters inoculated with any of the isolates compared to the control quarters (adjusted $P < 0.01$; **Table 3.2**). No significant difference was found between the SCC response in quarters challenged with *S. fleurettii*, and those challenged with either *S. chromogenes* strain (*S. chromogenes* IM and TA: adjusted $P = 0.43$ and $P = 1.00$). However, the SCC tended to be more pronounced in the quarters challenged with *S. chromogenes* IM than with *S. chromogenes* TA (adjusted $P = 0.06$). The evolution over time of the quarter milk SCC after challenge differed significantly between quarters (interaction inoculum x time of sampling: $P < 0.01$; **Table 3.2**). After 78h, the SCC continued to decline in the challenged quarters (**Figure 3.1**).

Bacterial shedding

The control quarters remained culture-negative throughout the trial. The bacterial shedding was significantly higher in the *S. chromogenes* TA- and *S. chromogenes* IM-challenged quarters than in the control quarters (adjusted *P*-values < 0.01). It was significantly more pronounced in *S. chromogenes* IM than *S. chromogenes* TA (adjusted $P \leq 0.01$). The bacterial shedding was so low in the *S. fleurettii*-inoculated quarters, that there was no significant difference with the control quarters (adjusted *P*-value = 0.25). The evolution over time of bacterial shedding differed significantly between quarters (interaction inoculum x time of sampling: $P < 0.01$; **Table 3.2**). In each cow, *S. fleurettii* was eliminated from the mammary gland within 12h, whereas *S. chromogenes* IM and TA remained present for a longer period of time (**Figure 3.2**). No CNS were found in the inoculated quarters after 144h p.i. though.

Milk PMN apoptosis and necrosis

The highest proportion of apoptotic and necrotic PMN was found in the control quarters. Compared to the control quarters, only the *S. fleurettii*- and *S. chromogenes* IM-challenged quarters yielded significantly less apoptotic milk PMN (adjusted *P*-value < 0.01 and 0.02 respectively; **Table 3.2**). No significant difference was found in the number of apoptotic PMN between the control quarters and *S. chromogenes* TA (adjusted *P*-value = 0.13). *Staphylococcus fleurettii* was the only strain that resulted in significantly less necrotic PMN than in the control quarters (pairwise comparison: P = 0.03; **Table 3.2**). No significant differences were found when comparing the different CNS isolates with each other in terms of PMN apoptosis or necrosis. The evolution over time of the proportion of apoptotic and necrotic PMN differed significantly between quarters though (interaction inoculum x time of sampling: P = 0.02 and P = 0.01; **Table 3.2**). It should also be noted that the proportion of apoptotic and necrotic PMN was reduced in all quarters immediately before the inoculation, compared to 24h previously (**Figure 3.3** and **Figure 3.4**).

Milk IL-1β, IL-8 and TNF-α

The IL-8 and IL-1β ELISA had an intra- and inter-assay coefficient of variation (CV) of < 6% and < 15%, respectively. The TNF-α ELISA showed lower precision (intra- and inter CV: 13% and 28%, respectively). Since the inoculation of the CNS strains generally elicited a minimal, or non-detectable cytokine response, the IL-1β, IL-8 and TNF-α levels were not statistically analyzed. Instead, descriptive statistics were generated. In the *S. fleurettii*- and *S. chromogenes* IM-challenged quarters, a low, transient IL-8 response (< 30 pg/mL) was observed within 28h after inoculation (**Figure 3.5**). These quarters also showed limited, erratic TNF-α peaks (< 10 ng/mL; **Figure 3.6**). *Staphylococcus chromogenes* IM caused a higher, long-lasting IL-1β response compared to the TNF-α response, starting at 12h p.i. (**Figure 3.7**). *Staphylococcus chromogenes* TA did not evoke a detectable IL-8 response, but the strain caused a major increase in IL-1β (peaking at 300 pg/mL 60h p.i.) in one heifer. This particular heifer was also the only animal that demonstrated a TNF-α reaction to *S. chromogenes* TA peaking at 60 h p.i.. Notably,

the data of the *S. fleurettii* quarter of the same heifer were omitted from the analysis 12h p.i. due to the occurrence of a spontaneous, natural infection in that quarter. Another heifer showed no discernable cytokine response in any challenged quarter. No cytokines were found in the control quarters.

Table 3.2. Linear mixed regression model for somatic cell count (Ln SCC), bacterial count (log[CFU/ml+1]), apoptotic neutrophils (Ln Apoptotic PMN) and necrotic neutrophils (Ln Necrotic PMN) in quarter milk after experimental infection with *Staphylococcus fleurettii*, the teat apex strain of *S. chromogenes* (TA), the intramammary strain of *S. chromogenes* (IM) and phosphate-buffered saline (control).

Predictor Variables	Ln SCC (cells/µL)				Log(CFU/ml + 1)				Ln Apoptotic PMN (%)				Ln Necrotic PMN (%)			
	β [1]	SE[2]	LSM[3]	*P*	β	SE	LSM	*P*	β	SE	LSM	*P*	β	SE	LSM	*P*
Intercept	3.43	0.34	-	<0.01[4]	0.52	0.16	-	<0.01[4]	3.16	0.19	-	<0.01[4]	2.72	0.11	-	<0.01[4]
Inoculum				<0.01[4]				<0.01[4]				<0.01[4]				<0.01[4]
Control	Ref.[5]	-	3.91	-	Ref.	-	-0.03	-	Ref.	-	3.48	-	Ref.	-	3.15	-
S. fleurettii			6.07	<0.01[6]			0.22	0.25[6]			2.85	0.01[6]			2.79	0.03[6]
S. chromogenes TA			5.78	<0.01[6]			0.50	<0.01[6]			3.06	0.13[6]			2.90	0.29[6]
S. chromogenes IM			6.71	<0.01[6]			0.89	<0.01[6]			2.92	0.02[6]			2.85	0.10[6]
Time of sampling	0.06	0.01	-	<0.01[4]	-0.05	0.01	-	<0.01[4]	0.01	0.00	-	<0.01[4]	0.02	0.15	-	<0.01[4]
Quadratic term of time of sampling	0.00	0.00	-	<0.01[4]	0.00	0.00	-	<0.01[4]				NS[7]				NS
Inoculum x time of sampling	-	-	-	<0.01[4]	-	-	-	<0.01[4]	-	-	-	0.02[4]	-	-	-	0.01[4]

[1] Regression coefficient; [2] Standard error; [3] Least square means; [4] *P*-value for overall effect; [5] Reference; [6] Bonferroni-adjusted *P*-value for comparing different inocula with control; [7] Not significant.

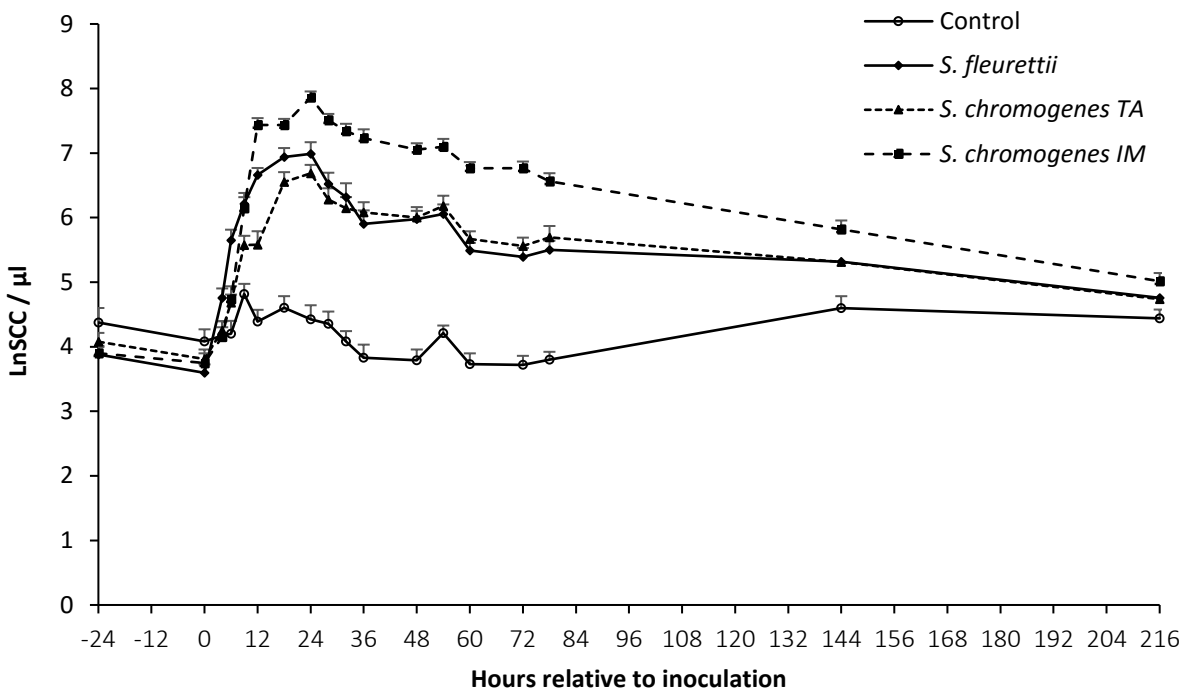

Figure 3.1. The average somatic cell count (SCC) (expressed as a natural logarithm of SCC; Ln SCC/µL) in quarter milk after intramammary challenge of 8 dairy heifers with *Staphylococcus fleurettii*, *S. chromogenes* TA (teat apex strain), *S. chromogenes* IM (intramammary strain) and phosphate-buffered saline (control). The error bars represent the standard error of the mean (+ SEM).

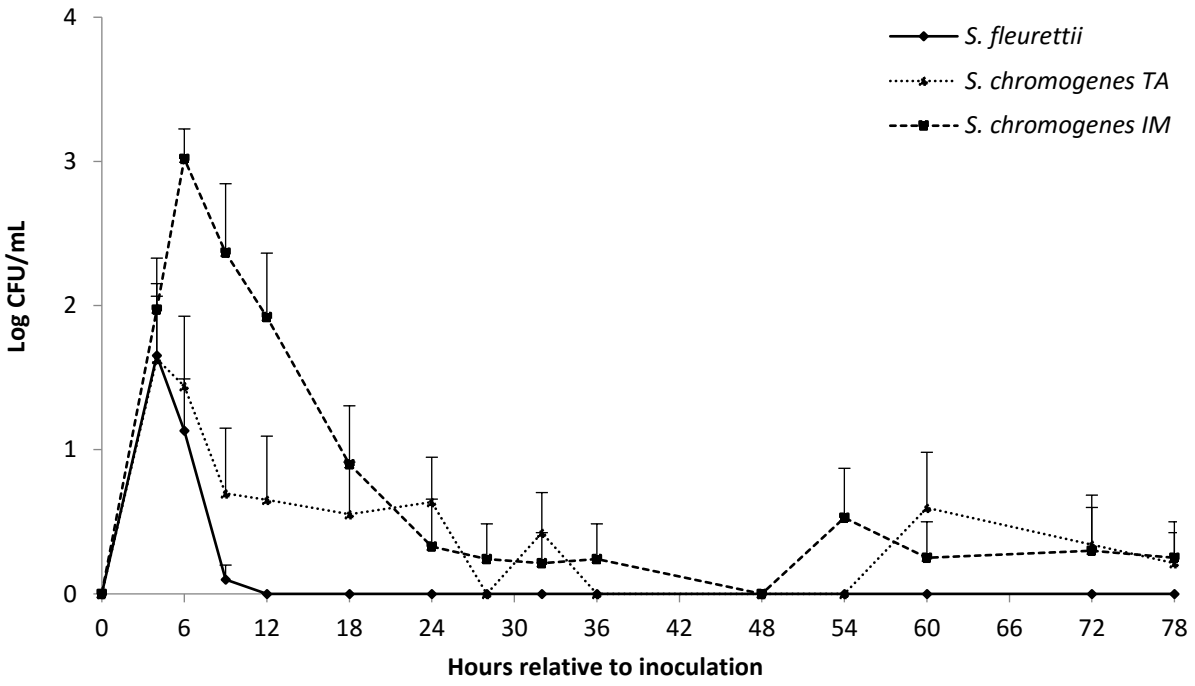

Figure 3.2. The average bacterial count Log CFU/mL in quarter milk following experimental intramammary challenge of 8 dairy heifers with *Staphylococcus fleurettii*, *S. chromogenes* TA (teat apex strain), *S. chromogenes* IM (intramammary strain) and phosphate-buffered saline (control). The error bars represent the standard error of the mean (+ SEM). The control quarters remained culturally negative throughout the study.

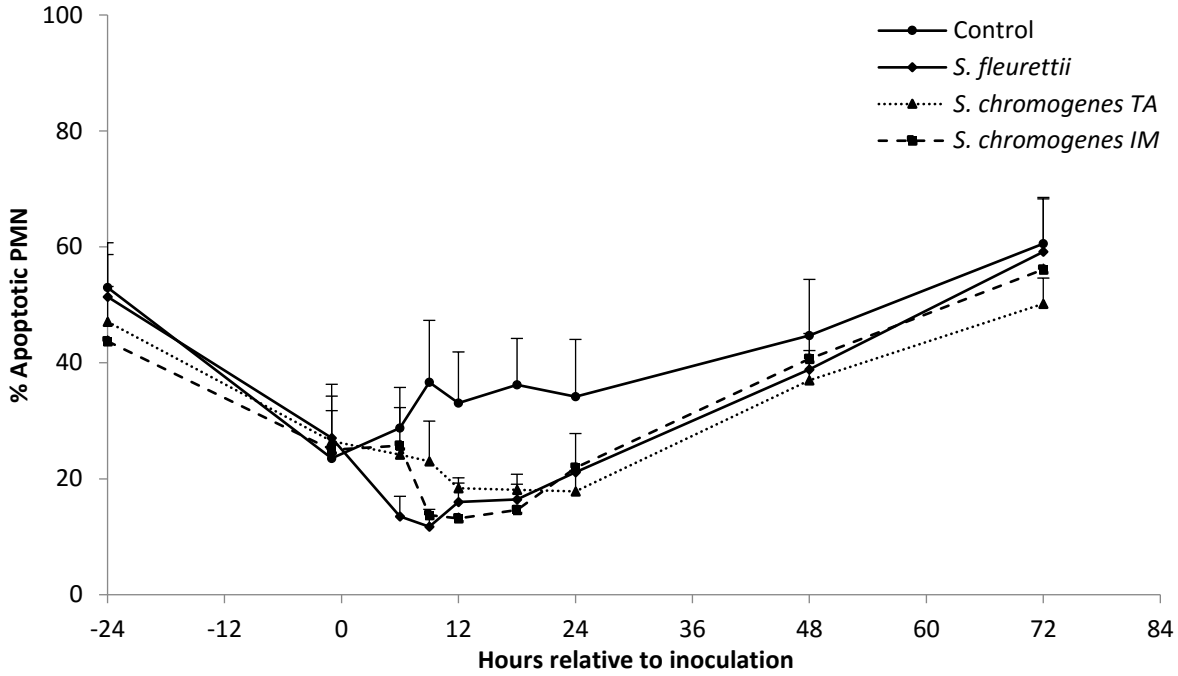

Figure 3.3. Proportion of apoptotic neutrophils (% Apoptotic PMN) in quarter milk following experimental intramammary challenge of 8 dairy heifers with *Staphylococcus fleurettii*, *S. chromogenes* TA (teat apex strain), *S. chromogenes* IM (intramammary strain) and phosphate-buffered saline (control). The error bars represent the standard error of the mean (+ SEM).

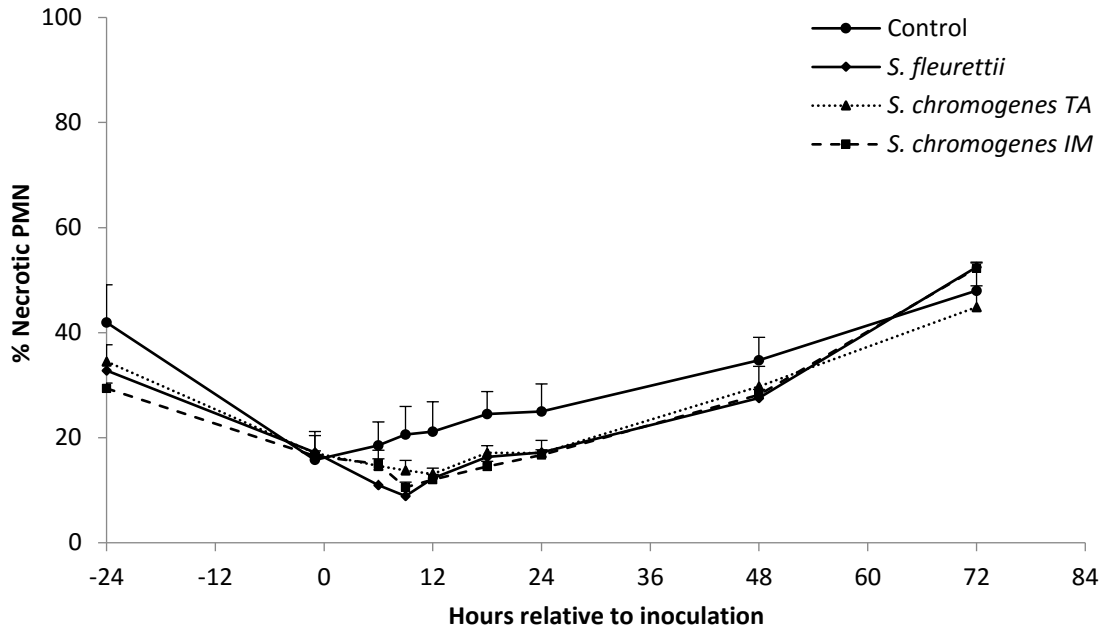

Figure 3.4. Proportion of necrotic neutrophils (% Necrotic PMN) in quarter milk following experimental intramammary challenge of 8 dairy heifers with *Staphylococcus fleurettii*, *S. chromogenes* TA (teat apex strain), *S. chromogenes* IM (intramammary strain) and phosphate-buffered saline (control). The error bars represent the standard error of the mean (+ SEM).

