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Molecular epidemiology and immune evasion strategies of ruminant *Staphylococcus aureus*

Moleculaire epidemiologie en immuun evasie strategieën van *Staphylococcus aureus* verantwoordelijk voor mastitis in herkauwers

(met een samenvatting in het Nederlands)

Proefschrift

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CHAPTER 1

General Introduction

Introduction

The dairy sector is the second largest agricultural sector within the European Union (1). In 2018, there were an estimated 22.9 million dairy cows in the EU, producing 165 million tons of raw milk (1). Mastitis, the inflammation of the mammary gland, is the biggest single cause of financial loss for the sector (2–4). These losses result from several cost-components, such as reduced milk yield and quality, costs of medication and diagnostic testing, reduced reproduction, culling and the replacement of animals (2–4). In addition, mastitis reduces animal welfare (5) and treatment of mastitis is the main reason for the use of antimicrobials and associated antimicrobial resistance within the dairy industry (6). Therefore, it is in the best interest of the industry to control mastitis.

Mastitis in ruminants is often characterized as clinical or subclinical and both of which may turn into a chronic state (7,8). During clinical mastitis (CM), at least one of the five classical signs of inflammation are present in the mammary gland (swelling, pain, erythema, warmth and loss of function), as well as alterations in the composition of secreted milk (7). Cases of CM can be classified as mild, moderate or severe. Mild CM cases display only abnormalities in secreted milk, whereas in moderate cases, this is accompanied by swelling or redness of the udder. During severe CM cases, animals also suffer from systemic symptoms such as anorexia, dehydration and fever (7). In The Netherlands, the herd incidence rate of CM is around 30 cases per 100 cows per year (9,10). The largest cost-components of CM on a herd-level are animal mortality and culling of sick animals, animal replacement and the reduction in milk yield (11).

Subclinical mastitis (SCM) is defined as the presence of infection without local inflammation. These cases lack any clinical sign or visible change in milk composition (7). The incidence of SCM is higher than that of CM and is around 70 cases per 100 cows per year on Dutch dairy farms (12). Diagnosis of SCM is based on positive microbiological culture of milk and determination of somatic cell count (SCC): the concentration of (primarily) leukocytes per mL of milk (7). The SCC of uninfected cows is < 100,000 cells / mL and an increase in SCC indicates inflammation (13). In The Netherlands, a threshold of SCC > 250,000 cells / mL is commonly used to diagnose SCM (10). The estimated herd-level costs of SCM are higher than those of CM, and the largest cost-component of SCM is the reduction in milk yield and quality (11).

Intramammary infections (IMI) with microorganisms are the main cause of mastitis and the clinical outcome of these infections depends on the interactions between the infecting pathogen, host and environment (8). This thesis focusses on the pathogenicity of the mastitis pathogen *Staphylococcus aureus* and this bacterium will first be introduced in the following section. A key feature of *S. aureus* is its ability to produce many different types of immune evasion factors, that are expected to influence to pathogenicity of *S. aureus* during IMI (14,15). In order to understand how these immune evasion factors function, this chapter will also give a brief overview of the immune defenses of the mammary gland. Then, different immune evasion strategies of *S. aureus* are discussed. Finally, the aim of the thesis is given and the outline of the thesis is presented.

Staphylococcus aureus as a pathogen of ruminant mastitis

One of the most prominent bacterial species responsible for ruminant mastitis is *Staphylococcus aureus* (16). This gram-positive bacterium is a commensal, opportunistic pathogen (8,17) with a wide host range that includes humans and mammalian, reptilian and avian species (18). In dairy animals, the reservoirs of *S. aureus* are chronically infected mammary glands, colonized teat skin, nares and (wounded) hocks (8,19). The bacterium can spread to uninfected quarters or animals by contact with colonized udder skin of lactating cows, teat cup liners or through flies (20). In cattle, infections with *S. aureus* mostly result in SCM but can also cause CM that are often chronic (8,21). In contrast, *S. aureus* is predominantly associated with CM in dairy goats and sheep, and these CM cases are often more severe than in cattle (8,16). Cure rates of *S. aureus* infections depend on animal, pathogen, and treatment factors but results of treatment are generally poor (8,16,22). In addition to its impact on ruminants, *S. aureus* mastitis also represents a threat to public health due to its zoonotic potential (23) and the risk of bacterial food poisoning from raw milk or cheese products (24,25).

Staphylococcus aureus has a clonal population structure and although the bacterium can be con-sidered a generalist pathogen based on its wide host range, specific clones of *S. aureus* are associated with certain host species (18). Evolutionary analysis demonstrated that humans have acted as the major donor species of animal associated *S. aureus* clones (18,26). After an initial jump to a new host species, *S. aureus* can adapted to its new host through allelic diversification, gene mutation and the uptake of mobile genetic elements (MGEs) from the host' microbiota, such as plasmids, prophages, phage-related pathogenicity islands (18,26). These MGEs are often host associated and contain genes with a specific functionality that in-

creases survival of *S. aureus* within their new host, with many MGE encoded proteins being involved in immune evasion (27,28). However, restriction-modification (R-M) systems limits the transfer of MGEs between different clones of *S. aureus* (29) and once MGEs have been incorporated in the bacterial genome, the elements are very stable and will be transferred vertically to subsequent generations of *S. aureus* (30,31). The variable part of the *S. aureus* genome is known as the accessory genome and contains around 25% of the total number of genes of the bacterium (27,32). In addition to MGEs, this accessory genome also contains genes of the "core-variable genome", a lineage-specific set of genes that are only transmitted ver-tically and many of these genes are also involved in immune evasion (27,33). Altogether, *S. aureus* clones found on different animal species can vastly differ in their genetic profile, and in particular in their carriage of virulence factors (27).

The differential carriage of immune evasion (and other virulence) factors by *S. aureus* clones is ex-pected to influence their pathogenicity during infection, and therefore it is it important to be able to dif-ferentiate between *S. aureus* clones. This is done using several genotyping techniques and genotyping of microorganisms forms the basis of the field of molecular epidemiology, which combines molecular biology with epidemiology by associating molecular characteristics of infection strains (lineage, gene carriage) to epidemiological data such as clinical parameters of disease (34,35). Common sequence-based typing tech-niques of *S. aureus* are multi locus sequence typing (MLST), Staphylococcal protein A gene (*spa*) typing and whole genome sequence (WGS) typing (35–37). The MLST technique uses single nucleotide polymorphisms (SNPs) within a 450 base pairs (bp) region of seven household genes of *S. aureus* to differentiate between strains. Based on the combination of gene alleles, a sequence type (ST) is assigned. In addition, related STs are often grouped together in Clonal Complexes (CC) based on BURSTs (Based Upon Related Sequence Types) analysis and these CCs consist of a founder ST and their single locus and double locus variants (35,38). Using this technique, it was identified that specific CCs are associated with different host species (18), with CC151, CC97 being the dominant bovine-associated CCs and CC133, CC130 and CC522 *S. aureus* are associated with goats and sheep (39–41). The resolution of MLST is relatively low as there can still be considerable genetic variation within the same CC or ST. Therefore, *spa*-typing, a technique using variable-number tandem repeats within the *spa* gene, is often used to differentiate between closely related *S. aureus* strains (37,38). Based on the variable repeats, a *spa*-type can be assigned and often, a combination of both MLST and *spa*-typing is used distinguish *S. aureus* strains (37,38). In recent years, the use of WGS typing is increasing due to the constant decrease in sequencing costs and the advantages of this technique over other typing methods (34,42). This technique allows comparison between bacteria based on their entire genome,

giving WGS typing the highest resolution of genotyping possible. This eliminates the need for identification of MLST, *spa*-type or the presence of specific genes of interest as this can be inferred in silico from the sequence data (42). Although the use of WGS can be considered the new gold standard for studies on the molecular epidemiology of *S. aureus*, the costs and complexity of data analysis of WGS data are still higher than for MLST and *spa*-typing (34,42).

Immune defenses of the ruminant mammary gland against *Staphylococcus aureus*

The mammary gland has a well-developed immune system that is able to react to invading pathogens using both innate and acquired immune defense mechanisms (21,43). The first line of defense is the physical barrier of the teat canal, which can be closed by the teat sphincter muscle to separate the exterior and the interior of the mammary gland (21,43). In addition, the teat canal is lined with keratin that can bind and immobilize microorganisms, preventing them from reaching the lumen of the mammary gland (44,45). Between milkings and during the dry period, a keratin plug is formed that creates an additional physical barrier (41,42).

When a pathogen manages to overcome these barriers and enters the lumen of the mammary gland, the innate immune defense mechanisms are activated to deal with the invading bacteria (21). In the initial phase of immune responsiveness, invading microorganisms are sensed by resident macrophages and mammary epithelial cells (MEC) (46). These cells are able to detect bacterial components, known as pathogen associated molecular patterns (PAMPs), using multiple pattern recognition receptors (PRRs), such as toll-like receptor (TLR) 1, TLR-2, TLR-6 and others, nucleotide-binding oligomerization domain-like receptor (NLR) 1 and NLR-2 (21,46,47). Activation of these PRRs by PAMPs induces the NF-κB and Mitogen-Activated Protein Kinase (MAPK) signaling pathway, which increases the transcription of several genes involved in the immune responses of the mammary gland (47,48). Antimicrobial peptides released into the lumen, like cathelicidins and β-defensins, as well as antibacterial proteins (e.g. lactoferrin), reduce bacterial survival, whereas acute phase proteins (e.g. serum amyloid A3 (SAA3), proinflammatory cytokines, such as Interleukin (IL)-1 β, IL-6, IL-8 and tumor necrosis factor (TNF)α, are the initial factors released in the inflammatory response (21,43,49). Release of IL-6 and IL-8 by MEC and macrophages triggers the recruitment of blood granulocytes (mainly neutrophils) to the site of infection (43,50). Neutrophils are the key effector cells of the host immune defense against *S. aureus* and kill bacteria through phagocytosis (50,51). This process is facilitated through opsonization of the

pathogen by Immunoglobulins (Ig)G and complement component 3 (C3), that contributes to elimination of the bacteria by neutrophils and other leukocytes (43,50). Pathogens internalized into phagosomes are eliminated after fusion with lysozomes containing reactive oxygen species, antimicrobial peptides and hydrolytic enzymes (52,53). Although these factors kill the bacteria, they are also harmful for the host when released into the lumen of the mammary gland. To avoid this, neutrophils undergoing apoptosis express specific proteins that label them for removal by host macrophages (43,53).

