Intramammary immunity against *Staphylococcus aureus* in cattle

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COLOFON

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(met een samenvatting in het Nederlands)

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Chapter 1

General introduction

With a total global cow milk production of over 585 million tonnes per year [1], the dairy industry is indispensable to our food chain. Over the last decades, improved management, better nutrition, and intense genetic selection have steadily increased the milk production per cow in order to meet the growing global demand. To guarantee milk production and quality, animal and udder health are of utmost importance. One of the diseases threatening a cow's health is mastitis; an inflammation of the mammary gland. Mastitis has detrimental effects on the cows' well-being and comprises a major economic impact due to reduced milk production and quality, premature culling, and veterinary interventions [2-5].

Bovine mastitis

Bovine mastitis is an inflammation of the mammary gland with a clinical, subclinical, or chronic representation. Clinical mastitis is initially defined by visible changes in the milk appearance. As the extent of the inflammation increases, udder swelling, redness, heat, and pain become apparent. In cases of severe clinical mastitis, symptoms are not restricted to the udder and become systemic with signs of fever, shock, and depression. Since subclinical mastitis is presented without visible signs of local inflammation or systemic involvement, detection of this asymptomatic form of mastitis usually occurs via somatic cell counts (SCC). The number of SCC is positively correlated with the presence of inflammation and is considered a reliable indicator of udder health disturbances [6-7]. When infections become chronic they may persist throughout lactation. Although mastitis can be caused by various pathogens including viruses [8], yeasts [9-10], and algae [11-12], mastitis is primarily linked with bacterial infections [13-14]. One of the main mastitis causing pathogens is *Staphylococcus aureus*. Other bacteria that have been linked to bovine mastitis include *Escherichia coli (E. coli), Streptococcus uberis, and coagulase-negative staphylococci* (CNS), amongst others [15].

Staphylococcus aureus

Staphylococcus aureus (S. aureus) is an opportunistic, contagious Gram-positive bacterium. Although *S. aureus* is known to cause diseases in humans ranging from minor skin infections like abscesses and impetigo to systemic diseases like endocarditis and osteomyelitis, strains are largely host specific and are rarely associated with transfer between species [16-19]. In cattle, *S. aureus* is generally associated with intramammary infections.

The capacity of *S. aureus* to infect a host is mediated by the expression of various virulence factors which are able to compromise and hamper the effectiveness of the innate and adaptive immune response, for example killing immune effector cells and

mediating immune evasion. Since many virulence factors are encoded on mobile genetic elements the loss or acquisition of these elements contribute to virulence of *S. aureus* [20-21]. Virulence factors include surface proteins like microbial surface component recognizing adhesive matrix molecules (MSCRAMMs), iron transporter motif proteins, members of the three-helical bundle motif family, and G5–E repeat proteins [22], capsular polysaccharides [23-24], secreted toxins like alpha- and beta-toxins, Panton-Valentine Leukocidins (PVLs), and various super antigens [25-29]. Apart from the expression of virulence factors to withstand the host's immune response, *in vitro* data shows the ability of *S. aureus* to invade and survive within mammary epithelial cells and neutrophils [30-32]. This mechanism might allow *S. aureus* to hide from components of the host's immune system as well as from antimicrobial agents. In addition, *in vitro* studies indicate that *S. aureus* is able to form a biofilm [33-35]. Although this may also contribute to intramammary survival, there is no direct evidence for biofilm formation of *S. aureus in vivo.*

In stables, reservoirs of *S. aureus* have been associated with housing, bedding, feedstuffs, equipment, hands and nostrils of milking personnel, non-bovine animals including insects, air in the milking parlour, and water [36-39]. Furthermore, *S. aureus* is frequently isolated from the bovine teat skin and external orifices [40-41]. The frequency by which *S. aureus* can be reisolated from a cow's environment and body sites suggests that contact between the bovine host and this major mastitis causing pathogen is almost inevitable.

Bovine immune responsiveness against intramammary *Staphylococcus aureus*

Initial *S. aureus* encounter is likely to occur early in life via the environment, the teat skin during suckling, or via ingestion of contaminated milk [41-42], which results in the induction of an immune response in the young calve. However, despite the presence of an immune response against *S. aureus*, intramammary invasion later in life when the mammary gland has evolved, often results in mastitis. Recovery from mastitis does not confer immunity to reinfection with the same pathogen [43] and has even been associated with an increased risk for reinfection [44]. Interestingly, quarters of the same cow can differ in their susceptibility to *S. aureus* mastitis [45-47] indicating that susceptibility is defined on the quarter level rather than the cow level. Quarter differences in acquiring new infections may be attributed to parameters involved in bacterial entrance into the mammary gland, such as quarter position and teat morphology [44,48]. However, quarter differences can also be observed post experimental inoculation when these parameters are bypassed [49-53]. Quarters can be divided in three categories: I) quarters able to resolve *S. aureus* without signs of inflammation, II) quarters with signs of inflammation able to resolve *S. aureus*, and III) quarters with signs of inflammation unable to resolve *S. aureus* (Figure 1).

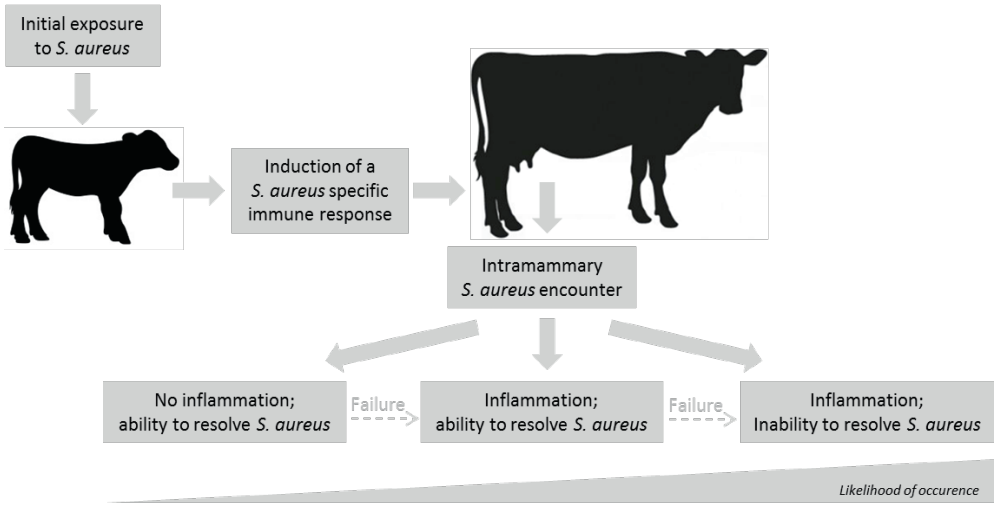

Figure 1: Quarter classification post-intramammary Staphylococcus aureus encounter; a schematic representation. *Initial exposure to S. aureus early in life does not result in the induction of a protective immune response in the mammary gland, which develops later in life. Quarters can be classified according to the consequences of S. aureus encounter. Experimental intramammary inoculation usually results in the onset of inflammation and the inability of a cow to resolve S. aureus indicating that this consequence is the most likely to occur post-encounter.*

When a pathogen is encountered, entrance into the mammary gland is prevented by a physical barrier that separates the internal milieu of the mammary gland and its environment, the teat canal. If a pathogen manages to pass the teat canal, it reaches the mammary gland cistern and the alveoli. Along the way various host innate and adaptive immune components are present to rapidly detect and combat invading pathogens. These immune components include antimicrobial peptides, antibodies, and various immune cells. Antimicrobial peptides in milk include lactoferrin, β-lactoglobulin, and lacto-peroxidase which are known for their protective antibacterial properties [54-55]. The increased activity of the enzymes lactate dehydrogenase, N-acetyl-β-d-glucosaminidase, and alkaline phosphatase has also been associated with bovine mastitis [56-59]. In addition, differences in fat and urea concentrations may influence bacterial growth in milk [60-61]. Since day-to-day variation in milk composition at the quarter level has been described [62-63] levels of milk components may contribute to susceptibility to bovine *S. aureus* mastitis. Furthermore, antibodies in milk are thought to contribute to the cows' defence mechanism by neutralizing toxins secreted by *S. aureus* and promoting phagocytosis by phagocytic cells [64]. Since the presence of low numbers of immune cells, a low SCC, at the moment of bacterial encounter have been associated with increased risk and severity of mastitis [65-66], immune cells are likely to promote bacterial clearance.

When the immune response in a quarter at the time of bacterial entrance fails to resolve *S. aureus*, an inflammatory response is induced. The infection leads to reduced blood–milk barrier integrity allowing blood-derived substances to enter the mammary gland [67-69]. With the help of these recruited components some quarters are able to resolve *S. aureus*, while other quarters remain infected (Figure 2). However, it is unknown which immune components contribute to clearance of intramammary *S. aureus*.

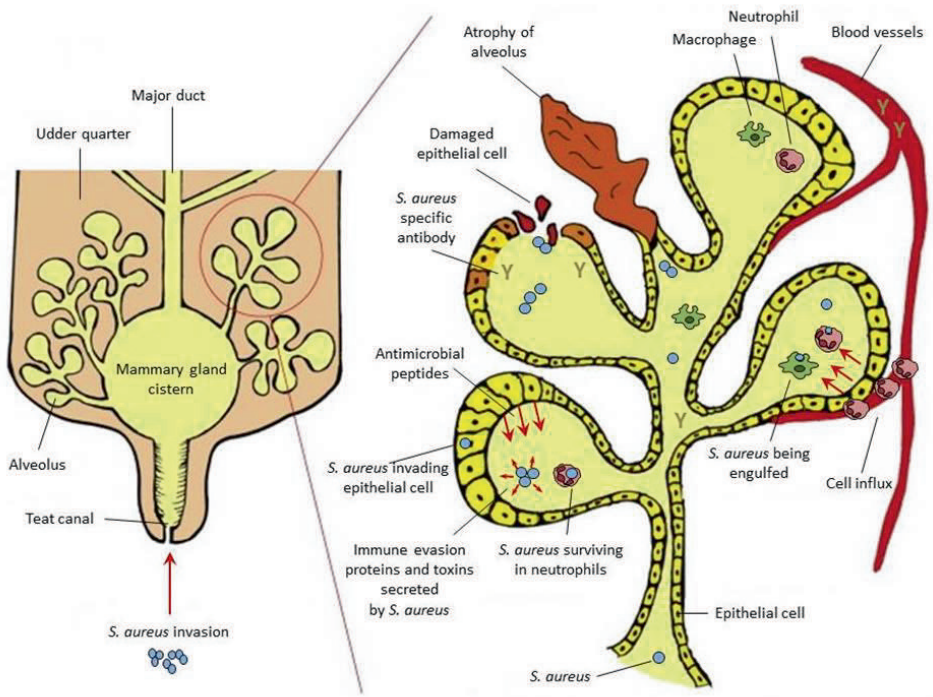

Figure 2: Schematic representation of mastitis development in an infected mammary gland. *Staphylococcus aureus invades the mammary gland through the teat canal and moves upwards to reach the mammary gland cistern and alveoli. Along the way, S. aureus faces various immune components present in milk to combat the invading pathogens; e.g. antimicrobial agents, antibodies, and immune cells. Severe infections may result in tissue damage. Adapted from Viguier et al., 2009 [70].*

Treatment of *Staphylococcus aureus* mastitis

To maintain animal and udder health, removal of the infected animal from the herd to prevent spreading of the pathogen and efficient treatment to eliminate the pathogen is required. Currently, cows affected by mastitis are treated with antibiotics of which efficacy has been shown to vary between strains [71-72], farms [73], cows [74], lactation status [75-77], clinical status [75], as well as by the dose [78], timing [79], duration [80] and route of administration [73]. Generally, the use of antibiotics in lactating cows has shown to provide low efficacy in the treatment of *S. aureus* mastitis and does not protect cattle

against recurrent infections [81-84]. Increasing public concerns about antibiotic residues present in the milk supplied and the emergence of antibiotic resistant bacteria in our food chain urges the need for alternative treatment or preventive strategies [85-86]. Although several alternative antimicrobial agents have recently been tested for their efficacy to cure *S. aureus* bovine mastitis with various levels of success [87-95], the main focus has been shifted from treatment towards prevention of mastitis.

Prevention of *Staphylococcus aureus* mastitis

Prevention of pathogen encounter and entrance into the mammary gland are important first steps in strategies to avoid occurrence of mastitis. Important strategies to minimize *S. aureus* encounter include hygienic farm facilities and herd management practices [96-98]. Proper milk equipment to prevent teat-end lesions and post-milking teat dipping are widely accepted measures to prevent bacterial entrance into the mammary gland [99-100]. Intensive research has focused on the development of a prophylactic vaccine to prevent bovine *S. aureus* mastitis. Experimental vaccines based on whole cells or cell lysates of (multiple) *S. aureus* strains have been evaluated for their efficacy against bovine mastitis [51-52,101-106]. Others studies used recombinant proteins as vaccine antigens [50,107]. The efficacy of vaccines based on biofilm associated proteins or DNA constructs has also been subject of investigation [53,108-109]. In addition, various combinations of the above have been described [53,109-110]. Beside a variety of antigens, differences between these trials further include the route of vaccination, the number of booster injections administered, the type of adjuvant used and the lactation stage of the cow.
The ability of experimental vaccines to induce an immune response is usually assessed by measuring increases in specific antibody levels in milk and serum [50-53,101-102,104,106-110]. In addition, some studies analysed the ability of these vaccines to increase the functionality of the immune response by analysing the neutralizing capacity of the antibodies in *in vitro* settings [110]. Antibodies were also shown to promote phagocytosis of *S. aureus* by milk macrophages and to prevent internalization of *S. aureus* into mammary epithelial cells [52, 101-102,110]. Little data is available on the cell mediated immune responses induced following immunization against *S. aureus* [53,109]. Some experimental vaccines show promising results in preventing udder infections [50,103,107]. Other vaccines failed to reduce the number of intramammary infections but promoted better post-challenge conditions as shown by a reduction in severity and longevity of clinical mastitis [51-53,104,108]. On the European market, only one vaccine against *S. aureus* mastitis is currently commercially available [111]. However, the efficacy of this vaccine, comprised of the inactivated *S. aureus* SP 140 strain and a slime associated antigenic complex prepared from *S. aureus* cultures (and an *E. coli* J5 bacterin) in an oil-based adjuvant, has been subject of debate [112-115], and efforts to develop a more efficacious vaccine against *S. aureus* bovine mastitis continue.

Scope of this thesis

This thesis aimed to gain a better understanding of milk composition parameters determining susceptibility or resistance to *S. aureus* mastitis and focused on improved vaccination protocols.

Since quarters of the same cow intrinsically differ in their susceptibility to *S. aureus* we first set out to identify quarter specific differences in milk composition parameters. Diurnal changes in the concentrations of these parameters were also analysed. In addition, we investigated the relation between these milk composition parameters and *in vitro* growth of *S. aureus* and *E. coli* (**Chapter 2**).

Next, we analysed the association between milk composition parameters and *in vivo* growth of *S. aureus* by determining milk composition just prior to experimental bacterial inoculation of the mammary gland and bacterial shedding dynamics post-inoculation (**Chapter 3**).

Driven by the observation that increased levels of specific antibodies in milk were associated with reduced *S. aureus* growth both *in vitro* (Chapter 2) and *in vivo* (Chapter 3), we explored immunization strategies to increase the presence of antibodies in the bovine mammary gland. Therefore, we determined the impact of vaccine administration via different routes (**Chapter 4**) and via different sites (**Chapter 5**), using various adjuvants on the magnitude of the antibody isotype responses in both milk and serum. Furthermore, the neutralizing capacity of these antibodies was analysed in *in vitro* assays.

Finally, the results reported in this thesis are summarized and discussed in **Chapter 6**.

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Chapter 2

2

Diurnal differences in milk composition and its influence on *in vitro* growth of *Staphylococcus aureus* and *Escherichia coli* in bovine quarter milk

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Abstract

In experimental intramammary inoculation studies it has been observed that mastitis susceptibility is influenced amongst others by cow factors. To identify milk characteristics leading to these differences, quarter milk samples of morning and evening milk were collected and analysed for their composition i.c. protein, fat, lactose, urea, lactoferrin, lactoperoxidase, and β-lactoglobulin concentrations, somatic cell count, and antibodies against *Staphylococcus aureus* (*S. aureus*). Furthermore, *in vitro* growth of *S. aureus* and *Escherichia coli* (*E. coli*) in fresh quarter milk samples was determined. All measured parameters differed significantly between quarters and also between morning and evening milk with the exception of lactose levels. In addition, quantitative growth of *S. aureus* and *E. coli* was significantly different in morning milk compared to evening milk. Mixed model analysis revealed that replication of *S. aureus* was negatively associated with the presence of fat, *S. aureus* specific IgG1 antibodies, contamination of the milk sample and morning milk. Replication of *E. coli* was negatively associated with fat concentrations, and positively associated with morning milk. The significant difference between morning and evening milk supports the theory that changes in milk composition influence bacterial growth. Although all determined milk components differed significantly between quarters and in time no significant association with bacterial growth could be identified with the exception of fat for both studied species and IgG1 titers for *S. aureus*. The negative association of fat with bacterial growth was assumed to occur due to activation of lipolysis by milk handling and can most likely be neglected for *in vivo* relevance. The fact, that *S. aureus* specific IgG1 titers were negatively associated with *S. aureus* growth *in vitro* encourages the ongoing effort to develop a vaccine against *S. aureus* induced mastitis.

Introduction

Mastitis in cattle is a worldwide problem in dairy farming with a major impact on animal welfare, life span and milk production [1-2]. When mastitis occurs, treatment with antibiotics is often necessary. However, prevention of the invasion of the udder rather than curing the subsequent infection is the ultimate goal to restrain the development of antibiotic resistance [3]. Cow factors as age, lactation stage, teat anatomy and condition, leaking milk and previous infection have been identified as risk factors for mastitis under field conditions most likely by influencing bacterial invasion of the udder and the general immune function of the cow [4-6]. Improvement of management factors and function of the milking equipment are important efforts to decrease the incidence of mastitis [4,7]. In addition, experimental studies have shown that invasion of a quarter by bacterial species does not always lead to clinical infection, implicating differences in susceptibility between cows and quarters [8-9]. In observational as well as experimental studies quarter specific susceptibility has also been observed when position of the quarter and anatomical differences were associated with susceptibility [5,10]. Milk composition may also influence the susceptibility to infection. Milk specific risk factors identified under experimental conditions are somatic cell count (SCC) [8,9], fat concentration and, for *Staphylococcus aureus* (*S. aureus*), the concentration of *S. aureus* specific immunoglobulin G1 (IgG1) [17]. In addition, lactoferrin, β-lactoglobulin, and lactoperoxidase are known for their antibacterial and protective properties in milk, [8,9,11-13] and differences in protein, fat, lactose and urea concentrations may influence bacterial growth in milk by changing the local environment. Normally, milk composition is measured per cow per day by analysing a sample consisting of equal amounts of morning and evening milk of all four quarters. As a result, quarter and time specific variation levels off. However, it is known that for example SCC change in a circadian rhythm and also differ between quarters which lead to differences in quarter susceptibility to infection [9,14,15]. Since the local defence system of the udder does not only consist of leukocytes present in milk but also includes molecular factors of the innate and adaptive immune system, concentrations of these factors are, most likely, also different between quarters and change over time. Therefore, the objective of this study was to identify quarter specific differences in milk composition and differences between morning and evening milk. In addition, *in vitro* growth of *S. aureus* and *Escherichia coli* (*E. coli*), two major mastitis causing pathogens, in fresh milk samples was measured and their growth patterns in relation to levels of milk factors potentially involved in altering bacterial growth compared.

Materials and Methods

Milk Collection

The dairy herd of the Faculty of Veterinary Medicine (Utrecht, The Netherlands) consisting of 47 cows, mainly of the Holstein breed, was housed in a free stall housing system. Cows were fed a diet based on grass and corn silage, beet pulp and concentrate which was formulated to meet the dietary requirements for lactating dairy cows. Corn silage was administered after milking twice daily (8 am and 7 pm). Concentrate was administered via an automated feeding system whereas grass silage and drinking water were supplied ad libitum. Milk volume was determined by the automatic milking system. Quarter milk samples of all cows were collected during evening and morning milking (with a 13 hr time interval) in parallel with Test Day samples (TD). Quarter milk samples (50 mL) were collected by hand after fore milking. Conventional TD samples were collected automatically and consisted of a representative composite sample of the four quarters and an equal volume of morning milk and evening milk. Both quarter samples and TD samples were analyzed for milk composition (fat percentage, total protein percentage, lactose level, and urea concentration) and quality (SCC) by a commercial milk quality assurance laboratory (Qlip, Zutphen, The Netherlands). Aliquots of the quarter milk samples were stored at -20 °C until determination of lactoferrin, lactoperoxidase, β-lactoglobulin concentrations and levels of *S. aureus* specific antibodies. Aliquots of fresh milk were used for the *in vitro* growth assay and for bacterial culture. For IgG purification colostrum of four cows of the dairy herd was collected on the day of parturition and analysed for the presence of *S. aureus* specific IgG1 antibodies as described below.

In vitro Growth Assay

For the *in vitro* growth assay *S. aureus* (strain Newbould 305) and an *E. coli* field isolate were used. Both bacteria were grown in sterile brain-heart infusion medium (OXOID, Wessel, Germany) overnight under aerobic conditions shaking gently at 37 °C. The overnight culture was centrifuged twice at 4000 x *g* for 15 min at room temperature and the pellet was resuspended in 20 mL sterile phosphate buffered saline (PBS; Lonza; Basel Switzerland). Of the bacterial suspension, aliquots of 300 µL were stored at -20 °C in microcentrifuge tubes. To determine bacterial load, three aliquots were thawed and 10-fold serial dilutions in PBS were prepared. The serial dilutions (50 µL) were plated on sheep blood agar plates and incubated overnight at 37 °C and 5 % CO_2. Then, colonies were counted and CFU/mL were calculated. For the *in vitro* growth assay, an aliquot of *S. aureus* and *E. coli* suspension was thawed and diluted as determined earlier to achieve a bacterial concentration of 600 CFU/mL.

For *in vitro* growth assessment 100 µL of each quarter milk sample was added into a U-bottom 96-well polystyrene microtiter plate (Costar® 9018, Corning B.V. Lifesciences, Amsterdam, The Netherlands). The remaining milk was stored at 4 °C until bacterial culture. Bacterial suspensions (50 µL) were added to milk samples and plates were incubated for 3 hrs at 37 °C and 5 % CO_2. After incubation, samples were mixed by pipetting and 50 µL from each well was plated on a sheep blood agar plate. In addition, 50 µL of each fresh milk sample were plated on sheep blood agar plates to check for bacterial contamination present prior to the *in vitro* growth assay. Following an overnight incubation at 37 °C and 5 % CO_2, colonies were counted. To identify the final number of CFU added to the milk samples 50 µL of the initial bacterial suspension was directly plated on sheep blood agar plates and incubated overnight under the same conditions. Bacteria were presumptively identified by colony size, morphology, pigmentation and type of hemolysis. The outcome was expressed as CFU/plate. Growth of bacteria other than *S. aureus* or *E. coli* was noted and expressed as a binomial value (0 = no other bacteria grown; 1 = other bacteria grown).

To study the specific influence of immunoglobulin concentration on *in vitro* growth of *S. aureus*, fresh quarter milk of three healthy (absence of clinical mastitis and other signs indicating disease, such as abnormal behavior, lack of appetite and lameness) mid-lactation donor cows was spiked with purified IgG in a concentration ranging from 0.08 - 5 mg/mL and used as a template in the *S. aureus in vitro* growth assay. Of spiked and original milk samples, 100 µL milk were pipetted into a U-bottom 96-well polystyrene microtiter plate and *in vitro* growth was studied as described above. Analyses were performed in triplicate.