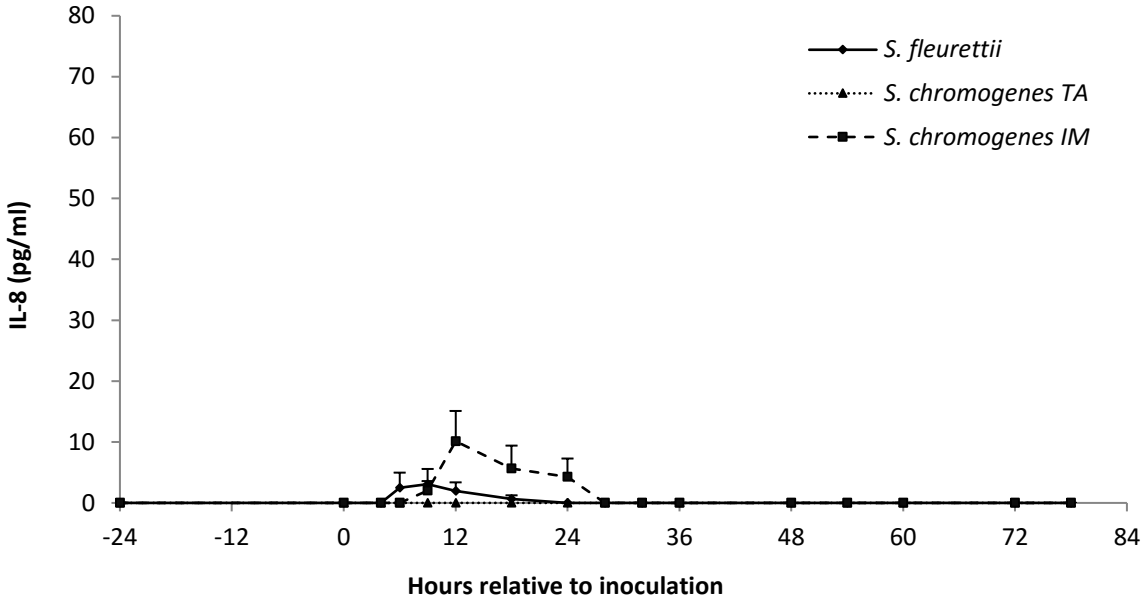

Figure 3.5. The average concentration of IL-8 (pg/mL) in quarter milk following experimental intramammary challenge of 8 dairy heifers with *Staphylococcus fleurettii*, *S. chromogenes* TA (teat apex strain), *S. chromogenes* IM (intramammary strain) and phosphate-buffered saline (control). The error bars represent the standard error of the mean (+ SEM). IL-8 was not detected in the control quarters.

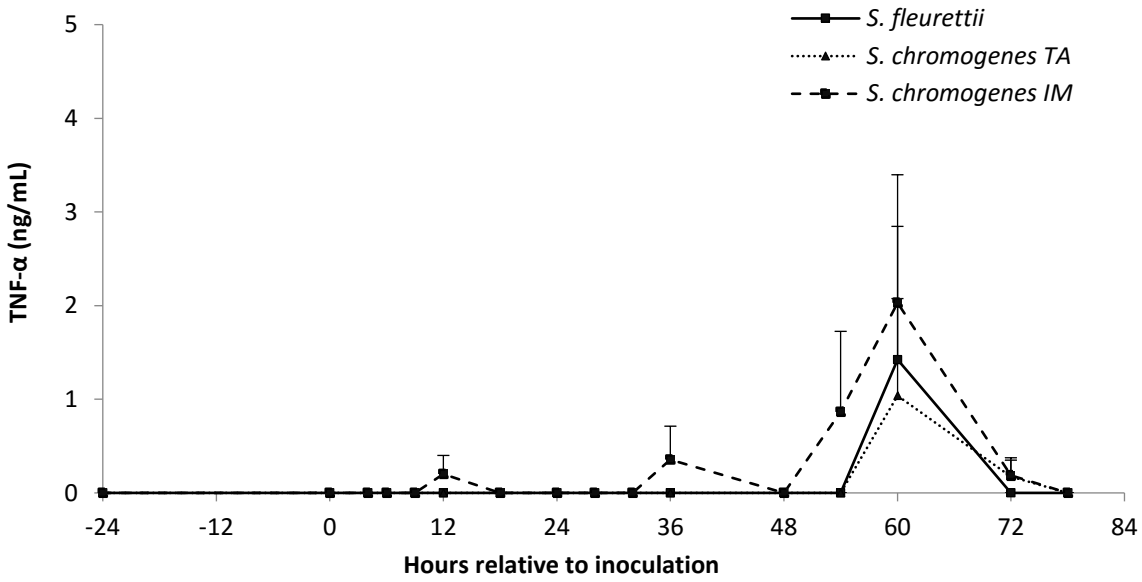

Figure 3.6. The average concentration of TNF-α (ng/mL) in quarter milk following experimental intramammary challenge of 8 dairy heifers with *Staphylococcus fleurettii*, *S. chromogenes* TA (teat apex strain), *S. chromogenes* IM (intramammary strain) and phosphate-buffered saline (control). The error bars represent the standard error of the mean (+ SEM). TNF-α was not detected in the control quarters.

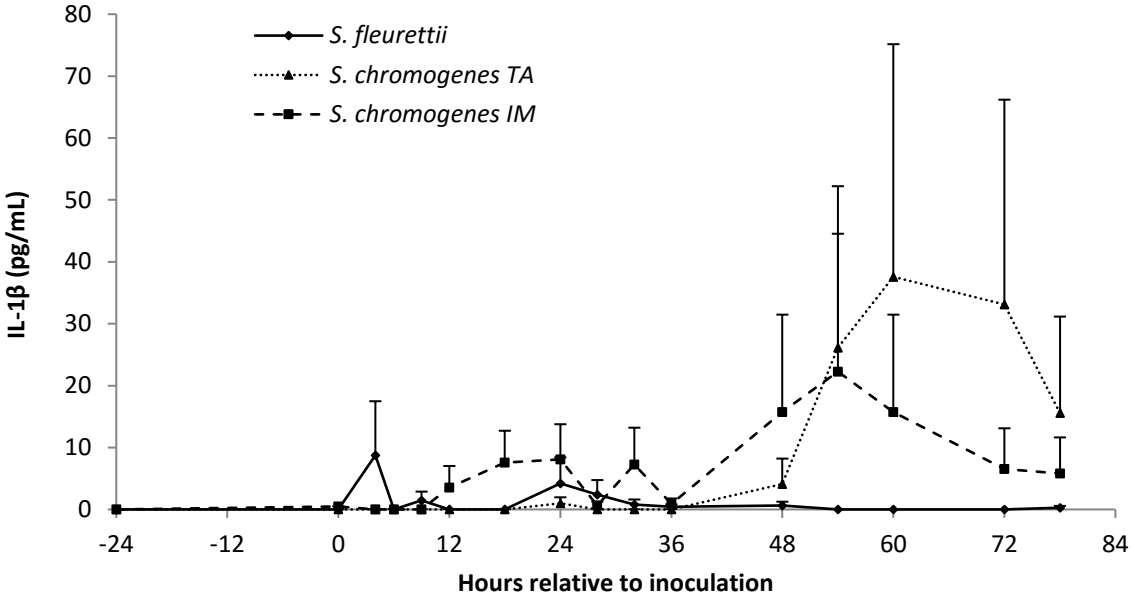

Figure 3.7. The average concentration of IL-1β (pg/mL) in quarter milk following experimental intramammary challenge of 8 dairy heifers with *Staphylococcus fleurettii*, *S. chromogenes* TA (teat apex strain), *S. chromogenes* IM (intramammary strain) and phosphate-buffered saline (control). The error bars represent the standard error of the mean (+ SEM). IL-1β was not detected in the control quarters.

3.6 Discussion

First, we wanted to compare the host responses in dairy cattle with subclinical mastitis caused by representative isolates of either an "environmental" or a "host-associated" CNS species. For this purpose, *S. fleurettii* was selected as the environmental species, and *S. chromogenes* as the host-adapted species. The second objective was to study the host's reaction to different strains of the same species, in this case *S. chromogenes*. Due to the split-udder design, which partially circumvents variation between heifers, the host response could be studied using only a limited number of experimental animals. In contrast to other experimental CNS trials (Simojoki et al., 2009; Simojoki et al., 2011), all CNS species found in the milk samples were identified with tDNA-PCR, in addition to conventional culturing techniques. Through the molecular identification of the CNS species, a natural infection with *S. epidermidis* was detected. The data of this particular quarter were subsequently discarded from the analysis.

Based on the changes in SCC and bacterial shedding, all quarters were successfully challenged with the different CNS strains, whereas the control quarters remained culture-negative throughout the entire trial. *Staphylococcus fleurettii* was fairly quickly (i.e. within 12h) eliminated from the mammary gland, and the bacterial shedding was lowest for this species. The latter finding was also observed in an experimental trial in mice using the same CNS isolates (Breyne et al., 2015). Both *S. chromogenes* strains, on the other hand, persisted in the mammary gland for at least 3 days, accentuating the host-adapted nature of this species. Altogether, this is still a short period, considering that *S. chromogenes* IM was originally isolated from a cow suffering from a persistent IMI lasting over 300 days (Piessens et al., 2011). The bacterial count of S. *chromogenes* TA was significantly lower than *S. chromogenes* IM though. In fact, *S. chromogenes* IM seemed to be the only strain able to multiply in the mammary gland in the first 6 hours after inoculation. The *S. chromogenes* IM strain also elicited a significantly larger increase in SCC than *S. chromogenes* TA. The lower cellular response in the *S. chromogenes* TA-infected quarters could possibly explain the slower bacterial clearance. These results might indicate a difference in pathogenicity

and *in vivo* growth capacity between the different strains. Interestingly, in the murine experimental trial, no differences in bacteriological shedding nor in neutrophil influx were observed between *S. chromogenes* IM and *S. chromogenes* TA (Breyne et al., 2015).

The intramammary challenge had a significant effect on the apoptosis of PMN in milk, which was likely the result of an influx of young, activated PMN from the blood stream to the site of infection along with a potentially delayed PMN apoptosis. Aging PMN eventually undergo apoptosis (or programmed cell death), impairing the functionality of the cells (Whyte et al., 1993; Van Oostveldt et al., 2002). Delayed apoptosis might therefore contribute to a faster clearing of the bacterial infection (Mehrzad et al., 2004). However, PMN apoptosis and subsequent phagocytosis by macrophages is a physiological necessity to curb unbridled inflammatory responses that result in tissue damage (Whyte et al., 1993). Since the lactation stage and parity may affect the survival of PMN (Van Oostveldt et al., 2001; Mehrzad et al., 2004), only mid-lactation heifers were used in this experiment, allowing us to study the effect of the infection (Boutet et al., 2004). *Staphylococcus fleurettii* evoked the greatest decrease in PMN apoptosis during the first 48 p.i., at least partly explaining the higher (albeit insignificant) SCC increase compared to *S. chromogenes* TA. Still, the PMN apoptosis was already decreased in all quarters right before the inoculation. The pre-inoculation drop in apoptosis might have been caused by stress (Liles et al., 1995) associated with the beginning the experiment, although this could not be confirmed. A longer adaptation period (> 48h) in the tie-stall facility prior to the inoculation might have mitigated these findings.

Pro-inflammatory cytokines, such as TNF-α and IL-1β, are involved in a plethora of immune functions on a local and systemic level (e.g. the endothelial adhesion of immune cells, induction of fever or the production of other cytokines) (Bannerman et al., 2004a; Schukken et al., 2011). The further recruitment of PMN to the infection site is mediated by IL-8 and other chemokines (Harada et al., 1994). In contrast to *E. coli* infections, *S. aureus* mastitis does not evoke a significant IL-8 or TNF-α response, which might partially explain the chronic nature of *S. aureus* IMI (Riollet et al., 2000; Bannerman et al., 2004b). As demonstrated in previous research (Simojoki et al., 2011), other CNS species (*S. epidermidis* and

Staphylococcus simulans) appear to induce a clear pro-inflammatory response with TNF-α, IL-8 and IL-1β nonetheless.

In this study, *S. chromogenes* IM induced the largest overall pro-inflammatory cytokine response, starting with an increase in IL-8 at 9h p.i.. The production of IL-1β occurred later at 12h p.i., but lasted longer (> 78h p.i.). The IL-1β response was less pronounced in the *S. fleurettii*-challenged quarters than in the *S. chromogenes* IM quarters. The IL-8 response induced by these strains seemed to be smaller than the response described for *S. epidermidis* or *S. simulans* (Simojoki et al., 2011). However, a higher infection dose was used in the latter study. Furthermore, the authors transformed the IL-8 data by multiplying it by 100, since they observed that the human IL-8 ELISA measures bovine IL-8 100-times less efficiently. That might explain why their IL-8 results fall in the ng/mL range, whereas our results are found in the pg/mL range. Also, we did not find a distinction between early (peaking at 12h) or late (peaking at 30h) IL-8 responders as seen in (Simojoki et al., 2011). In fact, none of our animals displayed any IL-8 response after 30 h p.i..

Staphylococcus chromogenes TA did not elicit any detectable cytokine response, except in one particular heifer. In a similar murine infection trial, some mice displayed a disproportionate reaction to *S. chromogenes* TA as well, resulting in a high IL-1β response and an intense clinical reaction (Breyne et al., 2015). Another heifer showed no cytokine response at all to any CNS strain. Everything considered, the pro-inflammatory cytokine response appeared to vary greatly between individual animals in our research.

As discussed in other research (Sipka et al., 2014), the split udder design used in this experiment is founded on within-cow comparisons. On the one hand, this study design partly circumvents high variation between individual animals, and reduces the needed number of research animals substantially. On the other hand, it assumes that all mammary quarters are completely separate anatomical entities within the udder. This is not necessarily the case, as demonstrated by previous research that illustrated that the immune response of the neighboring (uninfected) quarters is affected by an experimental challenge with major mastitis pathogens (Jensen et al., 2013). This should be kept in mind when

interpreting the results of this study. Nonetheless, we were still able to demonstrate different host responses to each CNS strain while using the split-udder design.

None of the heifers in our study showed any local signs of clinical mastitis (such as clots in the milk or swelling of the quarters), although 3 animals experienced a brief bout of fever in the first 12h after inoculation. In a similar challenge study with *S. chromogenes*, the heifers only developed very mild clinical signs of inflammation, even though the inoculation dose was more than double ours (2.1×10^6 CFU versus 1.0×10^6 CFU) (Simojoki et al., 2009). In the preliminary challenge trial conducted prior to this study (Verbeke et al., 2015) higher doses of the same *S. chromogenes* strain evoked a more pronounced immune response as opposed to lower doses with the appearance of mild clinical signs at a dose of 2×10^6 CFU. Observational research has demonstrated that approximately half of all cows with an intramammary *S. chromogenes* infection showed clinical signs of mastitis (Taponen et al., 2006; Waller et al., 2011). Notwithstanding a different infection dose or host-immune status, this could suggest that not all *S. chromogenes* strains are equally pathogenic. This is in accordance with *S. aureus*, where certain strains have also been linked to a more severe clinical outcome, with a reduced persistence in the mammary gland (Haveri et al., 2005). When extrapolating the current results to practice, it should be noted that natural infection doses might vary from doses used here, and that the experimental strains were directly infused into the teat cistern (as opposed to natural infections, where CNS have to overcome the teat barrier.) The results of our experimental trial with dairy heifers should be interpreted with caution, since the outcome of an IMI may vary with parity, lactation stage and other cow factors.

3.7 Conclusion

Even in case of large inoculation doses, bovine-associated *S. chromogenes* and environmental *S. fleurettii* strains trigger a similar, relatively mild local response. The environmental CNS species, *S. fleurettii*, evokes a profound cellular response in dairy heifers nonetheless, akin to the host-adapted

species *S. chromogenes*. However, *S. fleurettii* is eliminated more rapidly from the mammary gland than *S. chromogenes*. This might indicate that certain bovine-associated CNS species, like *S. chromogenes*, are better able to withstand and thrive in the environment of the mammary gland. Still, the present *in vivo* study also suggests that not all *S. chromogenes* strains exhibit the same degree of pathogenicity. The clinical outcome of a natural *S. chromogenes* mastitis might therefore not only depend on the infection pressure and the resistance of the cow, but also on the pathogenicity of the particular strain.

3.8 Acknowledgements

The authors would like to acknowledge Lars Hulpio, Marina Stevens and Dimitri Valckenier for their assistance during the sampling. The work of Lydia Bommelé, Karel Vermeulen and André Turtelboom at Biocentrum Agri-Vet is also greatly appreciated.

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Chapter 4

The role of milk prolactin in intramammary infections with coagulase-negative staphylococci

Chapter 4.1

Milk prolactin response and quarter milk yield after experimental infection with coagulase-negative staphylococci in dairy heifers

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Journal of Dairy Science, 2015, 98:1–8

4.1.1 Abstract

Coagulase-negative staphylococci (CNS) are the most common bacteria involved in subclinical mastitis in dairy cows. Remarkably, CNS-infected dairy heifers produce more milk than uninfected heifers. Because the lactation hormone prolactin (PRL) is also involved in mammary gland immunity, we investigated the milk PRL response and the mammary quarter milk yield following experimental CNS challenge. Eight healthy Holstein-Friesian heifers in mid-lactation were experimentally infected using a split-udder design with 3 different CNS strains: one *Staphylococcus fleurettii* (from sawdust bedding) and 2 *Staphylococcus chromogenes* strains (one isolate from a teat apex, the other isolate from a chronic intramammary infection). Three mammary quarters per heifer were simultaneously inoculated with 1.0×10^6 CFU, whereas the remaining mammary quarter was infused with sterile phosphate-buffered saline, serving as a control. An existing radioimmunoassay was modified, validated, and used to measure PRL frozen-thawed milk at various time points until 78 h after challenge. The mean milk PRL level tended to be higher in the CNS-challenged mammary quarters compared with the control mammary quarters (7.56 and 6.85 ng/mL, respectively). The increase in PRL over time was significantly greater in the CNS-challenged mammary quarters than in the control mammary quarters. However, no difference was found in the PRL response when comparing each individual CNS strain with the control mammary quarters. The mean mammary quarter milk yield tended to be lower in the CNS-infected mammary quarters than in the control mammary quarters (1.73 and 1.98 kg per milking, respectively). The greatest milk loss occurred in the mammary quarters challenged with the intramammary strain of *S. chromogenes*. Future observational studies are needed to elucidate the relation between PRL, the milk yield, and the inflammatory condition, or infection status, of the mammary gland.

Key words: coagulase-negative staphylococci, dairy heifer, experimental mastitis, prolactin

4.1.2 Introduction

Bovine mastitis, an inflammation of the mammary gland, creates a huge economic burden on the global dairy industry (Bradley, 2002). Coagulase-negative staphylococci are the predominant group of bacteria involved in subclinical mastitis (Pyörälä and Taponen, 2009) and can cause clinical mastitis with mild symptoms (Taponen et al., 2006). Thus far, more than 10 species of CNS have been isolated from bovine milk (Piessens et al., 2011) with documented species-specific differences in putative virulence (Vanderhaeghen et al., 2014), ecology, and epidemiology (Vanderhaeghen et al., 2015). Contrary to what one might expect, various studies have observed a higher test-day milk yield in CNS-infected dairy heifers and multiparous cows com- pared with noninfected cows (Compton et al., 2007; Schukken et al., 2009; Piepers et al., 2010). Some studies have attributed a protective effect to pre-existing CNS IMI against IMI with more virulent mastitis pathogens (e.g. Piepers et al., 2010). A meta-analysis could not confirm this finding in observational studies, but nonetheless revealed a pronounced protective effect in challenge trials (Reyher et al., 2012). Still, the positive effect on milk yield could be an indirect result of the reduced incidence of clinical mastitis observed in CNS-infected animals (Piepers et al., 2010). High-producing dairy cows might also be more susceptible to CNS IMI than low-yielding animals (Compton et al., 2007). However, even after correcting for these factors, an unexplained difference in milk yield of 2.0 kg/d remained between CNS-infected and uninfected herd mates (Piepers et al., 2013), leaving the exact mechanism to be determined.