In parallel with innate immune responsiveness, the adaptive immune response is activated and the initial neutrophil infiltration is gradually partly replaced with an influx of T and B lymphocytes activated in regional lymph nodes (21,43). However, neutrophils remain an important effector of the immune response during mastitis (52). Lymphocytes can recognize and respond to various antigenic structures through receptors that define the specificity of the acquired immune response (46). The B lymphocytes produce opsonizing antibodies that enhance phagocytosis by neutrophils (54). CD8+ cytotoxic T lymphocytes eliminate old and damaged host cells and may act as immune suppressors (46). They are the most abundant type of T lymphocytes in milk of healthy animals, but during inflammation, helper (Th) CD4+ T lymphocytes become the dominant type of lymphocytes in the mammary gland (43). These cells regulate the adaptive immune response by a wide array of inflammatory cytokines (55). There are multiple lineages of Th lymphocytes and the Th17 lineage is considered the major actor of the mobilization of neutrophils and a modulator of innate and antigen-specific inflammation during IMI through the release of cytokines IL-17A, IL-17F, IL-22 and IL-26 (54,56).

Immune evasion strategies of *Staphylococcus aureus*

As discussed above, the mammary gland has multiple immune defenses against microorganisms but *S. aureus* can manipulate almost every aspect of these defenses using a diverse repertoire of immune evasion factors (28,57). These factors include both cell wall anchored factors and secreted factors, also known as exotoxins (28,58). Secreted immune evasion factors can be grouped into different categories based on their mechanisms of immune evasion (33). Several exotoxins (leukocidins, phenol-soluble modulins), weaken the immune defenses by actively killing leukocytes (33). Other secreted factors, such as superantigens, modulate immune activation and therefore reduce the effectiveness of the immune response (28,59). Multiple exotoxins (e.g. Staphylococcal Superantigens-like proteins, Staphylococcal complement inhibitor), block host or receptor proteins, interfering with pathogen

recognition of *S. aureus* and several other immune functions (28,33). The next sections introduce several cell wall anchored and secreted factors, representing the different strategies of immune evasion of *S. aureus* (57), that may affect the immune responsiveness of the ruminant mammary gland during IMI.

Cell wall anchored immune evasion factors

The *S. aureus* cell envelope is a complex network of glycopolymers, phospholipids, peptidoglycan and cell wall anchored proteins (60). Although peptidoglycan and lipoteichoic acid act as PAMPs that can activate inflammatory responses in mammary gland (61), other components of the bacterial cell envelope offer protection against the host's immune response, such as wall teichoic acids (WTA), capsular polysaccharides (CP), Staphylococcal protein A (SpA) and the second immunoglobulin-binding protein (Sbi) (51,60,62).

The production of WTA by *S. aureus* has multiple functions, it is involved in cell wall biosynthesis and maintenance, interaction with phages and antimicrobial resistance (60,63). Furthermore, WTA increases the protection of *S. aureus* against defensins and contributes to bacterial adhesion and skin abscess induction (60,64). The formation of protective capsules using CPs reduces the interaction of complement and antibodies with *S. aureus*, rendering the bacteria more resistant to opsonophagocytic killing by neutrophils (65). In addition, encapsulated *S. aureus* are weaker activators of TLR-2 as compared to non-capsulated *S. aureus* (66), likely by shielding of PAMPs from PPRs and reducing shedding of PAMPs into the extracellular environment. The immunoglobulin-binding SpA also reduces opsonization by binding to IgG and is able to induce non-specific activation of B lymphocytes through binding to their receptors (57,67). Since this activation takes place without co-stimulatory signals, this results in the death and deletion of B lymphocytes (67). The Sbi is unable to bind to B lymphocyte receptors but instead binds to IgG and C3, further reducing the effectiveness of *S. aureus* opsonization (51,57). Both Sbi and SpA can be released from the bacterial cell surface into the extracellular space to further interfere with immune function (28).

Killing of neutrophils by leukocidins and phenol-soluble modulins

Leukocidins are pore-forming, bi-component cytotoxins that specifically target immune cells and so far, seven different sets of leukocidins have been identified (HlgAB, HlgCB, LukAB/HG, LukED, Panton-Valentine leukocidin (PVL), LukPQ and LukMF') which all consist of two subunits (a S- and a F-component) (68,69). Whereas the genes encoding for HlgAB, LukAB and HlgCB are part of the *S. aureus* core genome, the other leukocidins genes are located on prophages (PVL, LukPQ and

LukMF') or pathogenicity islands (LukED) and the presence of these leukocidins is therefore variable between *S. aureus* (68,69). The specificity of leukocidins towards immune cells is determined by the binding of the S or F- component to specific receptors or proteins on the cell-surface of leukocytes (68,70,71). This initial binding recruits the other leukocidin component and together these components forms a dimer on the cell surface. Oligomerization of four dimers ultimately results in the formation of a β-barrel pore spanning the lipid bilayer of the cell membrane, resulting in lysis and cell death (68). In addition to their specificity for immune cells, PVL, LukPQ, and LukMF' most strongly affect leukocytes from a limited range of species (68,69,72). The leukocidin LukMF' is a potent and specific killer of neutrophils from ruminant species, such as cattle, goats and sheep, but not of human neutrophils (71,73,74). The molecular basis of this host specificity is the binding of LukMF' to chemokine receptor CCR1, which is expressed in ruminant neutrophils and absent on the cell surface of human neutrophils (14). Since neutrophils are key effector cells of the immune response during mastitis, the killing of neutrophils by LukMF' is expected to increase the survival of *S. aureus* within the mammary gland (14). In addition, lysed neutrophils release their harmful content that could damage the mammary tissue (41).

In addition to leukocidins, *S. aureus* can also produce phenol-soluble modulins (PSMs) to kill leukocytes (76). There are several groups of PSMs but the short sized (20- to 25-amino-acid) α types of PSMs are identified as being the most effective PSM type (76,77). Bacteria caught inside phagosomes within neutrophils can free themselves from the phagosome by releasing PSMα, that acts as an intracellular toxin to kill neutrophils from within (76,77). In addition, PSMα aggregation on cell membranes triggers cell deformation and death of T lymphocytes (76,77). In addition, PSMs impair the release of IL-6, IL-8, IL-32 by bovine MEC in response to stimulation with *S. aureus* (78).

Modulation of immune response by *Staphylococcus aureus*

Superantigens (SAs) are a class of exotoxins that are potent mitogens for T lymphocytes as they induce an uncontrolled activation of these cells that results in the release of proinflammatory cytokines (79). The hyperactivation of T lymphocytes interferes with the proper buildup of the adaptive immune response (57) and some SAs can trigger apoptosis in MEC (80) or inhibit phagocytosis by neutrophils (79). The SAs are also known as Staphylococcal enterotoxins, as they have long been identified as a source of Staphylococcal food poisoning from raw milk or cheese products (81). Several SAs have a host-specific functional activity (79,82) and the carriage of SA genes varies greatly between *S. aureus* clones since almost

all identified SAs are located on MGEs or a part of the core-variable genome (82,83). Accordingly, there is major variation in the number of different SAs produced by *S. aureus*, with some clones producing up to 25 different SAs, whereas other lack SA genes (82,84).

Other *S. aureus* exotoxins that modulate the immune response are staphylocoagulase and the von Willebrand factor binding protein (vWFbp), and these factors bind and activate prothrombin, resulting in the conversion of fibrinogen to fibrin that promotes the clotting of plasma or blood (57). Although both vWFbp and staphylocoagulase are less effective in inducing clotting of ruminant plasma compared to human plasma, there are also specific phage encoded variants of vWFbp found exclusively in ruminant *S. aureus* strains that coagulate ruminant plasma (85).

Blocking of immune functions by *Staphylococcus aureus*

The family of Staphylococcal Superantigens-like proteins (SSLs) is structurally similar to the SA family, but SSLs lack superantigen activity and instead block multiple aspects of immune responsiveness, such as opsonophagocytosis, neutrophil function and pathogen recognition (15,84). So far, 14 different SSLs (SSL-1 to SSL-14) have been identified and SSLs encodings genes are located on pathogenicity island vSaα (SSL-1 to SSL-11) and immune evasion cluster 2 (SSL-12 to SSL14) within the core-variable genome of *S. aureus* (57). The SSL-2 and SSL-3 proteins block TLR-2 receptors, preventing the activation of the NF-κB pathway in TLR-2 expressing lymphocytes or MEC (86,87). SSL-7 interferes with the complement system by reducing the generation of complement C5a, an important chemoattractant and activator of neutrophils (88,89). The SSL-1 and SSL-5 proteins interfere with the activation and migration of neutrophils through inhibition of neutrophil matrix metalloproteases (90). Furthermore, SSL-11 blocks neutrophil migration by inducing adhesion of neutrophils, locking the cells in place and reducing the spread of neutrophils to the site of infection (91). Several SSLs have a specificity towards the human immune system and are less functional in animal host species (15). The SSL-10 protein is able to bind to human and primate IgG, preventing phagocytosis of IgG-opsonized bacteria, but lacks functionality against cattle, sheep or mouse IgG (92). Furthermore, SSL-3 and SSL-4 are not able to bind the bovine (and caprine) TLR-2 due to structural differences between the human and ruminant TLR-2 proteins (86). Nevertheless, the SSL-3 and SSL-4 genes are still found in *S. aureus* obtained from ruminants and this could suggest these SSLs have yet undiscovered functions in ruminants since several SSLs have multiple binding partners and functions (57,86).