***Staphylococcus aureus* specific Antibody ELISA**

The *S. aureus* Reynolds CP-strain is lacking a capsule and therefore lacking protein A [16]. This strain was chosen for the antibody ELISA to prevent non-specific reactions of immunoglobulins with protein A. *Staphylococcus aureus* Reynolds was grown in trypticase soy broth for 20 hrs at 39.5 °C and inactivated by overnight incubation with 0.5 % formalin. Bacteria were harvested by centrifugation and resuspended in PBS. Then, NUNC MaxiSorpTM plates (eBioscience, Hatfield, UK) were coated overnight at 4 °C with 0.1 µg bacterin in 0.05 M carbonate-bicarbonate buffer (120 µL; MSD-AH, Boxmeer, The Netherlands) and subsequently blocked with casein (200 µL; MSD-AH, Boxmeer, The Netherlands). Samples and positive control serum were tested in two-fold serial dilutions (100 µL) and incubated for 1 hr at 37 °C. An in-house negative control serum was taken along in octaploid. As secondary antibody, horseradish-peroxidase-conjugated sheep anti bovine IgG1 (Bethyl Laboratories, Inc., Montgomery, Texas, USA) was used in a 1:2000 dilution (100 µL). Tetramethylbenzidine (TMB; MSD-AH, Boxmeer, The Netherlands) was

used as a substrate (100 µL) and reactions were stopped after an incubation of 10 min in the dark by adding 2 M sulphuric acid (50 µL). Absorbance (450 nm) was measured on a Tecan SUNRISE device (Tecan Group Ltd., Männedorf, Germany) using XFluor4 Software Version V4.51-I4. Antibody titers were determined using CaSpEx Software AbendVertical version 0.11 V1 (MSD, Proprietary Software) and defined as the Log_2 dilution of the sample that would give the same absorbance as the predefined cut-off. The cut-off was defined as two times the average OD of the negative controls. Antibody titers of all milk samples were determined.

Detection of Lactoferrin, β-Lactoglobulin and Lactoperoxidase

Lactoferrin (LF) and β-lactoglobulin (bLG) concentrations were determined using commercially available ELISA kits (Bovine Lactoferrin and Bovine β-Lactoglobulin ELISA Quantification Set, Bethyl). ELISA's were performed according to the manufacturer's manual and outcomes expressed as ng/mL. Lactoperoxidase (LP) was detected using an in-house quantitative ELISA which was performed as follows. A standard curve was prepared using bovine LP in a 2-fold dilution ranging from 0.078 - 5 U/mL (Sigma-Aldrich). Milk and standard samples were diluted 1:50 in blocking buffer (Roche, Sigma-Aldrich) and 50 µL were added into a flatbottom microtiter plate (Costar). TMB was added (100 µL) and reactions were stopped after an incubation of 15 min in the dark by adding 2 M sulphuric acid (50 µL). The LP concentration was determined by measuring the absorbance at 450 nm using a spectrophotometer (Thermo Fisher Scientific Inc., Waltham, US). The outcome was expressed as U/mL.

Immunoglobulin G Purification from Colostrum and Titration in Milk Samples

IgG was purified from delipidated colostrum by liquid affinity chromatography using HiTrapTM Protein G columns (GE Healthcare, Eindhoven, The Netherlands). The final concentration step, resulting in a concentration of 67 mg/mL, was performed using a Vivaspin500 centrifugal concentrator (Sigma-Aldrich) according to the manufacturers' protocol.

Statistical Analysis

Data editing was done using Microsoft Excel (Microsoft office 2010, Microsoft, Redmond, USA). The descriptive- and statistical analyses were done using the SPSS statistical software package (IBM SPSS statistics 20.0, IBM, Armonk, USA). For graphical presentation of the data GraphPad Prism (GraphPad Software Inc. 6.01, La Jolla, USA) was used. Differences were defined to be significant at the $p < 0.05$ level.

To compare bacterial growth between quarters, growth was expressed as a binomial variable with the number of CFU present after incubation smaller than, or the same as, at

the beginning of incubation coded as 0, and samples with CFU above the number of added colonies before incubation coded as 1. The expected number of quarter milk samples with bacterial growth per cow was calculated and compared with the number of CFU observed using a chi-square statistic assuming a binomial distribution. Intra class correlations were determined using the ANOVA method.

Differences between quarters and between morning and evening milk were analyzed using a mixed model analysis with each milk trait as dependent variable, quarter (ranked from low to high for each milk trait) and time of milking (morning/evening) as explanatory variables, and cow as a random factor. The variation coefficient between triplicates and between cows of milk samples used to detect the effect of IgG titration on *in vitro* growth of *S. aureus* was determined. In addition, the correlation between spiked IgG concentration and *S. aureus* growth was determined.

Univariable linear analysis was used for every variable against *S. aureus* CFU and *E. coli*-CFU and only variables with $p < 0.2$ were used in the mixed model analysis to determine whether milk specific factors influenced *in vitro* growth of *S. aureus* and/or *E. coli*. To include milk cultures with no detectable growth in the analysis these cultures were set to 1. Fat, protein, lactose, urea, *S. aureus* Reynolds specific IgG1 antibodies, LF, LP, bLG and SCC were included as continuous variables. Milk contamination (yes/no) and milking time (morning/evening) where included as factorial variables. To correct for repeated observations quarter was nested within cow and used as a random effect. Variables were assessed for their contribution to the model by the Akaike's information criterion (AIC). The model with the lowest AIC was chosen as best predictor of the data. For all models, residuals were assessed for normal distribution, constant variance and a mean of 0. Whenever necessary, data were log transformed to achieve normality.

Results

Analysis of Milk Components

A total of 47 cows were sampled during the morning and the evening milking. Since one cow had only three functioning quarters, samples of 187 quarters were available. An overview of the herd characteristics displayed as the rolling year average as well as the herd and milk characteristics on the day of sampling is given in Table 1. Milk composition of foremilk (lower fat, protein and lactose concentration) differs as compared to the representative milk sample used for the test day analysis, Since the automated data processing algorithm performed at the milk quality insurance laboratory automatically discarded aberrant values for fat (< 1.5 %) for protein (< 2.0 %) and for lactose (< 2.5 %), only 152 and 121 values for fat, protein and lactose were reported for quarters of the morning milking and the evening milking, respectively. The amounts of LF, LP and bLG, as

well as *S. aureus* specific IgG1 concentrations in milk were determined for all quarters. The colostrum of the four cows sampled at the day of parturition contained *S. aureus* specific IgG1 (S/P ratios ranging from 0.8 - 1.2).

Table 1: *Herd characteristics and milk composition at the day of sampling.*

	Rolling year average at day of sampling							
	Milk yield[a]	Fat[a]	Protein[a]	Lactation	DIM	Fat[b]	Protein[b]	SCC[c]
Mean	4306.2	180.72	143.13	1.9	180.1	4.27	3.55	56.3
SD	3206.6	131.59	106.90	1.4	126.6	0.68	0.36	78.0
Min	318.0	13.00	11.00	1.0	17.0	2.85	2.77	5.0
Max	13738.0	490.00	432.00	6.0	461.0	6.04	4.37	469.0

DIM: days in milk; SCC: somatic cell count; SD: standard deviation; Min: minimum; Max:maximum.

[a] *Displayed in kilograms*

[b] *Displayed in percentage*

[c] *SCC x 10^3 cells/mL*

In vitro growth assay

The actual mean number of CFU's added to the quarter milk samples in the *in vitro* growth assay was 40 CFU per sample (range 22 - 63) for *S. aureus* and 50 CFU (range 35 - 68) for *E. coli*. CFU detected after *in vitro* growth per quarter (ranked per cow from lowest to highest) per milking time are presented in table 2 as median (min;max). Culturing fresh milk samples on sheep blood agar plates did not reveal bacterial contamination of the collected milk samples. After the *in vitro* growth assay 29 milk samples showed growth of other bacterial species on the blood agar plates next to the expected bacterial species indicating that the *in vitro* growth assay acted as an enrichment medium for low abundance contaminants. Data regarding milk composition are summarized as mean (SD; Table 2). Significant differences between morning and evening milk were detected for all measures with the exception of levels of lactose (Table 3). Protein, fat, urea, LF, *S. aureus* specific IgG1 and *in vitro* growth of *E. coli* were higher and bLG, LP, SCC and *in vitro* growth of *S. aureus* were lower in morning milk compared to evening milk with protein showing the smallest difference (factor 0.15) and LP the biggest difference (factor 14.3) between milkings. Significant differences between quarters were detected for all parameters (Table 3). Comparison between the highest and the lowest quarter revealed quarter differences ranging from a factor of 0.04 for protein up to a factor of 27.86 for LF. Figure 1 presents the observed numbers of quarter milk samples per cow that showed *in vitro* bacterial growth together with the expected number of quarters under a binomial distribution. Numbers of observed and expected quarter milk samples with bacterial growth differed significantly as determined by Chisquare statistic. The calculated intra-cow correlation coefficient for bacterial growth in quarter samples was 0.267 for *S. aureus* and 0.107 for *E. coli*.

Table 2: *In-vitro growth of Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli) in fresh quarter milk samples and quantities of milk components in morning milk and evening milk samples. Quarters of each cow were ranked from low (Q1) to high (Q4) for each milk trait and grouped accordingly before analysis. The table summarizes data of all quarters collected at morning and evening milking. Data for in vitro growth of S. aureus and E. coli are summarized as median (min;max) and the mean (+/- SD) for milk components.*

	S. aureus **(CFU/mL)**		***E. coli*** **(CFU/mL)**		**SCC (x10^3 cells/mL)**	
	Morning	**Evening**	**Morning**	**Evening**	**Morning**	**Evening**
Q1	640	2560	40	20	23.5	24.0
	(0;4000)	(0;17280)	(0;720)	(0;300)	(45.7)	(46.4)
Q2	1280	4480	110	120	38.6	45.5
	(0;6080)	(880;30720)	(0;2400)	(0;600)	(70.1)	(87.3)
Q3	1840	5760	180	200	59.5	66.6
	(20;10880)	(1520;33280)	(0;3760)	(0;1000)	(108.2)	(118.5)
Q4	2960	6880	810	470	97.7	105.9
	(640;13440)	(2160;42880)	(20;24000)	(0;2080)	(124.1)	(141.3)

	Fat (%)		**Protein (%)**		**Lactose (%)**		**Urea (mg/L)**	
	Morning	**Evening**	**Morning**	**Evening**	**Morning**	**Evening**	**Morning**	**Evening**
Q1	2.2	2.0	3.7	3.5	4.5	4.5	15.7	12.0
	(0.7)	(0.5)	(0.4)	(0.3)	(0.3)	(0.3)	(5.1)	(3.5)
Q2	2.4	2.3	3.7	3.6	4.6	4.6	16.7	13.3
	(0.7)	(0.6)	(0.4)	(0.4)	(0.2)	(0.3)	(4.9)	(3.9)
Q3	2.7	2.6	3.9	3.7	4.6	4.6	17.2	13.9
	(0.8)	(0.7)	(0.4)	(0.4)	(0.2)	(0.2)	(4.9)	(3.7)
Q4	3.0	2.9	3.9	3.8	4.6	4.6	18.2	14.9
	(0.8)	(0.8)	(0.3)	(0.3)	(0.2)	(0.2)	(5.1)	(3.9)

	β-lactoglobulin (ng/mL)		**Lactoperoxidase (U/mL)**		**Lactoferrin (ng/mL)**		***S. aureus*** **IgG1 titer**	
	Morning	**Evening**	**Morning**	**Evening**	**Morning**	**Evening**	**Morning**	**Evening**
Q1	3.1	4.9	26.1	38.5	66.5	64.3	4.8	4.6
	(0.9)	(1.4)	(6.3)	(9.5)	(52.5)	(52.6)	(1.7)	(1.7)
Q2	3.6	5.7	27.8	41.6	73.6	70.5	5.1	4.9
	(1.0)	(1.4)	(6.3)	(9.9)	(54.9)	(54.8)	(1.7)	(1.7)
Q3	3.9	6.3	28.8	43.9	84.8	79.4	5.4	5.2
	(1.1)	(1.3)	(6.4)	(10.8)	(55.5)	(56.3)	(1.7)	(1.7)
Q4	4.6	7.0	30.6	46.5	97.5	87.2	5.7	5.6
	(1.3)	(1.5)	(7.2)	(11.2)	(57.9)	(59.8)	(1.8)	(1.6)

Table 3: *Differences between quarters and morning and evening milk estimated for each milk trait using mixed model analysis. Quarters (Q) of each cow were ranked from low to high for each milk trait and grouped accordingly before analysis.*

		S. aureus **(Log_2)**	**CI**	***E. coli*** **(Log_2)**	**CI**	**SCC (Log_2)**	**CI**
Q	1	-2.9*	-3.42; -2.47	-4.5*	-5.10; -3.90	-2.23*	-2.54; -2.02
	2	-1.34*	-1.81; -0.86	-2.73*	-3.33; -2.17	-1.54*	-1.76; -1.34
	3	-0.64*	-1.12; -0.16	-1.51*	-2.11; -0.90	-0.96*	-1.18; -0.74
	4	rc		rc		rc	
Time	Morning	-2.0*	-2.34; -1.67	0.9*	0.48; 1.33	-0.24*	-0.39; -0.08
	Evening	rc		rc		rc	

		Fat (%)	**CI**	**Protein (%)**	**CI**	**Lactose (%)**	**CI**	**Urea (mg/L)**	**CI**
Q	1	-0.65*	-0.77; -0.52	-0.09*	-0.1; -0.07	-0.12*	-0.14; -0.09	-2.7*	-3.29; -2.11
	2	-0.42*	-054; -0.29	-0.06*	-0.08; -0.05	-0.07*	-0.09; -0.04	-1.59*	-2.19; -1.00
	3	-0.21*	-0.34; -0.08	-0.03*	-0.05; -0.01	-0.04*	-0.06; -0.01	-0.85*	-1.44; -0.25
	4	rc		rc		rc		rc	
Time	Morning	0.19*	0.11; -0.29	0.15*	0.14; 0.17	-0.01	-0.03; -0.01	3.45*	3.03; 3.87
	Evening	rc		rc		rc		rc	

		bLG (ng/mL)	**CI**	**LP (U/mL)**	**CI**	**LF (ng/mL)**	**CI**	***S. aureus*** **IgG1 titer**	**CI**
Q	1	-1.77*	-1.96; -1.58	-6.28*	-7.28; -5.27	-27.86*	-33.4; -22.3	-0.97*	-1.07; -0.87
	2	-1.17*	-1.36; -0.98	-3.90*	-4.91; -2.89	-21.24*	-26.8; -15.7	-0.63*	-0.73; -0.53
	3	-0.72*	-0.91; -0.52	-2.15*	-3.17; -1.14	-12.25*	-17.85; 6.65	-0.31*	-0.41; -0.21
	4	rc		rc		rc		rc	
Time	Morning	-2.19*	-2.32; -2.05	-14.3*	-14.9; -13.6	5.70*	1.77; 9.64	0.19*	0.12; 0.27
	Evening	rc		rc		rc		rc	

bLG: β-Lactoglobulin, LP: Lactoperoxidase, LF: Lactoferrin, CI: Confidence interval, rc: reference category.

**: Significant difference with the reference group.*

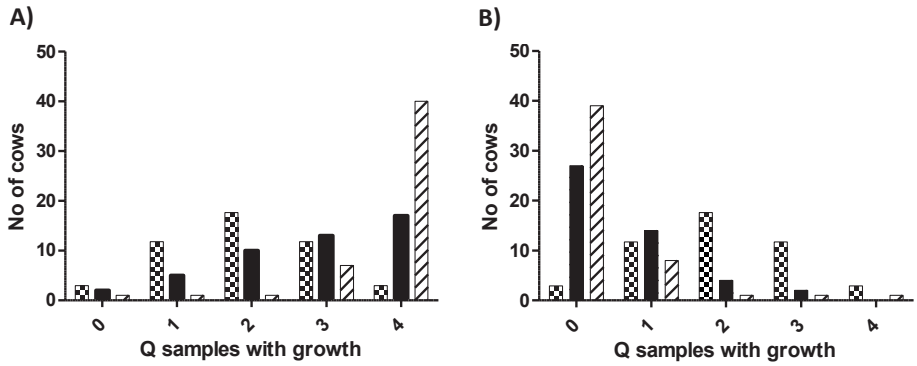

Figure 1: *Cows grouped according to the number of quarters milk samples (0 to 4) with growth of S. aureus (A) or E. coli (B) in the in vitro growth assay. Growth is presented as a binary indicator (0 = CFU after incubation ≤ added CFU; 1 = CFU after incubation > added CFU). Bars represent expected number of quarters with bacterial growth under a binomial distribution (checkered); observed number of quarters with bacterial growth in morning milk (black); and observed number of quarters with bacteria growth in evening milk (striped).*

Univariate analysis revealed that fat, protein, lactose, urea, SCC, LP, *S. aureus* specific IgG1, contamination of the milk sample, and milking time were important predictors for the growth of *S. aureus* and were therefore incorporated into the mixed model analysis. The milking time (morning/evening) was the strongest single predictor ($p < 0.000$). For the growth of *E. coli* fat, protein, lactose, urea, *S. aureus* specific IgG1, and milking time were the most important predictors. In this analysis, *S. aureus* specific IgG1 was the strongest single predictor ($p < 0.022$). The multivariate model best explaining *in vitro* growth of *S. aureus* (Table 4) contained four variables: time of milking, contamination of milk with other bacteria, percentage of fat, and *S. aureus* specific IgG1 titer. All four variables were negatively associated with growth of *S. aureus*. Morning milk was associated with a factor 0.29 decrease as compared to evening milk, a 1 % increase in fat was associated with a factor 0.58 decrease, a Log_2 increase of *S. aureus* specific IgG1 was associated with a factor 0.73 decrease and the presence of other bacteria in the milk sample with a factor of 0.25 decrease in growth of *S. aureus*. The multivariate model best explaining *in vitro* growth of *E. coli* contained the variables time of milking and percentage of fat. The morning milk was positively associated with growth (factor 1.48 increase compared to evening milk) of *E. coli* while the fat percentage showed a negative association (decrease by a factor 0.79).

Table 4: *Estimation of the association between in-vitro bacterial growth and milk components. Estimates are gained by mixed model analyses.*

Strain	Variable		Estimate	CI
S. aureus	Time of milking	Morning	-0.541	-0.665; -0.045
		Evening	rc	
	Fat (%)		-0.233	-0.319; -0.147
	S. aureus specific IgG1		-0.134	-0.252; -0.016
		No	0.244	0.076; -0.412
		Yes	rc	
E. coli	Time of milking	Morning	0.171	0.034; 0.308
		Evening	rc	
	Fat (%)		-0.102	-0.205; 0.002

CI: confidence interval, rc: reference category.

Effect of IgG Titration on *In vitro* Growth of *S. aureus*

The correlation between IgG concentration and *in vitro* growth is presented in Figure 2. As the figure indicates, a significant correlation of IgG concentrations with CFU counts could not be identified. The intra cow variation coefficient of triplicates of CFU at different IgG concentrations varied from 0.2 to 0.9. The inter cow variation coefficient varied from 0.1 to 0.5.

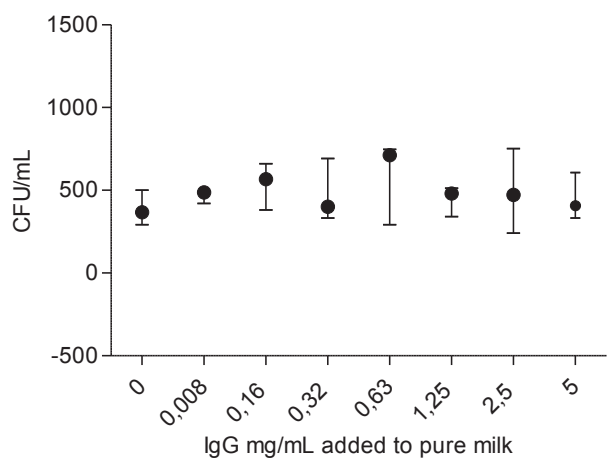

Figure 2: *Replication of S. aureus in milk spiked with purified IgG on top of natural IgG levels (< 1 mg/mL). Data represent the median S. aureus replication with the interquartile ranges of milk samples of three cows.*

Discussion

This study revealed significant differences in milk composition between quarters of a cow. In addition, *in vitro* bacterial growth was found to be significantly different in quarter milk samples between cows, and between morning and evening milk. Since quarter differences described in this study were identified in foremilk samples, the magnitude of variation among quarters might be different when using representative milk sample. Experimental inoculation studies circumventing the natural barrier of the teat duct also reported that mastitis susceptibility varies between cows and among quarters of cows [8] indicating that milk differences may also play a role in mastitis susceptibility *in vivo* and not only *in vitro* in striped foremilk samples. These variations have been attributed to SCC [9], the presence of other bacteria [5], and *S. aureus* Reynold specific IgG1 titers [17]. In *in vivo* inoculation studies it is difficult to distinguish whether the differences in susceptibility are influenced by factors directly present in milk or by antigen-host-interactions inducing an immune response influencing susceptibility. The observed associations between milk components and the deviating growth pattern *in vitro* of *S. aureus* and *E. coli* in this study occurred in an *in vitro* situation and therefore have to be attributed to diurnal differences in morning and evening milk.

When comparing the morning milk and evening milk, significant differences were found in most milk parameters with the exception of lactose. Although we analyzed foremilk instead of representative milk samples, the occurrence of diurnal changes in milk composition are consistent with literature; however, the magnitude of changes described differs between published studies [18-22]. The diurnal pattern of SCC is thought to be caused by a dilution effect through milk production between milkings and as a reaction to milking [14,20,23-25]. Although the milking interval between evening and morning milking was only slightly longer (13 hrs) than the interval between morning and evening milking (11 hrs), we observed the lowest SCC at morning milking and this has also been reported in other studies [14,24,26]. Other studies reported the lowest SCC prior to the evening milking [25] or failed to identify a consistent pattern [22]. Different milking intervals or the use of different type of milk samples might have led to these contrary findings. In high yielding dairy cows diurnal changes in urea concentration detected in milk have been shown to be related to feed uptake in relation to milking with an increase of milk urea several hours after feeding [21]. Although the interval between milking and feed intake in our study was similar a significant differences between morning and evening milk was detected. Fat concentration in milk has been found to be lower in morning milk when using a composite milk sample, and lower in fore milk samples after a longer interval between milkings [22-24]. Interestingly, in milk samples analyzed in this study, morning milk samples showed a higher fat concentration compared to evening milk samples

although the interval between both milkings was relatively similar and foremilk samples were used for analysis. We could not explain this finding.

Lactoferrrin, bLG and LP produced locally in the udder tissue [11,27-29] are known for their protective antibacterial properties [8,12-13]. We found LF to be present in higher concen-trations in morning milk while LP and bLG were higher in evening milk. So far, explanations for differences in concentrations given in literature were restricted to age, lactation status and SCC [12,27,30-31]. How these diurnal differences occur is unclear. Although *in vitro* bacterial growth in milk differed significantly between cows and between morning and evening milk samples, statistical analysis revealed no association with LF, bLG or LP.

Immunoglobulins present in milk during lactation are mainly of the IgG1 isotype that are selectively translocated from serum into the mammary gland [32]. IgG concentrations are not influenced by quarter location or blood serum concentration when measured once a day [17,33]. However, in this study we showed that the IgG1 antibody titer specific for *S. aureus* was statistically different among quarters of a cow and, when measured twice a day, we detected diurnal differences with concentrations higher in evening milk compared to morning milk. A significant negative association between *S. aureus* specific antibody concentrations in milk and replication of *S. aureus* was identified. Since we did not perform a vaccination against *S. aureus* titers are thought to be induced following natural exposure to *S. aureus*. Our data support findings of an *in vivo* study where susceptibility to experimentally induced *S. aureus* mastitis was analyzed and shown to be negatively associated with *S. aureus* specific IgG1 levels in milk [17]. These findings support the ongoing efforts of developing a vaccine against *S. aureus* mastitis. However, our attempt to influence bacterial growth by enhancing IgG concentrations in milk up to five times the natural level by simple titrations of purified antibodies did not successfully reproduce the growth inhibitory effect. Although the final *S. aureus* specific IgG1 concentration in spiked milk samples was not determined an increase must have been achieved since colostrum samples used for purification were confirmed to contain high levels of *S. aureus* specific antibodies and colostrum contains mainly antibodies of the IgG1 isotype [32]. It is likely that other factors not measured in this study such as complement components for example should have been present in enhanced concentrations as well to let immuno-globulins effectively hamper *S. aureus* growth.