Prolactin has been associated with over 300 different biological actions, including lactation and mammary gland development (Bole-Feysot et al., 1998). In dairy cattle, PRL is required to initiate (Akers et al., 1981) and maintain the milk production after parturition (Lacasse et al., 2012). The protein hormone also acts as a cytokine on molecular and functional levels (Goffin et al., 2002). The ubiquitous PRL receptor belongs to the class I cytokine receptor superfamily, which also includes the receptors of several interleukins and hematopoietic growth factors (Bazan, 1989; Bazan, 1990). The hormone promotes the activity of macrophages (Edwards et al., 1987), inhibits the apoptosis of T-

lymphocytes caused by glucocorticoids (Krishnan et al., 2003), and stimulates the production of tumor necrosis factor-α and IL-12 (Brand et al., 2004). Considering the immunomodulatory actions of PRL, several studies have focused on its potential involvement in bovine mastitis. The periparturient PRL peak coincides with the principal risk period for developing mastitis (Burton et al., 2001). The hormone induces the *in vitro* synthesis of several cytokines in bovine MEC through the activation of nuclear factor kappa B (Boutet et al., 2007). Although the circulating PRL level is not affected by acute, clinical mastitis (Hockett et al., 2000; Vanselow et al., 2006), a positive correlation was found between SCC and PRL concentration in milk of chronically infected mammary quarters (Boutet et al., 2007).

Because PRL is recognized as a pro-inflammatory cytokine, we hypothesize that milk PRL increases in response to an IMI with CNS. Furthermore, we hypothesize that the quarter milk yield (QMY) also increases after CNS IMI, assuming PRL stimulates the production of milk. To investigate this, an experimental challenge trial was set up using 8 clinically healthy, mid- lactating dairy heifers using 3 different CNS strains. An existing radioimmunoassay for fresh milk was modified and subsequently validated to measure bovine PRL in frozen-thawed milk samples. To assess the epithelial integrity of the blood-milk barrier, the sodium, potassium, and chloride levels were also determined in milk.

4.1.3 Materials and methods

The study is in compliance with the European Directive 2010/63/EU and was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC2012/73).

Animals

The study took place between December 2012 and May 2013 at the research dairy farm of Ghent University (Biocentrum Agri-Vet, Melle, Belgium). Eight clinically healthy Holstein-Friesian heifers in mid-lactation (78– 278 DIM) were selected. Heifers with a known history of clinical mastitis or

persistent high SCC (>150,000 cells/mL) were excluded from the trial. Milk samples were cultured according to NMC guidelines 48 and 24 h before inoculation to ensure all mammary quarters were free from IMI (NMC, 1999).

CNS Strains

All heifers were inoculated with 2 different wild strains of *Staphylococcus chromogenes* and 1 *Staphylococcus fleurettii* strain. The *S. fleurettii* isolate was recovered from sawdust bedding in a dairy barn (Piessens et al., 2011; Breyne et al., 2015). The first *S. chromogenes* strain originated from a cow suffering from a persistent IMI (hereafter referred to as *S. chromogenes* IM; Supré et al., 2011; Breyne et al., 2015), whereas the second *S. chromogenes* isolate was cultured from the teat apex of a heifer (hereafter referred to as *S. chromogenes* TA; De Vliegher et al., 2004; Breyne et al., 2015). The *S. chromogenes* TA strain has the ability to inhibit the growth of several major pathogens under laboratory conditions (De Vliegher et al., 2004), whereas the *S. chromogenes* IM strain does not. The 2 strains also elicit a different immune response in mice (Breyne et al., 2015), and the TA strain is unable to grow in anaerobic iron-depleted medium unlike the IM strain (Souza et al., 2016). An inoculum of 1.0×10^6 CFU of each strain was prepared to induce an experimental infection. The live number of CFU was determined by plating serial dilutions of the bacterial stock on tryptic soy agar.

Experimental Study Design

A split-udder design was used. The concept of the split-udder model is grounded on within-heifer comparisons to reduce individual variation (Sipka et al., 2014). Following the morning milking, 3 mammary quarters of each heifer were instantaneously inoculated with the 3 aforementioned CNS strains (one per mammary quarter) diluted in 5 mL of PBS using a sterile catheter (Vygon, Ecouen, France). The fourth mammary quarter, serving as a control, was infused in the same manner with 5 mL of sterile, pyrogen-free PBS. Milk samples for PRL analysis and microbiological culturing were collected from each mammary quarter at 0, 4, 6, 9, 12, 18, 24, 28, 32, 36, 48, 54, 60, 72, and 78 h post-inoculation

(PI). Milk samples for ion analysis were collected at 0, 24, and 48 h PI. The milk SCC was determined using a DeLaval Cell Counter (DeLaval, Tumba, Sweden). Bacteriological culturing was performed according to NMC guidelines (NMC, 1999). The milk samples for the PRL and ion analysis were stored at −20°C. The cows were milked twice a day with 12-h intervals, and QMY was registered using a mammary quarter milking device. The cow were examined clinically at each sampling. Rectal temperature, heart rate, respiratory rate, rumen motility, fecal consistency, and milk appearance were registered.

Milk Analysis

Prolactin RIA. Milk PRL was determined by a double antibody, homologous RIA adapted from Malven and McMurtry (1974). Because this protocol was developed for fresh, whole milk, we first modified and validated the RIA for frozen milk samples in particular. For the validation, mammary quarter milk samples (n = 4) were collected from randomly selected, multiparous Holstein-Friesian cows at a Belgian commercial dairy farm. The samples were then stored at −20°C for 96 h. Afterward, the milk samples were thawed in a warm water bath at 40°C for 30 min (Chew et al., 1977) and centrifuged at 1,800 × g for 15 min at 20°C (Sigma 2–16K, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). Bovine PRL (NIH-B5) was used both as a standard and tracer. To generate the tracer, bovine PRL was radiolabeled with 125I using the lactoperoxidase technique (Thorell and Johansson, 1971). A standard curve ranging from 0.8 to 100 ng/ mL was prepared in Tris buffer (25 mM Tris; 0.01 mM MgCl2; 1.5 mM NaN3) containing 0.1% (wt/vol) BSA at pH 7.5. To analyze each standard concentration, 100 µL was added to duplicate tubes containing 200 µL of Tris-BSA buffer. A sample volume of 50 µL was used for milk to minimize potential incubation damage to the labeled antigen (Malven and McMurtry, 1974). Afterward, 100 µL of tracer of approximately 30,000 cpm was added, followed by 100 µL of antiserum (R#144). This antiserum was collected from a rabbit after injection with biological bovine PRL (NIH-B5; Boutet et al., 2007). An antibody titer of 1:120,000 was previously selected for this assay, resulting in

the binding of circa 30 to 40% of the total amount of 125I-PRL. The amount of radiolabelled bPRL bound to the antiserum in tubes containing no bPRL (B_0) was designated as 100% 125I bound. After an overnight incubation at room temperature, the antibody-bound fraction of PRL was precipitated using a secondary antibody polyethylene glycol solution (Ayad et al., 2007). The radioactivity of the precipitated 125I-PRL was measured with a gamma counter (LKB Wallac 1261 Multigamma automatic counter, Breda, the Netherlands). Five parameters were taken into account to validate the RIA: (1) inter- and intra-assay coefficient of variation, (2) minimal detection limit (MDL), (3) parallelism, (4) accuracy, and (5) specificity. Three milk samples with a low (2.4 ng/mL), medium (12.9 ng/mL), and high (19.7 ng/mL) PRL concentration were used to calculate the inter- and intra-assay coefficient of variation. To assess the intra-assay coefficient of variation, the samples were assayed 10 times within the same run. The interassay coefficient of variation was evaluated by measuring each sample in 10 consecutive assays. The MDL was calculated by measuring the mean concentration of 20 B_0 (zero standard) replicates minus twice the standard deviation within the same assay. For the evaluation of parallelism, a sample dilution curve of a frozen-thawed sample was generated to detect any potential interference of the sample matrix. The accuracy or spike-recovery of the assay was studied by adding a series of known amounts of PRL to a sample with a low endogenous PRL concentration. To evaluate potential cross-reactivity (specificity), 2 structurally similar compounds were tested: native ovine growth hormone (oGH) and bovine placental lactogen (bPL). The validated RIA was then applied to all mammary quarter milk samples from the 8 heifers in the present experimental trial.

Indicators of Epithelial Integrity. The milk samples were thawed and subsequently centrifuged at 16,000 × g for 30 min at 4°C. The sodium, potassium, and chloride concentrations were analyzed in the fat-depleted fraction using an ion-selective electrode analyzer (Roche, Basel, Switzerland).

Statistical Analyses

The response of mammary quarter milk PRL after CNS challenge was evaluated using a linear mixed regression model (SPSS 22.0, Chicago, IL), with the time of sampling, quadratic term of time of sampling, and inoculum as predictor variables. Time of sampling and its quadratic term were included as a continuous variable, whereas inoculum was considered as a categorical variable. The interaction between inoculum and time of sampling was also tested, but only kept in the model when significant. A similar model was constructed to assess the association between QMY, and time of sampling, its quadratic term, and inoculum as predictor variables. For both outcome variables, the inoculum was initially considered a dichotomous variable (challenged versus control). In a second approach, the effect of all different CNS strains was examined (*S. chromogenes* IM, *S. chromogenes* TA, and *S. fleurettii*). To determine the relationship between inoculation (challenge versus control) and the milk ion concentration, comparable linear mixed regression models were constructed for sodium, potassium, and chloride. Sampling time was included as a 3-level categorical variable (0, 24, and 48 h). A natural logarithmic transformation of sodium and chloride was performed to obtain a normalized distribution of the residuals. In all aforementioned analyses, heifer and mammary quarter were included as random effects to account for the correlated nature of the data. Compound symmetry was selected as a covariance pattern to account for the clustering of repeated samplings within mammary quarter. Statistical significance was set at $P \leq 0.05$.

4.1.4 Results

Experimental Infection

The data of one mammary quarter were omitted from the analysis due to a naturally occurring IMI. Another mammary quarter was also excluded from the analysis for the same reason, but only 12 h after inoculation. An increase in SCC was observed in all challenged mammary quarters, indicating the establishment of IMI, whereas the SCC in the control mammary quarters remained low [geometric

mean of 47,000 cells/ mL; interquartile range (IQR) of 31,000–71,000 cells/ mL]. Twenty-four hours PI, the *S. fleurettii*, *S. chromogenes* TA, and S. *chromogenes* IM challenged mammary quarters had a geometric mean SCC of 2,400,000 cells/ mL (IQR: 442,000–3,420,000 cells/mL), 669,000 cells/ mL (IQR: 441,000–1,681,000 cells/mL), and 2,596,000 cells/mL (IQR: 1,662,000–4,775,000 cells/mL), respectively. The challenged mammary quarters did not show any visual signs of clinical mastitis. However, 3 heifers did experience a short bout of fever (>39.5°C) between 9 and 12 h PI. The *S. chromogenes* IM and TA strains were recovered from milk up to 78 h PI. In all mammary quarters, the *S. fleurettii* strain was eliminated within 9 h PI. No pathogens other than CNS were isolated during the study. The PBS-infused mammary quarters remained free from infection during the entire study period as demonstrated by bacteriological culture.

Prolactin

RIA Validation. The intra-assay coefficient of variation for samples with a low, medium, and high PRL concentration was 9.7, 8.3, and 8.1%, respectively. The interassay coefficient of variation for the corresponding samples was 11.9, 14.4, and 16.1%, respectively. The MDL of the assay was 0.536 ng/mL. Serial dilutions showed a dose-response curve parallel to the standard curve (data not shown). The recovery ratio of the spiked PRL was consistently >85%. The binding of radiolabeled PRL remained unaltered when testing the cross-reactivity, except for very high concentrations of oGH (starting at 6.2 × 103 ng/mL). This slightly interfered with the assay ($B/B_0 < 0.80$), resulting in an apparent increase of the PRL concentration.

Milk PRL. The overall mean PRL concentration tended to be higher in the challenged mammary quarters than in the control mammary quarters (LSM = 7.56 and 6.85 ng/mL, respectively) throughout the study (P = 0.10, **Table 4.1.1**). The PRL level varied over time in both challenged and control mammary quarters, although not in a linear manner (P < 0.001). The evolution of PRL over time was different between the challenged and the control mammary quarters (interaction inoculum × time of sampling: P = 0.05, **Figure 4.1.1**). No significant difference was observed in the PRL response between

each of the 3 CNS strains and the control mammary quarters (P = 0.77), nor in the evolution over time between strains (interaction inoculum × time of sampling: P = 0.14).

Quarter Milk Yield

The overall mean QMY per milking tended to be lower in the CNS challenged mammary quarters than in the control mammary quarters (LSM = 1.73 and 1.98 kg, respectively) throughout the study (P = 0.06, **Table 4.1.2**). The decline of the QMY over time was greater in the CNS-challenged mammary quarters than in the control mammary quarters (interaction inoculum × time of sampling: P < 0.001, **Figure 4.1.2**). The difference in QMY compared with the control mammary quarters was more pronounced in the mammary quarters challenged with *S. chromogenes* IM (−0.38 kg per milking) than with *S. chromogenes* TA (−0.21 kg per milking) or *S. fleurettii* (−0.17 kg per milking; **Table 4.1.2**).

Blood-Milk Barrier

The concentration of potassium in milk was not significantly influenced by the challenge with CNS strains (P = 0.37). A small, but significant increase in the natural logarithmic of sodium and chloride was noted in the challenged quarters (interaction inoculum × time of sampling P < 0.05). However, the sodium and chloride levels did not exceed the upper limit of the normal reference ranges (**Figure 4.1.3**; Gaucheron, 2005).

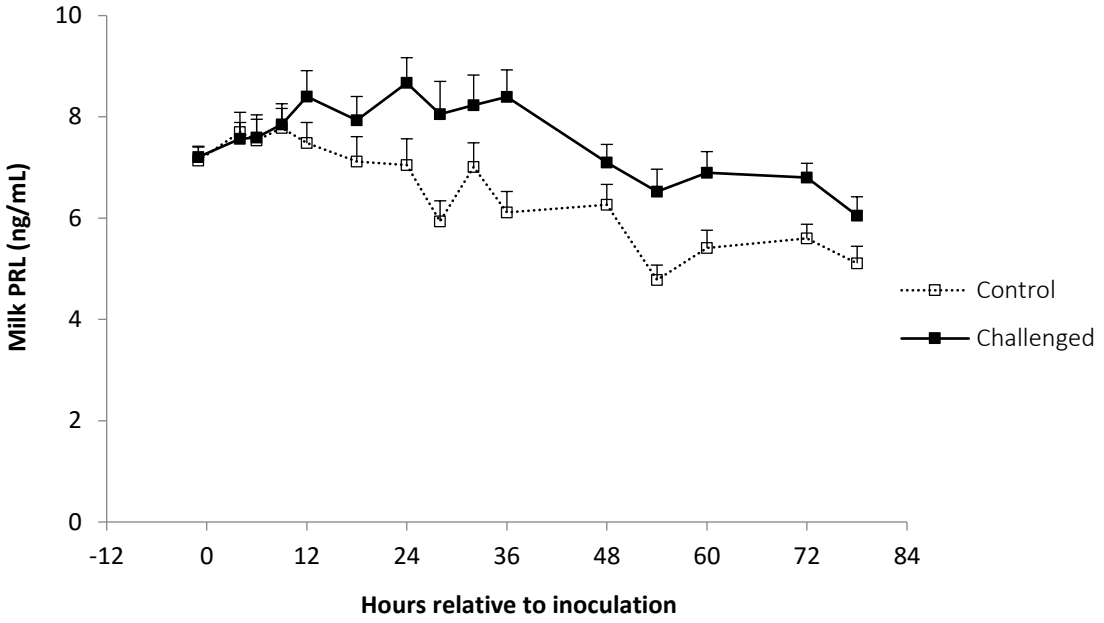

Figure 4.1.1. The average prolactin (PRL) (± SEM, standard error of the mean) concentration in milk following experimental inoculation in the challenged quarters (-■-) versus the control quarters (···□···). The challenged quarters were inoculated with *Staphylococcus fleurettii*, the teat apex strain of *S. chromogenes* and the intramammary strain of *S. chromogenes*.

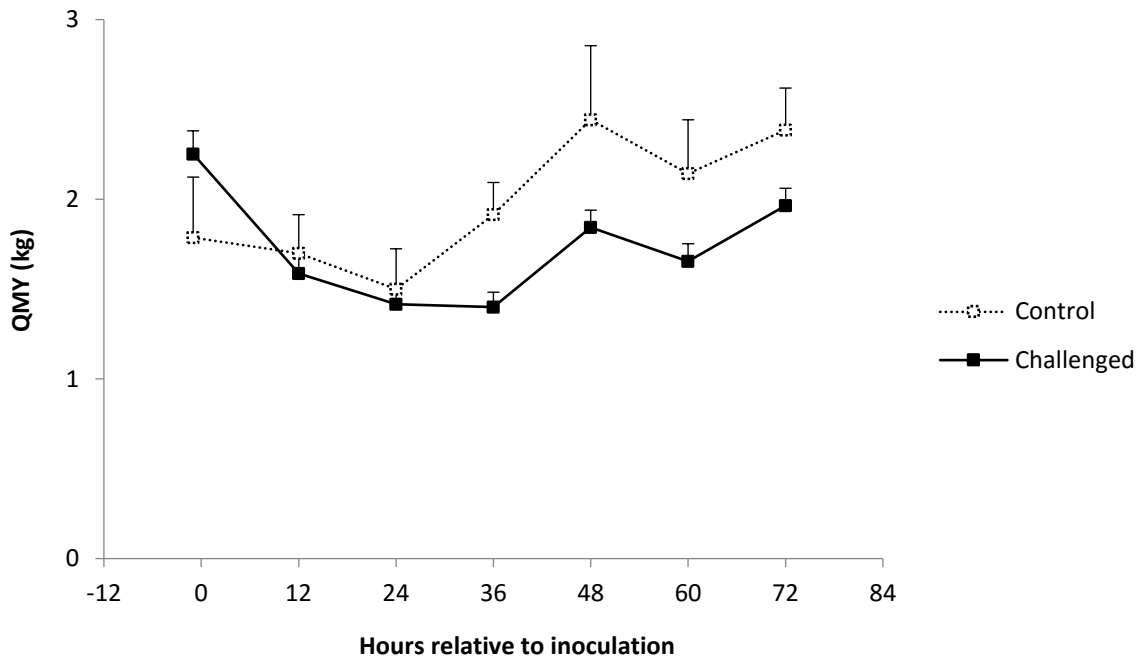

Figure 4.1.2. Mean quarter milk yield (QMY) (±SEM, standard error of the mean) following experimental inoculation in the challenged quarters (-■-) versus the control quarters (¨□¨). The challenged quarters were inoculated with *Staphylococcus fleurettii*, the teat apex strain of *S. chromogenes* and the intramammary strain of *S. chromogenes*.