The Staphylococcal complement inhibitor (SCN) blocks C3 convertases that play an essential role in the formation of functional C3, reducing the effectiveness of the

complement system and likely influencing the rate of phagocytosis by neutrophils (93). The initially discovered SCN protein is only effective in blocking human C3 convertases and likely because of this, the SCN encoding prophage (ϕSa3) is absent in animal-associated *S. aureus* strains (94). Recently, a phage-encoded variant of SCN was identified with a wider host range which can block C3 convertases of horses, humans, and pigs (93).

Aim and outline of thesis

In summary, infections with *S. aureus* are a common cause of ruminant mastitis and cause both CM and SCM. The pathogenicity of *S. aureus* during IMI depends on the interaction between pathogen, the hosts immune response and the environment but it is still not fully understood to what extent the clinical outcome of infection is driven by host or pathogen factors. Although a large number of different immune evasion factors have been identified for *S. aureus*, there is an uneven distribution of these factors among *S. aureus* clones. This suggests these clones evade the immune responses using different strategies and therefore, possibly also differ in their pathogenicity and host-pathogen interactions during ruminant mastitis. The research in this thesis explores the molecular epidemiology of *S. aureus* mastitis and aims to identify if genetic characteristics of *S. aureus* are associated with the clinical outcome of IMI. In addition, the second aim of this thesis was to investigate the interaction between *S. aureus* belonging to different CCs and ruminant MEC.

Leukocidin LukMF' was previously identified as a potential virulence factor that affects the pathogenicity of *S. aureus* during ruminant mastitis, and the study described in **Chapter 2** compares the LukMF' carriage, LukMF' production and genotype of *S. aureus* isolates obtained from bovine CM and SCM in The Netherlands. In **Chapter 3**, associations between bacterial genetic factors, CC and clinical outcome of *S. aureus* IMI are investigated in a collection of European *S. aureus* mastitis isolates. In addition, this chapter describes key differences in the carriage of immune evasion factors between ruminant-associated CCs and the distribution of these CCs in Europe. Different *S. aureus* CCs are associated with mastitis in small ruminants compared to cattle, and **Chapter 4** describes a study that compares genetic characteristics and LukMF' production levels of CM and SCM *S. aureus* isolates collected from dairy goats and meat sheep. Differences between isolates from different host species are also compared.

Besides the carriage and production of immune evasion factors by *S. aureus*, the interactions between host and pathogen also plays a role in the pathogenesis of

mastitis. A critical interaction in the early immune response is the activation of ruminant MEC by *S. aureus*. The research presented in **Chapter 5** investigates the activation of the immortalized bovine MEC line PS by *S. aureus* belonging to different ruminant-associated CCs. In **Chapter 6**, a study is described that compared the activation of primary bovine and caprine MEC by a caprine-associated and bovine-associated *S. aureus* strain. Finally, the results of the studies described in this thesis are summarized and integrated in **Chapter 7**. It will describe the key characteristics of ruminant-associated *S. aureus* CCs and discusses their differences in immune evasion strategies during mastitis.

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CHAPTER 2

High production of LukMF' in *Staphylococcus aureus* field strains is associated with clinical bovine mastitis

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Abstract

Staphylococcus aureus, a major cause of bovine mastitis, produces a wide range of immune-evasion molecules. The bi-component leukocidin LukMF' is a potent killer of bovine neutrophils *in vitro*. Since the role of LukMF' in development of bovine mastitis has not been studied in natural infections, we aimed to clarify whether presence of the *lukM-lukF'* genes and production levels of LukMF' are associated with clinical severity of the disease. *Staphylococcus aureus* was isolated from mastitis milk samples (38 clinical and 17 subclinical cases) from 33 different farms. The *lukM-lukF'* genes were present in 96% of the isolates. Remarkably, 22% of the *lukM-lukF'*-positive S. *aureus* isolates displayed a 10-fold higher *in vitro* LukMF' production than the average of the lower-producing ones. These high producing isolates were cultured significantly more frequently from clinical than subclinical mastitis cases. Also, the detection of LukM protein in milk samples was significantly associated with clinical mastitis and high production *in vitro*. The high producing LukMF' strains all belonged to the same genetic lineage, *spa*-type t543. Analysis of their global toxin gene regulators revealed a point mutation in the Repressor of toxins (*rot*) gene which results in a non-functional start codon, preventing translation of *rot*. This mutation was only identified in high LukMF' producing isolates and not in low LukMF' producing isolates. Since *rot* suppresses the expression of various toxins including leukocidins, this mutation is a possible explanation for increased LukMF' production. Identification of high LukMF' producing strains is of clinical relevance and can potentially be used as a prognostic marker for severity of mastitis.

Introduction

Mastitis, inflammation of the mammary gland, is a major cause of economic losses in the dairy industry associated with costs of treatment, reduced milk yield, discarded milk and premature culling of animals (1,2). In addition, mastitis has severe impact on animal welfare (3). *Staphylococcus aureus* is one of the major causes of mastitis in cows (4). These infections are mostly subclinical and often chronic, but may also result in clinical mastitis (5). *Staphylococcus aureus* possesses many virulence factors, some of which enable it to manipulate the innate and adaptive immune responses of the host (6). These virulence factors vary widely between lineages (7) and include immunomodulatory proteins (8), proteases (9), factors that impede phagocytosis (10) and cytotoxins (11). Some virulence factors are phage-encoded which allows horizontal transfer of virulence genes between bacteria, resulting in genetic diversity between *S. aureus* lineages (12).

An important group of S. aureus immune evasion molecules is that of the leukocidins: pore-forming, bi-component toxins that specifically target immune cells (13). So far, seven different leukocidins have been described (13,14), of these, the phage-encoded leukocidins Panton-Valentine leukocidin (PVL), LukPQ, and LukMF' most strongly affect immune cells from a limited host species range (6,13,14). LukMF' is almost exclusively present on prophages carried by *S. aureus* strains of ruminant origin (15–17) and it is a potent killer of bovine neutrophils, macrophages and monocytes, but not of human neutrophils (18,19). LukM binds to the CCR1 receptor, which is highly present on bovine neutrophils, but absent on human neutrophils (19). Since neutrophils are key players in initial immune responsiveness during inflammation of the mammary gland (20), it is expected that potent killing of neutrophils by LukMF' reduces its effectiveness and, therefore, influences the clinical outcome of infection. Indeed, experimental intramammary challenge with high LukMF' producing *S. aureus* strains resulted in more severe mastitis compared to challenge with intermediate producing *S. aureus* strains (21). Also, high LukM levels in milk have been associated with clinical mastitis (21). It is, however, unclear whether differences in LukMF' production are of clinical significance in natural infections.

In this study, we investigated the impact of LukMF' on clinical severity of bovine mastitis under field conditions. We identified a genetic lineage of *S. aureus* with increased LukMF' production that is associated with clinical rather than subclinical mastitis. In this lineage, we found a nonsense mutation in the start codon of the global expression regulator Repressor of toxins (*rot*). This mutation is the likely cause of increased toxin production associated with severity of clinical signs of mastitis

Results

Prevalence of *lukM-lukF'* Genes among Bovine Mastitis Isolates

Fifty-Five *S. aureus* isolates were collected at 33 Dutch dairy farms by bacteriological culture of milk samples from clinical (n = 37) and subclinical (n = 18) cases of bovine mastitis and the presence of the *femA*, *lukM*, and *lukF'* genes was detected using PCR. All isolates were positive for the *S. aureus*-specific gene *femA*, confirming that the selected bacteria were *S. aureus*, and 53/55 (96%) of isolates were positive for *lukM* and *lukF'*. The proportion of *lukM-lukF'* positive *S. aureus* was similar for clinical (37/38, 97%) and subclinical (16/17, 94%) mastitis cases.

Production of LukM *In Vitro* and *In Vivo*

The *in vitro* LukMF' production potential was investigated by growing *S. aureus* isolates for eight hours under controlled conditions and measuring the LukM concentration in supernatant using ELISA. All *lukM-lukF'* positive *S. aureus* isolates produced LukM *in vitro*, with levels ranging from 1.5 to 60 µg/mL. The production levels had a clear bimodal distribution (**Figure 1A**). Based on this distribution, isolates were categorized into two groups: LukMF' high producers (>10 µg/mL) and LukMF' low producers (<10 µg/mL). Most *S. aureus* (n = 41) were LukMF' low producers (mean = 2.2 µg/mL LukM, SD = 0.6). The remaining isolates (n = 12) were LukMF' high producers (mean = 39.6 µg/mL LukM, SD = 12.6) and were significantly more often isolated from cases of clinical mastitis (Fisher's exact test, p = 0.011). Although some of the 55 *S. aureus* isolates originated from the same farm, the 12 high producing isolates were cultured from cows on 12 different farms. To compensate for a possible farm effect, we randomly selected one isolate per farm (n = 33) and found the same association between the LukMF' high producing isolates and clinical mastitis (Fisher's exact test, p = 0.033).