Observed and expected bacterial growth of both *S. aureus* and *E. coli* in quarter milk samples between cows differed significantly (Figure 1) indicating the presence of cow specific differences which was supported by the intra-cow correlation coefficients. Cow characteristics influencing susceptibility to mastitis were previously identified *in vivo* where cows were experimentally infected with *S. aureus* and clustering within cows occurred [9]. The *in vitro* bacterial growth data presented here indicate that milk

composition without antigen-host interaction plays a role in altering bacterial growth. To adjust for the intra-cow correlation in statistical analysis cow was used as a random factor. Contrary to data of *in vivo* growth of *S. aureus* after experimental inoculation where fat concentration was positively associated with bacterial replication [17], in this study fat was associated negatively with replication of *S. aureus* as well *E. coli*. A 1% increase in fat was associated with a significant decrease of the number of CFU and this was stronger for *S. aureus* than for *E. coli*. Two explanations can be given for the direct inhibitory effect of fat on bacterial growth. First, a higher fat concentration in milk has been described to be accompanied by a higher amount of complement [34-35]. Presence and function of complement factors in milk were not analyzed in this study and therefore its role in the *in vitro* growth assay could not be determined. Secondly, agitation of milk leads to activation of lipolysis leading to the presence of fatty acids with antimicrobial properties [36-38]. Handling of milk during sampling and assay preparation most likely activated lipolysis by disrupting the milk fat globule and thereby exposing the lipid substrate to the lipase [38-39]. It is unlikely that the inhibitory effect on replication caused by lipolysis occurs *in vivo*. The contrary time effect, *S. aureus* replicating in lower numbers in morning milk whereas *E. coli* replication was higher, was surprising and could not be explained.

Contamination of milk samples incorporated as a binary variable (other bacteria present yes/no) was negatively associated with replication of *S. aureus* in milk *in vitro* which is consistent with literature where inhibition of staphylococcal replication by different bacterial species in the mammary gland as well as *in vitro* has been described [9,40-42]. *In vivo*, the presence of other staphylococci and *Corynebacterium bovis* has been found to reduce the risk of mastitis [43-44]. *In vitro*, the presence of commensal bacteria in human breast milk was shown to reduce growth of *S. aureus* and natural background flora has been considered as a useful tool to prevent redundant growth of staphylococci in food products [45-46]. For *E. coli* replication, a negative association with the presence of other bacteria has not been described so far and was also not present is this study.

In conclusion, almost all measured milk components differed significantly between quarters and showed a diurnal pattern. Although morning and evening milk showed a clear difference in bacterial replication none of the determined milk parameters were clearly associated with the exception of fat. Confirming that *S. aureus* specific immunoglobulins reduce *S. aureus* growth in milk of unvaccinated cows is emphasizing approaches to enhance udder immunity against *S. aureus* by vaccination. The exact mechanism leading to the association between *S. aureus* specific IgG1 concentration and decreased *S. aureus* replication could not be identified. Future studies should look into milk composition in more detail to identify natural antibacterial substances that could be used to enhance udder health.

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Chapter 3

3

Reisolation of *Staphylococcus aureus* from bovine milk following experimental inoculation is influenced by fat percentage and specific immunoglobulin G1 titer in milk

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**Jetta and Susanne contributed equally to this work*

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Abstract

The associations of management parameters, herd characteristics, and individual cow factors with bovine mastitis have been subject of many studies. The present study aimed to evaluate the association between milk composition parameters, including fat, protein, lactose, urea, and specific immunoglobulin levels, at the time of experimental bacterial inoculation of the mammary gland and subsequent shedding dynamics of *Staphylococcus aureus*. Sixty-eight cows were experimentally infected with *S. aureus* and closely monitored for 3 wk. Mixed model analyses were used to determine the influence of management and herd characteristics (farm and experimental group), individual cow factors (days in milk, milk yield, and quarter position), and a challenge related parameter (inoculation dose) in combination with either the milk components fat, protein, lactose and urea, or the *S. aureus*-specific antibody isotype titers at the time of bacterial inoculation, on the number of *S. aureus* reisolated from milk after inoculation. A positive association was observed between the milk fat percentage and the number of *S. aureus* reisolated from quarter milk, and a negative relationship between the *S. aureus*-specific IgG1 titer in milk and the number of *S. aureus*. These findings should be considered in the development of a vaccine against *S. aureus*-induced bovine mastitis.

Introduction

Mastitis is a major concern in the modern dairy industry because it is the most costly infectious disease due to milk losses, the need for veterinary interventions, and early culling of cows. The risks associated with increasing antibiotic resistance due to the current treatment of mastitis with antibiotics call for preventive strategies rather than treatment. Apart from management parameters and hygiene procedures, exploring the potential of vaccination as a preventive strategy is an ongoing effort [1-3]. For evaluation of potential vaccine candidates and vaccines, experimentally induced mastitis is often used as a model system [4,5]. However, in experimental infection studies, it has been observed that inoculation of bacteria leads to highly variable bacterial shedding dynamics between cows and even between quarters of the same cow, indicating the presence of cow- or quarter-dependent susceptibility [6,7]. A substantial amount of research has been dedicated to the identification of individual cow factors and their association with mastitis under field conditions. Frequently studied parameters include farm, herd, milking systems, season, parity, lactation stage, quarter position and physiology, and history of mastitis [8-13]. Other studies have focused on the somatic cell count (SCC) and, more in depth, the different cell types observed in milk pre- and post-infection [6,14-17]. In this study, we analyzed the association between milk composition, including immunoglobulin levels, at the time of experimental bacterial inoculation of the mammary gland and the bacterial shedding dynamics thereafter using data from cows of multiple independent challenge experiments. Measuring the concentrations of fat, protein, lactose, and urea in milk is part of the routine screening of all cows on farms in many management systems. If milk components influence the growth characteristics of mastitis-inducing pathogens, they can contribute to susceptibility or resistance to mastitis, and cows at risk may be quickly identified through these screenings. Although immunoglobulin levels are usually not measured on a regular basis, associations between bacteria-specific immunoglobulin isotype levels and susceptibility or resistance to mastitis are of interest in the development of a vaccine against mastitis. For vaccine efficacy testing, knowledge about the contribution of these factors to a cow's susceptibility or resistance to mastitis will enable defined animal selection and group stratification, which may contribute to a robust and reproducible animal model. The present study specifically focused on experimentally induced intramammary infections with *Staphylococcus aureus* (*S. aureus*), one of the main mastitis-causing pathogens in bovines. The *S. aureus* Newbould strain was used for experimental inoculations because this strain is strongly associated with, and known to be well adapted to, the bovine host [18-19]. Intramammary infections with *S. aureus* are often subclinical and persistent in nature. *Staphylococcus aureus* Newbould has been shown to typically induce subclinical, persistent, and in most cases reproducible infections

[4,6,20]. Furthermore, this strain possesses biofilm-associated genes, colonization factors, and genes encoding secreted toxins [21], which are target proteins in various vaccine development studies [22,23].

Materials and Methods

Animals

Data obtained from 68 clinically healthy, first-lactation Holstein Frisian cows purchased from commercial dairy herds were used in this study. Cows were enrolled in 7 independent challenge experiments between October 2012 and July 2014. Selection of the cows was based on similarity in age (2.5 year ± 3 month), low SCC (< 100,000 cells/mL), and no history of mastitis. Cows were housed in freestall barns at 1 of 2 experimental animal facilities of MSD-Animal Health (farms A and B; Boxmeer area, The Netherlands), and fed a mixed ration containing maize silage, grass clover silage concentrate, and minerals minerals (For Farmers Hendrix, Lochem, The Netherlands) according to their requirements [24]. Water was supplied ad libitum. Before experimental intramammary *S. aureus* inoculation, cows were allowed an acclimatization period of at least 2 month and were kept under regular supervision of a veterinarian. Cows were milked twice a day at 12-hr intervals using a traditional pipeline milking system. Pre-milking teat disinfection was practiced routinely with 70 % alcohol and post-milking teat disinfection with a 0.5 % iodine disinfectant. Daily milk yield was recorded throughout the study. The design of these studies was approved by an independent ethical committee. The experiment was performed in accordance with European Community guidelines and national laws on animal experiments.

Bacterial strain

Animals were experimentally infected with the *S. aureus* Newbould 305 strain (ATCC 29740). A freeze-dried stock of these bacteria was resuspended in sterile water and plated onto sheep blood agar plates (BioTrading, Mijdrecht, The Netherlands). Following overnight incubation at 37 °C, bacteria were cultured in trypticase soy broth (BioTrading) for 5 hr at 37 °C. Before intramammary inoculation, bacteria were washed twice with 0.9 % physiological saline and, following microscopic counting, diluted to the desired concentration in 0.9 % physiological saline. Cultures were plated onto sheep blood agar plates and, following an incubation of 16 to 24 hr at 37 °C and 5 % CO_2, numbers of colony-forming units (CFU) were counted to determine the actual number of viable cells per inoculation dose.

Experimental intramammary inoculation

Animals were experimentally infected by administering 1 mL of inoculum containing 2.41 to 2.93 Log_{10} CFU of *S. aureus* into the teat streak canal of 2 mammary quarters per cow, and the other 2 quarters were left untreated. Inoculation was performed 1 to 3 hr after the morning milking using a sterile plastic 5-mL syringe and individual plastic infusion cannulas (Bovivet Animal Healthcare, Bengaluru, Karnataka, India). Before inoculation, teat ends were disinfected with 70 % alcohol. Generally, both front quarters were experimentally inoculated and the rear quarters were left untreated. Only quarters clear of major mastitis-causing pathogens, free of infection by other bacteria, and with an SCC < 100,000 cells/mL at 2 samplings within 7 days before inoculation were included in the study. When a front quarter did not meet these criteria, a rear quarter was inoculated. The 2 inoculated quarters were always located on opposite sites (left and right) of the mammary gland. Experimental infections were conducted on 7 groups of animals (n = 68 cows; 136 quarters).

Sampling and data collection

Quarter milk samples were aseptically collected by trained animal caretakers at multiple time points following inoculation during morning milking. Milk samples for scoring of clinical symptoms, bacterial growth analysis, and SCC determination were collected just before inoculation (day 0) and 1, 2, 3, 7, 14, and 21 days post-inoculation. The first milk squirts were used to score for clinical symptoms as described below. Next, milk samples of 10 mL for laboratory analyses and 30 mL for SCC determination and milk component measurements were taken. Milk samples for laboratory analyses were transported to the laboratory at ambient temperature, and stored at 4 °C. Within 4 hr of sample collection, bacteriological analysis was performed as described below. Following bacteriological analysis, pre-inoculation milk samples were stored at −20 °C until antibody titer measurements were performed. Milk samples were added to vials pre-loaded with a preservative (sodium azide) and transported to a commercial milk quality assurance laboratory (Qlip, Zutphen, The Netherlands) for SCC determination. Pre-inoculation samples were also used for determination of milk composition with regard to fat percentage, total protein percentage, lactose level, and urea concentration. Determination of the SCC by flow cytometry and compositional parameters by Fourier transform infrared analyses were performed on a CombiFoss device (Foss, Hillerød, Denmark). Before inoculation, blood was collected from the coccygeal vein using a sterile blood collection system (BD Vacutainer, Becton Dickinson B.V., Breda, The Netherlands) and, after coagulation, centrifuged for 10 min at 3,000 × *g* to collect serum. Serum samples were stored at −20 °C until antibody titers were determined.

Clinical symptoms

Following inoculation of bacteria, inoculated quarters were scored for local, clinical signs of mastitis once daily at the morning milking for the remainder of the experiment. Clinical scores were assigned to each quarter Following inoculation of bacteria, inoculated quarters were scored for local, clinical signs of mastitis, where 0 = no abnormalities; 1 = slight swelling of the udder; 2 = moderate swelling; and 3 = severe swelling. Milk alterations were also scored using a 4-point scale, where 0 = normal milk; 1 and 2 = milk with a few or many clots, respectively; and 3 = serous, watery milk. *Staphylococcus aureus* infection was defined as the presence of *S. aureus* in milk post-inoculation independent of the occurrence of clinical signs.

Bacteriological analysis of quarter milk

Staphylococcus aureus in milk was determined as colony-forming units per milliliter by plating 50 µL of milk onto sheep blood agar plates using the EddyJet spiral plater (LabScientific Inc., Livingston, NJ). Milk from highly infected quarters was serially diluted (10-fold) in 0.9 % physiological saline before plating. After an incubation of 16 to 24 hr at 37 °C and 5 % CO_2, numbers of colony-forming units were determined and colonyforming units per milliliter milk calculated. Bacteria were presumptively identified by colony size, morphology, and type of hemolysis, and, when required, further identified using Gram stain, and coagulase tests.

***Staphylococcus aureus*-specific antibody titers assessed by whole-cell ELISA**

Antibodies directed against *S. aureus* in milk and serum were determined by ELISA. Because *S. aureus* Newbould expresses protein A that results in nonspecific binding of antibodies, the capsule-negative Reynolds strain [25] that lacks protein A was used. Staphylococcus aureus Reynolds was grown in trypticase soy broth for 20 hr at 39.5 °C without stirring and subsequently inactivated by overnight incubation with 0.5 % formalin. Bacteria were harvested by centrifugation for 10 min at 5,000 × *g* and resuspended in PBS. Then, Nunc MaxiSorp plates (eBioscience, Hatfield, UK) were coated overnight at 4 °C with 1 µg/well of *S. aureus* in 0.05 M carbonate-bicarbonate buffer and subsequently blocked with 24 µg of casein/well. Samples and positive control serum were tested in 2-fold serial dilutions and incubated for 1 hr at 37 °C. Eight replicates of negative control serum were included. As secondary antibodies, horseradish peroxidase-conjugated sheep anti-bovine IgG monoclonal (MSD-AH, Boxmeer, The Netherlands) or IgG1, IgG2, and IgA (Bethyl Laboratories Inc., Montgomery, TX) were used in 1:3,000, 1:500, 1:3,000, and 1:2,000 dilutions, respectively. Tetramethylbenzidine was used as a substrate and reactions were stopped after 10 min by adding sulfuric acid. Extinctions (450 nm) were measured on a Tecan Sunrise device (Tecan Group Ltd., Männedorf, Germany) using XFluor4 Software version V4.51-I4 (Tecan Group Ltd.). Antibody titers were determined using CaSpEx

Software AbendVertical version 0.11 V1 (proprietary software, MSD-AH) and defined as the Log_2 dilution of the sample that would give the same absorbance as the predefined cut-off. The cut-off was defined as 2 times the average optical density of the negative controls.

Statistical analysis

Data management was done using Microsoft Excel (Office 2010, Microsoft Corp., Redmond, WA). The descriptive and statistical analyses were performed using the statistical software package R (version 2.9.1, R Foundation for Statistical Computing, Vienna, Austria). For graphical presentation of the data GraphPad Prism software (version 5; GraphPad Software Inc., La Jolla, CA) was used. Two mixed model analyses were performed to identify factors influencing the number of *S. aureus* that were reisolated from quarter milk post-inoculation. Model 1 assessed the effect of cow-related factors and the milk components fat, protein, lactose, and urea on the number of *S. aureus*. To fulfill assumptions, the numbers of *S. aureus* were Log_{10}-transformed. Different experimental condition such as season, feeding batch, and other conditions in the stable, were taken into account by (forced) adding experimental group as a categorical variable to the model. Time was added as fixed factor as the samples were taken at fixed time points. In the full model, farm (A or B), quarter position (front or rear), and inoculum doses were included as categorical variables. As continuous variables, days in milk (DIM; per 28 days), milk yield (kg/day; per 5 L), fat (%), protein (%), lactose (%), and urea (mg/L) were included.

Model 2 assessed the effect of cow-related factors and specific antibody titers on the number of *S. aureus* reisolated from quarter milk post-inoculation. In this model, as in model 1, experimental group (forced) and time were included. As continuous variables, DIM (per 28 day), milk yield (kg/day; per 5 L), milk IgG1, milk IgG2, milk IgA, and serum IgG concentrations were included. Quarter position was excluded from this model because it was the same for all quarters analyzed in this model. Farm and inoculum dose were excluded because they were incorporated in the variable experimental group.

For both models, residuals were assessed for normal distribution and constant variance. Cow was added to the models as random effect to take repeated measurements within a cow into account, as well as a random slope for time within a cow. For the linear mixed effect models, the R package nlme was used. Function dredge (R package MuMIn) was used to find the best model between al competing models based on the Akaike's information criterion.

To confirm the significant associations observed in the mixed model analyses, differences in the milk fat percentage and the IgG1 titer in milk between quarters with different *S. aureus* shedding patterns were calculated using the 2-tailed t-test. P-values < 0.05 were considered significant.

Results

Intramammary infection

Following intramammary inoculation, 135 of 136 quarters became infected with *S. aureus*, as measured by the detection of *S. aureus* in quarter milk 1 day post-inoculation. *Staphylococcus aureus* was undetectable in milk from 1 quarter throughout the entire experimental period of 3 wk. The number of *S. aureus* reisolated from milk of 67 of 136 quarters (of 48 cows) was below the detection limit of 20 CFU/mL on at least one sampling occasion post-inoculation (day 1, 2, 3, 7, 14, or 21). Most of these quarters were positive again at one of the other consecutive samplings. The numbers of *S. aureus* reisolated from milk of 20 of 67 quarters (of 18 cows) were below the detection limit on at least 2 consecutive sampling occasions and remained negative until the end of the experimental period. Overall, the shedding dynamics of *S. aureus* could be divided into 2 patterns: quarters with a decreasing number of *S. aureus* (n = 60) and quarters with a constant or increasing number of *S. aureus* (n = 76; Figure 1). The SCC pattern post-inoculation was similar among all cows, regardless of the *S. aureus* shedding pattern observed (Figure 2). The mean Log_{10} SCC (cells per mL/1,000) of quarters used in model 1 was 1.06 ± 0.29 cells/mL, and the mean SCC of quarters used in model 2 was 1.02 ± 0.35 cells/mL before inoculation. Experimental inoculation of *S. aureus* resulted in subclinical mastitis, as reflected by generally low milk and udder scores and only a slight decrease in the mean daily milk yield during the first 4 days post-inoculation (Figure 3).

Factors influencing the numbers of *Staphylococcus aureus* reisolated from quarter milk post-inoculation

A total of 68 cows (n = 136 quarters) received intramammary inoculation of *S. aureus*. For model 1, which aimed to identify associations between milk components and the number of *S. aureus* reisolated from milk post-inoculation, data of 52 challenged quarters were excluded because the amount of the milk sample was insufficient to perform all tests accurately, resulting in one or more missing values per milk sample.

In total, 136 quarter milk samples were analyzed for fat percentage, total protein percentage, lactose level, and urea concentration. Overall, 84 of 544 milk parameter concentrations (15%) could not be obtained. In the statistical analyses used, all data of a quarter were excluded when a single value was missing. Quarters with missing values were equally divided over the groups with a decreasing and constant or increasing shedding pattern (18 of 60 and 20 of 76, respectively).

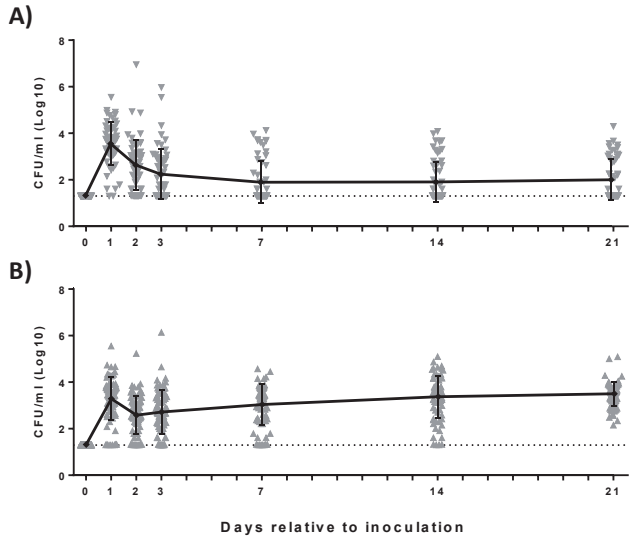

Figure 1: Staphylococcus aureus shedding patterns postinoculation. *Two milked-out quarters per cow were experimentally inoculated with S.aureus and bacterial shedding (expressed as Log_{10} CFU/mL) was measured over a 21-day period. The graph represents 2 different shedding patterns: (A) quarters with decreasing numbers of S. aureus (n = 60), and (B) quarters with constant or increasing numbers of S. aureus (n = 76). Lines represent the mean ± SD. The detection limit is 20 CFU/mL (1.3 Log_{10} CFU/mL; dotted line)*

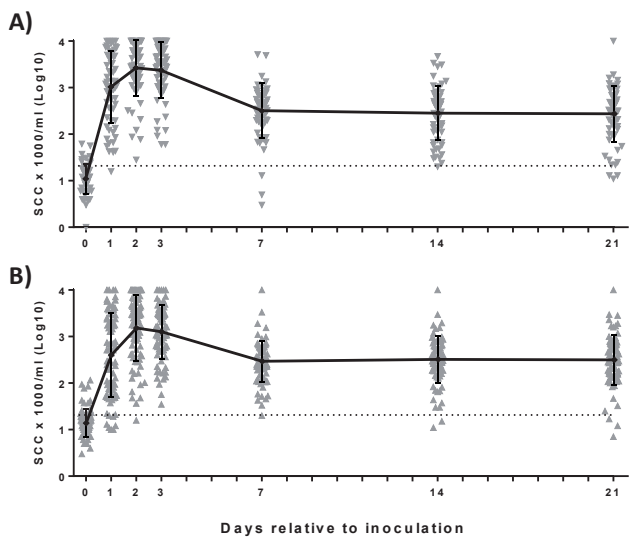

Figure 2: Quarter milk SCC post-inoculation. *Following experimental inoculation of Staphylococcus aureus, the SCC was determined. Thegraph represents SCC (mean ± SD) in cows with different S. aureus shedding patterns: (A) quarters with decreasing numbers of S. aureus (n = 60), and (B) quarters with constant or increasing numbers of S. aureus (n = 76). The detection limit is 4 Log_{10}.*

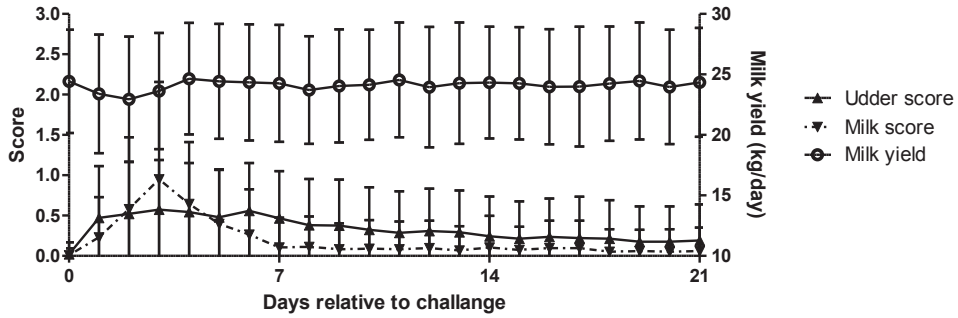

Figure 3: Udder and milk scores and daily milk yield post-inoculation. *Following experimental inoculation of Staphylococcus aureus, a scoring system was used to monitor (sub)clinical mastitis. A score of 0 indicates a lack of clinical signs of mastitis; the maximum udder score of 3 indicates severe udder swelling, and the maximum milk score of 3 indicates serous, watery milk. Results are expressed as mean ± SD. The graph represents data from 136 challenged quarters.*

Because data on the immunoglobulin levels of the quarters with missing milk composition concentrations were available, quarters were not excluded from the entire study but included in model 2, which aimed to identify associations between specific antibody levels at the time of inoculation and the number of *S. aureus* reisolated from milk post-inoculation. An overview of the parameter characteristics used in model 1 is given in Table 1 (mean ± SD). An overview of the parameter characteristics used in model 2 is given in Table 2 (mean ± SD). Data of *S. aureus*-specific antibody titers were available for 63 quarters of 32 cows.

In model 1, the variables quarter position, inoculation dose, DIM, milk yield, protein, and lactose were removed from the full model because they did not improve the fit significantly. The final model, including time, experimental group (forced), farm, fat percentage, and urea concentration, identified a positive association between the milk fat percentage and the number of *S. aureus* reisolated from quarter milk. Results indicate that an increase in the fat percentage of 1 resulted in a 2.5-fold increase in the numbers of *S. aureus* (p = 0.03). A direct comparison of the milk fat percentage between quarters with a decreasing and pattern and a constant or increasing *S. aureus* shedding pattern showed no significant differences (p = 0.09; Figure 4A).