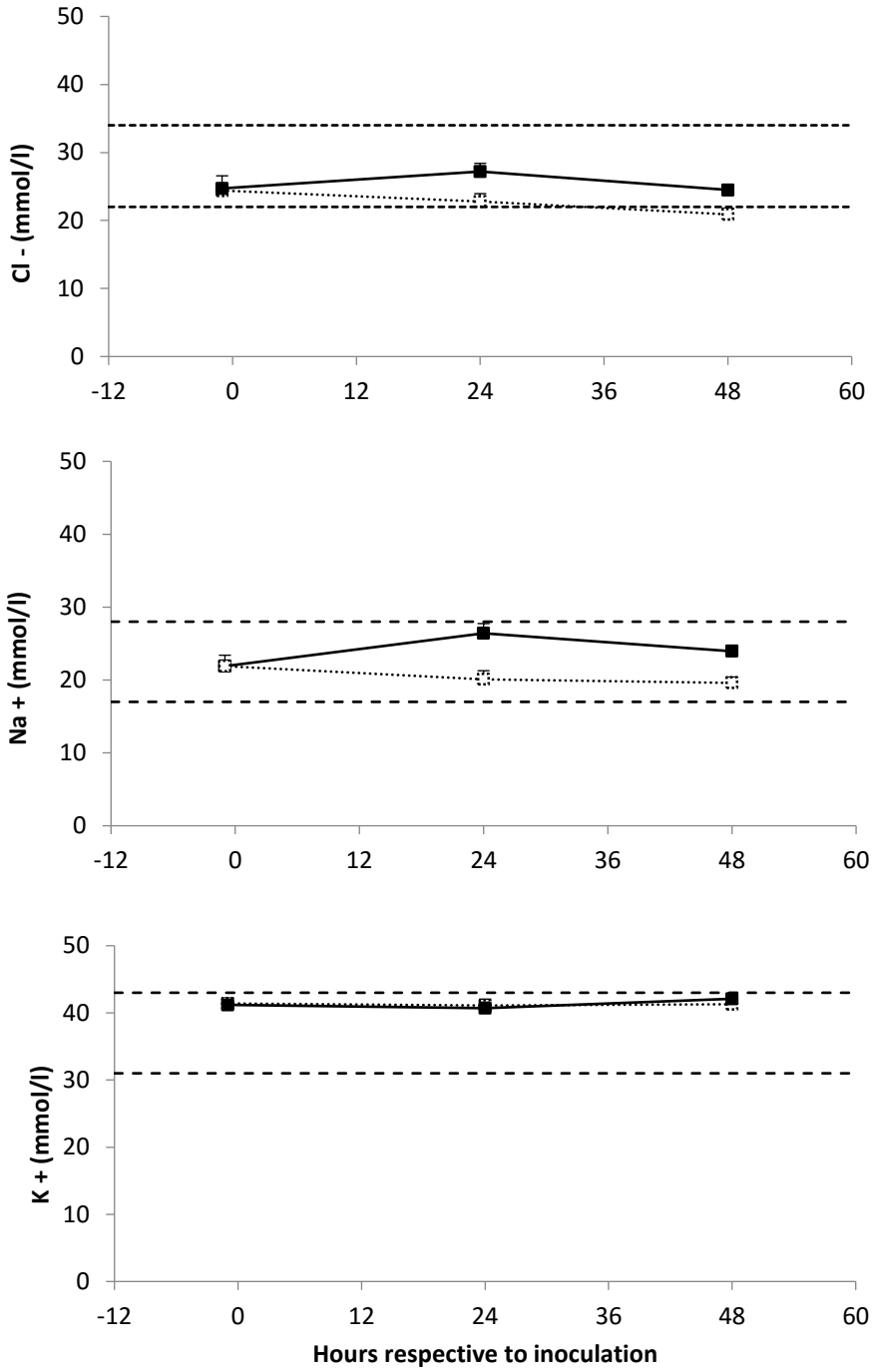

Figure 4.1.3. The average (± SEM, standard error of the mean) milk sodium (Na^+), potassium (K^+) and chloride (Cl^-) levels in the control (···□···) and challenged quarters (–■–). The dashed horizontal lines represent the upper and lower reference range of the respective ions in bovine milk from non-infected mammary glands. The challenged quarters were inoculated with *Staphylococcus fleurettii*, the teat apex strain of *S. chromogenes* and the intramammary strain of *S. chromogenes*.

Tabel 4.1.1. Linear mixed regression model for milk prolactin after experimental infection, including all three CNS strains combined (model 1; left) and with *Staphylococcus chromogenes* TA[1], *S. chromogenes* IM[2] and *S. fleurettii* considered seperately (model 2; right).

Predictor variable	Prolactin (ng/mL)				Prolactin (ng/mL)			
	β[3]	SE[4]	LSM[5]	*P*-value[6]	β	SE	LSM	*P*-value
Intercept	7.56	0.01	…	…	7.56	0.71	…	…
Inoculum				0.10				0.77
Control	Ref.	…	6.85	…	Ref.	…	6.85	…
Challenge[7]	0.21	0.48	7.56	…	…	…	…	…
S. fleurettii	…	…	…	…	0.38	0.59	7.60	…
S. chromogenes TA	…	…	…	…	-0.11	0.59	7.45	…
S. chromogenes IM	…	…	…	…	0.37	0.59	7.62	…
Time of sampling	0.005	0.01	…	0.28	0.018	0.013	…	0.17
Quadratic term of Time of sampling	- 0.001	< 0.001	…	< 0.001	-0.001	< 0.001	…	< 0.001
Inoculum x Time of sampling	…	…	…	0.05	…	…	…	0.14

[1] Teat apex strain of *S. chromogenes*; [2] Intramammary strain of *S. chromogenes;* [3] Regression coefficient; [4] Standard error; [5] Least square means; [6] *P*-value for overall effect; [7] Quarters challenged with *S. chromogenes* TA, *S. chromogenes* IM and *S. fleurettii* combined.

Tabel 4.1.2. Linear mixed regression model for quarter milk yield after experimental infection, including all three CNS strains combined (model 1; left) and with *Staphylococcus chromogenes* TA[1], *S. chromogenes* IM[2] and *S. fleurettii* considered seperately (model 2; right).

Predictor variable	Quarter milk yield (kg)				Quarter milk yield (kg)			
	β[3]	SE[4]	LSM[5]	*P*-value[6]	β	SE	LSM	*P*-value
Intercept	1.86	0.18	…	…	1.86	0.18	…	…
Inoculum				0.06				0.44
Control	Ref.	…	1.98	…	Ref.	…	1.98	…
Challenge[7]	0.15	0.17	1.73	…	…	…	…	…
S. fleurettii	…	…	…	…	0.29	0.20	1.81	...
S. chromogenes TA	…	…	…	…	0.15	0.20	1.77	…
S. chromogenes IM	…	…	…	…	0.02	0.20	1.60	…
Time of sampling	-0.02	0.004	…	< 0.001	-0.02	0.005	…	0.001
Quadratic term of Time of sampling	< 0.001	< 0.001	…	< 0.001	< 0.001	< 0.001	…	< 0.001
Inoculum x Time of sampling	…	…	…	< 0.001	…	…	…	0.001

[1] Teat apex strain of *S. chromogenes;* [2] Intramammary strain of *S. chromogenes;* [3] Regression coefficient;[4] Standard error; [5] Least square means;[6] *P*-value for overall effect;

[7] Quarters challenged with *S. chromogenes* TA, *S. chromogenes* IM and *S. fleurettii* combined.

4.1.5 Discussion

Because CNS-infected cows appear to produce more milk than non-infected cows (Compton et al., 2007; Piepers et al., 2010; Schukken et al., 2009), we investigated the milk yield and the response of milk PRL after CNS challenge in clinically healthy dairy heifers. There is a plethora of evidence implicating PRL as a immunomodulating factor (Edwards et al., 1987; Krishnan et al., 2003; Brand et al., 2004; Boutet et al., 2007). Therefore, we hypothesized that PRL increases in the milk after CNS infection. Milk PRL might then simultaneously stimulate the secretion of milk as a galactopoetic hormone (Lacasse et al., 2012) in a paracrine or autocrine manner (Clevenger et al., 1997), potentially explaining the aforementioned milk yield increases in CNS-infected cows. First, the modified PRL radioimmunoassay for frozen-thawed milk samples proved to be reproducible, accurate and specific. Only high concentrations of GH interfered with the assay, resulting in an apparent increase of PRL. This might be due either to cross-specificity, or to the presence of trace amounts of pituitary PRL in the native GH preparation. Either way, any cross-reactivity is irrelevant since the endogenous GH amount is limited in bovine milk (Burton et al., 1994).

This study confirmed that milk PRL indeed increases in challenged mammary quarters compared to control mammary quarters after CNS inoculation. This corresponds with the results from Boutet et al. (2007), demonstrating that the PRL level is elevated in chronically infected mammary quarters with high SCC (Boutet et al., 2007). Even though others have observed an increased milk production in naturally CNS-infected cows (Schukken et al., 2009; Piepers et al., 2013), this study reported a substantial milk loss in both challenged and control mammary quarters. Then again, this observation is not entirely unexpected. Unlike the previous observational studies, our experimental trial only monitored the milk yield for a short period of time during the acute phase of inflammation. This experimental challenge might not necessarily reflect a natural infection, based on the high infection dose and the direct intracisternal inoculation of CNS. Also, the aforementioned observational studies (Schukken et al., 2009; Piepers et al., 2013) focused on milk production at animal level, where

uninfected glands could compensate for the production loss in CNS-infected mammary quarters (Leitner et al., 2004). Our study measured the production at the mammary quarter level, and found transitory milk loss in all mammary quarters. The extent of production loss in the unchallenged mammary quarters has previously been used to score the severity of experimental mastitis (Burvenich et al., 2003). Since the production of the control mammary quarters was practically restored in all cows after 48 hours, we conclude that the systemic and long-term effects of the induced CNS IMI -even when using a high inoculum dose- were limited. As seen in other experimental infection trials, the CNS in this study evoke a mild inflammatory response despite the high infectious dose (Simojoki et al., 2009; 2011). It should, however, be noted that the overall milk yield during the experimental trial was unexpectedly low in all mammary quarters for Holstein-Friesian heifers. We believe this could be related to the different housing conditions and milking routines during the experimental trial.

Under physiological conditions, circulating pituitary PRL is transported from the bloodstream to the milk compartment via transcytosis. After binding on the membrane receptor, PRL is internalized by the mammary epithelial cell and subsequently released into the milk through secretory vesicles (Ollivier-Bousquet, 1998). Mastitis increases tight junction permeability, hereby enabling the paracellular transport of blood-borne components (Nguyen and Neville, 1998). Changes in milk ion concentration can indicate the disruption of the blood-milk barrier (Stelwagen et al., 1994). In this study, the sodium and chloride levels increased significantly in the challenged mammary quarters. This might imply that the PRL merely leaks from the bloodstream into the milk compartment, as a result of an increased tight junction permeability. Whether the milk PRL increase is entirely due to passive diffusion, can neither be confirmed nor denied in this experimental set-up. The biological significance of plasma-borne proteins in the milk could be questioned. Future research should address the origin of PRL in milk. Other ruminants, such as sheep and goats (Le Provost et al., 1994), are able to produce extra-pituitary PRL in the mammary gland. So far, this has never been demonstrated in cattle, but it would support the hypothesis of PRL as a pro-inflammatory cytokine and autocrine lactation hormone. Although Boutet et al. (2007) found no association between bacterial species and PRL concentration, we also

wonder if the release of PRL during IMI is pathogen dependent. Additional longitudinal studies on mammary quarter level will shed more light on the true association between QMY and CNS infections, and clarify the role of PRL in IMI.

4.1.6 Conclusion

This study demonstrated that milk PRL increases after an experimental intramammary CNS challenge in dairy heifers. The mechanism behind this PRL response, and whether it is biologically relevant, remains to be determined.

4.1.7 Acknowledgements

We would like to thank Dr. A.F. Parlow of the National Hormone and Peptide Program (NHPP) for kindly providing the bovine PRL, as well as Aline Flora Tchimbou from the University of Liège and Lars Hulpio from Ghent University for their excellent technical assistance.

4.1.8 References

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Chapter 4.2

Prolactin gene expression in bovine mammary epithelial cells after a challenge with coagulase-negative staphylococci

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Submitted to the Journal of Dairy Research

4.2.1 Abstract

This study addresses the hypothesis that the expression of the prolactin (PRL) gene increases in bovine mammary epithelial cells (MEC) following an infection with coagulase-negative staphylococci (CNS). Various studies have demonstrated in the past that dairy cattle with an intramammary infection caused by CNS have a higher milk yield than non-infected dairy cows. It has been suggested that this could be the result of an increased synthesis of the autocrine lactation hormone PRL, since PRL is also involved in the inflammatory response of MEC during mastitis. Bovine MEC (more specifically MAC-T cells) were therefore inoculated using three well-defined CNS strains from varying habitats: one strain of *Staphylococcus fleurettii* originating from sawdust, and two different strains of *Staphylococcus chromogenes* (one isolated from a heifer's teat apex [*Staphylococcus chromogenes* TA], the other from a persistent intramammary infection [*S. chromogenes* IM]). Although PRL was expressed in all samples, the expression was not higher in CNS-challenged cells compared to the unchallenged control cells. The elevated PRL level previously observed in milk from cows with CNS infection might rather be the result of a disruption of the blood-milk barrier, although this needs further substantiation.

Key words: prolactin – expression – mammary – coagulase-negative staphylococci

4.2.2 Introduction

Coagulase-negative staphylococci (CNS) are one of the most frequently isolated bacteria in dairy cattle with subclinical mastitis (Pyörälä and Taponen, 2009; Vanderhaeghen et al., 2014). Bovine-associated CNS occupy a diverging range of habitats, varying from environmental sites in the dairy barn to host-specific sites, such as the mammary gland and udder skin (Pyörälä and Taponen, 2009; De Visscher et al., 2014). Even though it seems counterintuitive, several studies indicate that dairy cattle infected with CNS have a higher milk yield than non-infected cows (Schukken et al., 2009; Piepers et al., 2010; Piepers et al., 2013). Various hypotheses for this unexpected observation have been put forward. On the one hand, high-producing dairy cows might be more prone to develop CNS mastitis compared to low-yielding cows (Gröhn et al., 2004). On the other hand, the presence of CNS might protect the mammary gland against major mastitis pathogens, such as *S. aureus* or *E. coli*, through competitive exclusion (Hibbing et al., 2010), the activation of the immune system (Schukken et al., 1999), or the production of antibacterial components (Braem et al., 2014). We previously postulated that the lactation hormone prolactin (PRL) might stimulate the milk production in an autocrine manner in response to a CNS infection (Piccart et al., 2015).

Besides being a key hormone in lactation (Akers et al., 1981; Lacasse et al., 2012), PRL also exhibits immunomodulatory characteristics. For instance, PRL can activate human macrophages (Edwards et al., 1987), stimulate the production of pro-inflammatory cytokines (Brand et al., 2004), and alter the expression of multiple genes regulating the activity of leukocytes (Dogusan et al., 2001). Even though the blood PRL level of dairy cows is not affected by intramammary infection (IMI [Hockett et al., 2000]), the PRL concentration is higher in the milk of infected mammary quarters compared to healthy, uninfected quarters (Boutet et al., 2007). Although the anterior pituitary gland is the major source of PRL, the hormone can also be synthetized in the mammary gland of humans, rodents and small ruminants (Fields et al., 1993; Le Provost et al., 1994; Lkhider et al., 1997). Gene expression profiling data previously submitted to the NCBI Gene Expression Omnibus (GDS4406, GDS4437, GDS4009) show

that PRL is also expressed in the MEC of dairy cattle, even in cells challenged with the major mastitis pathogen *S. aureus* (Brand et al., 2011; Günther et al., 2011).

Recent work has shown that the milk PRL concentration also increases when mammary quarters of dairy heifers are inoculated with strains of *S. chromogenes* and *S. fleurettii* (Piccart et al., 2015). While *S. fleurettii* is mostly found in the environment of dairy cattle, *S. chromogenes* is able to colonize the skin of the cow's teats (Piessens et al., 2011; De Visscher et al., 2014). At the same time, *S. chromogenes* is one of the more common CNS species involved in IMI (Vanderhaeghen et al., 2014). It has been demonstrated that these CNS strains originating from diverging habitats elicit a clearly differential host response in mice (Breyne et al., 2015) and dairy heifers (Piccart et al., 2016), and differ in their capacity to adhere to and invade MEC (Souza et al., 2016).

The objective of the present study was to determine whether the well-studied, epidemiologically diverging strains of *S. chromogenes* and *S. fleurettii* can promote the *PRL* expression in bovine MEC.

4.2.3 Materials & Methods

Bacterial strains

Three different field strains of CNS were used to challenge the MAC-T cells: one *S. fleurettii* strain and two *S. chromogenes* strains. The *S. fleurettii*-strain was isolated from sawdust bedding in a dairy barn, whereas both *S. chromogenes*-strains were isolated from dairy cattle: one strain originating from the skin of a heifer's teat apex (referred to as *S. chromogenes* TA), and another strain from a cow with persistent IMI (*S. chromogenes* IM). These particular strains have been used in other studies as well (Breyne et al., 2015; Piccart et al., 2016; Souza et al., 2016). The growth conditions are described in Souza et al., 2016.

Bovine mammary epithelial cells

An immortalized line of bovine MEC (MAC-T), originally established from mammary alveolar cells to mimic bovine lactation (Huynh et al., 1991), was used to study the *in vitro* expression of PRL. The MAC-T cells were cultured according to Souza *et al*., 2016 and incubated overnight in a humidified incubator with 5% CO_2 at 37°C.

Inoculation

Three replicate 6-well plates were prepared per inoculum (including three replicate unchallenged controls) per sampling time. Each well was inoculated with 4 mL of the respective staphylococcal suspension (3 x 10^5 CFU/mL; 1:1 ratio of staphylococci to cells). A negative, unchallenged control was set up in parallel. Following the inoculation, the cell cultures were incubated at 37°C in 5% CO_2. The MAC-T cells were harvested after 0.25% trypsin treatment (Gibco, Paisley, UK) at 1h, 3h, 6h and 12h. Then, 1mL of Dulbecco's modified Eagle's medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum was added to each well. The MAC-T cell suspensions were pooled accordingly (per sampling time), centrifuged at 400 x *g* for 8 minutes and adjusted to 5 x 10^6 cells using trypan blue staining and a Neubauer chamber. The pooled samples were stored for less than 3 months at -20°C in 1 mL of total RNA isolation reagent (TRIR; Ambion, Austin, USA).