Next, *in vivo* LukMF' production during mastitis was assessed by measuring LukM in the milk samples corresponding to each of the *S. aureus* isolates. Eight milk samples (seven clinical and one subclinical) could not be tested for LukM because the milk was clotted and therefore not fit for use in the ELISA, or because insufficient milk was available. LukM was detected in 15/45 milk samples, with concentrations ranging from 0.31 ng/mL to 96 ng/mL, significantly more often in milk from clinical mastitis cases (13/29 = 45%) than from subclinical cases (2/16 = 12%, Fisher's exact test, p = 0.046) and in samples that yielded LukMF' high producing isolates (9/11 = 82%) compared to samples that yielded LukMF' low producing isolates (6/34 = 18%, Fisher's exact test, $p < 0.001$). After again selecting one isolate per farm, the association between high *in vitro* production and LukM in milk, *ex vivo*, remained (Fisher's

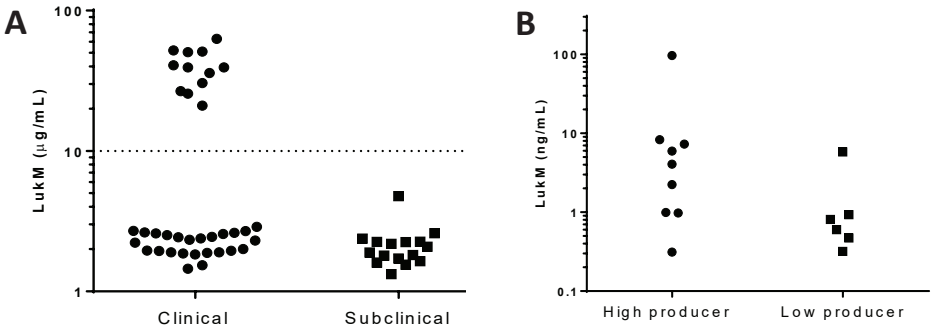

Figure 1. (A) LukM levels *in vitro* after eight hours of culture of 53 *lukM-lukF'* positive *S. aureus* isolates from milk of cows with clinical or subclinical mastitis, measured by ELISA. **(B)** LukM concentration in milk samples of mastitis cases (*in vivo*) caused by LukMF' high (>10 µg/mL production *in vitro* after eight hours of culture) and low (<10 µg/mL production *in vitro* after eight hours of culture) producing *S. aureus*

exact test, $p = 0.007$), but the association between LukM in milk and clinical severity was no longer statistically significant (Fisher's exact test, $p = 0.22$). The average LukM concentration in milk of cows that hosted LukMF' high producing isolates (mean = 14.2 ng/mL LukM, SD = 31.1) was higher than of those carrying LukMF' low producing isolates (mean = 1.49 ng/mL LukM, SD = 2.12) (Mann–Whitney test, $p = 0.049$) (**Figure 1B**).

Analysis of the *lukM-lukF'* Operon and *saeS*, *saeR*, *rot* Genes

To identify genetic factors that were associated with the LukMF' production phenotype, we compared whole genome sequences of three high producing and four low producing S. aureus isolates. First, the prophages containing *lukM-lukF'* were identified using PHAST (22). Prophages encoding *lukM-lukF'* were indeed present in all sequenced isolates, and were identified by PHAST to be most similar to reference phage phiPV83 (Genbank accession NC_002486.1). Prophages from CC479 and CC133 isolates were very similar to each other in size and gene content, but both differed from the smaller CC151 prophage. Variations were observed in lukM (four synonymous and five non-synonymous single nucleotide polymorphisms (SNPs)) and lukF' (three synonymous and six non-synonymous SNPs) among the different CCs (**Supplementary Figure S1A,B**). The putative promoter region of the *lukM-lukF'* op-

eron (up to 200 bp upstream from the start codon) contained five SNPs, but none were exclusively present in high producing CC479 strains (**Supplementary Figure S1C**).

Next, we examined genes putatively involved in the regulation of *lukM* expression. Genes involved in LukMF' expression are unknown, but analogous to the regulation of the expression of PVL, we investigated Repressor of toxins (*rot*) and the exoprotein two-component system SaeRS (*saeS* and *saeR*) (23,24). These genes were present in all sequenced strains and no variation was found in the *saeR* gene, whereas a single non-synonymous SNP (not associated with CC479) was found in the *saeS* gene (data not shown). Two non-synonymous SNPs (position 2 and 452) were found in the *rot* gene, exclusively present in the CC479 isolates. The SNP at position 2 renders the *rot* gene non-functional due to the loss of a start codon. To corroborate these findings, the region surrounding the start codon of *rot* was identified in five additional *S. aureus* isolates (three LukMF' high producing isolates and two LukMF' low producing isolates) using PCR and sequencing. In these additional isolates, the mutation in the *rot* start codon was also only present in the high producing *S. aureus* isolates (**Supplementary Figure S1D**).

Table 1. *Spa*-types and *in vitro* LukMF' production of field isolates from bovine clinical and subclinical mastitis cases

Spa-type	Isolates N	Clinical Mastitis N (%)	*lukM-lukF'* postive isolates N (%)[1]	High LukMF' production isolates N (%) [2]	Clonal Complex[3]
t529	40	23 (58)	40 (100)	0 (0)	CC151
t543	12	12 (100)	12 (100)	12 (100)	CC479
t524	1	1 (100)	0 (0)	0 (0)	ND[4]
t1403	1	1 (100)	1 (100)	0 (0)	CC133
t015	1	0 (0)	0 (0)	0 (0)	ND[4]
Total	55	37 (68)	53 (96)	12 (22)	

1 Number of *lukM-lukF'* positive isolates. Genes detected by PCR.

2.Number of samples with > 10 µg/mL LukM in 8h culture supernatant measured by ELISA.

3 Clonal complex determined by MLST based on whole genome sequences of subset of isolates from this spa-type

4.Not determined

Discussion

We observed a high *lukM-lukF'* carriage (96%) among *S. aureus* field isolates cultured both from cases of clinical and subclinical mastitis. A subpopulation of *S. aureus*, *spa*-type t543-ST479, with a very high *in vitro* LukMF' production was significantly associated with clinical rather than subclinical mastitis. A mutation in the *rot* gene, leading to loss of the primary start codon, found exclusively in *spa*-type t543-ST479 isolates, may be functionally linked to the increased LukMF' production.

All sequence types and *spa*-types identified in this study have previously been associated with bovine mastitis (25,26,27,28,29). Several authors have reported about sequence types and *lukM-lukF'* carriage of *S. aureus*, and this reveals that it is strongly associated with specific ruminant-associated clonal complexes, namely CC151, CC133, CC705, and CC479 (16,30,31,32,33,34) (see **Supplementary Table S1** for a detailed overview). Within the bovine associated CC97, only one specific lineage (ST352-CC97) showed a high prevalence of *lukM-lukF'* (30,31). This demonstrates that the carriage of the *lukM-lukF'* harboring (pro)phage (17) is lineage specific, since the genes are only present in certain sequence types and absent in others within the same CC.

LukMF' is a potent killer of bovine neutrophils, which play a critical role in the innate immune defense against *S. aureus* (5,19). We hypothesize that killing of neutrophils by LukMF' reduces the overall phagocytic activity in the mammary gland, resulting in survival of *S. aureus*, and hence pro-inflammatory responsiveness increasing the clinical severity of mastitis. In our study, LukMF' high production *in vitro* by ST479 *S. aureus* is indeed associated with clinical rather than subclinical mastitis. High production was also strongly correlated with substantial levels of LukM present in milk *in vivo*. Still, also the presence of LukMF' low producing *S. aureus* in milk may lead to detectable levels. Although differences in bacterial load of LukMF' producing bacteria in the mammary gland could also explain differences in milk LukM levels, we assume that ST479 are also LukMF' high producers *in vivo* during the course of infection. In a recent study, cattle were challenged intramammary with LukMF' high and low producing *S. aureus* strains (21). Quarters challenged with the high producer developed more severe clinical symptoms and higher bacterial loads compared to the other, low producing, strains (21).

Although, to our knowledge, the regulation of LukMF' expression has not been studied, it is plausible to assume that the regulation system of LukMF' expression is similar to that of other leukocidins, which are controlled by global gene regulators Agr, Rot and SaeRS (13). We observed a mutation in the rot start codon that was strongly associated with high LukMF' production. Rot is a global regulator of

S. aureus virulence gene expression and can directly bind to the promotor region of various toxin genes, such as *hla*, *hlgC-hlgB, lukE-lukD*, and *lukA-lukB* (23,35). Mutations in *rot* can both activate or repress gene expression, depending on the site of the mutation and the target gene (35). The expression of leukocidins (LukAB, LukED, PVL) by *S. aureus* increases when the *rot* gene is made inoperative (23,36). The hypervirulence of the community-associated methicillin-resistant *S. aureus* (CA-MRSA) clone USA500 is believed to be a result of increased leukocidin (LukAB, LukED, hlgCB) production compared to other CA-MRSA, induced by an insertion in the promotor region of *rot* that prevents expression of this gene (36). The LukMF' high producing strain used in a previous study (21) also contained the same mutation in rot as the one identified in our work. This demonstrates that the absence of, or impaired or altered function of rotlikely explains the increased LukMF' production of ST479 strains observed in our study. To further substantiate this, ST479 could be complemented with a functional rot copy which should lead to decreased LukMF' production compared to the WT ST479. Likewise, *rot* knockout strains of ST151 or ST133 are expected to produce higher amounts of LukMF' compared to the WT. These strains could also be used to identify how this mutation in rot affects the regulation of other leukocidins. Because of the limited geographic range from which our samples originated, it is unclear how prevalent the mutation in rot is in CC479 isolates cultured from cattle in other countries. Still, the strain S1444 (21), a high producing CC479 isolate was originally cultured from a German sample, suggesting that the mutation is not restricted to the region of this study.

Mastitis milk samples used in our study as source of *S. aureus* isolates were submitted by farmers, sometimes in consultation with their veterinarian, hence not randomly collected and not likely to be representative for the population in the field. Due to that approach, under- or overestimation of the actual proportions of LukMF' high producing isolates in the population as well as of isolates belonging to the various *spa*-types cannot be excluded. The association between high LukMF' production and clinical versus subclinical mastitis is, however, not likely to be affected by this sampling bias, but the clinical importance of this association in terms of the population attributable fraction depends on the prevalence of LukMF' high producing isolates in the field and cannot be calculated from our data.