Table 1: *Overview of cow and milk characteristics of animals (means with SD in parentheses) enrolled in model 1.*

Group	No. of quarters	Farm[1]	Quarter position[2]	Inoculum dose (Log_{10} CFU)	DIM	Milk yield (kg/day)	Fat (%)	Protein (%)	Lactose (%)	Urea (mg/L)
1	14	A	F	2.53 (1.8)	183.9 (32.9)	20.8 (3.9)	2.80 (0.89)	3.69 (0.32)	4.69 (0.14)	14.1 (2.9)
2	10	B	F(8)/R(2)	2.62 (1.9)	186.4 (8.9)	23.4 (4.3)	2.42 (0.46)	3.91 (0.19)	4.68 (0.13)	19.4 (4.4)
3	8	B	F(7)/R(1)	2.86 (2.1)	190.3 (3.5)	27.2 (3.4)	2.00 (0.40)	3.66 (0.16)	4.75 (0.04)	21.4 (2.6)
4	8	B	F(7)/R(1)	2.76 (2.1)	205.3 (3.6)	20.0 (5.8)	2.34 (0.46)	3.73 (0.39)	4.66 (0.10)	18.1 (2.0)
5	14	B	F(12)/R(2)	2.93 (2.0)	204.5 (5.8)	23.7 (4.3)	2.30 (0.47)	3.72 (0.26)	4.68 (0.11)	20.7 (3.4)
6	14	A	F(7)/R(7)	2.63 (1.7)	159.1 (6.3)	23.3 (4.2)	2.23 (0.51)	3.51 (0.19)	4.73 (0.09)	14.9 (3.8)
7	16	B	F	2.41 (2.0)	135.1 (15.0)	25.4 (3.6)	2.08 (0.38)	3.26 (0.17)	4.77 (0.13)	18.1 (2.5)
Total	84	-[1]	-[2]	-	177.5 (29.3)	23.6 (4.5)	2.34 (0.58)	3.61 (0.32)	4.71 (0.12)	18.1 (4.1)

[1] *A = 28/84; B = 56/84*

[2] *F (front) = 71/84; R (rear) = 13/84*

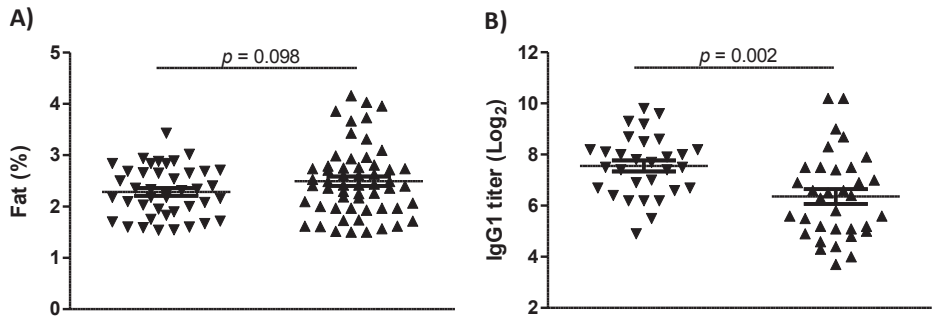

Figure 4: Differences in milk fat percentage and Staphylococcus aureus-specific IgG1 titers between quarters with different shedding patterns. *Differences in the milk fat percentages (A) and S. aureus-specific IgG1 titers (B) before inoculation between quarters showing a decreasing (▼) and a constant or increasing (▲) S. aureus shedding pattern post-inoculation are depicted (mean ± SEM).*

In model 2, the variables DIM, milk yield, milk IgG2, milk IgA, and serum IgG were removed from the full model because they did not improve the fit significantly. The final model, including time, experimental group (forced), and milk IgG1 antibody titers, identified a negative association between the *S. aureus* specific IgG1 titer in quarter milk and the numbers of *S. aureus*. An increase in IgG1 titer of 1 Log_2 was associated with a 0.7-fold reduction in the numbers of *S. aureus* (p = 0.04). A direct comparison of specific IgG1 titers between quarters with different *S. aureus* shed ding patterns showed a significantly higher IgG1 titer in quarters with a decreasing shedding pattern compared with quarters with a constant or increasing shedding pattern (p = 0.002; Figure 4B).

Table 2: *Overview of cow and milk characteristics of animals (means with SD in parentheses) enrolled in model 2.*

Group	No. of quarters	DIM	Milk yield (kg/day)	Milk[1] IgG1	Milk[1] IgG2	Milk[1] IgA	Serum[1] IgG
1	14	183.9	20.8	6.5	3.1	5.6	13.1
		(32.9)	(3.9)	(1.8)	(0.7)	(0.4)	(1.1)
6	18	159.4	22.4	6.7	3.7	6.6	14.1
		(6.2)	(4.5)	(1.7)	(1.1)	(0.9)	(1.1)
7	31	138.1	25.5	7.3	4.0	5.2	16.7
		(15.8)	(3.4)	(1.4)	(1.0)	(1.0)	(0.7)
Total	63	154.5	23.6	6.9	3.7	5.7	15.2
		(26.0)	(4.3)	(1.6)	(1.0)	(1.1)	(1.8)

[1] *Staphylococcus aureus Reynolds specific antibody titers (Log_2)*

We observed a significant difference in the *S. aureus* specific serum IgG titer between cows housed at the 2 experimental farms ($p < 0.00$). The mean Log_2 serum IgG titers of cows housed at farm A was 13.6, whereas the mean Log_2 serum titers of cows housed at farm B was 16.7. This difference was not reflected in milk IgG1 or IgG2 titers (Supplementary Figure S1).

No association between the quarter position and the numbers of *S. aureus* reisolated from quarter milk was observed. A total of 115 front quarters and 21 rear quarters were inoculated in this study. The 2 different shedding patterns (Figure 1) were represented in both front and rear quarters with almost equal distribution. Rear quarters represented decreasing numbers of *S. aureus* (7/60; 11.6%) and quarters with constant or increasing number of *S. aureus* (14/76; 18.4%) post-inoculation. To analyze whether the association between the milk fat concentration and the *S. aureus*-specific IgG1 titer in milk and the numbers of *S. aureus* reisolated from milk was cow or quarter related, the correlation between these 2 factors and the 2 inoculated quarters of the same cow were analyzed. Both the milk fat percentage ($R_2 = 0.794$) and *S. aureus*-specific IgG1 titers ($R_2 = 0.867$) at the time of inoculation were comparable between the 2 quarters of the same cow (Supplementary Figure S2).

Discussion

This study aimed to evaluate the association between milk composition parameters and the shedding dynamics of *S. aureus* following experimental intramammary inoculation. The enrollment criteria in this study in combination with the experimental infection provided a unique study population of cows with similar management and herd characteristics, enabling analysis of individual cow- and quarter-related factors, including the milk components fat, protein, lactose, urea, and *S. aureus*-specific antibody titers, with the severity of experimentally induced *S. aureus* bovine mastitis.

In the studies presented here, experimental inoculations with *S. aureus* resulted in subclinical mastitis with > 99% infected quarters post-inoculation. Both a decreasing shedding pattern and a constant or increasing *S. aureus* pattern were observed during the experimental period. Statistical models revealed a significant positive and negative contribution to the numbers of *S. aureus* reisolated from quarter milk post-inoculation for milk fat percentage and *S. aureus*-specific IgG1 titer in milk, respectively. In agreement with these results, quarters with a decreasing *S. aureus* shedding pattern showed a significantly higher IgG1 titer pre-inoculation compared with quarters with a constant or increasing *S. aureus* shedding pattern. In contrast, no differences in the milk fat percentages were observed between quarters with different *S. aureus* shedding patterns.

A key component of the bovine innate intramammary immune response against *S. aureus* is the ability of immune cells, which are resident in the milk, to phagocytose *S. aureus*. For phagocytosis to occur, recognition between bacteria and phagocytes is conferred by immunoglobulins and complement components. In the normal healthy mammary gland, macrophages constitute the predominant cell type [26]. Phagocytosis by macrophages can be enhanced by IgG1 antibodies, which is the main antibody isotype in normal bovine milk [27,28]; IgG1 also plays a role in complement activation [29]. However, in the healthy bovine mammary gland, the alternative, antibody-independent complement pathway has been shown to be the sole operating complement pathway [30-32]. The classical complement pathway, which does require the presence of antibodies because C1q activation is obtained by antigen:antibody complex recognition, was shown to be inactive due to limited concentrations of C1q [30]. This suggests that the negative relationship between the *S. aureus*-specific IgG1 titer in milk at the time of inoculation and the numbers of *S. aureus* reisolated from quarter milk post-inoculation observed in this study is likely due to an increased phagocytic capacity of macrophages rather than increased complement activation.

Staphylococcus aureus prevents opsonophagocytosis by expressing capsule polysaccharides. Capsule polysaccharide serotype-specific antibodies are required to increase phagocytosis [33]. Thus, for opsonophagocytosis of the *S. aureus* Newbould 305 CP5 (capsule-positive) strain used for experimental inoculations, CP5-specific antibodies are required. In the present study, IgG1 antibody titers were determined using the capsule-negative *S. aureus* Reynolds strain to prevent nonspecific binding of antibodies and protein A in the ELISA. Because CP5-specific antibody levels were not determined in the current study, the actual effect of IgG1 on phagocytosis might have been underestimated. Although it is known that IgG2 antibodies also enhance the activity of phagocytic cells, an association between IgG2 antibodies and the numbers of *S. aureus* in milk could not be identified in this study. This is in line with previous results indicating that receptors for bovine IgG2 were either absent from or present at a low level on the surface of macrophages but abundantly expressed on polymorphonuclear monocytes (PMN) [28]. Both IgG2 levels and the number of PMN are relatively low in normal milk but rapidly increase following recruitment after infiltration of the mammary gland [26,34,35]. The cows used for this study were selected based on the absence of intramammary infections, resulting in relatively low IgG2 levels and PMN concentrations in their milk at the time of inoculation, which might explain the lack of association between IgG2 levels and the numbers of *S. aureus*. Post-inoculation, the recruitment of PMN (among other cell types) is reflected by increased quarter milk SCC. Therefore, IgG2 might play a role in the host's defense against *S. aureus* during later stages of infection.

In this study, we observed a significant difference in preexisting *S. aureus*-specific serum IgG titers between cows housed at the 2 experimental farms. Because cows were not immunized against *S. aureus* before enrollment, specific antibody titers are thought to be a result of natural exposure to the pathogen at various body sites such as the teat skin and external orifices [36]. The differences in serum antibody titers between cows on the different farms were not reflected in the milk antibody titers. This might indicate that enhancing systemic antibodies might not lead directly to an increase in local immunoglobulin levels in the milk. Therefore, immunization techniques that specifically improve antibody levels in milk should be considered [37].

Whereas the phagocytic capacity of macrophages and neutrophils is enhanced by IgG1 antibodies, phagocytosis can be inhibited by the presence of milk fat globules. When encountering fat globules within the mammary gland, phagocytes start to ingest these globules. As a result, the ability of the cell to lower the pH of phagosomes is impaired, affecting digestion by lysosomal enzymes and thereby reducing the phagocytic capacity of the phagocyte [38]. Reduced phagocytosis and subsequent killing of *S. aureus* might explain the positive association observed in this study between milk fat percentage preinoculation and the numbers of *S. aureus* reisolated from quarter milk post-inoculation. The effect of fat on phagocytosis has previously been observed in a murine model, where chemotaxis of neutrophils was decreased after a high fat diet was provided, resulting in impaired phagocytosis of *S. aureus* [39]. High concentrations of fat in milk preinoculation may benefit *S. aureus* in more than one way because *S. aureus* is able to form a biofilm type of growth by binding to milk fat globules [40]. Biofilm formation is thought to play an important role in the pathogenesis of *S. aureus* infections because it contributes to the evasion of the cows' immunological defense mechanism [41,42].

On many farms, milk fat percentages are measured routinely at the bulk tank level but also on an individual cow basis. The fat percentages measured during these routine analyses might not be directly comparable to the fat percentages measured in this study because of differences in sampling methods. To associate milk fat percentages with susceptibility to intramammary *S. aureus* growth, it is important to avoid variation and to standardize the sampling time and method because milk fat concentrations are highly variable and influenced by season, nutrition, genetic variation, and even the time of milking [43-45]. Therefore, using milk fat percentage as an indicator for health disturbances is challenging. In addition, although the milk fat percentage pre-inoculation was associated with the numbers of *S. aureus* reisolated post-inoculation, there was no association with the observed shedding patterns. This suggests that milk fat percentage is influenced during the course of the infection. Future research focusing on the dynamics of milk fat percentage over the course of infection is required to conclude whether fat percentage is indeed subject to changes by intramammary growth of *S. aureus*.

It has been suggested that the position of a quarter on the mammary gland affects its susceptibility to naturally acquired *S. aureus* infections [13,17,46]. Results of this study show no association between front or rear quarters and the numbers of *S. aureus* reisolated from quarter milk. The position of the quarter is bypassed under the experimental inoculation conditions applied in this study, suggesting that, once the organism is inside, the position of the quarter is insignificant to *S. aureus*. This is in line with the observation that, at least when sampled at the same time via the same procedure, both milk fat percentage and IgG1 titers are comparable between quarters of the same cow regardless of their positions. Thus, quarter position and the physiological condition of the teat are likely to play a role in susceptibility to bacterial entrance, rather than in the establishment of an infection once the bacteria has penetrated the mammary gland. This underlines the importance of udder health management and improved milking routines in the prevention of mastitis [47,48].

The fact that milk fat concentrations and IgG1 titers in milk are similar between the 2 quarters of the same mammary gland and interquarter differences in bacterial shedding dynamics are frequently observed, it is unlikely that susceptibility to *S. aureus*-induced mastitis is solely dependent on these 2 milk components. Therefore, the possible influence of milk factors that were not analyzed in this study, such as cytokines, complement components, and lactoperoxidase [49], the milk microbiome [50], and extracellular, cell-derived vesicles [51,52], on the bacterial growth of *S. aureus* in milk needs to be elucidated in future studies. Besides focusing on specific preselected compounds, nontargeted proteomics approaches may also be considered [53].

Conclusions

The aim of this study was to identify whether routinely measured milk components and *S. aureus*-specific antibody titers influence the numbers of *S. aureus* that can be reisolated from quarter milk following experimental inoculation. Our results revealed a significant positive association of the milk fat percentage pre-inoculation and the numbers of *S. aureus* reisolated from milk post-inoculation. In addition, results showed that the *S. aureus*-specific IgG1 titer pre-inoculation is negatively associated with the numbers of *S. aureus* reisolated from milk post-inoculation. These findings support the ongoing effort to develop a vaccine against bovine *S. aureus*-induced mastitis and indicate that stimulation of the humoral immune response in milk against this bacterium might contribute to protection.

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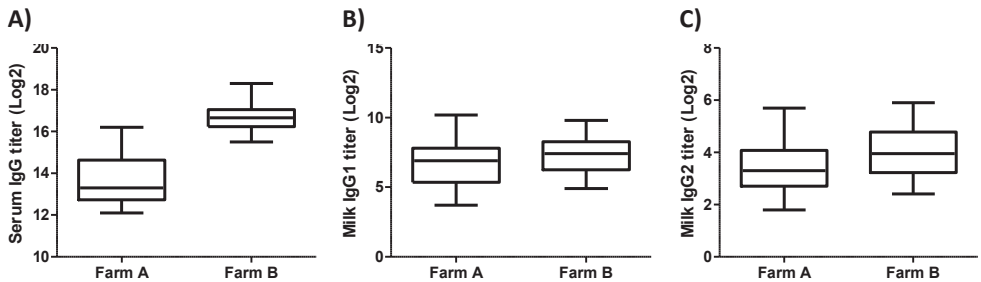
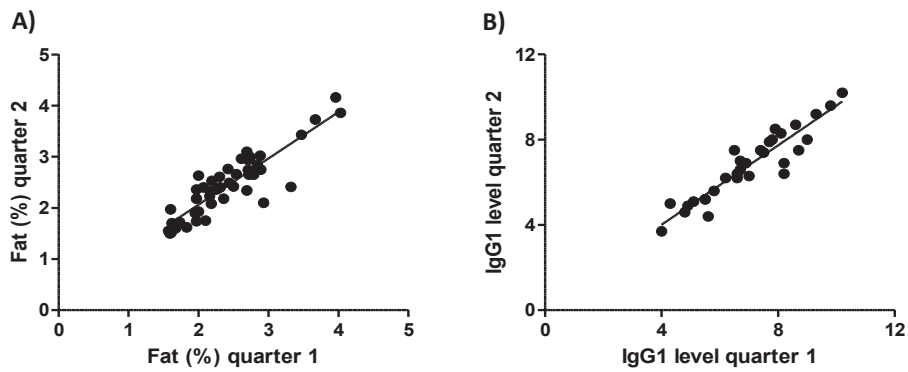
Supplementary Figures



Supplementary Figure 1: Between-farm differences in S. aureus specific antibody titers. *Serum IgG (A), milk IgG1 (B), and milk IgG2 (C) antibody titers as measured by ELISA. Results are presented as the median with the 25th to 75th percentiles. Whiskers indicate the min to max titer.*



Supplementary Figure 2: Inter-quarter correlation. *Correlations between the milk fat percentages (A) and specific IgG1 titers (B) of two inoculated quarters of the same mammary gland prior to S.aureus inoculation are presented. Both the milk fat percentage (R_2 = 0.891) and S. aureus specific IgG1 titers (R_2 = 0.931) were highly correlated.*

Chapter 4

Immunization routes in cattle impact the levels and neutralizing capacity of antibodies induced against *Staphylococcus aureus* immune evasion proteins

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Abstract

Vaccines against *S. aureus* bovine mastitis are scarce and show limited protection only. All currently available vaccines are applied via the parenteral (usually intramuscular) route. It is unknown, however, whether this route is the most suitable to specifically increase intramammary immunity to combat *S. aureus* at the site of infection. Hence, in the present study, immunization via mucosal (intranasal; IN), intramuscular (triangle of the neck; IM), intramammary (IMM) and subcutaneous (suspensory ligament; SC) routes were analyzed for their effects on the quantity of the antibody responses in serum and milk as well as the neutralizing capacity of the antibodies within serum. The experimental vaccine comprised the recombinant *S. aureus* immune evasion proteins extracellular fibrinogen-binding protein (Efb) and the leukotoxin subunit LukM in an oil-in-water adjuvant combined with a hydrogel and alginate.

The highest titer increases for both Efb and LukM specific IgG1 and IgG2 antibody levels in serum and milk were observed following SC/SC immunizations. Furthermore, the harmful effects of Efb and leukotoxin LukMF' on host-defense were neutralized by serum antibodies in a route-dependent manner. SC/SC immunization resulted in a significant increase in the neutralizing capacity of serum antibodies towards Efb and LukMF', shown by increased phagocytosis of *S. aureus* and increased viability of bovine leukocytes. Therefore, a SC immunization route should be considered when aiming to optimize humoral immunity against *S. aureus* mastitis in cattle.

Introduction

Infections with Staphylococci are common among humans and animals [1-3]. In cattle subclinical intramammary infections with *Staphylococcus aureus* (*S. aureus*) are common. Infections may lead to severe mastitis and/or chronic persistent infection with detrimental effects for the cows' well-being, lifespan and milk production [4,5]. The current treatment of *S. aureus* infections with antibiotics often fails to completely clear the infection, due to specific cow or pathogen related risk factors [6]. Ineffective treatment may result in increased antibiotic resistance in *S. aureus*. Therefore, the availability of an effective vaccine would be of great value [7]. However, despite the numerous attempts to develop a highly efficacious vaccine, commercially available vaccines against *S. aureus* mastitis are scarce and evaluation under field conditions have shown to result in limited protection only [8]. All current vaccines are applied parenterally inducing a systemic immune reaction, which is reflected by an increase in specific antibodies in serum [9]. To reach the site of infection, antibodies induced by parenteral immunization need to be translocated to the milk and hence pass the blood-udder barrier, an effective, physiological separation between the systemic circulation and the udder tissue [10-13]. This does only occur once infection has been established, therefore the goal of preventing new intramamamary infections has not been reached so far [14,15]. To develop an effective vaccine against *S. aureus* mastitis, it may be essential to increase intramammary, rather than systemic, humoral immunity. To date, little information is available regarding the impact of immunization routes on humoral immune responses in the bovine mammary gland [16,17].

From an immunological point of view, it is not clear whether the udder is part of the mucosal immune system or the skin immune system [18,19]. In addition, the environment of antigen uptake, processing and presentation may influence the magnitude of the antibody response as well as the neutralizing capacity of these antibodies. *Staphylococcus aureus* expresses and secretes many immune evasion proteins [20]. Two of these proteins, extracellular fibrinogen-binding protein (Efb) and the leukotoxin subunit LukM, are suitable experimental antigens for the assessment of antibody quantity and their neutralizing capacity after immunization via different routes. Furthermore, both proteins are potential vaccine candidates since they are known to be involved in the pathogenesis of many *S. aureus* strains [21-24]. Efb is known to generate a capsule-like shield around the surface of *S. aureus* through a dual interaction with complement C3 and fibrinogen to mask surface-bound C3b and antibodies thereby escaping recognition by phagocytic cells like neutrophils [25]. LukM is the binding subunit of the bi-component leukotoxin LukMF`, competent of killing bovine peripheral blood leukocytes (PBLs) in a highly efficient manner [26,27]. Antibodies induced by immunization may prevent the interaction of Efb with C3,

fibrinogen, or both, thereby preventing the formation of a capsule-like shield. In addition, neutralization of LukMF' may be accomplished by antibodies blocking the interaction of LukM with its target receptor on the surface of neutrophils [28,29], or by antibodies blocking the required interaction between LukM and LukF', thereby preventing pore formation [30]. Since it is thought that a delay in neutrophil lysis will allow these cells to phagocytose *S. aureus*, increased levels of neutralizing antibodies to both Efb and LukM are likely to improve vaccine efficiency [30,31].

The objective of this study was to analyze the impact of vaccine administration via different routes on the quantity of the antibody responses as well as the neutralizing capacity of these antibodies in dairy cattle using Efb and LukM as vaccine antigens.

Materials and methods

Animals

Sixteen clinically healthy mid-lactation dairy cows of the Holstein Frisian breed purchased in the Netherlands were housed at the Faculty of Veterinary Medicine (Utrecht, The Netherlands). After arrival, somatic cell counts (SCC) were determined at cow level by a commercial milk quality assurance laboratory (Qlip, Zutphen, The Netherlands). Only cows with a SCC < 100.000 cells/mL were enrolled and were allowed an acclimatization period of two weeks to get used to the daily routine before sampling and immunization. Cows were fed a diet based on grass and corn silage, beet pulp and concentrate for the entire study period which was formulated to meet the dietary requirements for lactating dairy cows [32]. Concentrate was administered via an automated feeding system and irregular concentrate uptake was monitored. Cows were milked twice a day and milk yield was recorded with an automated milk recording system. Following immunization, cows were daily monitored for signs of general and local reactions to the immunization by a veterinarian. The injection site was palpated to detect any swelling and painfulness. In cases where local reactions at the site of injection were observed a clinical examination of the cow was performed to identify general reactions. When immunization related changes in the udder tissue were detected quarter level SCC was determined using the California mastitis test. When abnormalities in the milk were observed, bacterial culture was performed by plating 50 µL of milk onto sheep blood agar plates. After an overnight incubation at 37 °C bacterial growth was determined. Bacteria were presumptively identified by colony size, morphology, pigmentation and type of hemolysis.

The use of animals in this study was approved by the Ethical Committee for Animal Experiments of the Utrecht University (DEC2012.II.09.136) and conducted according to national regulations.

Vaccine composition and recombinant proteins

The experimental vaccine consisted of an oil-in-water adjuvant combined with a hydrogel and alginate (proprietary adjuvant, MSD-AH). As antigenic proteins, Efb and LukM, were used (50 µg/dose each). Cows were immunized with *S. carnosus* derived Efb and *E. coli* derived LukM. *Staphylococcus carnosus* derived Efb was also used for ELISAs, while neutralization assays were performed with *E. coli* derived Efb, LukM and LukF'. For expression in *S. carnosus*, the gene encoding efb from the *S. aureus* Newbould 305 strain (ATCC29740) was amplified by PCR and ligated into a pXR100 derived vector. *Staphylococcus carnosus* culture supernatant was 0.2 µm filtered, analyzed on gel for Efb purity and concentration, and stored at -20 °C. For expression in *E. coli*, Efb, LukM and LukF' proteins were generated as described previously [33,34]. Briefly, the efb gene of the *S. aureus* Newman strain and the lukm and lukf gene sequences of the *S. aureus* field isolate S1444 were amplified by PCR and ligated into the pRSETB vector (Invitrogen). The proteins were expressed with a six-residue N-terminal HIS-tag and purified by nickel-chelating chromatography (GE Healthcare) according to the manufacturer's manual. Purified proteins were dialyzed against PBS and stored at −20 °C.