RNA extraction

Following extraction and DNase treatment with the Aurum Total RNA Fatty and Fibrous Tissue kit (Bio-Rad, Hercules, USA), the RNA concentration and purity was measured using a ND-1000 spectrophotometer (Nanodrop, Wilmington, USA). The RNA concentration ranged between 108 ng/µL and 1.626 ng/µL, whereas the $A_{260/280}$ ratio was ≥ 2.00 (range 2.09 – 2.18) for all RNA samples. The RNA integrity of all samples was visually assessed using agarose gelelectrophoresis. Additionally, a representative subset of RNA samples was subjected to microfluidic analysis (Experion; Bio-Rad, Hercules, USA), revealing an RQI number of ≥ 7.5. All samples were free of gDNA contamination after

DNase treatment, as demonstrated by the minus reverse transcriptase (RT) control. The minus RT control was performed using a PCR mix of 10 µL, containing 1 µL of diluted sample (corresponding with approximately 30 ng RNA), 1 µL 10 × FastStart Taq DNA Polymerase Buffer (Roche Applied Science, Indianapolis, USA), 0.2 µl dNTP Mix (10 mM each; Bioline, London, UK), 0.1 µL Taq DNA Polymerase (5 U/µl, Roche Applied Science, Indianapolis, USA), 0.5 µL forward primer [BtauPRL +1, 5 µM, 5'-TGGAGCCAAAGAGACTGAGC-3'] (Integrated DNA Technologies, Leuven, Belgium) and 0.5 µL reverse primer [BtauPRL -1, 5 µM, 5'-GCAGTTGTTGTTGTAGATGATTCTG-3'] (Integrated DNA Technologies, Leuven, Belgium). The PCR program consisted of an initial 4-min denaturation step at 95°C, followed by 40 cycles of denaturation (10s at 95°C), annealing (10s at 58°C) and synthesis (20s at 72°C), with a final extension step of 2 minutes at 72°C. The final product was examined using gel electrophoresis on ethidium bromide-stained agarose (2%) gel (150 V, 30 min).

cDNA synthesis

cDNA was synthetized with approximately 1 µg RNA using the enzyme Improm-II reverse transcriptase (Promega, Fitchburg, USA). The reaction further contained 0.8 µL random hexamer primers (10 µM; IDT, Coraville, USA) and 0.8 µL oligo(dT)15 primer (10 µM, IDT, Coraville, USA). The mix was incubated for 5 min at 70°C, and subsequently incubated for 5 min on ice. Afterwards, 1 µL Improm-II reverse transcriptase (20 U/µL), 4 µL Improm-II 5x reaction buffer, 2.4 µL MgCl2 (25 mM), 1 µL dNTP Mix (10 mM each; Bioline, London, UK) was added to the mix. The solution (20 µL) was incubated for 5 min at 25°C for primer annealing, followed by 60 min at 42°C for first-strand cDNA synthesis and heat-inactivation of the reverse transcriptase at 70°C for 15 min. The cDNA samples were diluted 10 times and stored at -20°C. The cDNA integrity was assessed using the same PCR conditions and primers as described above for the minus RT control.

Quantitative real-time PCR

The relative expression level of PRL was determined using qPCR on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA) with FastStart Essential DNA Green Master (Roche Diagnostics, Basel, Switzerland). Information on the primers, amplicons and amplification efficiency is listed in **Table 4.2.1**. A PCR mix (10 µL) was prepared for each sample with 5 µL ready-made FastStart Essential DNA Green Master (Roche Diagnostics, Basel, Switzerland), 1 µL forward and reverse PRL primer (5 µM) and 2 µL cDNA sample. The PCR program, performed on a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, USA), consisted of an initiation step of 10 min at 95°C, followed by 40 cycles of denaturation (20s at 95°C) and annealing-elongation (40s at the optimal annealing temperatures). A melt curve was then generated by heating the samples from 75°C to 95°C in 0.5°C increments while measuring the fluorescence. The optimal annealing temperature for the amplification of PRL and PCR efficiency was determined by gradient qPCR of a 4-fold dilution series of the cDNA of a bovine uterine tissue sample. Four reference genes (ubiquitin C [*UBC*], histone-2-alpha [*H2A*], succinate dehydrogenase [*SDHA*], and ribosomal protein S15A [*RPS15A*]) were selected based on previous research (Verbeke et al., 2015c) and evaluated via geNorm for normalization of the data from MAC-T cells (Vandesompele et al., 2002). The PRL primers were designed using Primer3Plus (Untergasser et al., 2007) based on the sequence available in NCBI Genbank. The pooled samples were analyzed in triplicate.

Data analysis

The raw quantification cycles (Cq) of each sample were transformed to a relative quantity (Q) using the ΔΔCt-method. The expression data of the CNS-inoculated cells were normalized against the control data for each sampling time, and a one sample t-test (SPSS 23.0, Armonk, USA) was used to determine if the mean PRL gene expression was affected by the challenge with CNS for each sampling time. The significance level was set at $P \leq 0.05$.

4.2.4 Results & discussion

The aim of this research was to determine if the *PRL* gene expression increases in cells challenged with *S. fleurettii*, *S. chromogenes* IM and *S. chromogenes* TA. RT-PCR revealed that PRL mRNA was present in all tested samples, including the unchallenged MAC-T cells. The geNorm analysis showed that *UBC, H2A* and *RPS15A* were the most stable reference genes in the MAC-T cells, and the geometric mean of the expression level of these three genes was used to normalize the *PRL* gene expression data.

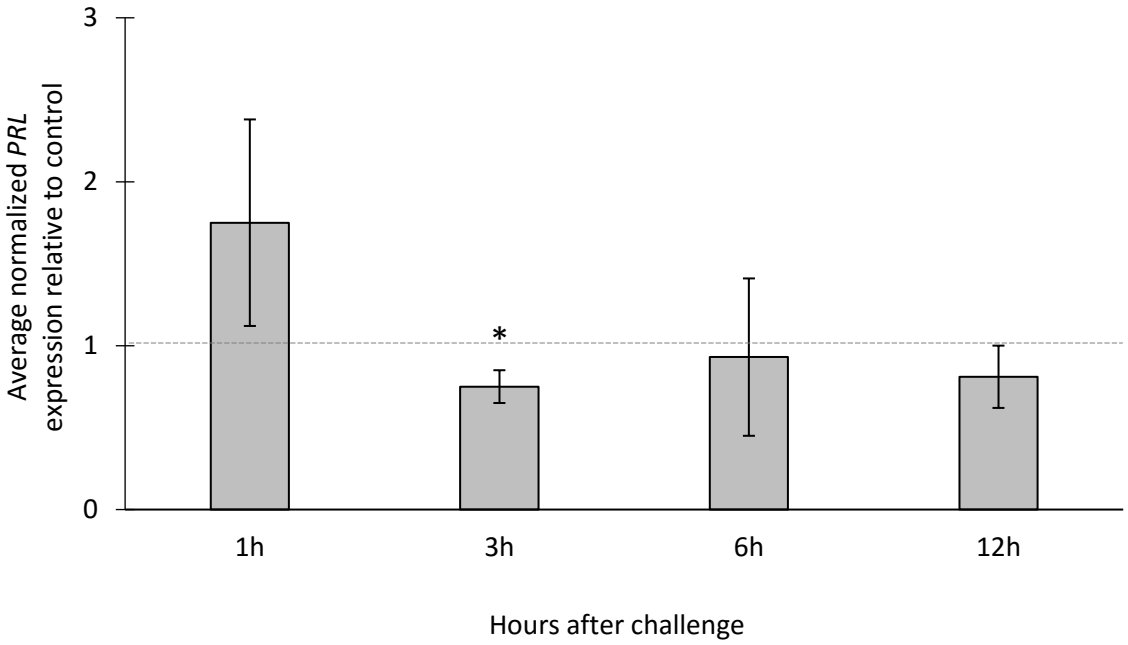

Figure 4.2.1. Overall average normalized *PRL* expression in mammary epithelial cells challenged with *Staphylococcus fleurettii*, *S. chromogenes* TA (teat apex strain) and *S. chromogenes* IM (intramammary strain). The mean is expressed relative to unchallenged control cells per sampling time. The dashed line corresponds with the expression level of the control cells. The error bars represent the standard deviation. The asterisk denotes a statistically significant difference (*P*-value ≤ 0.05) in the mean expression between challenged and unchallenged cells.

At 1h post-infection, the *PRL* expression was indeed higher in the CNS-challenged cells compared to the control, but the increase was not statistically significant (*P*-value = 0.17; **Figure 4.2.1**). Conversely, the *PRL* expression was slightly lower in the CNS-challenged cells at 3h, 6h and 12h after infection, although the difference was only significant at 3h (respective *P*-values = 0.05, 0.84 and 0.22). It should be noted that, due to the pooling of the 6-well plates per inoculum and per sampling time, this experimental set-up was not suitable for comparing the *PRL* expression between the individual CNS strains.

To our knowledge, this is the first study that demonstrates that MAC-T cells are capable of expressing *PRL*. The MAC-T cell line is a frequently used immortalized mammary cell line for *in vitro* studies on lactation or the mammary immune response (Jedrzejczak and Szatkowska, 2014; Günther et al., 2016). Although the immortalized cell line consists of a heterogeneous cell population (Zavizion et al., 1995) with a limited production of milk-specific proteins (Rose and McConochie, 2006), MAC-T cells respond with the same kinetic (albeit weaker) to mastitis pathogens as primary MEC (Günther et al., 2016).
However, the cell line used in this study cannot account for other external factors that might have otherwise influenced the *PRL* expression. For instance, the lactation stage and reproductive phase also affect the expression of *PRL* and its receptor in the mammary glands of other animals (Iwasaka et al., 2000; Morammazi et al., 2016). Prolactin and the PRL receptor might not only be synthetized by the epithelial cells, but also by the stromal tissue of the mammary gland (Camarillo et al., 2001; Zinger et al., 2003). Furthermore, the amount of mRNA does not necessarily correlate with the amount of protein that is produced (Guo et al., 2008).
Normally, pituitary-derived PRL is transported through the MEC via transcytosis following the binding on its respective membrane receptor (Ollivier-Bousquet, 1998). However, an intramammary inflammation promotes the paracellular passage of macromolecules due to the disruption of the blood-milk barrier (Nguyen and Neville, 1998). Based on the results of this study, we speculate that the elevated PRL level found in the milk of CNS-challenged dairy cattle (Piccart et al. 2015) might rather be the result of leaky tight junctions.

4.2.5 Conclusion

In conclusion, we observed that the *PRL* gene is indeed expressed in bovine MAC-T cells, but we did not find a higher *PRL* expression in cells challenged with *S. chromogenes* TA*, S. chromogenes* IM or *S. fleurettii*. The expression of *PRL* does not appear to be upregulated in MAC-T cells by these particular CNS strains.

4.2.6 Acknowledgements

The authors would like to express their gratitude to Carolien Rogiers of the Department of Comparative Physiology and Biometrics at the Faculty of Veterinary Medicine of the University of Ghent for her great support and advice in the laboratory.

Table 4.2.1. Gene, primer, amplicon and qPCR information.

Gene Symbol	Gene name	NCBI Gene ID	Genbank accession number	Sequence (5' to 3')	Product size (bp)	Ta (°C)	E (%)	R^2
H2A	Histone-2-alpha	506900	NM_001205596.1	GTCGTGGCAAGCAAGGAG GATCTCGGCCGTTAGGTACTC	182	60	91.3	0.999
RSP15A	Ribosomal protein S15a	337888	NM_001037443.2	AATGTCCTGGCTGATGCTCT GGGCTGATCACTCCACACTT	218	59	90.1	0.999
PRL	Prolactin	280901	NM_173953.2	TGGAGCCAAAGAGACTGAGC TGGAGCCAAAGAGACTGAGC	181	58	103.0	0.998
UBC	Ubiquitin C	444874	NM_001206307.1	AGTTCAGTCTTCGTTCTTCTGTG GGTTTTACCAGTGAGGGTCTT	88	58	89.2	0.995
ACTB	Actin, beta	280979	NM_173979.3	CCTCACGGAACGTGGTTACA TCCTTGATGTCACGCACAATTT	87	58	90.2	0.996

TA (°C) = Optimal annealing temperature; E (%) = PCR efficiency; R^2 = squared correlation coefficient

4.2.7 References

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Chapter 5

General discussion

5.1 Introduction

Coagulase-negative staphylococci are among the most prevalent bacteria involved in subclinical bovine mastitis (Barkema et al., 1999; Pitkälä et al., 2004; Sampimon et al., 2009; Tenhagen et al., 2009; Pankey et al., 2011; Piepers et al., 2011; Rall et al., 2014). Initially, bovine-associated CNS were considered as one uniform group (Hogan et al., 1987; Nickerson and Boddie, 1994), even though they cover a wide range of habitats; from the dairy cow's surrounding environment to the external and internal milieu of the mammary gland (De Visscher et al., 2014). Also, CNS-infected dairy cattle produce more milk than their non-infected herd mates according to certain studies (Schukken et al., 2009; Piepers et al., 2013).

Over the past decennium, numerous studies have shown that the clinical impact of CNS on bovine udder health varies considerably between different species. However, knowledge on intraspecies diversity is still limited. The main objective of this thesis was to develop a deeper understanding of how a dairy heifer's immune system responds to different CNS strains from diverging habitats by means of a live challenge study. In addition, we examined the role of the lactation hormone PRL in CNS mastitis. This work focused on three particular CNS strains, each representative for their particular habitat: *S. fleurettii* from the dairy barn environment, *S. chromogenes* IM from a persistent IMI and *S. chromogenes* TA from the teat apex of a heifer (**Chapter 2**). To characterize the host response of dairy heifers infected with these CNS strains, eight Holstein heifers were subjected to an experimental challenge (**Chapter 3**). The same experiment was used to study the milk PRL concentration in response to CNS-infection (**Chapter 4.1**). Finally, the *PRL* expression in MEC following CNS-infection was examined using an *in vitro* study with MAC-T cells (**Chapter 4.2**).

5.2 Response of heifers to host-adapted versus environmental CNS species

In terms of ecology, CNS can be roughly divided into "host-adapted" and "environmental" species or strains (Piessens et al., 2012a; Fry et al., 2014; De Visscher et al., 2014). As the name suggests, environmental CNS species are typically found at different sites in the farm environment (e.g. floors,

sawdust, air… [Piessens et al., 2011]), and they do not require a host for their survival (Zadoks and Schukken, 2006). Environmental CNS species are rarely associated with IMI (De Visscher et al., 2014). Still, for some mastitis-causing CNS species, the environment can act as a reservoir. For instance, species such as *S. haemolyticus* and *S. simulans* survive well in the environment, yet they are often implicated in IMI as an opportunistic pathogen (Piessens et al., 2011; De Visscher et al., 2014). A number of live challenge studies have sought to define the host response following an IMI with staphylococci (**Table 5.1**). This work is the first to study the host response of dairy heifers following an infection with the archetypical environmental CNS species *S. fleurettii*. However, since these studies all use a different inoculum dose or host species, it is difficult to compare their results. We chose to inoculate the primiparous cows with *S. fleurettii*, although other CNS species, like *S. equorum* (another typical environmental CNS species), would have been a suitable alternative (Vanderhaeghen et al., 2015).

The main question of this thesis comes down to why some CNS (such as *S. chromogenes*) are commonly found in bovine milk causing IMI, whereas other species (like *S. fleurettii*) only rarely appear in milk. In **Chapter 3**, we demonstrated that the *S. fleurettii*-strain is unable to survive in the mammary gland for a long period of time, indicating that it is not a matter of lack of exposure. *Staphylococcus fleurettii* was rapidly (i.e. within 12h) eliminated from the challenged quarters, as opposed to the two *S. chromogenes* strains. The limited bacterial growth is in line with findings of Breyne et al. (2015), who retrieved less CFU of *S. fleurettii* than of both *S. chromogenes* strains 28h and 48h after challenging the mammary glands of mice, using the exact same isolates. *Staphylococcus fleurettii* did not appear to grow in the mammary gland of mice, since the retrieved bacterial numbers were lower than the bacterial count of the initial inoculum. In **Chapter 3**, we showed that the challenge with *S. fleurettii* triggered a slightly faster immune response than *S. chromogene*s IM or TA. Although the overall magnitude of the SCC response did not differ between *S. fleurettii* and either *S. chromogenes* strain, the influx of PMN occurred somewhat earlier in the *S. fleurettii*-challenged quarters, as indicated by the higher SCC and lower proportion of apoptotic (Annexin V-FITC+/PI-) and necrotic (Annexin V-

FITC+/PI+) PMN at 6h after inoculation. This was in all likelihood the result of the earlier IL-8 response. The chemotactic cytokine IL-8 was seen to be increased more rapidly (i.e. within 6h) in some of the *S. fleurettii*-challenged quarters, although the cytokine response was generally very low and varied too much between individual animals and quarters to draw any definite conclusions.

One potential immune evasion tactic of the staphylococci is to invade the host cells (Dego et al., 2002). Based on the fast elimination of *S. fleurettii* in the mammary gland, one might expect that *S. fleurettii* would invade MEC slower than *S. chromogenes*. Indeed, Souza et al. (2016) -using the exact same isolates- observed that the adhesion and internalization capacity of *S. fleurettii* was lower than that of *S. chromogenes* IM after 12h of incubation, yet higher than that of *S. chromogenes* TA. The efficacy with which bacteria can attach to MEC is presumably also influenced by various host factors (Van Belkum et al., 2002). As such, Hyvönen et al., (2009) could not find any differences in the *in vitro* adhesion or internalization between CNS strains (including *S. chromogenes*, *S. epidermidis* and *S. haemolyticus*) causing persistent IMI and transient IMI.

All in all, *S. fleurettii* evoked a mild clinical response in the dairy heifers. For one, there were no visible, clinical signs of mastitis in any of the challenged quarters. Also, the overall loss in milk yield following inoculation was less pronounced in *S. fleurettii*-challenged quarters compared to quarters challenged with either *S. chromogenes* strain (**Chapter 4.1**).

Table 5.1. An overview of experimental challenge studies with staphylococci that outline the host response of ruminants.