Since the *rot* gene is part of the core genome of *S. aureus*, transfer of the ability of ST479 to produce high levels of LukMF' seems unlikely. It is unclear if increased production of LukMF' by ST479 also increases the transmission of this lineage in the population, as a severe clinical infection is expected to result in a quicker death or culling from the herd of the host or quicker treatment with antibiotics, reducing the chances to further spread in the population. In previous studies, ST479 made up

26% of Dutch (28) and 17% of German *S. aureus* mastitis isolates (16), suggesting that this lineage can persist within a population despite its association with clinical mastitis.

Screening tools, like loop-mediated isothermal amplification (LAMP) (37), currently exist that allow for quick identification of mastitis pathogens (38). However, these tools mostly identify the pathogen on a species level. Since our research shows that the sequence type of *S. aureus* is strongly associated to the type of mastitis, sequence typing of mastitis isolates would enable farmers to implement specific interventions in case of infection with the high LukMF' producing lineages.

Methods

Collection of *S. aureus* Bovine Mastitis Isolates

Quarter milk samples from cows with subclinical or clinical mastitis were aseptically collected by farmers belonging to the University Farm Animal Practice (Harmelen, the Netherlands) and sent in for bacteriological culture and species identification, which were performed according to National Mastitis Council protocols (39). As participating farms (n = 33) belonged to the same veterinary practice, the farms were geographically clustered around the Utrecht region in the Netherlands. Samples were collected between April 2014 and December 2015. Clinical or subclinical mastitis was diagnosed by the farmer, with clinical mastitis defined as visibly abnormal appearance of the udder, the milk or both. Subclinical mastitis was characterized by absence of clinical signs and generally were animals with a high somatic cell count. A total of 55 S. aureus positive milk samples from cases of bovine mastitis (38 clinical and 17 subclinical cases) were used for this study and stored at −18 °C before further use. After thawing of the milk samples, 200 µL aliquots of the *S. aureus* positive milk samples were plated on sheep blood agar plates and cultured overnight at 37 °C to isolate fresh bacterial colonies. Of milk samples showing signs of a clinical mastitis (clots, flakes, discolored milk), a smaller volume of 50 µL was used to prevent bacterial overgrowth of the plate. Single colonies were picked and added to 2 mL T1438 Todd Hewitt Broth (THB) (Sigma, St. Louis, MO, USA) and incubated overnight at 37 °C with agitation. Bacterial glycerol stocks (25% glycerol) were made by adding 0.5 mL of bacterial broth to 0.5 mL 50% glycerol solution in distilled water. Stocks were stored at −80 °C before use in further experiments.

DNA Extraction and Amplification of *lukM*, *lukF'*, *femA* and *rot* Genes

Bacterial isolates were plated from glycerol stocks on blood agar plates and cultured overnight at 37 °C. Single colonies were picked for DNA extraction and washed in 1 mL distilled water. After centrifugation (17,000× g for 1 min), bacteria were re-suspended in 200 µL distilled water and heated at 100 °C for 10 min, centrifuged at 17,000× g for 1 min, and diluted 1:10 in distilled water and stored at −20 °C.

Primers to amplify *femA*, *lukM*, *lukF'* and *rot* were designed or taken from literature (Table 2). The primers for *femA* and *lukF'* were used together in a duplex PCR, and the other primers (*rot*, *lukM*) in separate, single PCR. The reaction was performed in a total volume of 25 µL containing 10 µL 1:10 diluted boiled DNA sample, 5 µL GoTaQ Green buffer 5× (Promega, Madison, WI, USA), 1.5 mM MgCl2 (Promega), 0.2 mM dNTPs (Promega, Madison, WI, USA), 0.4 µM of the *lukF'* primer pair and 0.6 µM of the other primer pairs (Invitrogen, Carlsbad, CA, USA) and 0.625 U of GoTaQ DNA polymerase (Promega, Madison, WI, USA). After an initial denaturation step at 95 °C for 2 min, 35 cycles (30 s at 95 °C, 35 s at various annealing temperatures (Table 2, column 4) and 35–60 s at 72 °C) were performed in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Electrophoresis on 1.5% agarose gel was used to visualize PCR products.

***In Vitro* and *In Vivo* LukM Production**

Bacteria were cultured from glycerol stock on blood agar plates overnight at 37 °C. Single colonies were picked and added to 1.5 mL THB. Bacteria were incubated with agitation for 30 min at 37 °C. Optical density (OD) at 600 nm was measured and samples were diluted to an OD of 0.01 in 2.5 mL of THB. Next, bacteria were incubated with agitation for 8 h at 37 °C. After incubation, samples were centrifuged (4000× g for 10 min) and supernatant was collected. The supernatant was sterilized using a microfilter (0.20 µm; Corning Incorporated, Corning, NY, USA) and stored at −20 °C before use in further experiments. Bacterial supernatants were produced in triplicate using separate, single colonies from the same cultured plate.

LukM in supernatant and bovine mastitis milk samples was measured by ELISA, according to the method described by Vrieling *et al.* (21). In short, LukM is captured using LukM specific polyclonal bovine IgG isolated from the colostrum of a cow with high LukM antibody titers, and captured LukM is detected using the LukM specific monoclonal antibody LM43.F8 (21). Milk samples were heated to 95 °C for 10 min to prevent interference from antibodies in the milk.

Table 2. Primers and annealing temperature used in this study

Gene	Sequence	Product size (bp)	Annealing temperature (°C)	Reference
femA	f: 5'-accaatttagcctcattcggtttg-3'	142	59.5	(41)
	r: 5'-catcgtcaacaggacgctct-3'			
lukM	f: 5'-aaacgcgcagttaataaaaag-3'	975	55	This study
	r: 5'-agcattaggtcctcttgtcg-3'			
lukF'	f: 5'-cgagctactctgtctgccac-3'	472	59.5	This study
	r: 5'-cgagctactctgtctgccac-3'			
rot	f: 5'-accaatttagcctcattcggtttg-3'	705	55	This study
	r: 5'-accaatttagcctcattcggtttg-3'			

Genotyping of Mastitis Strains and Genomic Analyses

The polymorphic X-region of the Staphylococcal Protein A (spa) gene of all *S. aureus* isolates was amplified according to the Ridom StaphType standard protocol (www.ridom.org). PCR amplicons were purified using ExoSAP-IT PCR Cleanup Reagent (Affymetrix, Santa Clara, CA, USA) according to manufacturer's instructions and sequenced using Sanger sequencing (Baseclear, Leiden, The Netherlands). BioNumerics v7.5 software (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyze sequence data and to assign spa-types.

Whole genome sequencing was performed on seven isolates, selected from LukMF' high and low producers of different spa-type, and each selected isolate originated from a different farm. DNA was isolated with the Ultra Clean Microbial DNA isolation kit (Mo-Bio, Carlsbad, CA, USA). MiSeq sequencing (Illumina, San Diego, CA, USA) was performed at the Utrecht Sequencing Facility (UMC Utrecht, the Hubrecht institute and Utrecht University, the Netherlands), using 300 bp paired end reads. Reads were assembled into a scaffold genome using SPAdes v3.1.1. (41). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession QFCT00000000-QFCZ00000000. The version described in this paper is the first version. MLST was determined using the MLST tool of the Center of Genomic Epidemiology (accessible on https://cge.cbs.dtu.dk/services/MLST/) (42). Prophages containing the *LukM-lukF'* genes were identified using PHAST (accessible on http://phast.wishartlab.com/index.html) (22). The *lukM-lukF'* encoding gene sequences and putative promoter region (200 bp upstream of start LukM gene) were identified by BLASTN using reference sequences for *lukM* (GenBank accession: 1262967) and *lukF'* (GenBank accession: 1262954). Sequences for candidate LukMF' regulator genes were extracted from available genomes using reference gene se-

quences of rot (Genbank accession: AF189239.2) and saeRS locus (Genbank accession: AF129010.1). Nucleic acid sequences were translated to their corresponding protein sequences using EMBOSS Transeg software (accessible on http://www.ebi.ac.uk/Tools/st/emboss_transeq/). Gene and protein sequences were aligned using MegALIGN Pro software (DNAstar Incorporate, Madison, WI, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA). *In vitro* differences in LukM levels between clinical/subclinical isolates and *in vivo* LukM levels between LukMF' high/low producers were compared using the Mann–Whitney U test. The associations between in vitro LukMF' production levels, presence of detectable LukM in milk and mastitis type were tested using Fisher's exact test. A subset of the dataset with a single sample per farm was assembled using the random number generator function in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