Immunization

Cows were randomly assigned to four groups and immunized twice (1 mL/dose) with a six-week interval. Immunizations were administered intranasal (IN/IN), intramuscular in the injection triangle of the neck (IM/IM), intramammary (one dose per each of the four milked-out quarters) followed by a subcutaneous booster close to the suspensory ligament (IMM/SC), and subcutaneous with both injections close to the suspensory ligament (SC/SC). The rationale behind the IMM/SC route was that cells primed in the mammary tissue would migrate to the local lymph node and re-enter the mammary gland following a booster immunization thereby enhancing the local immune response. For intranasal administration, aerosolized inoculums with a variable size were injected directly into the nostril using a nasal spray pump. For intramuscular and subcutaneous administrations 21G needles (BD MicrolanceTM, Broek op Langedijk, The Netherlands) were used. For intramammary administration a sterile plastic 5 mL-syringe and individual plastic infusion cannulas (Bovivet Animal Healthcare, Konannkunte, Banglore) were used.

Sampling and sample preparation

Blood, milk, saliva and nasal secretion samples were collected at three time points: before immunization, three weeks after the priming immunization, and two weeks after the booster immunization. Blood was collected from the coccygeal vein using a sterile blood collection system (BD Vacutainer, Beckton Dickinson B.V., Breda, The Netherlands) and, after coagulation, centrifuged for 10 min at 1000 x *g* to collect serum. Milk samples,

collected before the morning milking, were centrifuged for 10 min at 1000 x *g* to obtain skimmed milk. Saliva and nasal secretions were collected by inserting a tampon into the cow's mouth or nostril. Tampons were removed after 1 min and transferred into a 20 mL syringe. To extract secretions, 4 mL of PBS (Lonza, Basel, Switzerland) were added and 1-2 mL of secretion was extracted from each tampon by compression within the syringe barrel. All samples were stored at −20 °C.

ELISA

The presence of Efb and LukM specific IgG1, IgG2 and IgA antibodies in serum, milk, saliva and nasal secretions was determined by ELISA. Plates (NUNC MaxiSorpTM, eBioscience, Affymetrix, Santa Clara, USA) were coated with 0.55 µg/mL Efb or 3 µg/mL LukM in 0.05 M sodium-bicarbonate buffer. Samples and positive control serum were tested in two-fold serial dilutions. An in-house negative control serum was taken along in eightfold. As secondary antibodies, horseradish-peroxidase-conjugated sheep α bovine IgG1, IgG2 and IgA (Bethyl Laboratories, Inc., Montgomery, USA) were used in 1:4000, 1:8000 and 1:5000 dilutions for the LukM ELISAs, respectively. For the Efb ELISAs these antibodies were used in 1:6000, 1:12,000 and 1:8000 dilutions, respectively. Tetramethylbenzidine was used as a substrate and reactions were stopped by adding 4 N sulphuric acid. Extinctions (450 nm) were measured on a Tecan SUNRISETM (Tecan Group Ltd., Männedorf, Switserland) spectrophotometer using the XFluor4 Software Version V4.51-I4.

Efb neutralization assay

The neutralizing capacity of specific Efb antibodies in serum was analyzed using a phagocytosis based assay described previously [25]. In short, 2.5 µg Efb was incubated with complement inactivated pre- or post-immunization serum or negative control serum for 10 min at room temperature (RT; 18-21 ^{o}C). Then, bovine lepirudin anti-coagulant plasma (5 % final concentration; CSL Behring GmbH, Marburg, Germany) and 0.6 x 10^7 CFU FITC-labeled *S. aureus* KV27 were added, followed by an incubation of 10 min at RT. Simultaneously, bovine PBLs of a blood donor where freshly isolated by adding 10 mL aquadest to 3 mL heparinized blood to lyse red blood cells. Then, 37 mL of RPMI medium (Gibco®, Paisley, Scotland) containing 0.05% human serum albumin was added followed by centrifugation for 4 min at 300 x *g*. Cells were washed twice and resuspended in medium. Leukocytes (5 x 10^5 cells) were then added to the assay and phagocytosis was allowed for 15 min at 37 °C. The reaction was stopped by 1 % phosphate-buffered formaldehyde fixation for 30 min at 4°C and analyzed by flow cytometry on a FACSCalibur (Beckton Dickinson B.V., Breda, The Netherlands). To determine changes in phagocytosis as a result of antibody addition, cells were gated based on their forward and side scatter. The fluorescence of 10,000 gated neutrophils was measured for each sample.

Phagocytosis was expressed as the percentage of neutrophils with a fluorescence above baseline (cells without fluorescent bacteria). Finally, phagocytosis ratio's post- and pre-immunization were determined.

LukM neutralization assay

Complement inactivated pre- and post-immunization sera were analyzed for their ability to neutralize pore-formation and cell lysis induced by LukMF'. Sera were incubated with 33 nM LukMF' for 30 min at RT and subsequently with 5 x 10^6 bovine PBLs (isolated as described above) in the presence of 1.8 µg/mL 4',6-diamidino-2-phenulindole (DAPI). The final concentration of LukMF' was 10 nM. DAPI fluorescence was measured in duplo for 30 min at 37 °C in a FLUOstar Omega microplate reader (BMG Labtech GMBH, Ortenberg, Germany). The time of lysis onset of the donor cells was defined as the time when DAPI-fluorescence reached three times the standard deviation of control samples that were incubated without LukMF'. The average time of lysis onset was calculated from three independent experiments.

Calculations and statistics

Antibody titers were determined using CaSpEx Software AbendVertical version 0.11 V1 (MSD, Proprietary Software) and defined as the dilution of the sample that would give the same absorbance as the predefined cut-off. The cut-off was defined as 2 times the average negative control. For the Efb neutralization assay, the average ratio between post- and pre-immunization per cow was calculated from four independent experiments using blood from different donor cows. Data analysis was performed in the SPSS statistical software package (version 20; IBM SPSS statistics 20.0; IBM Corp., Armonk, NY, USA). Data were checked for normality and Log_2 transformed to achieve normality when necessary. Success of randomization was checked by comparing initial group antibody titers using an ANOVA with Bonferroni correction. Total titer increases and changes in phagocytic activity were calculated by subtracting pre-immunization values of post-booster values. Differences in total titer increase and percentage phagocytosis between groups were analyzed using the One-way ANOVA with Bonferroni correction. Differences in lysis onset pre- and post-immunization in the LukM neutralization assay were analyzed per group using a repeated measures analysis with the lysis onset as dependent variable. P-values below 0.05 were considered significant. For graphical presentation of the data GraphPad Prism software (Version 5; GraphPad Software Inc., La Jolla CA, USA) was used.

Results

Clinical observations

The median lactation of enrolled cows was 2 (min 1; max 6). Cows produced on average 25.3 (SD ± 1.5) liters of milk per day throughout the study. According to the automated feeding system cows finished their daily concentrate ration during the entire study period.

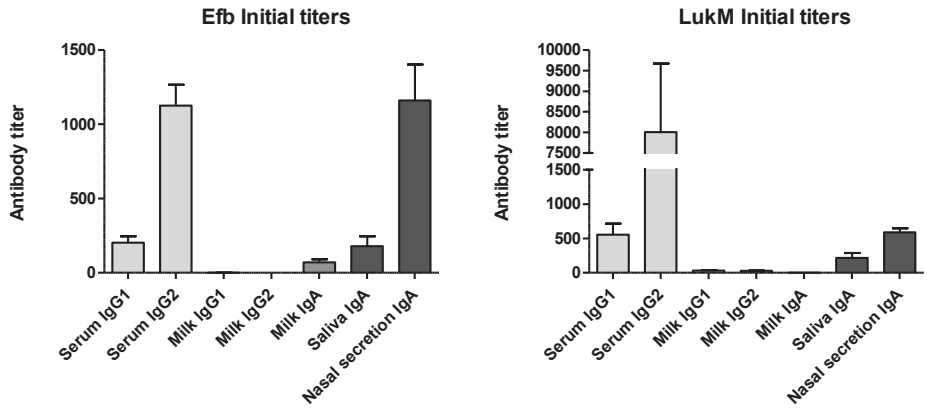

Figure 1: Pre-immunization titers of specific antibody isotypes directed against Efb and LukM. *Initial Efb (A) and LukM (B) specific antibody titers in serum, milk, saliva and nasal secretion samples were measured by ELISA. Each bar represents mean ± SEM (n = 16).*

A small nodule at the site of injection developed in all IM and SC immunized cows during the first three days post-immunization. In one IMM immunized cow a hardened quarter with an increased SCC was observed one day following treatment. However, bacterial culture did not reveal the presence of any bacteria. IN immunized cows did not display any symptoms at all. No systemic reactions, changes in appetite or changes in milk production were observed throughout the study. All nodules at the application sites disappeared within fourteen days following immunizations.

Quantitative analyses of the antibody responses

Prior to immunization, in all cows Efb and LukM specific antibodies were detected in serum, milk, saliva and nasal secretions, with the exception of Efb specific IgG1 and IgG2, and LukM specific IgA antibodies in milk (Figure 1). The levels of these initial titers were not significantly different between groups (Supplementary Table 1).

Figure 2 shows the IgG1 and IgG2 antibody titer increases in serum and milk. Efb specific antibody titers in serum increased from 100 up to 2000 (IgG1) and from 1000 up to 15,000 (IgG2). LukM specific antibody titers in serum increased from 300 up to 7000 (IgG1) and from 6000 up to over 70,000 (IgG2). Milk titer increases were minimal for Efb

specific IgG1 and IgG2 antibodies, whereas milk titer increases for LukM specific antibodies ranges from 50 to 600 (IgG1) and from 20 up to 130 (IgG2). No significant route specific increases in total antibody titers were observed over time. However, between routes statistically significant different titer increases were observed. Following priming and booster immunizations, the increase in Efb-specific IgG1 levels in serum was significantly higher in IM/IM, IMM/SC and SC/SC immunized animals than in IN/IN immunized cows (p = 0.037, p = 0.001, and p = 0.000, respectively; Figure 2A). The increase in Efb-specific IgG2 serum levels were higher in SC/SC immunized animals compared to IN/IN and IM/IM immunized cows (both $p < 0.001$; Figure 2B). Elevations in serum levels of LukM-specific IgG1 were also significantly higher following SC/SC immunizations compared to IN/IN immunizations (p = 0.041; Figure 2C). In contrast, route specific increases in LukM-specific IgG2 serum levels were not observed (Figure 2D). In milk, the increase in Efb-specific IgG1 was higher following SC/SC immunization than following IMM/SC immunization (p = 0.029; Figure 2E). These differences were not observed for Efb-specific IgG2 levels (Figure 2F). LukM-specific antibody titers in milk were only slightly affected by immunization via the different administration routes with the highest increases following SC/SC immunizations (Figure 2G-H). However, for all groups, increases in milk antibody levels compared to serum were only moderate and pre- and post-immunization titers were not statistically different. In both milk and nasal secretions, Efb- and LukM-specific IgA levels were not affected by immunization. IgA levels in saliva were highly variable between cows and both increases and decreases were observed within groups in response to immunization (data not shown).

Serum and milk IgG1/IgG2 ratios did not change over the course of the experiment for both Efb and LukM, indicating that antibody increases following immunization with the experimental vaccine were similar for IgG1 and IgG2.

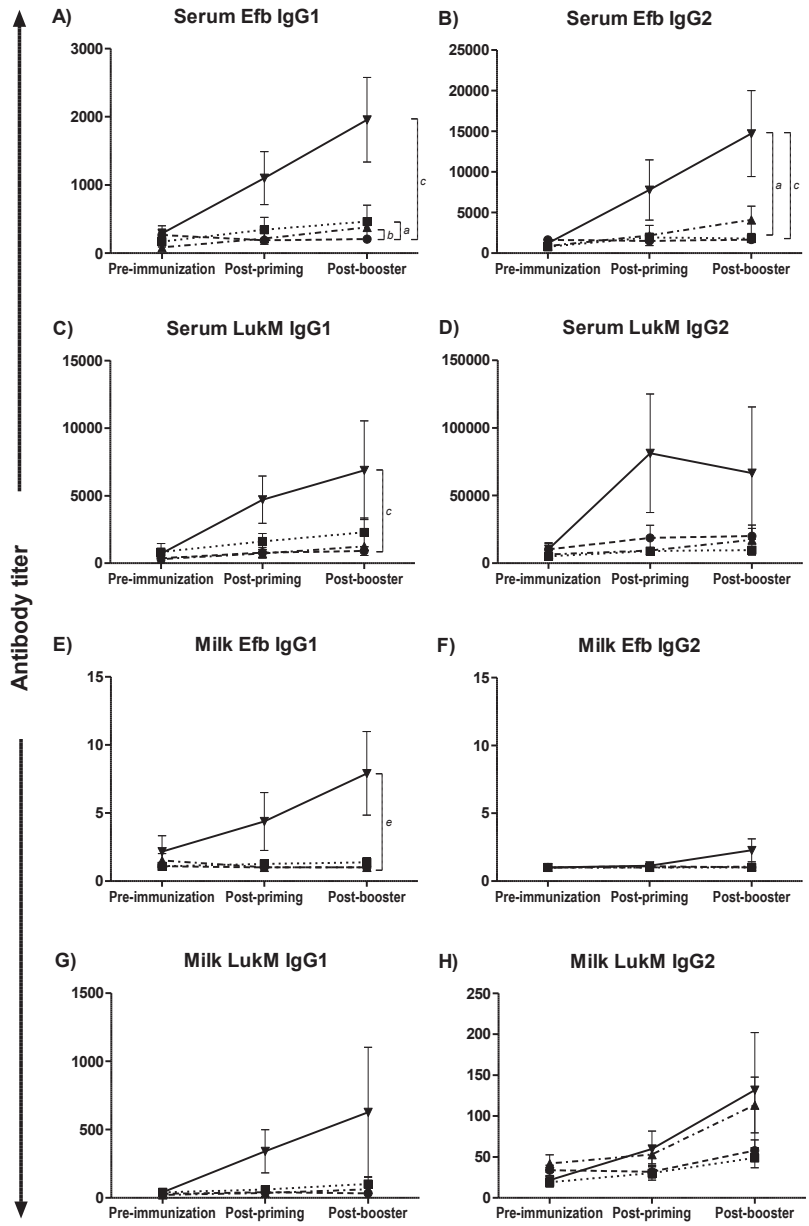

Figure 2: IgG1 and IgG2 antibody titer increases in serum and milk. *Isotype specific antibody titers in serum (A-D) and milk (E-H) following IN/IN (●), IM/IM (■), IMM/SC (▲) or SC/SC (▼) immunization with Efb (A-B, E-F) and LukM (C-D, G-H) were measured by ELISA. Results are expressed as the mean per group ± SEM. Differences in total titer increases following prime plus booster immu-nizations were analyzed using the One-way ANOVA. Letters indicate significant differences between IN/IN and IM/IM (a), IN/IN and IMM/SC (b), IN/IN and SC/SC (c), IM/IM and SC/SC (d), and IMM/SC and SC/SC (e) immunization routes.*

Neutralizing capacity of the antibodies

Antibodies within serum were tested for their ability to neutralize the inhibitory effects of Efb on phagocytosis. Figure 3 shows the differences in phagocytosis ratios of post- and pre-immunization serum. IM/IM and IMM/SC immunizations did not result in increased phagocytosis indicating that the induction of Efb neutralizing antibodies was limited. Only one cow immunized via IN/IN administration showed an increased percentage of phagocytosis post-immunization. In contrast, all SC/SC immunized animals showed an increase in neutralizing antibodies post-immunization. The increase in the Efb neutralizing capacity of serum antibodies was significantly higher following SC/SC immunization than after IM/IM and IMM/SC immunization (p = 0.015 for both groups).

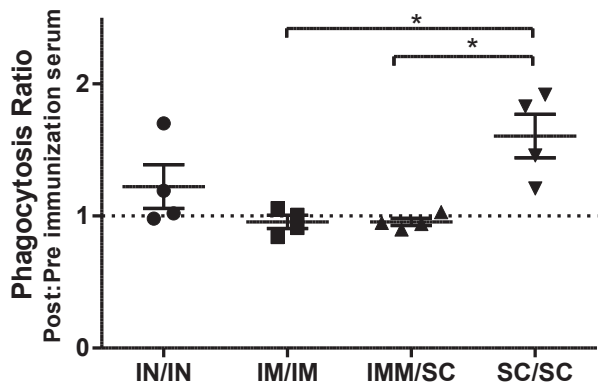

Figure 3: Efb neutralization assay. *The presence of Efb neutralizing antibodies in serum of cows from different immunization groups were analyzed in an Efb neutralization assay. For each cow, the phagocytosis ratio between post- and pre-immunization serum was calculated from four independent neutralization experiments. Results are expressed as the mean ratio per group ± SEM. Differences in neutralization ratios between groups were calculated using the One-way ANOVA. *p < 0.05.*

Serum antibodies were also tested for their ability to neutralize the effects of LukM on PBL lysis. Figure 4 shows that LukM specific antibodies in serum of SC/SC immunized cows significantly blocked the pore forming ability of LukMF' when compared to pre-immunization serum (p = 0.009). LukM specific antibodies in serum of IM/IM immunized cows also significantly decreased the pore formation by LukMF' compared to pre-immunization serum (p = 0.012). However, the differences in neutralization pre- and post-immunization were limited compared to the increase observed in SC/SC immunized cows. A trend in LukMF' neutralization was observed in serum of IMM/SC immunized cows (p = 0.058), whereas no differences in neutralization of LukMF' were observed in serum of cows immunized via the IN/IN route.

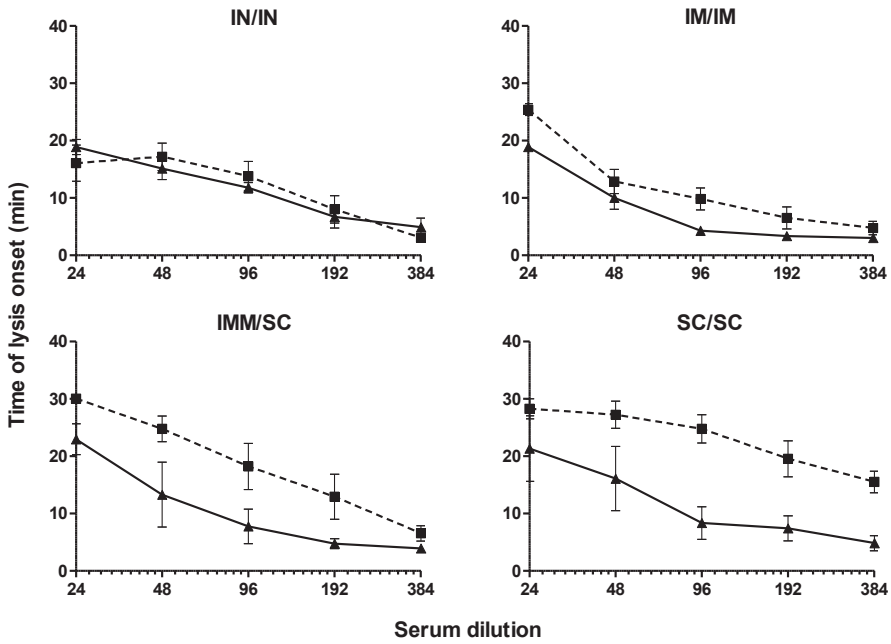

Figure 4: LukMF' neutralization assay. *The presence of LukMF' neutralizing antibodies in serum of cows from different immunization groups were analyzed in a LukMF' neutralization assay. For each group, the time of lysis onset between pre- (▲) and post- (■) immunization serum was calculated. Results are expressed as time of lysis onset ± SEM of three independent neutralization experiments. Differences in the time of lysis onset pre- and post-immunization within each immunization group were analyzed by repeated measures analysis. LukMF' neutralization post-immunization was significantly increased in cows immunized via SC/SC (p = 0.009) and IM/IM (p = 0.012) administration when compared to pre- immunization.*

Discussion

The aim of this study was to assess whether the route of immunization impacts the quantity of the antibody response as well as the neutralizing capacity of these antibodies, with an emphasis on intramammary immunity to *S. aureus*.

Antibodies directed against Efb and LukM could be detected in all cows prior to immunization. The induction of antibodies against *S. aureus* immune evasion proteins following natural and experimentally induced acute and chronic infections has previously been described in human, mice, goats and cows [35-38]. The presence of these antibodies demonstrates that *S. aureus* expresses and secretes both proteins *in vivo* and that their presence can elicit an immune response. Since recurrent infections with *S. aureus* frequently occur, it is unlikely that the antibodies induced by natural infections are protective against *S. aureus*. The lack of protection (among other factors) may be due to insufficient antibody levels at the site of infection. Even though high initial IgG1 and IgG2

levels were detected in pre-immunization serum their levels in milk were remarkably lower. A lack of protection may also occur when naturally induced antibodies do, for the main part, not target relevant (i.e. neutralizing) epitopes. In this study, a rise in mainly serum, but also in milk antibody levels following SC/SC immunizations was observed. Furthermore, serum of SC/SC immunized cows showed increased neutralization capacity in *in vitro* neutralization assays. The increased antibody levels are likely to contribute to the increased neutralization observed post SC/SC immunization. These results are in line with previous studies where SC administration of recombinant Efb or LukM resulted in the induction of antibodies with a neutralizing effect on Efb and LukM in mice and rabbits [22,23]. The neutralizing capacity of antibodies in milk could not be measured since milk components interfere with the fluorescence based assays and insufficient amounts of milk were available for antibody isolation. As antibodies which are translocated over the blood-udder barrier retain their specificity [39;40], it is likely that the increased neutralizing capacity of Efb and LukM specific antibodies is also present in milk. The influence of immunizations on IgA levels in saliva and nasal secretions were highly variable. This might be due to the sample collection technique used since the volumes of saliva and nasal secretions collected in the tampons were not controlled during sampling. This may have influenced the final antibody concentration of the analyzed fluids. Therefore, a more standardized method is required in order to analyze the IgA response following immunizations. Whether neutralizing antibodies are beneficial to the clearance of *S. aureus* from the udder at all has to be established in future research.

The immunization route and the applied adjuvant determine the immunological environment antigens are taken up and processed in [41]. Different responses may be elicited when antigens are administrated in different tissues [42,43]. Cytokines in the local environment influence the process of T cell stimulation by dendritic cells and eventually determine the antibody isotype produced by B-cells [40]. In this study, no shift in IgG1/IgG2 ratios was observed following immunization, indicating a similar production of both antibody isotypes regardless of the immunization route. These findings correspond with earlier studies where cows were immunized with an *S. aureus* capsular polysaccharide type 5 conjugate in combination with a mineral oil adjuvant or with a polysaccharide from Streptococcus agalactiae conjugated to ovalbumin in Freund's incomplete adjuvant [44-46]. However, two other studies found a more pronounced IgG2 response after immunization with an *S. aureus* CP5-ovalbumin conjugate in Freund's incomplete adjuvant or with a killed *S. aureus* cell-toxoid vaccine using dextran sulphate as an adjuvant [47,48]. Since alveolar macrophages in the udder have been shown to lack IgG2 receptors [49] it has been speculated that, in order to enhance local immunity, it is preferable to increase the IgG1 titer in milk. IgG1 is selectively transported to the mammary gland by the FcRn receptor [45]. During the development of infection IgG2 leaks

into the milk together with other serum components [50]. Therefore, IgG2 is thought to be more important in the second line of defense. Notwithstanding, in the study presented here immunization with antigens in a modified oil-in-water adjuvant via different administration routes did resulted in different levels and neutralizing capacity of antibodies. Of all administration routes applied, subcutaneous tissue exhibited the best environment to evoke a response against both Efb and LukM. Since all SC injections were administered close to the suspensory ligament it cannot be distinguished whether the subcutaneous route as such was responsible for this beneficial effect or whether it was the regional administration that influenced the humoral response. However, previous studies did not observe differences in serum and milk antibody titer increases in cows immunized via SC administration in the neck versus SC administration in the area of the supramammary lymph node indicating that the administration route might be more important than the location of administration [51,52].

Intra-nasal mucosal immunization resulted in a marginal response only, indicating that this route is less suitable for generating humoral protection against *S. aureus* immune evasion proteins in the presence of the chosen adjuvant. Although the classical IM route is widely used and described to stimulate the humoral immune response in a similar way as the SC route, in this study it only led to an intermediate increase in antibody titers as did the combined IMM/SC route [53,54].

In conclusion, this study showed that immunization routes impact the antibody response induced against the *S. aureus* immune evasion proteins Efb and LukM. A subcutaneous immunization in the suspensory ligament region resulted in higher antibody levels with increased neutralization capacities when compared to the other immunization routes.