Staphylococcus spp.	Inoculum dose	Animals	N[1]	DIM[2]	Host response	Authors
S. aureus	72 CFU	Holstein cows	8	214 ± 8.67	- Mild clinical symptoms - No detectable quantities of IL-8 or TNF-α - Increase in IL-1β, IL-10, IL-12, and IFN-γ - Increase in SCC within 24h - *S. aureus* recovered in 6/8 challenged quarters after 7 days	Bannerman et al., 2004
	50 – 100 CFU	Holstein cows	6	Mid-lactation	- Subclinical mastitis - No detectable quantities of IL-1β, TNF-α or IL-8 - Slow and moderate increase in SCC after 24h - Bacterial growth peaked at day 6	Riollet et al., 2000
S. simulans	5.7 x 10^6 CFU	Ayrshire & Holstein primiparous cows	8	Mid-lactation	- Mild to moderate clinical symptoms - Increase in IL-1β, IL-8 and TNF-α - After 2 weeks, the infection persisted in 6/16 quarters	Simojoki et al., 2011
	5.0 x 10^7 CFU	Welsh-Mountain & Dorset-Horn ewes	17	6 – 16	- Subclinical mastitis - Increase in SCC - *S. simulans* was isolated (intermittently) for at least 20 days after inoculation	Fthenakis and Jones, 1990
S. fleurettii	1.0 x 10^6 CFU	Holstein primiparous cows	8	78 – 278	- No visual symptoms of mastitis - Increase in SCC within 6h & significant increase in PMN viability - Infection eliminated within 12h	Chapter 3

Staphylococcus spp.	Inoculum dose	Animals	N[1]	DIM[2]	Host response	Authors
S. epidermidis	3.3 x 10^7 CFU	Merino ewes	5	3 months	- Mild clinical symptoms - Increase in IL-8 from 4h until day 3 - Significant elevation of IL-1β in 1 ewe - SCC increased after 4h and peaked after 1 day - *S. epidermidis* was found sporadically in the milk of 4 ewes until 10 weeks, while 1 ewe eradicated the infection after 8h	Winter et al., 2003
	5.7 x 10^6 CFU	Ayrshire & Holstein primiparous cows	8	Mid-lactation	- Mild to moderate clinical symptoms - Increase in IL-1β, IL-8 and TNF-α - After 2 weeks, the infection persisted in 5/16 quarters	Simojoki et al., 2011
	2.8 x 10^6 CFU	Merino ewes	5	3 months	- Mild clinical symptoms - Increased leukocyte count in milk - Increase in IL-6, IL-8, IL-1β - *S. epidermidis* was shed intermittently until the end of the trial (144h)	Winter and Colditz, 2002
S. chromogenes	2.1 × 10^6 CFU	Holstein primiparous cows	6	4 weeks	- Mild clinical symptoms - Increase in SCC within 12h, peaking at 30h - *S. chromogenes* was eliminated within 46h, except in 1 cow that developed a persistent infection	Simojoki et al., 2009
	1.0 x 10^6 CFU	Holstein primiparous cows	8	78 – 278	- No visual symptoms of mastitis - Increase in SCC within 9h & significant increase in PMN viability in *S. chromogenes* IM challenged quarters - *S. chromogenes* still present in milk at 78h	Chapter 3

[1] Number of challenged animals; [2] Days in lactation at the moment of inoculation.

5.3 Intraspecies diversity within *Staphylococcus chromogenes*

Thanks to the increasing availability of low-cost molecular identification techniques, we have recently gained more insight into the strain-specific virulence of mastitis pathogens. The heterogeneity of the mastitis pathogen *S. aureus* is well documented, in contrast to that of other staphylococci. Although most of the *S. aureus* strains causing bovine mastitis can be traced back to a limited number of lineages or clonal complexes (Kapur et al., 1995; Barkema et al., 2006; Delgado et al., 2011), the strains often differ in their ability to invade MEC, produce biofilm, respond to antibiotic treatment, affect milk yield, evoke a SCC response or spread from cow to cow (Barkema et al., 2006; Zadoks et al., 2011; Bardiau et al., 2014). While some studies conclude that extramammary *S. aureus* strains (e.g. isolated from skin, perineum, nares, etc.) are indistinguishable by molecular typing from strains involved in IMI (Haveri et al., 2008; Capurro et al., 2010), others do report the existence of tissue-specific strains (Zadoks et al., 2002; Van Leeuwen et al., 2005).

The intraspecies diversity of CNS, on the other hand, has long been overlooked. Although several studies have used molecular typing (e.a. pulsed field gel electrophoresis [PFGE]) for strain differentiation of bovine CNS (Thorberg et al., 2006; Mørk et al., 2012; Bexiga et al., 2014), there is not much known at the present time about the clinical importance of these different individual strains. **Chapter 3** of this thesis therefore focused on the host response and clinical impact of different strains of *S. chromogenes*, the most commonly isolated CNS species in dairy cattle with an IMI (Sampimon et al., 2009; Thorberg et al., 2009; Piessens et al., 2011). To address the potential tissue-specificity of *S. chromogenes*, two separate strains were selected; i.e. the so-called “teat apex strain” and “intramammary strain”. The *S. chromogenes* strains challenged the innate immune system in varying degrees (**Table 5.2**). The *S. chromogenes* IM strain appeared to be better equipped to invade and colonize the bovine mammary gland than *S. chromogenes* TA. Consequently, *S. chromogenes* IM induced the largest cellular response and resulted in the greatest production loss. Even though *S. chromogenes* IM was originally isolated from a cow with a persistent IMI lasting for more than 10 months, both *S. chromogenes*-strains were eliminated within 6 days. In another live challenge study with *S. chromogenes* (Simojoki et al., 2009),

one out of six heifers developed a persistent IMI, seeing that the bacteria were still present after 7 days in the challenged quarter. It should be emphasized that the duration of the infection not only depends on the virulence of the strain, but also on various host factors and the initial inoculum dose. The host response of the mammary gland also responds to pathogens in a dose-dependent manner (Günther et al., 2010). In case of experimental *E. coli* mastitis, lowering the inoculum dose results in a slower bacterial clearance, and is preceded by excessive bacterial growth (Shuster et al., 1996; Vangroenweghe et al., 2004). We assume that the high inoculum dose used in our experimental study (i.e. 1.0 x 10^6 CFU) might have triggered a more aggressive host response than is the case with natural infections, possibly shortening the duration of infection.

Yet, *S. chromogenes* is also able to colonize the host (i.e. the cow's body) without causing damage (i.e. mastitis). The species can be found on the teat skin, streak canal, hair coat, nares, vagina and perineum of healthy dairy cattle (White et al., 1989; De Vliegher et al., 2003; Taponen et al., 2008; De Visscher et al., 2016), though not necessarily on every cow (Braem et al., 2013; De Visscher et al., 2014). While *S. chromogenes* is rarely found on the teat skin of lactating cows (Braem et al., 2013; De Visscher et al., 2014), the species appears to be omnipresent on the teats of dry cows and pregnant heifers (De Visscher et al., 2016b). Certain pulsotypes of *S. chromogenes* are found both in the milk and on extramammary sites (Taponen et al., 2008). Some authors reported an association between the extramammary colonization by *S. chromogenes* and IMI (Taponen et al., 2008; Quirk et al., 2012; De Visscher et al., 2016a), while others found no such relation (De Vliegher et al., 2003). In fact, De Vliegher et al. (2003) stated that teat apex colonization with *S. chromogenes* might be protective against IMI in early lactation, although no strain typing was performed in this study.

Table 5.2. A basic summary of the known differences between strains of *S. chromogenes* isolated from a teat apex (TA) and from an intramammary infection (IM) in terms of their interaction with the host.

Parameter	Study object	*S. chromogenes TA*	*S. chromogenes IM*	Author
Adhesion capacity	MAC-T cells	+	+ +	Souza et al. (2016)
Invasion capacity	MAC-T cells	+	+ +	Souza et al. (2016)
SCC increase	Heifers	+	+ + N.S.	Chapter 3
% Apoptotic milk PMN	Heifers	+ + N.S.	+	Chapter 3
Bacterial growth in the mammary gland	Heifers	+	+ +	Chapter 3
Milk yield	Heifers	–	– –N.S.	Chapter 4.1
Milk PRL increase	Heifers	+	+	Chapter 4.1
Local IL-1β level	Mice	+ + N.S.	+	Breyne et al. (2015)
	Heifers	+ D.S.	+	Chapter 3
Local TNF-α level	Mice	–	–	Breyne et al. (2016)
	Heifers	+D.S.	+	Chapter 3
Local IL-8 level	Heifers	–	+ +D.S.	Chapter 3

N.S. No statistically significant difference.

D.S. Based on descriptive statistics.

5.4 The role of PRL in CNS mastitis

Although certain CNS species and strains are associated with persistent IMI and elevated SCC levels, some studies suggest that CNS-infected dairy cattle produce more milk throughout the lactation period than their non-infected herd mates (Compton et al., 2007; Schukken et al., 2009; Piepers et al., 2010). Several hypotheses have been put forward, but there is still no sound biological explanation for this unexpected observation (Piepers et al., 2013). In **Chapter 4.1**, the milk production was monitored for only three days following the experimental challenge with *S. fleurettii*, *S. chromogenes* TA and *S. chromogenes* IM. Even though the milk production was suppressed in all challenged quarters, the overall production losses were minimal. These results align with the work of Pearson et al., 2013 and Tomazi et

al., 2015, who observed little or no decline in milk yield in dairy cattle following a CNS IMI. Still, the experiment in **Chapter 4.1** only focused on the acute phase of inflammation, and is therefore not suitable for inferring definitive conclusions regarding the milk production throughout lactation following an IMI with the 3 CNS strains.

The hypothesis that PRL stimulates the milk production in response to an infection with CNS was examined in **Chapter 4**. It had previously been demonstrated that PRL is involved in the pathogenesis of bovine mastitis by stimulating the inflammatory response in the MEC through the activation of the NF-κB (Boutet et al., 2007). Consequently, this thesis postulated that the local production of PRL would increase in the mammary gland following an infection with CNS. The inflammation-induced PRL might then possibly stimulate autocrine milk production, although this association was not directly studied in this work. **Chapter 4.1** shows that, over time, PRL is higher in the milk of CNS-infected quarters compared to uninfected quarters. No significant difference was found in the PRL level between the three different CNS strains. Approximately 28 hours after the inoculation, the PRL concentration started to decline in both the challenged and control quarters. The final PRL levels at the end of the experiment, 78h after the inoculation, were lower than the initial, pre-challenge values. One possible explanation for the overall decline in milk PRL, is that the sampling interval lengthens as the experiment progresses. It has previously been demonstrated that a higher milking frequency leads to higher circulating PRL levels (Bar-Peled et al., 1995). Therefore, the overall higher PRL concentration at the beginning of the experiment might be the result of the higher sampling frequency. Another possibility is that the secretion of (pituitary) PRL is controlled through a classic negative feedback loop (Anderson et al., 2006), either by promoting the hypothalamic dopamine release or by directly inhibiting the release of PRL in the lactotrophs (Bernard et al., 2015).

Chapter 4.2 confirmed that the bovine mammary gland is indeed able to synthesize PRL, similar to humans, rats or goats. No increase in mRNA of PRL was found in MEC after challenging them with *S. chromogenes* TA, *S. chromogens* IM or *S. fleurettii*. The results imply that the expression of the PRL gene

is not directly altered by these CNS strains. To date, little is known about the physiological factors that steer the secretion of mammary PRL (Chen et al., 2012).

The elevated milk PRL concentration observed in **Chapter 4.1** might not be the result of a local response of the MEC. Normally, systemic PRL is transported to the milk via transcytosis (Ollivier-Bousquet, 1998), except when the epithelial integrity is disrupted (in this case, due to the intramammary infection). Hence, it is likely that PRL leaked from the capillaries of the challenged udder quarters into the milk compartment. The slightly increased levels of sodium and chloride found in the milk of challenged quarters also support this idea. One could question the biological significance of blood-borne proteins in the pathogenesis of mastitis. Still, it should be kept in mind that the general correlation between mRNA and the final protein levels is known to be poor (Maier et al., 2009). It is therefore difficult to infer definitive conclusions about the abundance of mammary PRL based only on the expression study in **Chapter 4.2.**

5.5 Evaluation of the experiments

5.5.1 In vivo experiment

During the *in vivo* experiment (**Chapter 3 & 4.1**), eight Holstein heifers received an intramammary infusion with either *S. fleurettii*, *S. chromogenes* TA, *S. chromogenes* IM or PBS in each udder quarter. Three quarters were inoculated with a bacterial dose of 1.0 x 10^6 CFU suspended in 5 mL PBS, whereas the fourth quarter –infused with an equal volume of sterile PBS- was used as an internal control. This type of experimental set-up is referred to as a "split-udder design", which has its inherent strengths and weaknesses.

One advantage of this particular study design is that the heifers act as their own control group, allowing for a smaller number of required experimental animals and a reduction in variability owing to individual differences. This is in agreement with the reduction principle of the “3Rs” (Replacement, Reduction and Refinement), the ethical framework for humane experimentation. A split-udder design assumes that all neighboring udder quarters are separate, individual anatomical entities within the same animal. However, previous research has demonstrated that this is not necessarily the case. An experimental infection of an udder quarter with *E. coli* or *S. aureus* results in changes in gene expression in neighboring, uninfected quarters (Mitterhuemer et al., 2010; Jensen et al., 2013). But even though genes associated with the local immune response are upregulated in untreated control quarters (Jensen et al., 2013), the SCC or the PMN viability does not seem to increase significantly in uninfected quarters compared to naturally infected quarters (Blagitz et al., 2015). In the experimental CNS-challenge described in **Chapter 3**, the SCC nor the viability increased considerably, and the cytokines TNF-α, IL-8 or IL-1β were not detected in uninfected control quarters following the challenge with sterile PBS. Therefore, we believe that the effect of any potential “cross-talk” between neighboring quarters on our results is of minor importance.

In vivo experiments in dairy cattle also have certain disadvantages. For one, the animals have to be taken out of their usual environment, which affects their stress level. It has been well documented that stress can modulate the immune response and circulating PRL levels of animals (Carroll & Forsberg, 2007;

García-Ispierto et al., 2009). Glucocorticoid hormones, associated with a stress response, can delay the apoptosis in human PMN (Liles et al., 1995). This might explain why the proportion of apoptotic PMN (**Chapter 3**) already decreased in all mammary quarters before the actual inoculation procedure had taken place. For future *in vivo* experiments, we suggest incorporating a longer (> 48h) adaptation period for letting the cows adjust to their new surroundings, or -if feasible- performing the experiment in their usual setting together with familiar herd mates.

Another limitation of our study, is that only heifers in mid-lactation were challenged with the CNS strains, making it difficult to extrapolate our results to multiparous cows, or cows in a different stage of lactation. As mentioned in **Chapter 2**, we chose to only include primiparous cows in our study, because the prevalence of CNS IMI is higher in primiparous cows than in multiparous cows (Sampimon et al., 2009; De Visscher et al., 2016). The heifers were knowingly selected according to their CXCR1 genotype for an additional experiment studying the effect of a polymorphism (c.980 A>G) in the CXCR1 gene on the innate immune response (Verbeke et al., 2015). Although the PMN migration was slightly slower in the *S. chromogenes* IM-infected quarters of the c.980AG heifers than the c.980GG heifers, it is unlikely to have had any effect on the conclusions of this thesis. Whether or not the heifers had previously been infected with either CNS strain under natural circumstances (and therefore might have established an antigen-specific memory through the adaptive immune system [Rainard et al., 2016]) can never be completely ruled out though.

Because of these limitations, alternative *in vivo* models for exploring bovine mastitis have been developed in mice (Brouillette and Malouin, 2005). They are less expensive, less cumbersome and easier to control. The mammary glands of mice and cows share certain anatomical characteristics. For one, both species have one teat opening and one primary duct per mammary gland. Similar to cows, the glands of mice are structurally independent from each other (Notebaert and Meyer, 2006). On the other hand, the composition of murine milk is fundamentally different (Gors et al., 2009), and mice have less resident phagocytic cells in their mammary glands (Notebaert and Meyer, 2006). All limitations

considered, we believe that an *in vivo* challenge in dairy cattle is still the most accurate method for mimicking natural IMI in bovines.

5.5.2 In vitro experiment

In vitro or *ex vivo* studies, using an immortalized cell line (as in **Chapter 4.2**) or primary bovine cells, have also contributed to our understanding of bovine mastitis. These types of studies are generally inexpensive, fast and easy to handle, making it a popular model for studying intramammary infections. However, *in vitro* studies cannot fully account for the various host factors that affect the course of an infection, such as parity (Mehrzad et al., 2002), stage of lactation (Mallard et al., 1998), and genetic variability (Rupp and Boichard, 2003). Primary cultures of bovine MEC, taken from mammary tissue, are thought to be a better model for studying the *in vivo* conditions of the mammary gland (Rabot et al., 2006). Although they reflect the physiological and metabolic processes of epithelial cells more closely, primary MEC quickly lose their original characteristics after a number of passages (Matitashvili et al., 1997). In **Chapter 4.2**, we chose to infect MAC-T cells to study the *PRL* expression in response to the 3 CNS strains. MAC-T cells, immortalized by originally transfecting mammary cells with Simian virus 40 (SV40), consist of a heterogeneous cell population (excluding myoepithelial cells [Zavizion et al., 1995]). The MAC-T cell line has the ability to express milk proteins (i.e. β-casein, α-lactalbumin, etc.), GH-receptors, and various cytokines in response to an infection (Zhou et al., 2008; Günther et al., 2016). Although the cytokine response of MAC-T cells is weaker than that of primary MEC, the cell line responds in a similar manner (Günther et al, 2016). Primary bovine MEC might have displayed a higher *PRL* expression than MAC-T cells in **Chapter 4.2**, but we assume that this would not have affected the conclusion of the study.

5.5.3 The inoculum dose

Simulating a natural IMI under experimental conditions is inherently difficult. One particular issue is selecting an inoculum dose that corresponds with natural infections. The *in vivo* study of **Chapter 3** and

Chapter 4.1 was preceded by a limited dose-response trial, in which a multiparous cow was challenged in each quarter with increasing doses of *S. chromogenes* IM (2.5 x 10^5, 5.0 x 10^5, 1.0 x 10^6 and 2.0 x 10^6 CFU in 5 mL PBS) as described by Verbeke et al. (2015). Only the highest dose, 2.0 x 10^6 CFU, resulted in local, clinical symptoms (i.e. a swollen, reddish mammary quarter). However, in the quarter that was challenged with the lowest inoculum dose, 2.5 x 10^5 CFU, the bacteria were eliminated within 20h. Unlike Simojoki et al. (2009), we did not necessarily want to induce clinical mastitis in the dairy heifers. Generally speaking, CNS infections are mostly associated with subclinical or mild clinical symptoms (Taponen et al., 2006; Supré et al., 2011). That is why we chose for the inoculum dose of 1.0 x 10^6 CFU. Still, only a limited number of strains were studied in this thesis, which is insufficient to represent entire communities of CNS within the host-associated or environmental niche. Furthermore, considering the strain differences we found in *S. chromogenes*, we cannot exclude that *S. fleurettii* strains are equally diverse in terms of pathogenicity.