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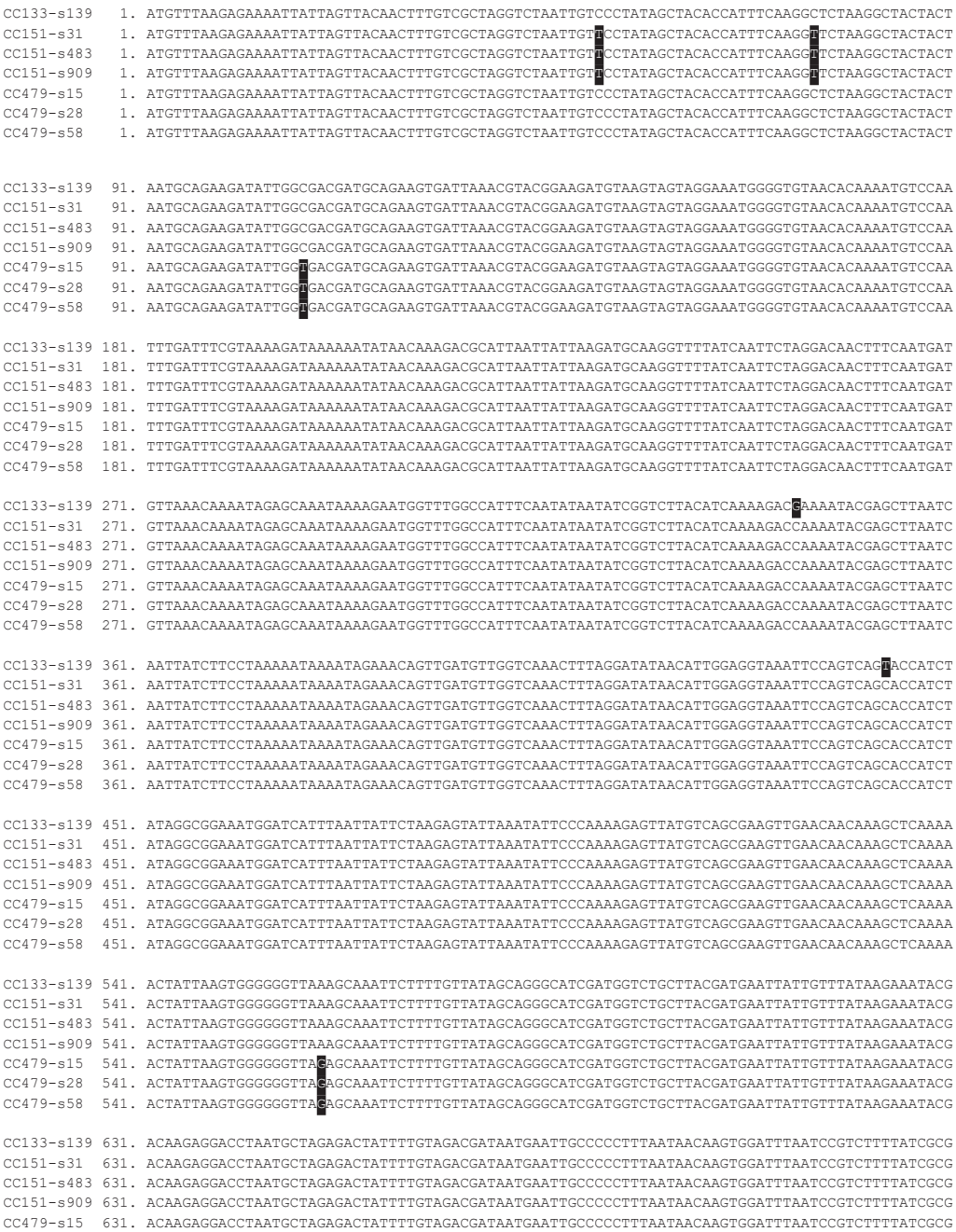
Supplementary Figures

Fig S1A. Alignment of *lukM* of seven *S. aureus* isolates obtained from cases of bovine mastitis[1]



1.Sequences obtained from whole genome sequence (WGS) from seven isolates using reference lukM sequence (GenBank accession: 1262967). Isolates were selected based on CC and LukMF' production potential (high LukMF' production = CC479, low LukMF' producing = CC151, CC133)

Fig S1B. Alignment of *lukF'* of seven *S. aureus* isolates obtained from cases of bovine mastitis[1]

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CC133-s139 361. AATATTGTTGACTACGCGCCTAAAAATCAAAATGAAGAATTTCAAGTTCAACAAACATTAGGTTATTCATATGGCGGAGATATTAATATA
CC151-s31  361. AATATTGTTGACTACGCGCCTAAAAATCAAAATGAAGAATTTCAAGTTCAACAAACATTAGGTTATTCATATGGCGGAGATATTAATATA
CC151-s483 361. AATATTGTTGACTACGCGCCTAAAAATCAAAATGAAGAATTTCAAGTTCAACAAACATTAGGTTATTCATATGGCGGAGATATTAATATA
CC151-s909 361. AATATTGTTGACTACGCGCCTAAAAATCAAAATGAAGAATTTCAAGTTCAACAAACATTAGGTTATTCATATGGCGGAGATATTAATATA
CC479-s15  361. AATATTGTTGACTACGCGCCTAAAAATCAAAATGAAGAATTTCAAGTTCAACAAACATTAGGTTATTCATATGGCGGAGATATTAATATA
CC479-s28  361. AATATTGTTGACTACGCGCCTAAAAATCAAAATGAAGAATTTCAAGTTCAACAAACATTAGGTTATTCATATGGCGGAGATATTAATATA
CC479-s58  361. AATATTGTTGACTACGCGCCTAAAAATCAAAATGAAGAATTTCAAGTTCAACAAACATTAGGTTATTCATATGGCGGAGATATTAATATA

CC133-s139 451. ATAAATGGATTAACTGGTGGATTGAATGGGTCAAAATCATTTTCAGAAACGATTAATTATAAGCAAGAAAGCTACAGAACTACGATTGAT
CC151-s31  451. ATAAATGGATTAACTGGTGGATTGAACGGGTCAAAATCATTTTCAGAAACGATTAATTATAAGCAAGAAAGCTACAGAACTACGATTGAT
CC151-s483 451. ATAAATGGATTAACTGGTGGATTGAACGGGTCAAAATCATTTTCAGAAACGATTAATTATAAGCAAGAAAGCTACAGAACTACGATTGAT
CC151-s909 451. ATAAATGGATTAACTGGTGGATTGAACGGGTCAAAATCATTTTCAGAAACGATTAATTATAAGCAAGAAAGCTACAGAACTACGATTGAT
CC479-s15  451. ATAAATGGATTAACTGGTGGATTGAATGGGTCAAAATCATTTTCAGAAACGATTAATTATAAGCAAGAAAGCTACAGAACTACGATTGAT
CC479-s28  451. ATAAATGGATTAACTGGTGGATTGAATGGGTCAAAATCATTTTCAGAAACGATTAATTATAAGCAAGAAAGCTACAGAACTACGATTGAT
CC479-s58  451. ATAAATGGATTAACTGGTGGATTGAATGGGTCAAAATCATTTTCAGAAACGATTAATTATAAGCAAGAAAGCTACAGAACTACGATTGAT

CC133-s139 541. AGGAAAACAAATCATAAATCAATCGGCTGGGGTGTCGAGGCACATAAAATCATGAATAATGGTTGGGGGCCATATGGCAGAGATAGTAGT
CC151-s31  541. AGGAAAATAAATCACAAATTAATCGGCTGGGGTGTCGAGGCACATAAAATCATGAATAATGGTTGGGGACCATATGGCAGAGATAGTAGT
CC151-s483 541. AGGAAAATAAATCACAAATTAATCGGCTGGGGTGTCGAGGCACATAAAATCATGAATAATGGTTGGGGACCATATGGCAGAGATAGTAGT
CC151-s909 541. AGGAAAATAAATCACAAATTAATCGGCTGGGGTGTCGAGGCACATAAAATCATGAATAATGGTTGGGGACCATATGGCAGAGATAGTAGT
CC479-s15  541. AGGAAAACAAATCATAAATCAATCGGCTGGGGTGTCGAGGCACATAAAATCATGAATAATGGTTGGGGACCATATGGCAGAGATAGTAGT
CC479-s28  541. AGGAAAACAAATCATAAATCAATCGGCTGGGGTGTCGAGGCACATAAAATCATGAATAATGGTTGGGGACCATATGGCAGAGATAGTAGT
CC479-s58  541. AGGAAAACAAATCATAAATCAATCGGCTGGGGTGTCGAGGCACATAAAATCATGAATAATGGTTGGGGACCATATGGCAGAGATAGTAGT

CC133-s139 631. GATTCATTATATGGAAACGAACTATTTTTAGGTGGCAGACAGAGTAGCTCGAATGCTAATCAAAATTTCTTACCAACACATCAAATGCCC
CC151-s31  631. GATTCATTATATGGAAACGAACTATTTTTAGGTGGCAGACAGAGTAGCTCGAATGCTAATCAAAATTTCTTACCAACACATCAAATGCCC
CC151-s483 631. GATTCATTATATGGAAACGAACTATTTTTAGGTGGCAGACAGAGTAGCTCGAATGCTAATCAAAATTTCTTACCAACACATCAAATGCCC
CC151-s909 631. GATTCATTATATGGAAACGAACTATTTTTAGGTGGCAGACAGAGTAGCTCGAATGCTAATCAAAATTTCTTACCAACACATCAAATGCCC
CC479-s15  631. GATTCATTATATGGAAACGAACTATTTTTAGGTGGCAGACAGAGTAGCTCGAATGCTAATCAAAATTTCTTACCAACACATCAAATGCCC
CC479-s28  631. GATTCATTATATGGAAACGAACTATTTTTAGGTGGCAGACAGAGTAGCTCGAATGCTAATCAAAATTTCTTACCAACACATCAAATGCCC
CC479-s58  631. GATTCATTATATGGAAACGAACTATTTTTAGGTGGCAGACAGAGTAGCTCGAATGCTAATCAAAATTTCTTACCAACACATCAAATGCCC

CC133-s139 721. ATATTAGCACGTGGTAATTTCAATCCAGAATTTATAAGCGTACTTTCTCACAAACAAAAGGATGTTAAAAAATCTAAAATTAAAGTGACT
CC151-s31  721. ATATTAGCACGTGGTAATTTCAATCCAGAATTTATAAGCGTACTTTCTCACAAACAAAAGGATGTTAAAAAATCTAAAATTAAAGTGACT
CC151-s483 721. ATATTAGCACGTGGTAATTTCAATCCAGAATTTATAAGCGTACTTTCTCACAAACAAAAGGATGTTAAAAAATCTAAAATTAAAGTGACT
CC151-s909 721. ATATTAGCACGTGGTAATTTCAATCCAGAATTTATAAGCGTACTTTCTCACAAACAAAAGGATGTTAAAAAATCTAAAATTAAAGTGACT
CC479-s15  721. ATATTAGCACGTGGTAATTTCAATCCAGAATTTATAAGCGTACTTTCTCACAAACAAAAGGATGTTAAAAAATCTAAAATTAAAGTGACT
CC479-s28  721. ATATTAGCACGTGGTAATTTCAATCCAGAATTTATAAGCGTACTTTCTCACAAACAAAAGGATGTTAAAAAATCTAAAATTAAAGTGACT
CC479-s58  721. ATATTAGCACGTGGTAATTTCAATCCAGAATTTATAAGCGTACTTTCTCACAAACAAAAGGATGTTAAAAAATCTAAAATTAAAGTGACT