Acknowledgements

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Supplementary Table

Table 1: *Initial antibody titers per group.*

			Administration route				
Antigen	**Specimen**	**Isotype**	**IN/IN**	**IM/IM**	**IMM/SC**	**SC/SC**	***p*-value**
Efb	Serum	IgG1	7. 8 (1.1)	7.0 (1.3)	6.1 (1.1)	7.5 (2.1)	0.41
		IgG2	10.7 (0.3)	9.4 (0.9)	9.5 (1.3)	10.1 (0.9)	0.24
	Milk	IgG1	0.1 (0.2)	0.1 (0.2)	0.4 (0.8)	0.6 (1.3)	0.30
		IgG2	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	-
		IgA	6.7 (1.5)	5.3 (1.2)	4.8 (1.0)	5.3 (1.0)	0.18
	Saliva	IgA	7.0 (1.5)	5.6 (1.8)	6.5 (1.3)	6.7 (2.3)	0.72
	Nasal secretion	IgA	8. 5 (0.4)	9.5 (0.3)	9.2 (0.9)	9.2 (0.4)	0.05
LukM	Serum	IgG1	8.3 (1.1)	8.5 (2.0)	7.9 (1.3)	9.1 (1.4)	0.68
		IgG2	13.0 (1.0)	12.1 (0.9)	12.4 (1.2)	12.8(1.5)	0.70
	Milk	IgG1	4.5 (1.2)	4.8 (1.5)	4.2 (1.1)	5.2 (0.9)	0.62
		IgG2	5.0 (0.6)	3.8 (1.6)	5.3 (0.6)	4.4 (0.6)	0.90
		IgA	1.2 (0.2)	1.5 (0.8)	1.8 (0.9)	1.7 (0.8)	0.63
	Saliva	IgA	7.4 (1.8)	6.6 (1.9)	7.7 (1.6)	5.5 (1.1)	0.28
	Nasal secretion	IgA	8.3 (1.8)	9.7 (1.2)	9.4 (1.0)	11.1 (0.6)	0.10

Chapter 5

The antibody response in the bovine mammary gland is influenced by the adjuvant and the site of subcutaneous vaccination

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Abstract

Intramammary infections in cattle resulting in mastitis have detrimental effects for cows' well-being, lifespan and milk production. In the host defense against *Staphylococcus aureus* (*S. aureus*) mastitis antibodies are thought to play an important role. To explore potential ways to increase antibody titers in the bovine mammary gland the effects of various adjuvants on the magnitude, isotype, and neutralizing capacity of antibodies produced following subcutaneous vaccine administration at different immunization sites were analyzed. In this study, α-toxoid was used as a model antigen and formulated in three different alum-based adjuvants: Alum-Saponin, Alum-Oil, and Alum-Saponin-Oil. Vaccines were administered near the suspensory ligament of the udder (in this study referred to as the udder) or in the lateral triangular area of the neck (in this study referred to as the neck). At both immunization sites, immunization with α-toxoid in Alum-Saponin-Oil resulted in significantly higher specific antibody titers in milk and serum as compared to Alum-Oil and Alum-Saponin, without favoring an IgG1 or IgG2 response. Furthermore, the neutralizing capacity of milk serum and serum following immunization near the udder and in the neck was significantly higher when Alum-Saponin-Oil was used as adjuvant compared to Alum-Oil and Alum-Saponin. Prime immunizations near the udder effectively increased both antibody isotype titers and neutralization titers, while prime plus boost immunizations were required to induce similar effects following immunization in the neck. Results indicate that subcutaneous administration of an Alum-Saponin-Oil based vaccine near the udder could be a one-shot vaccination strategy to efficiently increase intramammary antibody responses.

Introduction

Intramammary infections in cattle often result in mastitis with detrimental effects on the cows' well-being, lifespan and milk production [1]. Vaccination represents one of the most studied and sought after tools to prevent bovine mastitis [2-4]. However, despite numerous attempts to develop an effective vaccine, commercially available vaccines are scarce and their evaluation under field conditions showed limited protection only [5,6]. *Staphylococcus aureus* (*S. aureus*) represents one of the major mastitis causing pathogens. Although immune correlates of protection against *S. aureus* mastitis have not yet been well established, high *S. aureus* specific IgG1 levels in milk have been associated with reduced growth of *S. aureus* both *in vivo* and *in vitro*, suggesting a role for this antibody isotype in the hosts defense against *S. aureus* [7-9]. IgG1 is the most abundantly present antibody isotype in the healthy mammary gland and it facilitates phagocytosis by macrophages, the predominant leukocytes in normal milk [10,11]. IgG2, which is recruited to the inflamed tissue, promotes phagocytosis by neutrophils and is thought to play a role during later stages of intramammary infection [12,13]. Several studies suggest that antibodies also play a role in the host defense by neutralizing immune evasion proteins which are secreted by *S. aureus* during infection [14,15]. The failure of presently available vaccines to protect cattle against intramammary infections might be due to an insufficient capacity to induce strong, neutralizing antibody responses in the mammary gland. Both the magnitude and neutralizing capacity of antibodies are influenced by the route and site of vaccine administration [16-18]. In cattle, subcutaneous (SC) immunization near the supramammary lymph node (SMLN) positively influences the antibody response in both milk and serum [16,19]. In addition, the ability of adjuvants to modulate antibody responses is generally appreciated and widely exploited in different immunization strategies [20-22].

The aim of this study was to analyze the magnitude, isotype, and neutralizing capacity of antibodies produced following SC immunizations near the suspensory ligament of the udder (in this study referred to as the udder) or in the lateral triangular area of the neck (in this study referred to as the neck) using the α-toxoid of *S. aureus* as model antigen formulated in alum-based adjuvants supplemented with either saponin, oil, or both.

Materials and Methods

Animals

A total of 48 clinically healthy mid-lactation Holstein-Frisian cows between 2 and 3 years of age and 5-6 months in lactation were used in this study. Cows were randomly divided over 6 groups of 8 cows. Animals were housed in free stall barns and fed according to their

requirements [23]. Water was supplied ad libitum. Cows were milked twice a day and the milk yield (kg/day) was recorded throughout the study. Rectal temperatures were measured pre-immunization, 4 hrs and 1, 2, and 3 days post-prime and boost-immunization. The design of the study was approved by an independent ethical committee. The study was performed in accordance with European Community guidelines and national laws on animal experiments.

Experimental vaccine and immunization procedure

To produce α-toxoid *S. aureus* strain DU1090, containing the pDU1212[H35R] plasmid encoding a genetically detoxified α-toxin [24], was cultured in Todd Hewitt Broth medium for 24 hrs at 37 °C. Culture medium was filtered (0.2 µm) to remove cells and α-toxoid was concentrated over a 10 kDa ultrafilter. Purity and concentration were checked by SDS-PAGE and Coomassie blue staining. The experimental vaccine consisted of 15 µg α-toxoid per dose (4 mL) formulated in a proprietary alum-based adjuvant supplemented with either saponin, mineral oil, or both (MSD-AH). Each vaccine was administered subcutaneously to two groups: one group received 2 mL of the vaccine on each the left and right side of the suspensory ligament of the udder at both prime and boost immunizations, while the other group received 4 mL of the vaccine in the lateral triangular area on the left side of the neck for the prime immunization, and 4 mL on the right side of the neck for the boost immunization. Prime and boost immunizations were administered 6 weeks apart. Vaccines were administered using 18G needles (BD Microlance).

Local reaction scores

Local reactions to the experimental vaccines were scored pre-immunization, and 1, 2, 3, 7, and 14 days post-prime and boost-immunization, based on swelling size estimations at the injection site and classified in 4 categories: swelling with a diameter of 1) 0-5 cm, 2) 5-10 cm, 3) 10-15 cm, or 4) > 15 cm. Since immunizations near the udder were administered on both the left and right side of the udder each time for prime and boost immunizations, the sum of the swelling size on both immunization sites was used as final score.

Milk and blood sampling

Milk and blood samples were collected pre-immunization, 4 weeks post-prime and 2 weeks post-boost immunization. A representative aliquot (10 mL) of the mixed morning milk yield was collected for each cow and transported to the laboratory at ambient temperature. Part of the milk sample was centrifuged for 15 min at 2000 x *g* to collect milk serum as whole milk interfered with the α-toxin neutralization assay. Blood was collected from the coccygeal vein using a sterile blood collection system (Becton Dickinson).

Following coagulation, blood was centrifuged for 10 min at 3000 x *g* to collect blood serum (in this study referred to as serum). Milk, milk serum, and serum samples were stored at -20 °C until further analysis.

Titers and isotypes of α-toxin specific antibodies measured by ELISA

α-Toxin specific antibody isotype titers were determined by ELISA. NUNC MaxiSorp plates (eBioscience) were coated overnight at 4 °C with 1 µg/mL α-toxin (Sigma-Aldrich). Samples were added in three-fold serial dilutions and incubated for 1 hr at 37 °C. Eight replicates of an in-house negative control serum were taken along. Horseradish-peroxidase-conjugated sheep-anti-bovine IgG1 and IgG2 conjugates (Bethyl Laboratories Inc.) were used in 1:500 and 1:2000 dilutions, respectively. Tetramethylbenzidine was used as a substrate and reactions were stopped by adding sulfuric acid. Extinctions (450 nm) were measured on a Tecan SUNRISE device using XFluor4 Software Version V4.51-I4 (Tecan Group Ltd.). Antibody isotype titers were determined using CaSpEx Software Version 1.12 (MSD, Proprietary Software) and defined as the Log_2 dilution of the sample that would give the same absorbance as 1.6 times the average OD of the negative controls.

α-Toxin neutralization assay

Samples were analyzed for their capacity to neutralize α-toxin, thereby preventing α-toxin mediated erythrocyte lysis, in two-fold serial dilutions. Rabbit blood was collected and immediately mixed 1:1 with Alsever's solution (MSD-AH) to prevent clotting. Erythrocytes were harvested by centrifugation for 10 min at 2000 x *g*. Erythrocytes were washed twice with PBS (MSD-AH), and dissolved in a volume of PBS equal to the volume of the original blood sample. Samples were incubated with α-toxin (1.6 µg/mL) for 30 min at 37 ^{o}C while gently shaking. Then, erythrocytes were added and samples were incubated for 1 hr at 37 ^{o}C. For maximal and minimal lysis, erythrocytes were incubated with and without α-toxin, respectively, in the absence of milk serum and serum. Following incubation, supernatant was transferred to a clean microtiter plate. Extinctions (OD 545nm) were measured on a Tecan SUNRISE device. Neutralization titers were determined using CaSpEx and defined as the Log_2 sample dilution that resulted in 25 % lysis inhibition based on the average OD of the samples with maximal and minimal lysis.

Statistical analyses

Statistical analyses were performed using the statistical software package SAS Version 9.3 (SAS Institute Inc.). An ANOVA linear mixed model was used to identify effects of the different adjuvants and/or immunization sites on titer increases post-immunization. A full interaction model including 'adjuvant' (Alum-Saponin/Alum-Oil/Alum-Saponin-Oil), 'site' (udder/neck), and 'time' (pre-immunization/post-prime/post-boost) was used.

Pre-immunization titers were included as covariates and 'cow' was added as a random effect. The likelihood ratio test was used to select the most parsimonious variance-covariance structure. An unstructured covariance structure was used for milk NT and a compound symmetry structure for all other analyses. Tests were two-sided using a significance level of 0.05. Correlations between titers were estimated by the Spearman's correlation coefficient. For graphical presentation of the data GraphPad Prism Software Version 5 (GraphPad Software Inc.) was used.

Results

Local reaction scores post-immunization

Immunizations had no effect on the body temperature and daily milk yield (data not shown). Local reactions scores of 4 and 3 were observed post-immunization with Alum-Saponin near the udder and in the neck, respectively. Scores decreased from one week post-immunization onwards. Local reaction scores post-immunization with Alum-Oil and Alum-Saponin-Oil resulted in scores of 4 near the udder and in the neck. Scores persisted throughout the experimental period (Supplementary Figure 1).

α-Toxin specific IgG1 and IgG2 antibody titers

Significant mean differences in milk and serum IgG1 and IgG2 antibody titers (AT) and neutralization titers (NT) post-immunization with different adjuvants and/or via different immunization sites are shown in Supplementary Table 1. Only significant differences in mean titers of at least 1.5 Log_2 (Table 1) were considered biologically relevant and are addressed below.

Pre- and post-immunization milk IgG1 and IgG2 AT are depicted in Figure 1A and 1B, respectively. Adjuvant effects on the total titer increase following prime and boost immunization were observed (adjuvant effect). The use of Alum-Saponin-Oil resulted in higher mean IgG2 AT with differences of 1.6 and 1.5 Log_2 for milk and serum, respectively, as compared to Alum-Oil. Time dependent adjuvant effects were also observed (adjuvant*time effect). Boost immunizations with Alum-Saponin-Oil resulted in 2.3 and 1.8 Log_2 higher mean IgG1 AT in milk and serum, respectively, as compared to titers induced by prime immunizations. When using Alum-Saponin-Oil as adjuvant, boost immunizations resulted in 2.6 and 1.8 Log_2 higher mean IgG1 AT in serum as compared to boost immunizations with Alum-Saponin and Alum-Oil, respectively. Furthermore, mean IgG1 AT in milk were 2.5 Log_2 higher post-boost immunization with Alum-Saponin-Oil as compared to Alum-Saponin. Some adjuvant effects were immunization site dependent (adjuvant*site effect). When administered near the udder, mean IgG1 AT in milk were 2.6 and 1.9 Log_2 higher post-immunization with Alum-Saponin-Oil as compared to Alum-Saponin and

Table 1: *Differences of least square means of α-toxin specific antibody isotype titers and neutralization titers in milk and serum.*

Effect	Adjuvant	Site	Time	# Adjuvant	# Site	# Time	Estimate	Lower*	Upper*	*p*-value	Sample	Titer
Adjuvant	Alum-Saponin-Oil	-	-	Alum-Saponin	-	-	1.6	0.7	2.5	0.0012	Milk	IgG2
				Alum-Oil	-	-	1.5	0.9	2.1	<0.0001	Serum	IgG2
Adjuvant * Time	Alum-Saponin-Oil	-	Prime	Alum-Saponin-Oil	-	Boost	-2.3	-3.1	-1.5	<0.0001	Milk	IGg1
							-1.8	-2.3	-1.3	<0.0001	Serum	IGg1
		-	Boost	Alum-Saponin	-	Boost	2.6	1.9	3.4	<0.0001	Serum	IGg1
							2.5	1.4	3.5	<0.0001	Milk	IGg1
		-	Boost	Alum-Oil	-	Boost	1.8	1.0	2.6	<0.0001	Serum	IGg1
Adjuvant * Site	Alum-Saponin-Oil	Udder	-	Alum-Saponin	Udder	-	2.6	1.4	3.8	<0.0001	Milk	IGg1
		Udder	-	Alum-Oil	Udder	-	1.9	0.6	3.3	0.0069	Milk	IGg1
	Alum-Oil	Neck	-	Alum-Saponin	Neck	-	2.1	0.9	3.2	0.0009	Milk	IGg1
		Udder	-	Alum-Oil	Neck	-	-2.1	-3.4	-0.9	0.0014	Milk	IGg1
Site * Time	-	Neck	Prime	-	Neck	Boost	-2.0	-2.4	-1.6	<0.0001	Milk	IGg2
							-1.9	-2.6	-1.3	<0.0001	Milk	IGg1
							-1.8	-2.2	-1.4	<0.0001	Milk	NT

This table lists only statistically significant comparisons with an effect size of at least 1.5 Log_2.
NT = Neutralization Titer
 # Compared to
** Confidence Interval (95 %)*

Alum-Oil, respectively. When administered in the neck, mean IgG1 AT in milk were 2.1 Log_2 higher post-immunization with Alum-Oil as compared to Alum-Saponin. Using Alum-Oil as adjuvant, vaccine administration in the neck resulted in 2.1 Log_2 higher mean IgG1 AT in milk compared to administration near the udder. Boost immunizations showed significant effects on milk IgG1 and IgG2 AT when administered in the neck with estimated mean titer increases of 1.9 and 2.0 Log_2, respectively (site*time effect). Although not significant, prime immunizations with Alum-Saponin-Oil near the udder increased AT and NT with greater magnitude as compared to prime-immunization in the neck. Similar results were obtained for serum IgG1 and IgG2 AT (Table 1, Supplementary Table 1 and Supplementary Figure 2A-B).

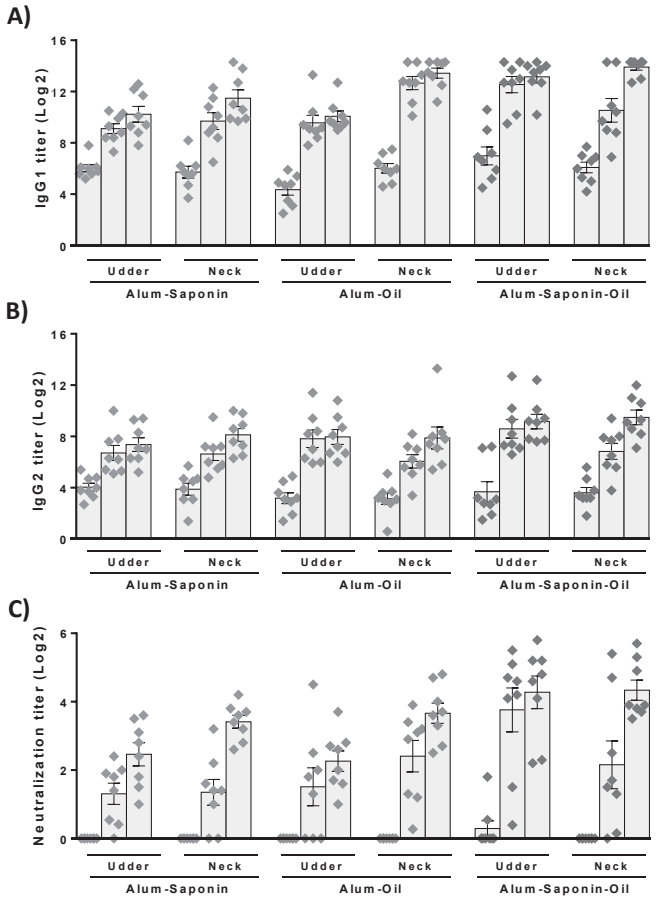

Figure 1: α-Toxin specific antibody isotype titers and neutralization titers in milk. *Specific antibody isotype titers were measured in an α-toxin specific ELISA. The neutralization capacity of milk serum was analyzed in an α-toxin neutralization assay. Milk IgG1 (A) and IgG2 (B) antibody titers and milk serum neutralization titers (C) pre-immunization (●), post-prime (▲), and post-boost (◆) immunization are expressed as the mean ± SEM per group. Significant mean titer differences are given in Table 1 and Supplementary Table 1.*

α-Toxin neutralization capacity of milk serum and blood serum

The neutralization titers (NT) of pre- and post-immunization milk serum are depicted in Figure 1C. No adjuvant dependent effect on the NT was observed. The site of vaccine administration influenced the milk serum NT in a time dependent manner (site*time effect). Boost immunizations resulted in higher milk serum NT when administered in the neck as compared to administration near the udder with an estimated mean titer difference of 1.8 Log_2. Comparable results were observed for the neutralization capacity of serum (Table 1, Supplementary Table 1 and Supplementary Figure 2C).

Titer correlations

The comparable dynamics of AT and NT in milk and serum post-immunization are reflected in the high correlation between milk and serum titers (Figure 2). IgG1 and IgG2 milk and serum AT showed strong Spearman correlation coefficients of 0.928 and 0.954, respectively. NT of milk serum and serum were also strongly correlated with a Spearman correlation coefficient of 0.876.

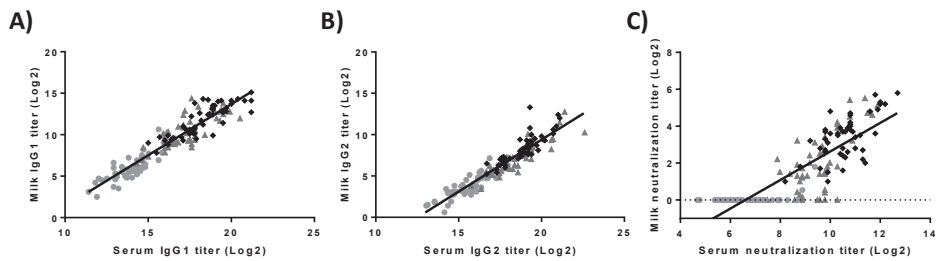

Figure 2: Correlation between milk and serum titers. *Plots represent correlations between α-toxin specific titers in milk and serum for IgG1 (A), IgG2 (B), and neutralization titers (C) as measured pre-immunization (●), post-prime (▲) and post-boost (◆) immunization. Spearman correlation coefficients were 0.928 (A), 0.954 (B), and 0.876 (C).*

Correlations between the IgG1 and/or IgG2 AT and their corresponding NT pre- and post-immunization were also analyzed. The highest correlation with NT was observed for IgG1 milk and serum titers with Spearman correlation coefficients of 0.838 and 0.893, respectively (Figure 3A-B). Spearman correlation coefficients for α-toxin specific IgG2 titers in milk and serum and their respective NT were 0.727 and 0.659 (Figure 3C-D). The IgG1:IgG2 ratios were not influenced by the type of adjuvant and/or site of SC vaccine administration and remained unchanged in the course of the experiment.

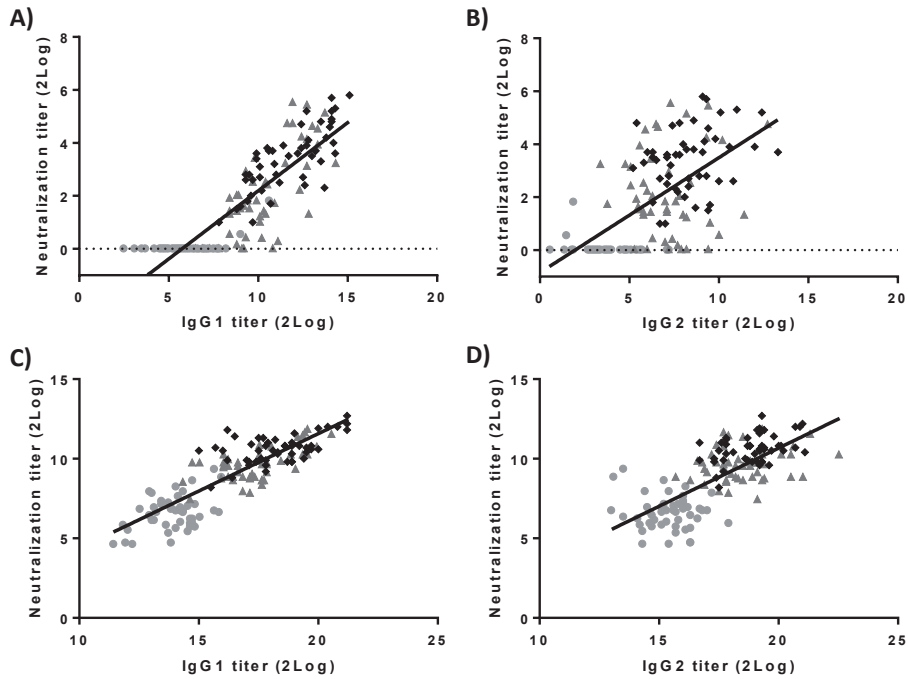

Figure 3: Correlation between α-toxin specific antibody isotype titers and neutralization titers. *Plots represent the correlation between milk IgG1 (A) and IgG2 (B) titers with their respective milk serum neutralization titers, and the correlation between serum IgG1 (C) and IgG2 (D) titers with their respective serum neutralization titers. Titers pre-immunization (●), post-prime (▲) and post-boost (◆) immunization are depicted. Spearman correlation coefficients were 0.838 (A), 0.727 (B), 0.893 (C), and 0.659 (D).*

Discussion

Alum based adjuvants are widely used in human and veterinary vaccines and known to induce effective, primarily antibody-mediated, immune responses [25-27]. In the present study, alum-based adjuvants were supplemented with saponin, a well-recognized potent stimulator of both humoral and cellular immune responses [28-30], or/and an oil emulsion, which is thought to provide a depot effect at the injection site resulting in sustained antigen release providing long term immune stimulation [31]. The synergistic effects of alum and saponin have previously been reported in sheep where immunization with a Bacteroides antigen formulated in Alum-Saponin induced higher AT and NT as compared to formulation in alum only [32]. Supplementation of an alum-based adjuvant with oil increased the humoral and cellular immune responses in mice with greater magnitude and better neutralizing capacity as compared to an adjuvant based on alum only [33]. Saponin and oil can also act synergistically as shown in a murine model where

increased AT were observed following supplementation of the oil adjuvanted commercial FMD vaccine and an experimental oil-based HIV-1 vaccine with saponin [29,34]. Results of the present study show that supplementation of an alum-based adjuvant with both saponin and oil resulted in higher AT and NT compared to immunization with Alum-Oil and Alum-Saponin, respectively.