5.6 Conclusions and practical implications

In this work, we focused on the host response in dairy heifers following three CNS field strains from diverging environments: *S. chromogenes* TA, *S. chromogenes* IM and *S. fleurettii*. The effects of these strains have been studied in MEC (**Chapter 4.2**; Souza et al., 2016), mice (Breyne et al., 2015) and dairy heifers (**Chapter 3** and **Chapter 4.1**). These results confirm that (quarters of) dairy heifers respond differently to host-adapted versus environmental CNS. *Staphylococcus fleurettii* (or at least the particular strain used in this research) is less accustomed to the bovine mammary gland than the typically host-adapted species *S. chromogenes*. Furthermore, strains within *S. chromogenes* can differ in their ability to cause damage to the host (**Chapter 3** and **Chapter 4.1**). The *S. chromogenes* strain derived from an extramammary site, i.e. the teat apex, is less able to grow in the mammary gland than the other *S. chromogenes* strain isolated from a mastitis case. Overall, *S. chromogenes* TA induced a less pronounced inflammatory response. The results in **Chapter 3**, combined with experimental challenge studies in mice and MEC, suggest that the strains within *S. chromogenes* show a variable degree of

pathogenicity and tissue-specificity. Of course, intraspecies diversity in terms of pathogenicity is not unique to *S. chromogenes*, and has been observed in other mastitis bacteria as well, such as *S. uberis* (Tassi et al., 2013), *S. aureus* (Middleton and Fox, 2002; Haveri et al., 2007) and *E. coli* (Dogan et al., 2006; Lippolis et al., 2014). The degree in which bacteria cause damage to their host, is the result of a complex interplay between both the microorganism and the host. Distinguishing pathogenic from less pathogenic strains or isolates within one species, and anticipating their clinical impact, is therefore not a straightforward process. In practice, it often boils down to the decision on whether or not to treat a case of bovine mastitis, and whether or not to cull a chronically infected cow. However, in case of the two *S. chromogenes* strains studied in this thesis, the difference in the clinical impact and loss in milk yield in dairy heifers is ultimately limited (**Chapter 3** and **Chapter 4.1**). Since the use of veterinary antimicrobials is under scrutiny in the European Union, subclinical and mild cases of mastitis are often left untreated, assuming that the infection is self-limiting. Spontaneous cure rates in CNS are generally thought to be as high as 60 – 70% (McDougall, 1998; Wilson et al., 1999), although more recent studies report lower numbers (i.e. 12 to 45% [Deluyker et al., 2005; Taponen et al., 2006]). Because of their limited clinical importance, CNS are currently not routinely identified in veterinary laboratories at the species level. Therefore, routine strain-typing of CNS in practice seems out of the question for now. Still, addressing CNS infections can be of great value for dairy farms with a low bulk milk SCC that specifically strive for an even lower bulk milk SCC. In herds with a bulk milk SCC below 200,000 cells/mL, CNS infections contribute on average 17.9% of all somatic cells in the bulk tank (Schukken et al., 2009).

It was beyond the scope of this thesis to assess the effect of CNS as a bacterial group on the milk yield. Although PRL does increase in the quarters challenged with the three CNS strains (**Chapter 4.1**), there is inconclusive evidence that this is the result of a local PRL production in the mammary gland (**Chapter 4.2**).

5.7 Future research

The effect of CNS on the milk yield of dairy cattle remains unclear. A longitudinal study throughout lactation on the association between the milk yield and CNS infections can offer more insights. It might be opportune to monitor the milk production at quarter level (for instance at farms equipped with automatic milking systems), so one can compare the milk production of CNS-infected quarters to healthy, uninfected quarters. This minimizes the effect of confounding variables at the cow or herd level (Pyörälä and Taponen, 2009; Tomazi et al., 2015). Future studies should ideally identify the involved CNS at the subtype or strain level using genotypic methods. Strain typing is required in observational studies to ascertain whether the CNS infections are transient or persistent in nature. Pulsed field gel electrophoresis used to be considered the "gold standard", although it is quite labor-intensive and expensive (van Belkum et al., 2007). Depending on the study objective, multi locus sequence typing or whole genome sequencing might be more appropriate to differentiate isolates (Smith et al., 2005; Köser et al., 2012; Vanderhaeghen et al., 2015).

Strain typing is required in observational studies to ascertain whether the CNS infections are transient or persistent in nature. The results from **Chapter 3** also clearly demonstrate the existence of pathogenic diversity within CNS species (in this case *S. chromogenes*). Species and strain differences might account for the seemingly contradicting research findings regarding the milk production of CNS-infected cattle (Gröhn et al., 2004; Piepers et al., 2010; Tomazi et al., 2015). Studies considering CNS as one homogeneous group inevitably fall prey to ecological fallacy, i.e. the error that arises when drawing conclusions about individuals based on the observations of groups (Morgenstern, 1982). The same reasoning may hold true for studies focusing on the species level of CNS, given the strain diversity we found in *S. chromogenes*.

Strain typing of *S. chromogenes* is essential to further elucidate the association between teat apex colonization and IMI, or the inhibition of IMI. The teat apex strain used in this work, *S. chromogenes* TA, is capable of inhibiting the *in vitro* growth of mastitis field isolates of *S. aureus*, *S. uberis* and *S. dysgalactiae* to some extent (De Vliegher et al., 2004). This is likely the result of extracellular bacteriocins

produced by the *S. chromogenes* TA strain, as has been demonstrated in other CNS (Nascimento et al., 2005; Braem et al., 2014). Bacteria produce these substances as a tool for defending their niche against similar, competing microorganisms (Kommineni et al., 2015). It is still uncertain though whether teat apex colonization with *S. chromogenes* TA, or any other strain with similar inhibitory properties, is actually able to prevent IMI caused by Gram-positive mastitis pathogens in dairy cattle. Additional challenge studies are needed to verify this assumption. One possible experimental set-up is an split-udder or split-herd trial where the cows receive a "protective strain" on the skin of their teats (for instance, through swabbing or dipping of the teats). By determining the incidence of new IMI in the challenged and control teats over a period of time, one could determine the efficacy of the protective strain. However, such a trial is cumbersome, and moreover, restricted by biosafety legislations (Van Vaerenbergh et al., 2010). Therefore, this type of trial cannot be performed in commercial dairy farms. Another possibility is to inoculate a protective strain of interest directly into the teat or gland cistern of dairy cows (as described in this thesis), followed by an experimental superinfection with a major mastitis pathogen.

All in all, the concept of using live microflora to combat bacterial infections is not new (Woodward et al., 1987; Bouchard et al., 2015). One could wonder if certain strains of CNS can be utilized as an alternative (preventive) therapy for bovine mastitis. At this time, there is insufficient scientific evidence to presume that one or more CNS strains might be suitable as an intramammary probiotic. For one, the purported protective effect *in vivo* has never been traced back to a particular species or strain. Furthermore, their safety cannot be warranted. Multiple antimicrobial resistance genes (e.g. *mecA, blaZ*) have been found in CNS from bovine milk (Frey et al., 2013), creating a potential reservoir for transferring resistance to other bacteria (Piessens et al., 2012b). Even so, the notion of intramammary probiotics remains a promising track to combat and prevent bovine mastitis, and should be further investigated.

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Summary

Mastitis, an inflammation of the mammary gland, is a major issue in the dairy industry. It not only undermines the welfare of cows, but it also leads to considerable financial losses for the dairy farmer. Furthermore, mastitis is the primary indication for antimicrobial use in dairy cattle. That is why this disease requires our continuous attention.

Chapter 1 illustrates the importance of coagulase-negative staphylococci (CNS), the most common causative agent of mastitis in dairy cattle. Although CNS were initially considered as one large, homogenous group of bacteria, recent epidemiological studies demonstrate that some CNS species have a more severe clinical impact than others. However, the relative importance of intraspecies differences in terms of pathogenicity is unexplored. All in all, little is known about the host response of dairy cattle to different CNS species and strains.

Ecologically speaking, CNS can be divided into "host-adapted" and "environmental" species. Host-adapted species, such as *Staphylococcus chromogenes*, are mainly found in the milk or on the cow's body. Environmental species, like *Staphylococcus fleurettii*, on the other hand, mostly occur in the immediate surroundings of the cow and rarely in milk. Why some species thrive in the mammary gland, as opposed to other body sites or the surrounding environment, is not entirely clear. Furthermore, there are a number of observational studies showing that CNS-infected cows, against all odds, produce more milk than their non-infected herd mates. However, the involved CNS were never identified at species or strain level. The underlying mechanism for this apparent increased milk yield is still unclear.

Chapter 2 states the objectives of the thesis. The general purpose of this work is to broaden the existing knowledge of the host-pathogen interactions between heifer and ecologically diverse CNS species and strains, in order to explain the dichotomy between host-adapted and environmental CNS. This work focused on 3 different field strains: one particular environmental *S. fleurettii* strain originating from sawdust and two representative host-adapted *S. chromogenes* strains. One of those strains (*S. chromogenes* IM) was initially isolated from the left hind quarter of a multiparous cow with a persistent (> 300 days), while the other strain (*S. chromogenes* TA) was isolated from the teat apex of a heifer. Furthermore, the latter strain is able to inhibit the *in vitro* growth of major mastitis pathogens. An

additional objective of this thesis is the examination of prolactin (PRL) as a potential autocrine lactation hormone and immunomodulatory factor in CNS mastitis.

Eight healthy Holstein heifers in mid-lactation were inoculated with the three respective CNS strains to examine the species differences between *S. fleurettii* and *S. chromogenes* on the one hand, and the strain differences within *S. chromogenes* on the other hand (**Chapter 3**). Each mammary quarter was inoculated with one strain, or a sterile phosphate-buffer saline solution as control. Despite the high inoculum dose (1.0×10^6 colony-forming units), the three CNS strains evoked a relatively mild and self-limiting inflammatory response. These results confirm that the environmental species *S. fleurettii* is less adjusted to the internal milieu of the mammary gland than the host-adapted *S. chromogenes*. Contrary to *S. chromogenes*, *S. fleurettii* was rapidly (within 12h) eliminated from the quarters. The influx of polymorphonuclear neutrophils occurred a little sooner (within 9h) in the *S. fleurettii*-infected quarters, evidenced by the slightly higher somatic cell count (SCC) and the lower proportion of apoptotic neutrophils. The two *S. chromogenes* strains also differed in pathogenicity. *Staphylococcus chromogenes* IM showed a more distinct bacterial growth than *S. chromogenes* TA, and induced a higher SCC response. Even though *S. chromogenes* IM was originally isolated from a chronically infected quarter, the same exact isolate was not capable of colonizing the mammary tissue in this study. After all, the strain was eliminated from the quarters within one week.

Chapter 4 evaluates the PRL level in the milk during an experimental challenge with the three respective CNS strains. **Chapter 4.1** is based on the same *in vivo* challenge described in the previous chapter. During this trial, the quarter milk yield was determined and the milk PRL level was measured using a radioimmunoassay in the following 78h after inoculation. This study showed that the milk production decreased compared the control quarters, which is not entirely unexpected, since we only measured the milk yield during the acute phase of inflammation. *Staphylococcus chromogenes* IM resulted in the largest numeric production loss. The PRL-concentration was higher in the milk of CNS-infected quarters, although we found no considerable difference between the CNS strains. After inoculation, the sodium and chloride content of the milk slightly increased in the infected quarters,

indicating that the blood-milk barrier was somewhat disintegrated. This might indicate that the increased PRL is the result of circulatory PRL leaking from the blood to the milk component.

Chapter 4.2 is consistent with these findings. We investigated in an *in vitro* study whether the MEC respond to a CNS challenge by expressing the *PRL* gene. An immortalized epithelial cell line (MAC-T) was challenged with the same three CNS strains (in a 1:1 ratio of staphylococci to cells). Next, the expression of the PRL gene was quantified using qPCR. We found no evidence for a higher *PRL* expression in the infected cells compared to the control cells. Although bovines can produce PRL locally in the mammary gland, the expression of *PRL* does not appear to be regulated by an infection with these strains. However, it should be noted that the general correlation between mRNA and the final protein can be low, and that MAC-T cells might possibly not be the best reflection of a dairy cow's biological mammary tissue.

Finally, **Chapter 5** provides a summary of the obtained results and relates these findings to the existing body of research on CNS.

Samenvatting

Mastitis, oftewel ontsteking van het melkklierweefsel, is een van de belangrijkste aandoeningen in de melkveesector. Het ondermijnt niet alleen het welzijn van de koeien, maar leidt ook tot zware economische verliezen voor de melkveehouder. Bovendien is mastitis de primaire indicatie van antimicrobiële middelen bij melkvee. Daarom verdient deze aandoening bijzondere aandacht.

In **Hoofdstuk 1** wordt er dieper ingegaan op de coagulase-negatieve stafylokokken (CNS), de meest voorkomende mastitisverwekkers bij melkvee. Hoewel de CNS aanvankelijk als één grote, homogene groep bacteriën aanschouwd werden, tonen recente epidemiologische studies aan dat sommige CNS-soorten een zwaardere klinische impact hebben dan andere. Of er binnen één species ook onderlinge verschillen bestaan tussen stammen, is niet duidelijk. Bovendien is er weinig geweten over de afweerreactie van vaarzen tijdens CNS-infecties.

Ecologisch gezien kunnen CNS ingedeeld worden in “gastheergebonden” en “omgevingsgebonden” soorten. Gastheergebonden CNS-soorten, zoals *Staphylococcus chromogenes*, worden voornamelijk teruggevonden in de melk of op het lichaam van de koe. Omgevingsgebonden soorten, zoals *Staphylococcus fleurettii*, worden daarentegen zelden geïsoleerd uit melk, maar komen voornamelijk voor in de stalomgeving. Waarom sommige CNS soorten vaker voorkomen in de uier dan anderen, is echter niet geheel duidelijk. Daarnaast blijkt uit een aantal observationele onderzoeken dat CNS-geïnfecteerde koeien -tegen alle verwachtingen in– een hogere melkgift hebben dan niet-geïnfecteerde koeien, ook al werden de betrokken isolaten in deze studies meestal niet nader geïdentificeerd op species- of stamniveau. Het onderliggende mechanisme achter deze zogenaamde productieverhoging is vooralsnog onduidelijk.

In **Hoofdstuk 2** worden de doelstellingen van de thesis aangehaald. Het overkoepelende doeleinde van dit werk is het verbreden van de bestaande kennis rond de gastheer-pathogeen interactie tussen vaarzen en ecologisch diverse CNS soorten en stammen, om de dichotomie tussen gastheer- en omgevingsgebonden CNS soorten te helpen verklaren. Er wordt gefocust op drie verschillende veldstammen, waarvan één specifieke omgevingsgebonden *S. fleurettii*-stam uit zaagsel en twee representatieve gastheergebonden *Staphylococcus chromogenes*-stammen. De ene *S. chromogenes*-

stam (IM) werd oorspronkelijk geïsoleerd uit het linker achterkwartier van een multipare koe met een chronische infectie (> 300 dagen), terwijl de andere stam (TA) teruggevonden werd op de speentop van een vaars. Tevens kan deze stam ook de groei van andere mastitiskiemen in cultuur inhiberen. Een bijkomende doelstelling van deze thesis is het bestuderen van de rol van prolactine (PRL) in CNS mastitis, als mogelijk autocrien lactatiehormoon en immunomodulerende factor.

Om enerzijds de soortverschillen tussen *S. fleurettii* en *S. chromogenes*, en anderzijds de stamverschillen binnen *S. chromogenes* te bestuderen, werden acht gezonde Holstein vaarzen in mid-lactatie geïnoculeerd met de respectievelijke stammen (**Hoofdstuk 3**). De vier uierkwartieren van elke vaars werden simultaan geïnoculeerd met één van de drie beschreven CNS-stammen, of een steriele fosfaatgebufferde zoutoplossing ter controle. Uit het experiment bleek dat, ondanks de hoge infectiedosis van 1,0 x 10^6 kolonievormende eenheden, de drie stammen een zelflimiterende infectie en milde ontstekingsreactie uitlokten. De resultaten bevestigen dat de omgevingsgebonden kiem *S. fleurettii* wel minder goed geadapteerd is aan het inwendige milieu van de uier dan de gastheergebonden *S. chromogenes*. *Staphylococcus fleurettii* werd immers snel (binnen 12u) geëlimineerd uit het kwartier, in tegenstelling tot *S. chromogenes*. Hoewel het celgetal (SCC) op dezelfde manier doorheen de tijd evolueerde, kwam de influx van neutrofielen in de *S. fleurettii*-geïnfecteerde kwartieren iets vroeger (binnen 9u) op gang. Dit blijkt uit het hogere SCC en het lagere aandeel apoptotische neutrofielen in de melk. Ook tussen beide *S. chromogenes*-stammen blijkt er een verschil te bestaan wat betreft de virulentie. *Staphylococcus chromogenes* IM vertoonde een meer uitgesproken bacteriële groei dan *S. chromogenes* TA, en wekte eveneens een hogere SCC respons op. Hoewel *S. chromogenes* IM oorspronkelijk geïsoleerd werd uit een chronisch geïnfecteerd kwartier, was hetzelfde isolaat in deze studie niet in staat om het melkklierweefsel te koloniseren. Ongeveer een week na inoculatie waren alle bacteriën immers verdwenen uit de geïnfecteerde kwartieren.

In **Hoofdstuk 4** wordt het PRL gehalte in de melk bestudeerd tijdens de experimentele infectie met de drie CNS-stammen. **Hoofdstuk 4.1** stoelt op dezelfde *in vivo* studie die reeds aangehaald werd in het vorige hoofdstuk. Tijdens deze proef werd ook de melkproductie per kwartier gemeten en het PRL

gehalte in de melk bepaald tot 78u na inoculatie via een radioimmunoassay. Uit deze studie bleek dat de melkproductie in de geïnfecteerde kwartieren daalde ten opzichte van de controlekwartieren. Dit lag in de lijn der verwachtingen, aangezien we de melkproductie enkel tijdens de acute fase van de inflammatie opgemeten hebben. Het productieverlies was op zich het grootst in de *S. chromogenes* IM-geïnfecteerde kwartieren. Het PRL gehalte lag ook hoger in de melk van de CNS-geïnfecteerde kwartieren dan in de niet-geïnfecteerde kwartieren, hoewel er geen noemenswaardig verschil aangetoond kon worden tussen de CNS-stammen onderling. Na inoculatie, steeg het Na^+ en Cl^- gehalte in de melk van de geïnfecteerde kwartieren, wat aangeeft dat de bloed-melk barrière enigszins aangetast werd. Dit kan er mogelijks op wijzen dat PRL vanuit de bloedbaan naar het melk-compartiment doorsijpelt.