CC133-s139 811. TATCAAAGAGAAATGGATCGGTATGAAAATTTTTGGAACAACTTGCACTGGATAGGTTATAATATTAAGAATCAAAAGAGAGCAACACAC
CC151-s31  811. TATCAAAGACAAATGGATCGGTATGAAAATTTTTGGAACAACTTGCACTGGATAGGTTATAATATTAAGAATCAAAAGAGAGCAACACAC
CC151-s483 811. TATCAAAGACAAATGGATCGGTATGAAAATTTTTGGAACAACTTGCACTGGATAGGTTATAATATTAAGAATCAAAAGAGAGCAACACAC
CC151-s909 811. TATCAAAGACAAATGGATCGGTATGAAAATTTTTGGAACAACTTGCACTGGATAGGTTATAATATTAAGAATCAAAAGAGAGCAACACAC
CC479-s15  811. TATCAAAGAGAAATGGATCGGTATGAAAATTTTTGGAACAACTTGCACTGGATAGGTTATAATATTAAGAATCAAAAGAGAGCAACACAC
CC479-s28  811. TATCAAAGAGAAATGGATCGGTATGAAAATTTTTGGAACAACTTGCACTGGATAGGTTATAATATTAAGAATCAAAAGAGAGCAACACAC
CC479-s58  811. TATCAAAGAGAAATGGATCGGTATGAAAATTTTTGGAACAACTTGCACTGGATAGGTTATAATATTAAGAATCAAAAGAGAGCAACACAC

CC133-s139 901. ACATCAATTTATGAAATTGATTGGGAAAAACACACGGTTAAATTAGTAGCTTCGCAATCTAGCGAATAA
CC151-s31  901. ACATCAATTTATGAAATTGATTGGGAAAAACACACGGTTAAATTAGTAGCTTCGCAATCTAGCGAATAA
CC151-s483 901. ACATCAATTTATGAAATTGATTGGGAAAAACACACGGTTAAATTAGTAGCTTCGCAATCTAGCGAATAA
CC151-s909 901. ACATCAATTTATGAAATTGATTGGGAAAAACACACGGTTAAATTAGTAGCTTCGCAATCTAGCGAATAA
CC479-s15  901. ACATCAATTTATGAAATTGATTGGGAAAAACACACGGTTAAATTAGTAGCTTCGCAATCTAGCGAATAA
CC479-s28  901. ACATCAATTTATGAAATTGATTGGGAAAAACACACGGTTAAATTAGTAGCTTCGCAATCTAGCGAATAA

CC479-s58  901. ACATCAATTTATGAAATTGATTGGGAAAAACACACGGTTAAATTAGTAGCTTCGCAATCTAGCGAATAA
```

1.Sequences obtained from whole genome sequence (WGS) from seven isolates using reference lukF sequence (GenBank accession: 1262954).

Fig S1C. Alignment of the putative promotor region[1] of *lukM-lukF'* operon of seven *S. aureus* isolates obtained from cases of bovine mastitis

```
CC133-s139   1. GTTTTTCAGGTATATTTCTATACAACTATAATGAACTTTTTTAATTATTGATCGTGTTTGAATGTTTTTTGGGTGTAACAATACTATTTT
CC151-s31    1. GTTTTTCAGGTATATTTCTATACAACTATAATGAACTTTTTTAATTATTGATCGTGTTTGAATGCTTTTTG-GTGTAAAAATACTATTTT
CC151-s483   1. GTTTTTCAGGTATATTTCTATACAACTATAATGAACTTTTTTAATTATTGATCGTGTTTGAATGCTTTTTG-GTGTAAAAATACTATTTT
CC151-s909   1. GTTTTTCAGGTATATTTCTATACAACTATAATGAACTTTTTTAATTATTGATCGTGTTTGAATGCTTTTTG-GTGTAAAAATACTATTTT
CC479-s15    1. GTTTTTCAGGTATATTTCTATACAACTATAATGAACTTTTTTAATTATTGATCGTGTTTGAATGTTTTTTGGGTGTAACAATACTATTTT
CC479-s28    1. GTTTTTCAGGTATATTTCTATACAACTATAATGAACTTTTTTAATTATTGATCGTGTTTGAATGTTTTTTGGGTGTAACAATACTATTTT
CC479-s58    1. GTTTTTCAGGTATATTTCTATACAACTATAATGAACTTTTTTAATTATTGATCGTGTTTGAATGTTTTTTGGGTGTAACAATACTATTTT

CC133-s139  91. AGAGATTTATTAACCTTATTGAATTTTTAATAAATTTTTAATTATATAATTATGAATTATGTAATAGTATAAATTTGTATTAAAAATATA
CC151-s31   90. AGGGATTCATTAACCTTATTGAATTTTTAATAAATTTTTAATTATATAATTATGAATTATGTAATAGTATAAATTTGTATTAAAAATATA
CC151-s483  90. AGGGATTCATTAACCTTATTGAATTTTTAATAAATTTTTAATTATATAATTATGAATTATGTAATAGTATAAATTTGTATTAAAAATATA
CC151-s909  90. AGGGATTCATTAACCTTATTGAATTTTTAATAAATTTTTAATTATATAATTATGAATTATGTAATAGTATAAATTTGTATTAAAAATATA
CC479-s15   91. AGAGATTTATTAACCTTATTGAATTTTTAATAAATTTTTAATTATATAATTATGAATTATGTAATAGTATAAATTTGTATTAAAAATATA
CC479-s28   91. AGAGATTTATTAACCTTATTGAATTTTTAATAAATTTTTAATTATATAATTATGAATTATGTAATAGTATAAATTTGTATTAAAAATATA
CC479-s58   91. AGAGATTTATTAACCTTATTGAATTTTTAATAAATTTTTAATTATATAATTATGAATTATGTAATAGTATAAATTTGTATTAAAAATATA

CC133-s139 181. AAGAGAAAGAAAGTGAAACT
CC151-s31  180. AAGAGAAAGAAAGTGAAACT
CC151-s483 180. AAGAGAAAGAAAGTGAAACT
CC151-s909 180. AAGAGAAAGAAAGTGAAACT
CC479-s15  181. AAGAGAAAGAAAGTGAAACT
CC479-s28  181. AAGAGAAAGAAAGTGAAACT
CC479-s58  181. AAGAGAAAGAAAGTGAAACT
```

1. Region of 200 base pairs before the start of lukM, containing putative Tata box and promoter elements.

Figure S1D. Alignment of the first 20 nucleotides of *rot* in 12 *S. aureus* isolates obtained from cases of bovine mastitis[1]