In the present study, saponin did not specifically favor the induction of IgG2 as shown by unchanged IgG1:IgG2 ratios in the course of the experiment. Since pre-immunization titers indicate previous contact with *S. aureus*, saponin may have been ineffective in redirecting the already established immune response [35]. Alternatively, the stimulating effects of alum on the humoral response might have prevented saponin to direct the immune system to a more cellular based response [36].

Previous studies in a murine model show that the type of immune response induced is influenced by the site of SC antigen presentation [18,37-38]. Previous studies in cattle describe no differences in specific AT in both milk and serum following vaccine administration near the udder or in the neck using a polymer-based adjuvant or an oil-in-water adjuvant [19,39]. In the present study, the type of immune response was not influenced by the site of vaccine administration and the IgG1:IgG2 ratios remained unchanged in the course of the experiment. Although the use of Alum-Oil resulted in increased IgG1 AT to a greater magnitude post-immunization in the neck compared to immunization near the udder, this effect was not reflected in the IgG2 titers and NT. For Alum-Saponin and Alum-Saponin-Oil, no immunization site specific effects on AT and NT were observed.

Independent of the adjuvant used, the effect of boost immunizations was strongest following administration in the neck. As shown in a murine model, the major reservoir of memory B-cells is the spleen, but subsets of memory B-cells are retained in draining lymphoid sites [40,41]. Following secondary antigen encounter, memory B-cells are rapidly reactivated and stimulated to proliferate and differentiate into antibody secreting plasma cells. Since *S. aureus* is frequently isolated from external orifices and the teat skin [42,43], SMLNs are likely to have acted as draining LN following natural contact with *S. aureus*. In contrast, since skin infections caused by *Staphylococcus* ssp. in the neck area, drained by the prescapular LNs, are highly uncommon [44], it is unlikely that prescapular LNs have previously encountered *S. aureus* antigens. Therefore, prime immunizations in the neck may have resulted in the generation and local presence of memory B-cells able to mount a recall response post-boost immunization while prime immunization near the udder already acted as a booster. Interestingly, a prime immunization near the udder with Alum-Saponin-Oil was sufficient to increase AT and NT with similar magnitude compared to prime plus boost immunizations in the neck, suggesting highly effective re-activation of memory B-cells by this adjuvant. Alternatively, the magnitude of the immune response might have been influenced by the antigen dose and number of LNs simultaneously

targeted as a consequence of our immunization strategy. Since the vaccine was divided over the left and right site of the udder for both prime and boost immunizations, a low antigen concentration per injection was administered, targeting both SMLNs. In contrast, immunizations in the neck resulted in the presentation of a higher antigen dose to the left (prime) or right (boost) prescapular LN. Although antigen presentation might not be exclusively restricted to the draining LN on the ipsilateral site, the induction of an immune response in the contralateral LN due to antigen relocation or migration of antigen presenting cells cannot be excluded [45]. Additional studies with similar antigen doses and number of injections are required to determine their possible role in the AT and NT dynamics observed in this study.

Regardless the adjuvant and immunization site, a high correlation between milk and serum α-toxin specific AT were observed suggesting antibody exchange between the systemic circulation and the mammary gland. Pre-immunization AT did not correlate with NT, suggesting either the absence of neutralizing antibodies or NT below the detection limit in the neutralization assay. Alternatively, immunizations might have induced antibodies with higher neutralizing capacities compared to antibodies induced following natural contact with *S. aureus* [16]. Correlation coefficients between AT and NT were higher for IgG1 as compared to IgG2, which might suggest that neutralization of α-toxin is predominantly mediated by IgG1. Further tests with purified IgG1 and IgG2 antibody isotypes are needed to provide additional proof.

Conclusions

In the attempts to develop an efficacious vaccine against bovine *S. aureus* mastitis, Alum-Saponin-Oil should be considered as adjuvant since it efficiently stimulates the induction of AT, favoring both IgG1 and IgG2 responses, and NT. While prime immunizations with Alum-Saponin-Oil near the udder resulted in high titer increases, immunization in the neck required a prime-boost regimen to induce similar titers. This implies that, when subcutaneously administered near the udder, a one-shot vaccination strategy with Alum-Saponin-Oil may be sufficient to efficiently increase intramammary antibody responses.

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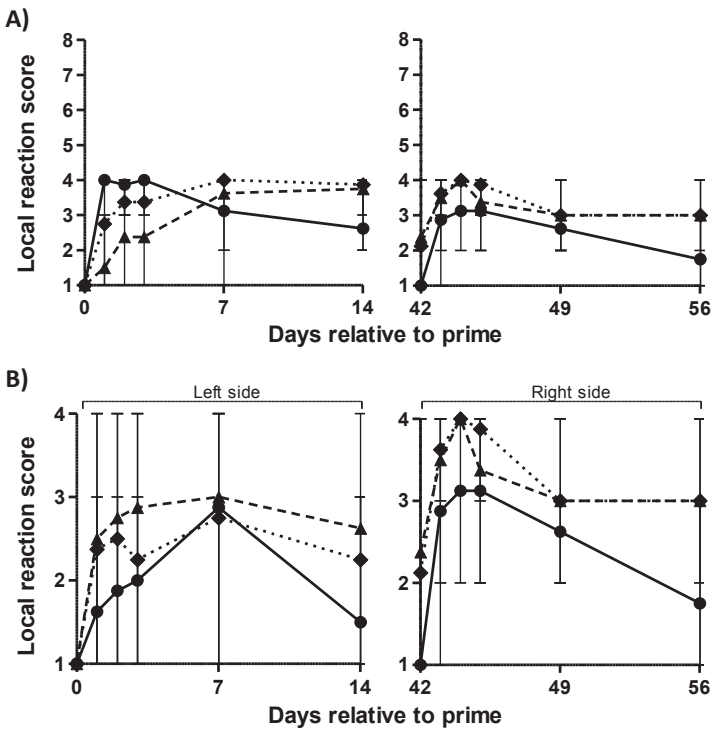
Supplementary Figures and Table



Supplementary Figure 1: Local reaction scores post-immunization. *Local reaction scores near the udder (A) and in the neck (B) following prime (day 0) and boost (day 42) immunizations are depicted. Figure A shows the sum of the local reaction scores observed at the immunization site on the left and right side of the udder. Figure B shows the local reaction scores observed on the left side of the neck post-prime immunization and on the right side of the neck post-boost immunization. Data is expressed as the mean ± range per group for Alum-Saponin (●), Alum-Oil (▲), and Alum-Saponin-Oil (◆).*

Supplementary Table 1: Differences of least square means of α-toxin specific antibody isotype titers and neutralization titers in milk and serum.

Effect	Adjuvant	Site	Time	# Adjuvant	# Site	# Time	Estimate	Lower*	Upper*	*p*-value	Sample	Titer
Adjuvant	Alum-Saponin-Oil	-	-	Alum-Saponin	-	-	1.6	0.7	2.5	0.0012	Milk	IgG2
							1.4	0.6	2.2	0.0012	Milk	NT
							1.1	0.6	1.5	<0.0001	Serum	NT
							0.8	0.2	1.3	0.0102	Serum	IgG2
	Alum-Saponin-Oil	-	-	Alum-Oil	-	-	1.5	0.9	2.1	<0.0001	Serum	IgG2
							1.1	0.3	1.9	0.0109	Milk	NT
							0.6	0.1	1.1	0.0198	Serum	NT
	Alum-Oil	-	-	Alum-Saponin	-	-	0.7	0.2	1.3	0.0156	Serum	IgG2
Adjuvant * Time	Alum-Saponin-Oil	-	Prime	Alum-Saponin-Oil	-	Boost	-2.3	-3.1	-1.5	<0.0001	Milk	IgG1
							-1.8	-2.3	-1.3	<0.0001	Serum	IgG1
	Alum-Oil	-	Prime	Alum-Oil	-	Boost	-0.9	-1.7	-0.1	0.0298	Milk	IgG1
							-0.6	-1.1	-0.1	0.0231	Serum	IgG1
	Alum-Saponin	-	Prime	Alum-Saponin	-	Boost	-1.1	-1.9	-0.3	0.0076	Milk	IgG1
							-0.5	-1.0	0.0	0.0349	Serum	IgG1
	Alum-Saponin-Oil	-	Prime	Alum-Saponin	-	Prime	1.4	0.6	2.1	0.0012	Serum	IgG1
							1.3	0.3	2.3	0.0125	Milk	IgG1
	Alum-Oil	-	Prime	Alum-Saponin	-	Prime	1.4	0.5	2.5	0.0058	Milk	IgG1
							0.8	0	1.6	0.0423	Serum	IgG1
	Alum-Saponin-Oil	-	Boost	Alum-Saponin	- -	Boost	2.6	1.9	3.4	<0.0001	Serum	IgG1
							2.5	1.4	3.5	<0.0001	Milk	IgG1
	Alum-Saponin-Oil	-	Boost	Alum-Oil	-	Boost	1.8	1	2.6	<0.0001	Serum	IgG1
							1.2	0.2	2.3	0.0266	Milk	IgG1
	Alum-Oil	-	Boost	Alum-Saponin	-	Boost	1.2	0.2	2.3	0.0175	Milk	IgG1
							0.9	0.1	1.7	0.0329	Serum	IgG1

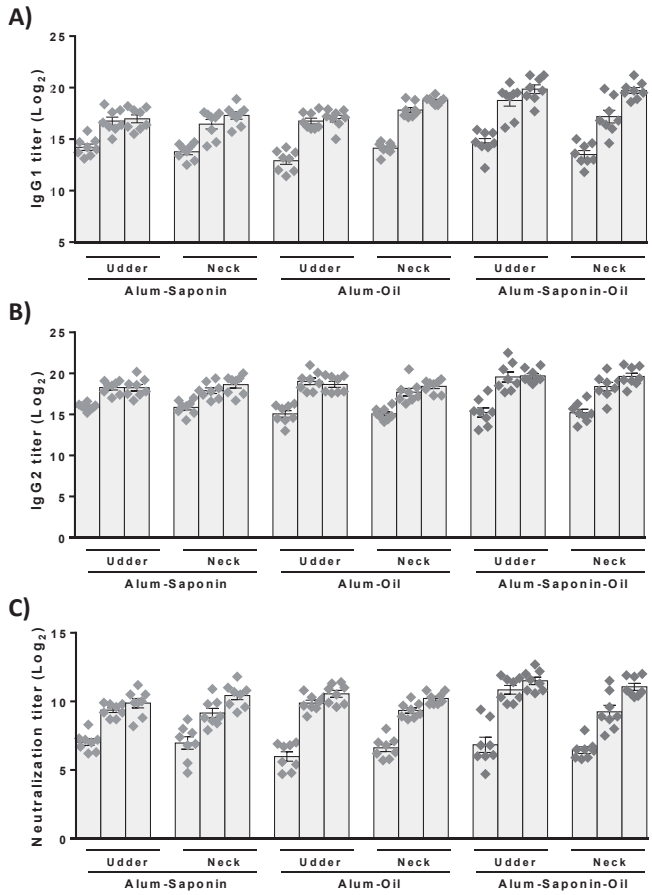
Adjuvant * Site	Alum-Saponin-Oil	Udder	-	Alum-Saponin	Udder	-	2.6	1.4	3.8	<0.0001	Milk	IgG1
		Udder	-	Alum-Oil	Udder	-	1.9	0.6	3.3	0.0069	Milk	IgG1
		Neck	-	Alum-Saponin	Neck	-	1.2	0.0	2.4	0.0441	Milk	IgG1
	Alum-Oil	Neck	-	Alum-Saponin	Neck	-	2.1	0.9	3.2	0.0009	Milk	IgG1
		Udder	-	Alum-Oil	Neck	-	-2.1	-3.4	-0.9	0.0014	Milk	IgG1
Site * Time	-	Udder	Prime	-	Neck	Prime	1.1	0.3	1.9	0.0068	Milk	IgG2
							0.9	0.4	1.5	0.001	Serum	IgG2
							0.8	0.4	1.3	<0.0001	Serum	NT
	-	Udder	Prime	-	Udder	Boost	-0.9	-1.6	-0.3	0.0068	Milk	IgG1
							-0.8	-1.2	-0.4	0.0003	Milk	NT
							-0.6	-0.9	-0.3	0.0004	Serum	NT
							-0.5	-0.9	-0.1	0.0132	Serum	IgG1
							-0.5	-0.9	0.0	0.0419	Milk	IgG2
	-	Neck	Prime	-	Neck	Boost	-2.0	-2.4	-1.6	<0.0001	Milk	IgG2
							-1.9	-2.6	-1.3	<0.0001	Milk	IgG1
							-1.8	-2.2	-1.4	<0.0001	Milk	NT
							-1.4	-1.8	-1.0	<0.0001	Serum	IgG1
							-1.3	-1.6	-1.0	0.0008	Serum	NT
							-0.9	-1.3	-0.5	<0.0001	Serum	IgG2
	-	Udder	Boost	-	Neck	Boost	-1.2	-2.0	-0.4	0.0048	Milk	IgG1
							-0.9	-1.4	-0.3	0.0028	Milk	NT
							-0.7	-1.3	-0.1	0.0364	Serum	IgG1

This table lists only statistically significant comparisons with an effect size of at least 1.5 Log_2.

NT = Neutralization Titer

 # Compared to

** Confidence Interval (95%)*



Supplementary Figure 2: α-Toxin specific antibody isotype titers and neutralization titers in serum. *Specific antibody isotype titers were measured in an α-toxin specific ELISA. The neutralization capacity of serum was analyzed in an α-toxin neutralization assay. Serum IgG1 (A) and IgG2 (B) antibody titers and serum neutralization titers (C) pre-immunization (●), post-prime (▲), and post-boost (◆) immunization are expressed as the mean ± SEM per group. Significant mean titer differences are given in Table 1 and Supplementary Table 1.*

Chapter 6

Summarizing discussion

Mastitis is an infectious disease with detrimental effects on the cows' well-being and an enormous economic impact due to reduced milk production and quality, and the need for veterinary interventions. A major mastitis causing pathogen in bovines is *Staphylococcus aureus (S. aureus)*. When *S. aureus* mastitis occurs, treatment with antibiotics is mostly practiced. However, prevention of invasion of the udder - for example through vaccination - rather than curing the subsequent infection is the ultimate goal. In order to develop an effective vaccine against bovine *S. aureus* mastitis it is important to understand the intramammary immune responsiveness against this pathogen. In the present chapter the research described in this thesis is summarised and the role of various milk immune components and mechanisms in the cows' defence against intramammary *S. aureus* infections is discussed.

In vivo differences in quarter susceptibility to *Staphylococcus aureus* mastitis

Quarters of a cow differ in their susceptibility - or resistance - to *S. aureus* mastitis. Although establishment of natural infections may depend on teat characteristics involved in bacterial entrance into the mammary gland, such as quarter position and teat morphology [1,2], quarter differences can also be observed post experimental inoculation when these teat characteristics are bypassed [**Chapter 3**; 3-6]. In the field, quarters that are susceptible to *S. aureus* can be identified by the onset of mastitis. It is more difficult to identify quarters that effectively resist the pathogen since, in the absence of inflammation, it is hard to define whether a quarter prohibited *S. aureus* establish an infection or whether bacterial entrance simply did not occur. In experimental inoculation studies, the time point of bacterial entrance is known and the outcome of infection is usually well defined. Post-inoculation, some quarters resolve *S. aureus* without showing clinical signs of inflammation, indicating protective immune reactivity [**Chapter 3**]. The quarters that do show clinical signs of inflammation, such as visible changes in the milk appearance, udder swelling, and increased somatic cell counts (SCC), can be subdivided in quarters able to resolve *S. aureus* and quarters which are unable to do so. When a quarter is unable to resolve *S. aureus*, infections often become chronic.

Although quarters without an onset of mastitis post experimental inoculation with *S. aureus* are frequently observed [6-12], often they are not given much attention by the researchers. Some researchers even failed to mention whether quarters that did not develop mastitis post-inoculation were observed [13-14], or classified these quarters as unsuccessfully inoculated and adapted a repeated inoculation method to achieve a 100% infection rate [15]. When clinical signs of inflammation are not observed in quarters of non-vaccinated cows post experimental inoculation, they negatively influence the statistic

power of the study which might explain this lack of attention. However, especially these quarters are of great interest. Quarters able to resolve *S. aureus* in the absence of inflammation are likely able to do so with help of components present in the mammary gland at the very moment of bacterial entrance and may provide essential information about immune or other components required to protect a quarter against *S. aureus* infection.

Influence of the amount of milk and milk composition on bacterial growth

Our results indicate that the total daily milk yield does not influence intramammary growth of *S. aureus* [**Chapter 3**]. However, the presence of milk in a quarter at the time of bacterial entrance might influence the ability of a cow to prevent *S. aureus* from establishing an infection. The amount of milk present in the mammary gland varies during the day. In general the amount of milk is low just after milking and increases until the next milking moment. Variation in the amount of milk between quarters can be introduced by incomplete milking out. If the amount of milk influences bacterial growth, the time of bacterial entrance relative to milking may play a role in the (in)ability of a cow to prevent *S. aureus* from establishing an infection (Figure 1a). Although it is hard, if not impossible, to define the time of bacterial entrance in the field, the timing of experimental inoculation is controlled. Experimental inoculation of *S. aureus* shortly after milking might provide a window for pathogen survival due to low amounts of milk present. Since only a few papers describe the timing of experimental inoculation [11,15] it is hard to compare the outcome of infection following inoculation at different time points relative to milking.

Not only the amount of milk present in a quarter varies over time. The concentrations of fat, lactose, urea, and protein (including: lactoferrin, lactoperoxidase, and β-lactoglobulin), were also shown to vary between quarters of the same cow, between days, and even between morning and evening milkings [**Chapter 2**; 16]. However, variation in the concentrations of these components - with the exception of the fat percentage - does not seem to have a major impact on the course of infection since their levels were not associated with *in vitro* and *in vivo* growth of *S. aureus* [**Chapter 2**; **Chapter 3**].

As for the milk fat percentage, we found it to be positively associated with *in vivo* growth of *S. aureus* (Figure 1b) [**Chapter 3**]. Since phagocytic cells ingest fat globules which comprise their phagocytic capacity, this might result from a decreased ability of the hosts' immune system to combat *S. aureus*. Furthermore, *S. aureus* is able to form a biofilm type of growth by binding to milk fat globules [17]. Biofilm formation is thought to play an important role in the pathogenesis of *S. aureus* since it allows the pathogen to evade the cows' immunological defence mechanisms [18-19]. In contrast, the milk fat percentage was negatively associated with *in vitro* growth of *S. aureus* [**Chapter 2**]. These

contradictory results might be explained by activated lipolysis due to agitation of milk during preparation for *in vitro* assays which leads to the presence of fatty acids with antimicrobial properties [20-23]. It is unlikely that lipolysis is activated *in vivo*. Furthermore, a higher milk fat percentage has been associated with higher amounts of complement [24-25]. The complement system plays a crucial role in the hosts' defence against invading pathogens and provides a functional bridge between innate and adaptive immune responses [26]. However, while the complement system in human milk is well characterised, there is little information on its presence and activity in bovine milk. Recently it was shown that, although the levels of complement factors present in milk of healthy bovine mammary glands are generally comparable to those in human milk, complement activity is lower [24]. The alternative complement pathway was shown to be the sole operating complement pathway [27-29], since the classical complement pathway is inactive due to limited concentrations of C1q in healthy bovine mammary glands [27,30]. Only when inflammation develops, the blood-derived complement components may contribute to higher complement activity in milk. Since the *in vitro* assays were carried out in milk of healthy bovine mammary glands, low complement activity is expected. Therefore, we do not think that increased complement activity can explain the contradictory results obtained in our *in vitro* and *in vivo* analyses. Further research is necessary to evaluate the contribution of the complement system to a quarters' defence against *S. aureus* mastitis (Figure 1c) [29,31-32].

Antibodies in intramammary immunity

Bovine milk contains antibodies directed against a broad range of *S. aureus* antigens. Various studies have shown that increased antibody levels promote phagocytosis of *S. aureus in vitro* [9,11,33-35]. Furthermore, *S. aureus* specific antibodies in milk and serum were shown to have a direct role in inhibiting the function of *S. aureus* virulence factors and toxins [**Chapter 4**; **Chapter 5**; 36]. This suggests that antibodies are involved in the hosts' defence mechanism against *S. aureus* and that they possess functional properties in milk. Indeed, the presence of specific IgG1 antibodies in milk has been associated with reduced growth of *S. aureus* both *in vitro* and *in vivo* [**Chapter 2**; **Chapter 3**]. However, despite the presence of antibodies in milk of non-vaccinated cows, *S. aureus* infections still frequently occur indicating that ubiquitously present milk antibodies fail to provide protection.

Antibodies from the systemic circulation are transported over the blood-udder barrier to the mammary gland by the FcRn receptor [37-38]. Immunoglobulins bind to this receptor at the basolateral surface of mammary epithelial cells. The receptor-bound immunoglobulin is then internalized via endocytosis, transported to the apical end of the cells and

released into the alveolar lumen [39]. In bovine serum the concentration of IgG1 and IgG2 is approximately equal [40]. However, in colostrum and normal milk antibodies of the IgG1 isotype predominate [39-40]. Cianga and co-workers showed that transportation of IgG subclasses to the mammary gland is inversely correlated with their affinity for the FcRn receptor [41]. Since IgG1 has lower affinity for the receptor compared to IgG2 [42], mammary epithelial cells secrete IgG1 into the mammary gland while they do not effectively pass IgG2 on but rather recycle this antibody subtype back into the circulation [39,42]. In contrast to IgG1, the presence of IgG2 in milk at the moment of bacterial encounter has not been associated with reduced intramammary growth of *S. aureus* [**Chapter 5**]. However, although levels rapidly increase following recruitment after infiltration of the mammary gland, IgG2 levels are generally very low in milk of healthy cows [39]. It cannot be excluded that the presence of increased IgG2 levels could contribute to protection during the early stages of inflammation (Figure 1d).

Predominant presence of IgG1 in the bovine mammary gland

The predominant transportation of IgG1 to the mammary gland might have an evolutionary function. In cattle, antibodies are not actively transferred from maternal serum across the placenta to the foetus, and calves rely on the passage of maternal antibodies to colostrum for passive immune protection until their own immune system is fully functional [43]. The massive transfer of IgG1 from serum to colostrum might suggest that antibodies of this isotype are essential to provide appropriate passive immunity to the calve [44]. Indeed, despite the abundant presence of *S. aureus* in the environment, systemic infections in calves are rare indicating sufficient protective immunity. The lack of protection of IgG1 against intramammary *S. aureus* encounter might be a negative side effect of a process which has the primary aim to protect the offspring. However, if IgG1 provides protection to the calve, why does it not protect a cow from intramammary infections with this pathogen? A possible explanation could be that, although *S. aureus* specific antibodies can be detected in milk of all cows [**Chapter 2**; **Chapter 3**; **Chapter 4**; **Chapter 5**], their levels may still be too low to provide protection in the mammary gland (Figure 1e).

Another explanation for the predominance of IgG1 in milk may be found in the 'danger model' [45-46]. This model suggests that the immune system responds to antigens which pose a potential threat rather than to antigens which are foreign, as suggested by the older 'self - non-self' model. Whereas the self – non-self model relies on the idea that the immune system promotes the effector class that matches the pathogen it is fighting, the danger model proposes that the ultimate control over the type of immune response induced is controlled by the tissue in which the response occurs. By controlling the type of

immune response, a tissue can promote the response which is locally most useful while protecting tissue integrity and preserve tissue function. Only when the response is unsuccessful in eliminating the invading pathogen a tissue might activate responses which could damage the tissue itself [45]. Thus, according to the danger model, we speculate that the mammary gland might allow the presence and induction of IgG1 while preventing extensive infiltration of IgG2. The production of IgG1 is promoted by cytokines produced by Th2 cells, while Th1 cell subsets secrete cytokines that support a switch towards an IgG2 antibody isotype. The induction of a cell-mediated Th2 response may provide a higher risk for tissue damage than a Th1 response. When the preferred immune response is unable to resolve the infection the integrity of the blood-udder barrier is lost, allowing the influx of additional antibody isotypes - as well as other immune components and effector cells - to cooperate in the battle against *S. aureus*.