Hoofdstuk 4.2 sluit aan op deze bevindingen. Of de epitheliale cellen van de melkklier überhaupt kunnen reageren op een CNS-infectie door middel van expressie van het *PRL*-gen werd onderzocht in een *in vitro* studie. Een geïmmortaliseerde epitheliale cellijn (MAC-T) werd namelijk geïnfecteerd met dezelfde 3 CNS-stammen (volgens een 1:1 ratio van stafylokokken op cellen). Vervolgens werd de expressie van het *PRL* gen gekwantificeerd via qPCR. Deze studie toont aan de *PRL*-expressie na infectie met de 3 CNS-stammen niet hoger is dan in de controle cellen. Dit suggereert dat de aanmaak van PRL weliswaar mogelijk is ter hoogte van de melkklier (zoals reeds aangetoond werd bij andere diersoorten), maar dat de expressie niet noodzakelijk beïnvloed wordt door deze 3 CNS-stammen. Het verhoogde PRL gehalte uit **Hoofdstuk 4.1** is wellicht dus niet het gevolg van een verhoogde PRL productie ter hoogte van de melkklier. Toch moet men zich er van bewust zijn dat de correlatie tussen mRNA en het uiteindelijke eiwit notoir slecht is, en dat de MAC-T cellen in deze studieopzet mogelijks niet de beste weerspiegeling zijn van het natuurlijke melkklierweefsel van koeien.

Hoofdstuk 5 is tenslotte een synthese van de verkregen onderzoeksresultaten, waarin deze geïntegreerd in en afgetoetst worden aan de bestaande wetenschappelijke kennis.

Curriculum vitae & publications

Curriculum vitae

Kristine Piccart werd op 12 mei 1986 geboren in Hasselt. In 2004 behaalde ze haar diploma hoger secundair onderwijs, meer bepaald in de richting Latijn-Wetenschappen, aan het Virga-Jesse College in Hasselt. Datzelfde jaar startte ze ook de studie Diergeneeskunde aan de Universiteit Gent, die ze in 2010 succesvol afrondde.

Na een korte periode in het klinisch onderzoek, keerde Kristine terug naar de Faculteit Diergeneeskunde en startte in 2011 een doctoraatsonderzoek bij de “Mastitis and Milk Quality Research Unit” van de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde. Het doctoraat werd gefinancierd door het Bijzonder Onderzoeksfonds (BOF). Kristine Piccart is (mede-)auteur van verschillende publicaties in wetenschappelijke “peer-reviewed” tijdschriften, en presenteerde haar werk op diverse internationale congressen en symposia. Als lid van het M-team, ondersteunde Kristine ook het onderwijs en het laboratorium voor bacteriologie.

Na haar tewerkstelling aan de Universiteit Gent, vervoegde Kristine het redactieteam van het agrarische nieuwsblad Landbouwleven. Sinds augustus 2015 is ze echter actief als wetenschappelijk medewerker aan het Instituut voor Landbouw-, Visserij- en Voedingsonderzoek (ILVO) in Merelbeke, waar ze zich toelegt op precisieveeteelt.

Publications

Piccart K., Verbeke J., Piepers S., De Sousa N.M., Beckers J.F., and De Vliegher S. 2015. Milk prolactin response and quarter milk yield after experimental infection with coagulase-negative staphylococci in dairy heifers. J Dairy Sci 98(7): 4593-600.

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Oral & poster presentations

Piccart K. and S. De Vliegher. 2012. Host response during experimental intramammary infection with *S. chromogenes* and *S. fleurettii*. Annual Meeting Mastitis Research Workers, November 2012, Chicago, USA.

Piccart K., Verbeke J., Piepers S. and S. De Vliegher. 2013. Host response during experimental intramammary infection in dairy heifers with *S. chromogenes* and *S. fleurettii* - Preliminary results. Dutch Mastitis Research Workers' Meeting, 16 April, Deventer, The Netherlands.

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Piccart K., Piepers S., Verbeke J., De Souza N.M., Beckers J.F. and S. De Vliegher. 2014. Milk prolactin response after induced intramammary infection with coagulase-negative staphylococci in dairy heifers. National Mastitis Council Regional Meeting, 4-6 August, Ghent, Belgium.

Van Weyenberg, S., Pluym L., **Piccart K**., Aernouts B., Huybrechts T., De Busser E., Mondelaers K. and W. Saeys. 2016. Farmers' preference towards heat detection sensor technology. Precision Dairy Farming 2016, 21-23 June, Leeuwarden, The Netherlands.

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Dankwoord - Acknowledgements

Elke koe is als kalf begonnen.

Uit “Dit is mijn Hof” – Chris De Stoop

Wanneer ik in wetenschappelijke, Engelstalige artikels verwijs naar "*we*" , "*us*" en "*ours*", dan maak ik geen gebruik van het koninklijk meervoud. Wetenschappelijk onderzoek is bovenal een groepsgebeuren. Zonder de inzet en het inzicht van vele anderen had dit werk nooit tot stand kunnen komen.

In de eerste plaats wil ik mijn promotoren bedanken die me van *Arcanobacterium* tot *Zymophilus* begeleid hebben. Sarne, bedankt voor het vertrouwen dat je me van meet af aan geschonken hebt. Na mijn studies vond ik de weg naar het M-team niet onmiddellijk, maar ik ben blij dat je de deur op een kier hebt laten staan. Je hebt me de vrijheid en de mogelijkheid gegeven om "zotte dingen" te doen, zoals koeien infecteren met levende bacteriën, naar Chicago reizen om m'n resultaten voor te stellen, een paar maanden verdwijnen om stalen te analyseren in Luik, enzovoort. Je hebt ooit eens gezegd dat ik "mijn kleinkinderen later nog zou vertellen over alle dingen die ik tijdens die proef gedaan heb". Toen lachte ik het misschien weg, maar nu begin ik te denken dat je wel eens gelijk kon hebben.

Sofie, bedankt voor alle uren die je in dit werk gestopt hebt, gaande van het melken van koeien in de vroege uurtjes, tot het bijschaven van presentaties en statistische analyses. Ik kan me niet herinneren dat ik je ooit "nee" heb horen zeggen op een vraag of een verzoek. Ik wens je dan ook veel succes met je onderneming MEX™. (De factuur voor deze sluikreclame mag je eerstdaags in de bus verwachten :))

Professor Haesebrouck, mijn interesse in microbiologie werd voor het eerst aangewakkerd tijdens uw lessen (inmiddels al vele jaren geleden). Bedankt om de deuren van uw labo open te stellen, en dank voor uw vakkundig inzicht bij het nalezen van deze thesis en andere artikels.

Mijn dank gaat tevens ook uit naar alle leden van de examencommissie, die tijd vrijgemaakt hebben om dit werk grondig na te lezen en te verbeteren. Thank you for your contributions Dr. Suvi Taponen, Dr. Otlis Sampimon, Prof. Evelyne Meyer, Dr. Bert Devriendt, Prof. Geert Opsomer en Prof. Catherine Delesalle. Bedankt aan de voorzitter van de examencommissie, Prof. Luc Duchateau, om de verdediging van dit doctoraat in goede banen te leiden.

In dit doctoraat heb ik gebruik gemaakt van verschillende analysetechnieken buiten de vertrouwde muren van DI08, en dat had ik natuurlijk nooit kunnen doen zonder de juiste begeleiding. Un grand

merci à toute l'équipe de Physiologie de la Réproduction à ULg, nottament à Professeur Jean-François Beckers, Noelita Melo de Sousa en Aline Flora Tchimbou. Dank aan de toffe bende van het labo voor dierlijke genetica, met onder andere Prof. Luc Peelman, Mario Van Poucke en ook Caro Rogiers. Dankjewel Kristel Demeyere en Koen Breyne van Biochemie voor alle flowcytometrische adviezen. Thank you, Fernando Nogueira de Souza, for sharing your cell culture and for your skillful, relentless aid. I also want to give a shout-out to the Honey group at the Division of Medical Microbiology at Lund University, with Alejandra Vásquez, Tobias Olofsson, Sepideh Lamei, Éile Butler and Anette Mårtensson. Tevens dank aan alle koeien die zo vriendelijk waren hun uier ter beschikking te stellen aan de wetenschap, en dank aan de mensen die ze verzorgd hebben: Karel, André en Lydia van Biocentrum Agri-Vet.

De mensen die me dag in dag uit gesteund hebben doorheen dit hele traject, zijn natuurlijk mijn oude bureaugenootjes Joren, Dimtri, Reshat, Anneleen en Marina. Joren, jij hebt me bij het M-team binnengeloodst (en indirect eigenlijk ook bij ILVO – is er sprake van een trend?). Jouw kurkdroge Brugse humor werd ten zeerste geapprecieerd (de ranzige bureaubladachtergronden die je steeds opnieuw op mijn pc instelde als ik het kantoor verliet misschien iets minder :p). Joren en Ilse, nog eens proficiat met jullie prachtige dochtertje Lisette. Dimitri, bedankt voor al je hulp tijdens de infectieproef. Misschien kan jij het prolactine-verhaal binnenkort wel ophelderen? Veel succes met je onderzoek, en alles daarbuiten. Reshat, bij deze mijn excuses voor de geluidsoverlast op de bureau. Het ga je goed in Canada!

En dan Anneleen en Marina, oftewel Visserke en Rina, mijn mede-musketiers. Het voelde vreemd aan toen onze alles behalve heilige Drievuldigheid doorbroken werd op de bureau, maar intussen is gebleken dat "uit het oog, uit het hart" niet altijd klopt. Rina -Limburger, Wereldburger- ik heb zoveel respect voor jouw kracht en doorzettingsvermogen. Hoeveel keer heb ik geen tranen gelachen met jouw verhalen… Ik kijk al uit naar de dag dat jij hier vooraan in dit auditorium zal staan. Die dag komt sneller dan je denkt. Ik wens jou en Ruben dan ook het allerbeste toe. En Visserke, waar moet ik beginnen met jou te bedanken? Jij bent één van de meest behulpzame mensen die ik ooit ontmoet

heb. Jij stond letterlijk op elk moment klaar met raad & daad (& koffie). Respect voor alles wat je de laatste jaren opgebouwd hebt, en veel succes in dit nieuwe luik van je leven met Anthony en “mini”. Ik wil gewoon nog één ding zeggen…. jij stinkende spons.

Ook dank aan de andere M-team leden (“emmertjes”) met wie ik al dan niet kortstondig een bureau gedeeld heb: Ameline, Justine, Zyncke, Katelijne, Wannes, Pieter en Bert. De opvolging is intussen ook al verzekerd met Chloë en Lisa. Bert, organisator van de befaamde wildfeestjes, ik kijk al uit naar de piek van het scheerseizoen en hoop dat mijn rug het dit keer uithoudt!

Maar de vakgroep beslaat meer dan enkel het M-team. Dank aan alle collega’s voor de fijne babbels, kerstquizzen, hilarische kettingmails (“zwaluwen: pro of contra?”), Eluna bezoekjes, en meer. Bedankt om epjes te helpen labelen, Johan, ik ben het nog niet vergeten! Ilse en Lotte, merci dat ik mee op uitstap mocht naar het slachthuis, een heuse afwisseling voor mij. Miel en Jenne, merci voor het aangename gezelschap in Kansas, inderdaad één van de tofste congressen en tevens ook huwelijksreizen die ik meegemaakt heb. Dan denk ik natuurlijk ook nog aan Vanessa, Mieke, Kristof, Steven, Bénédicte, Merel, Jeroen, Ann, Dominique, Catharina, Maarten, Jan, Hilde, Eline, Kim, Valérie, Rubèn, Osvaldo, en zovele anderen.

Het administratieve en technische personeel is echter de ruggengraat van de vakgroep. Dagelijks zetten zij zich in om de zaak draaiende te houden. Leïla & Sandra, bedankt voor de goede zorgen (zelfs nadat ik de Universiteit verlaten heb). Ria, bedankt voor je luisterend oor en spannende verhalen. Veronique, op jou kon ik ook altijd rekenen. Lars, wat had ik zonder jou aangevangen in het labo? Ik ken niet veel laboranten die bereid zijn om op zulke ongoddelijke uren melkstalen te gaan nemen voor een doctoraatsstudent! Bedankt om alle uren die ik in het labo doorbracht wat kleur te geven. Steven, jij was de steeds de redder in IT-nood. Els, jij knuffelbeer, bedankt voor alles en wie zien elkaar wel weer in de Eurotuin. Natuurlijk wil ik ook Willy, Marnik, Dirk, Petra en Isabel in de spreekwoordelijke bloemetjes zetten. De vakgroep is echter zo’n grote, dynamische groep… ik ben ongetwijfeld nog vele belangrijke mensen vergeten. Mea culpa.

Daarnaast wil ook het team van Landbouwleven en Sillon Belge vermelden. Bedankt voor de weliswaar korte, maar desalniettemin aangename samenwerking: André, Tim, Ivan, Marc, Delphine, Pierre-Yves, Jérémy et tous les autres. En natuurlijk mogen de ILVO collega's, die zich sinds augustus 2015 over mij ontfermd hebben, niet ontbreken in dit dankwoord. In de eerste plaats wil ik Stephanie bedanken, die altijd voor mij klaarstaat en mij de kans geeft om mezelf te ontplooien. Merci om me binnen te halen bij ILVO: ik ben serieus met mijn gat in de boter gevallen! Mijn nieuwe (maar intussen al niet meer zo nieuwe) bureaugenootjes –Jarissa, Tim, Olga, Zee – ik wil jullie bedanken voor alle dingen die ik dagelijks van jullie opsteek, gaande van het veilig verwijderen van hoornaars tot communicatie protocollen voor RFID. Fijn om deel te mogen uitmaken van zo'n diverse groep. Jürgen, Annelies, Ingrid, Philippe, Pieterjan, Katrien B., Eva, Simon, Caroline, Raphaël, Lieve H., Dieter, Ingrid, Veerle, Ruben, Gerlinde, Donald, Maarten, Jef, Ludwig, Tim D., Sophie, Elsy, Lieve D., Johan, Katrien V., Nathalie, Claudia, Loes, Eric, Bart L., Lorenzo, Bart E., Marie-José, Marleen, Peter, Bert B., Davy, Brecht, Olav, Els, Sofie, Ronny, Filip, Joren, Koen en alle andere collega's: bij deze beloof ik jullie om opnieuw meer tijd vrij te maken voor niet-werkgerelateerde personeelsactiviteiten. (Zei daar iemand "Feestpaleis"?)

Maar in de eerste plaats wil ik vanaf nu meer tijd maken voor mijn familie en vrienden. "Nee, ik moet nog werken", was zowat mijn standaardrespons in de afgelopen weken (maanden?). Aan mijn Limburgse vrienden, bij wie ik de navelstreng nooit zal doorknippen, *salute*! De "Tunesian Girls" – Hanne, Inge en Fee – van de kleuterklas tot in het rusthuis blijven jullie in m'n hart. (Ugh, zo melig.) Bedankt om de vriendengroep te verbreden met de tofste aanhangsels denkbaar: Marco, Zander en Fredje. Ook hier staat de volgende generatie al klaar met Alexander en Fema (een samentreksel van Federica & Marco, bij gebrek aan een gepaste naam voor jullie nog ongeboren spruit). Jeroen, Annelies en kleine Robin, ik kijk al uit naar het moment dat we samen de straten van Sint-Amandsberg gaan onveilig maken, Campo Santo-style. Abbie, Lieve en Pia, jullie mogen altijd aansluiten bij onze crew! Sofie en Joël, vanaf nu ga ik jullie deur platlopen, u weze gewaarschuwd. Stephen, het spijt me, maar helaas heb ik geen tickets voor wetenschappelijke congressen rond kernenergie te geef. Noami, jou bombardeer ik tot mijn gids in het Gentse nachtleven. Peter, jij wordt die voor Hasselt en daarbuiten.

Janneman en Sofie, waar blijft die dinner-date? Jullie gaan er niet onderuit kunnen muizen! Aan alle andere leden van de Orde van de Rode Draak -met Aurelia, Joost, Peter, Thomas, Lotje - *stay stong*. Brecht, onze logeerkamer is beschikbaar als je heimwee krijgt naar je studententijd in Gent. Merci ook aan de CAVA-werkgroep voor al het professionele advies dat ik krijg, en bedankt aan alle studiegenootjes die m'n tijd in Merelbeke zo plezierig maakten.

Dank aan mijn familie, een zotte collectie van tantes, nonkels, nichtjes en neefjes en een resem aanhangsels. Als ik jullie allemaal apart zou bedanken, dan moet ik een tweede volume aan dit doctoraat toevoegen :) Wel een speciale vermelding voor het allerliefste metekindje ter wereld, Oscar Ignoul, en mijn grootouders (oma "*van Ciet"*, oma "*van soep*", opa & Maria, en de opa die ik helaas nooit heb mogen kennen). Ook dank aan mijn lieve schoonfamilie – een hechtere schoonfamilie zou ik me niet kunnen inbeelden! Pieter en Rosalie, geniet nog van jullie studententijd– want voor je het weet sta je vooraan een auditorium één of ander obscuur onderzoek te verdedigen :) Eef en Kevin, ik hoop dat jullie binnenkort eens een weekendje plannen naar Gent (ook hier geldt het aanbod van het logeerbed uiteraard!). En Kevin, als je per se wilt koken, hou ik je niet tegen! Ivo… ondanks het feit dat je zelf door een enorm moeilijke periode gaat, sta je op ieder moment klaar om ons te helpen in het huis en daarbuiten. Ik weet niet hoe we je ooit kunnen bedanken voor alles dat je voor ons, en de andere kinderen, doet. Wat zou ik Carine er graag op deze dag bij gehad hebben. Ik ben er zeker van dat ze fier zou geweest zijn op ons!

Natuurlijk wil ik ook mijn ouders bedanken, zonder wie dit werk er helemaal niet zou geweest zijn. Dankuwel, mam & pap, voor alle financiële en morele steun, van de eerste dag dat jullie me gingen afzetten aan mijn kot in Gent tot nu, in het huisje te Sint-Amandsberg. Bedankt om mij in al mijn keuzes onvoorwaardelijk te steunen. Wie had kunnen denken dat het tot dit boek zou leiden? Ik ben blij dat mijn slaapkamer nog steeds in ere bewaard wordt, en dat wij op ieder moment welkom zijn. Het omgekeerde geldt vanaf nu ook, als jullie Gent eens willen bezoeken. (Ik blijf doorheen dit dankwoord maar de logeerkamer aanprijzen…. Misschien moet ik een B&B openen.)

Soit, tijd om dit dankwoord, en bijgevolg ook dit doctoraat, af te ronden. Er blijft nog één persoon over die ik per se wil bedanken, en dat is … Loopy!

Nee, mopje, natuurlijk ben jij dat, Hans! :) We hebben in die 14 jaar samen al zoveel meegemaakt, maar het lijkt alsof we de laatste 2 jaar in een exponentiële stroomversnelling terecht gekomen zijn. Ik ben zo ongelofelijk trots op al wat je doet, en wie je geworden bent. Wie had kunnen denken dat ik met “die knappe jongen aan de bibliotheek” de rest van mijn leven zou delen… Puber-Kristine heeft onmiddellijk de hoofdvogel afgeschoten! :p Bedankt voor alles wat je dag in dag uit voor mij doet. Hopelijk mogen wij nog enkele decennia aan ons verhaal verderbreien. Ik ben al benieuwd waar de toekomst ons heen zal leiden…