In order to investigate whether IgG1 is the primary antibody isotype required for intramammary protection against *S. aureus* or whether IgG2 is more beneficial for the host, strategies to increase specific antibody isotype levels in milk are required. The environment of antigen uptake, processing and presentation may influence the magnitude of the antibody response as well as the neutralizing capacity of these antibodies. In cattle, subcutaneous immunization near the supramammary lymph node positively influences the antibody response in both milk and serum [47]. In addition, the ability of adjuvants to modulate antibody responses is generally appreciated and widely exploited in different immunization strategies [48-49]. In this thesis, we show that a single subcutaneous immunization near the udder with a vaccine comprising an aluminium based adjuvant supplemented with saponin and mineral oil effectively increases the magnitude and neutralizing capacity of antibodies induced, both locally and systemically [**Chapter 4**; **Chapter 5**]. Interestingly, independent of the site and route of immunization, and the type of adjuvant used, IgG1 and IgG2 levels in milk and serum increase with similar magnitude indicating that vaccines are unable to induce a specific type of antibody isotype response. Although this phenomenon might be explained by the danger model, it is also possible that previous *S. aureus* encounter interferes with the ability of vaccines to induce a specific type of response.

Influence of initial *Staphylococcus aureus* encounter on vaccination

The first encounter between a cow and *S. aureus* antigens is likely to occur early in life. Calves may ingest *S. aureus* via the milk and teat skin during suckling. Furthermore, the presence of *S. aureus* in the calves' environment is likely to result in nasal and skin contact [3,50-54]. When a cow is immunized with a vaccine composed of an antigen previously encountered, activation of memory cells is likely to occur which might negatively impact

the desired response to vaccination later on. When the immune system is stimulated by antigens that have epitopes with a certain degree of homology to those of antigens encountered previously, memory T- and B- cell responses against the original epitopes may be recalled first instead of induction of primary responses against the homologous structure. This phenomenon has been termed original antigenic sin and has been widely described for virus infections, particularly influenza and dengue [55-56]. Little data are available regarding original antigenic sin concerning bacterial antigens [57].

Experimental vaccines against *S. aureus* are generally composed of whole cells or lysed bacteria with or without supplementation of recombinant proteins [8-9,33-34,58-60]. The presence of antigen specific antibodies in serum and milk of non-vaccinated cows indicates that exposure to *S. aureus* antigens frequently occurs [**Chapter 2**; **Chapter 3**; **Chapter 4**; **Chapter 5**]. Therefore, vaccination is likely to result in the activation of memory cells and the induction of an immune response similar to the initial type of response. Furthermore, since the initial priming of these memory cells in early life occurred in the absence of the mammary gland, which had not yet developed at that time, the class of immune response (isotypes and T cell effector types) as well as the homing properties of these cells might be dissimilar to the characteristics required for mammary gland homing.

Additional research is required to determine whether original antigenic sin interferes with the result of (experimental) vaccination against *S. aureus*. If so, strategies to redeem adaptive immunity from the consequences of antigenic sin are warranted. One strategy to prevent these problems might be to immunize calves prior to initial antigen encounter, i.e. shortly after birth. Currently, in most trials cows are vaccinated during pregnancy or in lactation when an immune response has already been established [**Chapter 4**; **Chapter 5**; 7-11,15,33-34,58-61]. Furthermore, although the alum-based adjuvants described in this thesis were not able to redirect the type of immune responses after pre-exposure to *S. aureus*, certain dendritic cell-activating adjuvants might be able to overcome the hurdle of original antigenic sin [62-63].

Cellular innate immune responses in the mammary gland

Differences in susceptibility to *S. aureus* mastitis between quarters have also been linked to the SCC. Herds with low bulk milk SCC manifested a higher incidence rate of new infections compared to herds with high bulk milk SCC [64]. The extensive recruitment of immune cells towards the site of infection also indicates that these cells are involved in the hosts' immune defence.

Cells of the innate immune system provide the first line of defence against invading pathogens. Mammary epithelial cells and alveolar macrophages play an important role in the recognition of *S. aureus* and in the release of various cytokines and chemokines which

triggers the massive influx of cells from the systemic circulation to the milk [65-68]. The main cell type recruited to the site of infection is neutrophils [69-70]. Neutrophils isolated from milk exhibit reduced antimicrobial capabilities compared to neutrophils isolated from blood since milk fat and proteins inhibit phagocytosis and oxidative burst [71-72]. However, neutrophils are also able to disarm and kill pathogens by the release of extracellular traps (NETs) [73-74]. These NETs, comprised of a mesh of DNA, histones, antimicrobial proteins and proteinases, entrap and inactivate the invading pathogen without requiring a direct contact or an engulfment by the host cell [75]. Interestingly, the formation of NETs is not inhibited by milk [76-78]. However, NETs can be degraded by nucleases expressed and secreted by *S. aureus* [79]. Blocking the effect of these nucleases results in more efficient NET mediated clearance of *S. aureus in vitro* [80]. Whether NETs have a functional significance in *S. aureus* mastitis remains to be determined (Figure 1f). Caution should be taken when analysing possibilities to promote local NET formation *in vivo*, since NETs were shown to directly induce epithelial and endothelial cell death [81].

There is accumulating evidence that cells of the innate immune system can remember pathogens to which they have previously been exposed and can respond to a subsequent exposure more efficiently, a phenomenon termed 'trained innate immunity' [82]. It has been demonstrated that trained innate immunity is directed by epigenetic reprogramming, broadly defined as sustained changes in gene expression that do not involve permanent genetic changes such as mutations and recombination, which are essential for adaptive immunity [83]. Although the specificity and memory of the innate immune system cannot compete with the adaptive response, the presence of trained innate immunity may contribute to clearance of *S. aureus* before the onset of inflammation. Differences in previous exposure to *S. aureus* between quarters may contribute to differences in effective memory responses of the innate immune system and thereby to variation in quarter susceptibility. Additional research regarding trained innate immunity and its possible beneficial effects in the hosts' defence mechanism against *S. aureus* mastitis is required.

Cellular adaptive immune responses in the mammary gland

Amongst the cells recruited to the mammary gland are also cells of the adaptive immune system. $CD8^+$ T-lymphocytes are the main sub-population in milk from healthy udder quarters [84-86]. However, during acute and chronic *S. aureus* mastitis $CD4^+$ T-cells are more extensively recruited and become the dominating sub-population [86-90]. Since *S. aureus* developed mechanisms to induce the differentiation of $CD8^+$ T cells towards a phenotype with suppressive activity against antigen-specific proliferative responses of

$CD4^+$ T lymphocytes [84,91], recruitment of $CD4^+$ T cells might be essential for battle against this pathogen.

The proportion of B-cells in infected quarters increases both in acute and chronic phases [86]. The preferred localization of B-cells is in the connective tissue, mainly in areas with no alveoli [92], suggesting that local antibody production occurs in the mammary tissue rather than in milk.

Th17 and γδ-T cells are known to express and secrete IL-17 which plays a pivotal role in the antigen-specific T cell recruitment of neutrophils during mastitis [93-94]. Up-regulation of IL-17 mRNA has been detected in tissues from the alveolar, ductal, gland cistern and teat canal regions of the bovine mammary gland [95-96], but not in blood mononuclear cells of infected cows indicating that the predominant role of IL-17 may be local, rather than systemic [96]. IL-17 can also be detected at the protein level in mammary glands infected by *S. aureus* [97-98]. Although cells of the different lymphocyte subtypes have all been associated with bovine *S. aureus* mastitis, it is as yet unknown of which cell type at what time a cow benefits most (Figure 1g).

Concluding remarks

Taken together, both innate and adaptive immunity are likely to play a role in the control of intramammary *S. aureus* infections. As discussed above, the immune correlates of protection in the hosts' defence against *S. aureus* are not yet completely understood. The data presented and discussed in this thesis contribute to our knowledge on immune components required to effectively combat this major mastitis causing pathogen. Our data show that levels of the routinely measured milk components lactose, urea, and protein (including lactoferrin, lactoperoxidase, and β-lactoglobulin), at the moment of bacterial entrance are not associated with the ability of a quarter to resolve *S. aureus*. In addition, we show a positive association between the milk fat percentage at the time of experimental inoculation and intramammary growth of *S. aureus*. However, since the milk fat percentage is highly variable, using its measures as an indicator for health disturbances is challenging.

Furthermore, we show an association between increased levels of *S. aureus* specific IgG1 in milk and reduced bacterial growth, both *in vitro* and *in vivo*. However, although IgG1 antibodies are ubiquitously present in milk, *S. aureus* mastitis often occurs. The levels of this predominant antibody isotype may be too low to efficiently combat *S. aureus*, or may not be the required isotype to provide protection. In this thesis, we show that a single subcutaneous immunization near the udder with a vaccine comprising an aluminium based adjuvant supplemented with saponin and mineral oil effectively increases the magnitude and neutralizing capacity of IgG1 and IgG2 antibodies, both locally and

systemically. Increased levels of functional antibodies allow the investigation of the role of antibodies in bovine intramammary immunity during *S. aureus* infections.

Although not analysed in this thesis, immunizations are likely to affect cellular immune responses as well. It was indicated that both (trained) innate and adaptive immune cells may play, as yet undefined, roles in the cows' defence against intramammary *S. aureus* infections. Additional research is required to unravel the presence of which cell type at what time benefits a cow the most and how immunization approaches can stimulate the required immune cells.

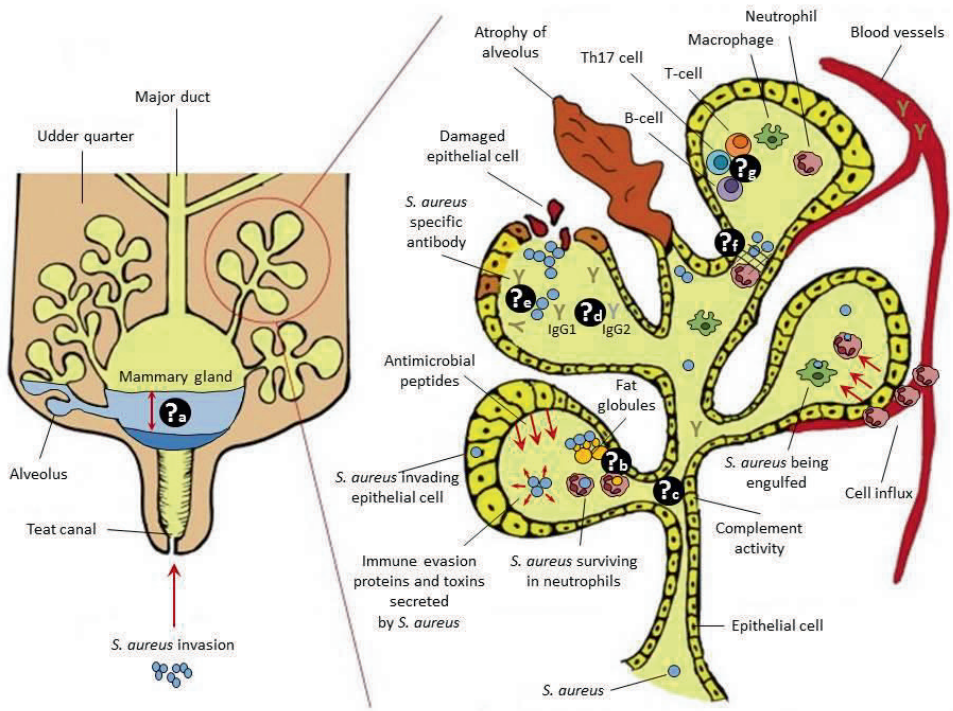


Figure 1: Schematic representation of immune mechanisms combatting S. aureus in the bovine mammary gland. *Several immune components and mechanisms in the mammary gland may be involved in the cows' defence against this major mastitis causing pathogen. The amount of milk, which contains molecular and cellular immune components, present in a quarter at the moment of bacterial encounter may influence the ability of a cow to prevent S. aureus from establishing an infection (a). Furthermore the milk fat percentages (b) and presence of complement components (c) may be involved in the cows' defence mechanism. In addition, antibodies present in the mammary gland are also thought to contribute to the cows' intramammary immune response against S. aureus. However, it is unknown which antibody isotype is required (d) and whether antibody levels in the mammary gland are sufficient (e). On a cellular level, S. aureus mastitis is characterized by an influx of neutrophils. Neutrophils may combat S. aureus by the formation of neutrophil extracellular traps (f). Although lymphocyte subpopulations (B-, T-, and Th17 cells) have also been linked to bovine mastitis, it is unknown of which cell type the host benefits most (g). Adapted from Viguier et al., 2009 [99].*

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Chapter 7

Nederlandse samenvatting

Dankwoord

Curriculum vitae

List of publications

Nederlandse samenvatting

Intramammaire infecties kunnen leiden tot een ontsteking van het uierweefsel, ook wel mastitis genoemd. Mastitis is één van de meest voorkomende gezondheidsproblemen in de melkveehouderij en kan ernstige gevolgen hebben voor het welzijn van de koe, de melk productie en de kwaliteit van de melk. Een veel voorkomende veroorzaker van bovine mastitis is de bacterie *Staphylococcus aureus* (*S. aureus*). Intramammaire infecties met *S. aureus* kunnen zich ontwikkelen tot klinische, subklinische of chronische mastitis. Klinische mastitis wordt gekenmerkt door visuele veranderingen in de melk. Daarnaast kan het geïnfecteerde kwartier warm en gezwollen of hard aanvoelen. In ernstige gevallen kunnen de symptomen systemisch worden en kan koorts optreden. Subklinische mastitis heeft vaak geen visuele verschijnselen en het vaststellen van subklinische mastitis gebeurd door gebruik te maken van een celgetal bepaling in de melk. Een verhoogd celgetal is positief gecorreleerd met de aanwezigheid van een ontstekingsreactie en wordt daarom vaak gebruikt als indicator voor uiergezondheid. Wanneer mastitis onopgemerkt blijft kunnen de infecties chronisch worden en gedurende de gehele lactatie periode aanwezig blijven.
In de melkveehouderij worden diverse maatregelen getroffen om intramammaire infecties met *S. aureus* te voorkomen. Zo is een goede hygiëne in de stal erg belangrijk in de preventie van mastitis. Goed werkende melkmachines en een gedegen melktechniek voorkomen het ontstaan van beschadigingen aan het uier waardoor de kans dat bacteriën de speen binnendringen afneemt. Ook vind na het melken dippen of sprayen van de spenen plaats om de speenhuid te desinfecteren. Indien er toch mastitis bij een koe wordt geconstateerd wordt deze vaak behandeld met antibiotica. Echter, over het algemeen heeft behandeling met antibiotica in melkgevende koeien een lage efficiëntie en bied het geen bescherming tegen herhaalde infecties. Bovendien is er vanuit de samenleving steeds meer vraag naar mogelijkheden om het antibiotica gebruik terug te dringen. Er wordt daarom veel onderzoek gedaan naar de ontwikkeling van een profylactisch vaccin tegen *S. aureus* mastitis.

Variatie in gevoeligheid voor mastitis

Niet alle koeien zijn even gevoelig voor mastitis. Interessant is dat er ook variatie zit in de gevoeligheid voor mastitis tussen de kwartieren van dezelfde koe. Dit impliceert dat er kwartier gebonden factoren aan deze variatie ten grondslag liggen. Voorbeelden van kwartier gebonden factoren zijn de morfologie en positie van een kwartier. Ook in experimentele inoculatie studies, waarbij *S. aureus* door het tepelkanaal heen wordt geïnjecteerd en de morfologie en positie van het kwartier geen rol spelen, worden

verschillen in gevoeligheid waargenomen. Het is nog onduidelijk hoe deze verschillen worden veroorzaakt.

Om te onderzoeken of de melksamenstelling een rol speelt in de gevoeligheid voor mastitis hebben we in **Hoofdstuk 2** gekeken naar variatie in de melk samenstelling tussen de kwartieren van dezelfde koe. We hebben hiervoor de vet, eiwit, lactose, ureum, lactoferrine, lactoperoxidase, en β-lactoglobuline concentraties, het celgetal en de *S. aureus* specifieke antilichaam titer bepaald. Tevens hebben we onderzocht of er verschillen bestaan in de samenstelling van de ochtend en avond melk. Om het effect van de melksamenstelling op de groei van twee veel voorkomende mastitis veroorzakers te bepalen hebben we *in vitro* de groei van *S. aureus* en *Escherichia coli* (*E. coli*) in verse ochtend en avond melk geanalyseerd. Onze resultaten laten zien dat, met uitzondering van lactose, alle gemeten parameters significant verschillend waren tussen kwartieren alsmede tussen de ochtend en avond melk. Ook de groei van *S. aureus* en *E. coli* was significant verschillend in ochtend en avond melk. Deze resultaten ondersteunen het idee dat variatie in de melk samenstelling bijdraagt aan verschil in bacteriële groei. Hoewel alle gemeten parameters significant verschilden tussen kwartieren en tussen ochtend en avond melk waren er slechts twee geassocieerd met de groei van *S. aureus* en *E. coli*: het vet gehalte en de *S. aureus* specifieke IgG1 titer. De groei van *S. aureus* was negatief geassocieerd met het vet percentage, de *S. aureus* specifieke IgG1 antilichaam titer en ochtend melk. De groei van *E. coli* was negatief geassocieerd met het vet gehalte in de melk en positief geassocieerd met de ochtend melk.

In **Hoofdstuk 3** hebben we onderzocht of de melksamenstelling ook een invloed heeft op de *in vivo* groei van *S. aureus*. Dit hebben we gedaan door de samenstelling van de melk vlak voor experimentele intramammaire inoculatie van *S. aureus* te bepalen en de uitscheiding van *S. aureus* in melk na inoculatie. Met behulp van gemengde model analyses hebben we onderzocht wat de invloed is van ofwel de melk componenten vet, eiwit, lactose en ureum, ofwel de *S. aureus* specifieke antilichaam titers, in combinatie met management en kudde gerelateerde parameters (boerderij en experimentele groep), individuele koe factoren (aantal dagen in melk, melk productie en de positie van het kwartier) en een pathogeen gerelateerde parameter (inoculatie dosis) op het aantal *S. aureus* dat kon worden terug gekweekt uit de melk na experimentele inoculatie. Onze resultaten lieten een positieve associatie zien tussen het melk vet gehalte en het aantal terug gekweekte *S. aureus* en een negatieve associatie tussen de *S. aureus* specifieke IgG1 antilichaam titer in de melk en het aantal terug gekweekte *S. aureus*.

Vaccin ontwikkeling

De observatie dat de *S. aureus* specifieke IgG1 antilichaam titer negatief geassocieerd was met de groei van *S. aureus*, zowel *in vitro* als *in vivo*, bemoedigd de pogingen om een vaccin tegen bovine *S. aureus* mastitis te ontwikkelen. Gedreven door deze observatie hebben we onderzoek gedaan naar vaccinatie strategieën om specifieke antilichaam titers in het uier te verhogen.

In **Hoofdstuk 4** hebben we gekeken of de toedieningsroute van het vaccin een invloed heeft op de antilichaam respons. Huidige (experimentele) vaccins worden veelal toegediend via de parentele route (meestal intramusculair). Het is echter niet bekend of dit de meest optimale vaccin toedieningsroute is om antilichaam titers in de melk te verhogen. We hebben daarom koeien via de intramusculaire, intranasale, intramammaire en subcutane route gevaccineerd om te bepalen welke route de grootste impact heeft op de stijging en kwaliteit van de antilichaam respons. Als vaccin is gebruik gemaakt van twee model antigenen, het extracellulaire fibrinogeen bindend eiwit (Efb) en de leukocidine subunit LukM, geformuleerd in een olie-in-water adjuvans gecombineerd met een hydrogel en alginaat. De resultaten van deze studie lieten zien dat een subcutane toediening van het vaccin resulteert in de hoogste antilichaam titer stijgingen in melk. Tevens waren de hoogste stijgingen in de neutraliserende capaciteit van melk en serum waarneembaar na subcutane vaccinatie.

Vervolgens hebben we in **Hoofdstuk 5** onderzocht of de locatie van subcutane vaccin toediening op het lichaam invloed heeft op de antilichaam respons. Ook hebben we gekeken welke impact het type adjuvans heeft op de geïnduceerde antilichaam respons. In deze studie is gebruik gemaakt van het model antigeen α-toxoïde dat geformuleerd werd in drie verschillende aluminium gebaseerde adjuvantia: Alum-Saponine, Alum-Olie, en Alum-Saponine-Olie. De koeien hebben een prime en een boost vaccinatie ontvangen via subcutane toediening nabij de ophangband van het uier of in de nek. Vaccinatie op beide lichaamslocaties resulteerde in significant hogere specifieke IgG1 en IgG2 antilichaam titers wanneer gebruik gemaakt werd van Alum-Saponine-Olie als adjuvans in vergelijk met de uit Alum-Olie of Alum-Saponine bestaande vaccins. Ook de neutraliserende capaciteit van melk en serum liet een significant hogere stijging zien wanneer gevaccineerd werd met een vaccin dat Alum-Saponine-Olie bevatte in vergelijking tot vaccins die op Alum-Olie of Alum-Saponine gebaseerd waren. Opmerkelijk was dat een prime vaccinatie nabij het uier resulteerde in vergelijkbare antilichaam- en neutralisatie titer stijgingen als een prime-boost vaccinatie in de nek. Deze resultaten suggereren dat een one-shot vaccinatie mogelijk voldoende is om op efficiënte wijze een antilichaam respons in de melk te induceren.

Conclusies

In **Hoofdstuk 6** zijn de resultaten van dit proefschrift in perspectief geplaatst en bediscussieerd. De resultaten hebben meer inzicht gegeven in de variatie die bestaat in de melksamenstelling tussen verschillende kwartieren en de impact die de melksamenstelling heeft op de groei van twee mastitis veroorzakende bacteriën. Daarnaast dragen de resultaten bij aan vaccin ontwikkeling door informatie te verschaffen over vaccin toedieningsroutes en adjuvantia die de antilichaam respons positief beïnvloeden.
Samenvattend concluderen we dat de routinematig gemeten melk componenten lactose, ureum en eiwit (inclusief lactoferrine, lactoperoxidase en β-lactoglobuline) op het moment van infectie niet geassocieerd zijn met het vermogen van een kwartier om de intramammaire groei van *S. aureus* tegen te gaan. In tegenstelling, een verhoogde *S. aureus* specifieke IgG1 antilichaam titer in melk heeft een negatieve invloed op de groei van *S. aureus*. Een efficiënte methode om specifieke, neutraliserende antilichamen te induceren, zowel lokaal als systemisch, is door middel van subcutane vaccinatie nabij het uier met een vaccin dat Alum-Saponine-Olie als adjuvans bevat. Verhoogde, neutraliserende antilichaam titers worden als noodzakelijk gezien om de rol van antilichamen in intramammaire immuniteit tegen *S. aureus* in koeien verder te kunnen onderzoeken.

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Curriculum Vitae

Eveline Myrthe Boerhout was born on January 23, 1986 in Heerlen, The Netherlands. In 2003 she started her studies in Biotechnology at the van Hall Institute, Leeuwarden. During her studies Eveline fulfilled an internship at the John Hughes Bennett Laboratories in Edinburgh, Scotland, where she studied the role of the Wnt signaling pathway in leukemia. During her second internship, Eveline worked at the Department of Environmental Biology at the Fachhochschule Aachen in Jülich, Germany, where she participated in studies aimed to unravel the association between the algae *Alexandrium ostenfeldii* and bacteria living in the North Sea. Eveline fulfilled a third internship at the Center for Genomic Regulation in Barcelona, Spain, where she focussed on the effects of the inhibition or over-activation of specific Aurora kinases in human cell lines.

After receiving her Bachelor degree of Applied Science in 2007, Eveline obtained a position as research technician at the Department of Human Genetics of the Radboud University Medical Center (UMC), Nijmegen, where she worked on the identification of downstream targets of the acute leukemia associated AF10 protein. In 2008 Eveline started her master studies Medical Biology at the Radboud University, Nijmegen. During these studies, she returned to the Department of Human Genetics of the Radboud UMC for an internship. During this internship she focused on the effects of various HDAC inhibitors on a human synovial sarcoma cell line. Eveline fulfilled her final internship at the department of Microbiological Research and Development (R&D) of MSD Animal Health (MSD-AH), where she analyzed the immune responses induced by *Neospora caninum* based DNA vaccines in a murine model. In 2011, she graduated with honours.

After her Master studies Eveline continued to work at the department of Microbiological R&D at MSD-AH where she started her PhD studies on *Staphylococcus aureus* mastitis in cattle in 2012. This research was conducted in collaboration with the divisions of Immunology and Biochemistry & Cell Biology of the Faculty of Veterinary Medicine of the Utrecht University. The results of this research are described in this thesis and are published in peer-reviewed scientific journals.

Currently, Eveline works at the Poultry R&D department of MSD-AH.

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** These authors contributed equally*