```
CC133-s139-WGS  1. ATGCATAAGTTAGCACATAC
CC151-s31-WGS   1. ATGCATAAGTTAGCACATAC
CC151-s483-WGS  1. ATGCATAAGTTAGCACATAC
CC151-s505-PCR  1. ATGCATAAGTTAGCACATAC
CC151-s605-PCR  1. ATGCATAAGTTAGCACATAC
CC151-s909-WGS  1. ATGCATAAGTTAGCACATAC
CC479-s15-WGS   1. AAGCATAAGTTAGCACATAC
CC479-s28-WGS   1. AAGCATAAGTTAGCACATAC
CC479-s58-WGS   1. AAGCATAAGTTAGCACATAC
CC479-s192-PCR  1. AAGCATAAGTTAGCACATAC
CC479-s245-PCR  1. AAGCATAAGTTAGCACATAC
CC479-s670-PCR  1. AAGCATAAGTTAGCACATAC
```

1.Sequences of seven isolates obtained from genome after whole genome sequencing (labeled WGS), five additional sequences where obtained from sequenced PCR products (labeled PCR).

Supplementary Tables

Table S1. Number of *S. aureus* of different lineages found among bovine isolates, with percentage of *lukM-lukF'* positive *S. aureus* among these lineages

	Portugal	Sweden[1]	Germany[1]	Japan	Germany / Switzerland	South Africa
Sample / Lineage	Nasal swabs	CM	SCM	SCM	Mastitis	SCM
ST133-CC133		20 (100)	21 (100)		6 (100)	
ST124-CC133	13 (0)					
ST352-CC97[3]	13 (100)			24 (92)		71 (100)
ST97-CC97[4]		13 (85)	9 (0)	16 (6)	23 (0)	63 (0)
ST151-CC151			98 (100)		61 (100)	
ST705-CC705[5]		29 (100)		9 (100)		10 (100)
ST479-CC479		1 (100)	33 (97)			
ST30-CC20[6]		1 (0)		14 (0)	9 (11)	
ST5 -CC5				4 (0)	2 (0)	
ST7-CC7[7]	8 (0)		1 (0)	1 (0)		
ST398-CC398			8(0)		2(0)	
ST8-CC8		1 (0)		6 (0)	11 (0)	1 (0)
ST508-CC45		3 (0)	5 (0)	3 (0)		1 (100)
Other CC		2 (0)	14 (0)	33 (0)	14 (0)	
Total	34 (38)	70 (90)	189 (80)	110 (29)	128 (53)	146 (56)
Reference	(34)	(32)	(16)	(31)	(33)	(30)

1. Only Clonal Complex (CC) data available in these studies

2. Number of *S. aureus* of this lineage found, with percentage of *lukM-lukF'* positive *S. aureus* among this lineage in brackets

3. Includes single locus variant (SLV) ST1366

4. Includes SLV ST124, ST1367

5. Includes SLV ST351, ST1363, ST1363, ST1365

6. Includes SLV ST1368, ST1370

7. Includes SLV ST789

CHAPTER 3

Genomic analysis of bovine S*taphylococcus aureus* from clinical versus subclinical mastitis in eleven European countries

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In preparation

Abstract

Intramammary infections (IMI) with *Staphylococcus aureus* are a common cause of bovine mastitis and can result in both clinical (CM) or subclinical mastitis (SCM). Although bacterial isolates of *S. aureus* differ in their virulence potential it is largely unclear which bacterial virulence factors are responsible for increased clinical severity. We performed a genome wide association study and used a generalized linear mixed model to investigate the correlation between gene carriage, lineage and clinical outcome of IMI in a collection of *S. aureus* isolates from cattle with CM (n = 125) and SCM (n = 151) from 11 European countries. An additional aim was to describe the genetic variation of bovine *S. aureus* in Europa. The dominant lineages in our collection were clonal complex (CC) 151 (81/276, 29.3%), CC97 (54/276, 19.6%), CC479 (32 / 276, 11.6%) and CC398 (19/276, 6.9%). Virulence and antimicrobial resistance (AMR) gene carriage was highly associated with CC. Among a selection of nine virulence and AMR genes, CC151, CC479 and CC133 carried more virulence genes than other CCs, and CC398 was associated with AMR gene carriage. Whereas CC151, CC97 were widespread in Europe, CC479, CC398 and CC8 were only found in specific countries. Compared to CC151, CC479 was associated with CM rather than SCM (OR = 3.62; 95% CI = 1.38 - 9.50) and the other CCs were not. Multiple genes were associated with CM, but due to the clustering within CC of carriage of these genes, it was not possible to differentiate between the effect of gene carriage and CC on clinical outcome of IMI. Nevertheless, this study demonstrates that characterization of *S. aureus* CC and virulence genes helps to predict the CM or SCM outcome of *S. aureus* IMI and is therefore helpful when formulating an appropriate intervention strategy in *S. aureus* mastitis control.

Introduction

Mastitis is responsible for significant financial losses on dairy farms due to reduced milk yield, milk unsuitable for consumption, treatment costs and culling of animals (1,2). The main causes of bovine mastitis are bacterial intramammary infections (IMI), with *Staphylococcus aureus* being one of the most relevant pathogens (3). Infections with *S. aureus* mostly result in subclinical mastitis (SCM), but can also lead to clinical mastitis (CM) (4).

The *S. aureus* clones responsible for bovine mastitis predominantly belong to bovine-associated clonal complexes (CC), such as CC151, CC97, CC133, CC479 and CC771 (5,6). The genomic content of *S. aureus* clones can differ greatly due to their accessory genome, which makes up approximately 25% of the total genome (7). The accessory genome of *S. aureus* predominantly consists of genes introduced by horizontal gene transfer (HGT), with these transferred genes often being phage genes, virulence and antimicrobial resistance (AMR) genes, expected to affect the pathogenesis of *S. aureus* IMI (8). A large number of virulence genes have been identified in *S. aureus* isolates obtained from bovine mastitis cases, several of which are involved in evasion of the host immune response during infection (8,9). Leukocidins, such as LukMF', can directly attack immune cells in the lumen of the mammary gland and superantigens (SAs), such as enterotoxins seA – seQ, or toxic shock syndrome toxin 1 (tsst-1), massively activate T lymphocytes and antigen-presenting cells, interfering with the buildup of a proper adaptive immune response (10). Furthermore, Staphylococcal superantigen-like proteins (SSLs) disrupt different pathways of the immune response, such as pathogen recognition by TLR-2 (SSL-3, SSL-4), neutrophil function and recruitment (SSL-1, SSL-5, SLL-7, SSL-13) and the opsonization of bacteria (SSL-7) (10,11). Some of these immune evasion factors are considered ruminant adapted10, such as LukMF' and the *S. aureus* pathogenicity island encoded variant of the Von Willebrand factor-binding protein (SaPI vWFbp) (12,13). Furthermore, *S. aureus* can carry AMR encoding genes, including *blaZ*, *tetM* and *mecA*, which reduce effectiveness of antimicrobial treatment of *S. aureus* mastitis (14). The majority of virulence and AMR genes are located on mobile genetic elements and *S. aureus* belonging to the same CC have specific virulence gene signatures due to clonal expansion (6,15,16) and restriction-modification systems that reduce HGT between different *S. aureus* lineages (17,18).

Several studies investigated CCs and virulence/AMR gene carriage among bovine *S. aureus* isolates responsible for IMI (6,9,19), but only few directly compare isolates from CM and SCM cases. Recently, we identified a specific bovine *S. aureus* lineage (CC479) that was associated with CM in The Netherlands (20). In addition,

carriage of certain genes (*lukM-lukF'*, *seO*) was overrepresented among CM isolates compared to SCM isolates (21,22). This suggests that some *S. aureus* lineages are more likely to cause CM than others. Although both CM and SCM come with financial losses for the farm, the costs of treatment of CM are substantially higher than of SCM and CM seriously affects animal welfare (1,23). Therefore, identification of *S. aureus* isolates with increased risk of developing into CM is likely valuable to better support farm health management decisions. However, studies comparing isolates from CM and SCM have been mainly of national scale (20–23), whereas the genetic makeup of bovine *S. aureus* likely differs between countries. Therefore, in this study we performed whole-genome sequencing (WGS) of 276 *S. aureus* isolates from cattle with CM or SCM in 11 European countries.

The primary aim of this study was to investigate differences in lineage and genomic content between *S. aureus* isolates responsible for CM and SCM. A second aim was to describe the variation in lineages, virulence gene and AMR gene carriage of bovine *S. aureus* isolates in Europe.

Results

Bovine *Staphylococcus aureus* CCs differ in their carriage of immune evasion and AMR genes.

After selection of isolates and quality control of WGS results, 276 genomes were available of *S. aureus* isolates obtained from bovine mastitis cases originating from 254 unique herds in eleven different European countries. There was an approximately even distribution of CM (125/276, 45%) and SCM (151/276, 55%) isolates, and *S. aureus* in the collection belonged to eighteen different CCs. The most prevalent CCs were CC151 (81/276, 29.3%), CC97 (54/276, 19.6%), CC479 (32/276, 11.6%), CC398 (19/276, 6.9%), CC1 (14/276, 5.1%), CC20 (11/276, 4.0%) and CC8 (11/276, 4.0%), and carriage of a selection of nine key virulence and AMR genes among isolates was related to CC (**Table 1**). The CC151, CC479 and CC133 strains had high carriage of virulence genes but lacked AMR genes. All these three CCs carried the *lukM-lukF'* genes and CC479, CC151 also possessed SA genes. In addition, CC479, CC133 *S. aureus* carried the SaPI encoded vWFbp gene (**Table 1**). In contrast, CC398 *S. aureus* lacked all these virulence factors but did had a high carriage rate of the AMR genes *blaZ* (8/19, 42%), *tetM* (19/19, 100%) and *mecA* (11/19, 58%) (**Table 1**). The CC97 displayed a moderate carriage of both virulence gene *lukM-lukF'* (16/54, 30%) and the AMR gene *blaZ* (16/54, 30%) (**Table 1**).

Table 1. Number and percentage of isolates positive for a selection of virulence and antimicrobial resistance genes per clonal complex and number and proportion of clinical mastitis of 276 *S. aureus* isolates obtained from bovine clinical and subclinical mastitis in 11 European countries.

			Virulence genes n (%)						Antimicrobial resistance genes n (%)			Manifestation of mastitis		
CC[1]	n	%	*lukM-lukF'*[2]	*scn*	*SaPI vWFbp*	*seI*	*seL*	*tsst-1*	*blaZ*	*tetM*	*mecA*	CM[3]	Odds Ratio[4] (95% CI)	P[7]
151	81	29.3	81 (100)	0 (0)	0 (0)	80 (99)	19 (23)	19 (23)	1 (1)	0 (0)	0 (0)	41 (51)	Ref[5]	Ref
97	54	19.6	16 (30)	7 (13)	0 (0)	0 (0)	0 (0)	0 (0)	16 (30)	0 (0)	0 (0)	23 (42)	0.75 (0.37 - 1.54)	0.44
479	32	11.6	30 (94)	0 (0)	32 (100)	32 (100)	0 (0)	0 (0)	0 (0)	1 (3)	0 (0)	25 (78)	3.62 (1.38 - 9.50)	< 0.01
133	25	9.1	23 (92)	21 (84)	25 (100)	0 (0)	2 (8)	2 (8)	0 (0)	0 (0)	0 (0)	13 (52)	1.05 (0.42 - 2.61)	0.92
398	19	6.9	0 (0)	10 (52)	0 (0)	0 (0)	0 (0)	0 (0)	8 (42)	19 (100)	11 (58)	5 (27)	0.41 (0.12 - 1.48)	0.17
1	14	5.1	9 (64)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (21)	0.30 (0.07 - 1.23	0.10
20	11	4.0	0 (0)	0 (0)	0 (0)	7 (64)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.34 (0.08 – 1.45)	0.15
8	11	4.0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	6 (54)	0 (0)	0 (0)	2 (18)	0.21 (0.43 – 1.10)	0.06
9	5	1.8	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (66)	0 (0)	0 (0)	2 (33)	NA[6]	NA
50	5	1.8	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)	1 (20)	NA	NA
49	4	1.4	1 (25)	0 (0)	0 (0)	0 (0)	1 (25)	0 (0)	1 (25)	0 (0)	0 (0)	1 (25)	NA	NA
7	4	1.4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (50)	NA	NA
5	4	1.4	0 (0)	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (75)	NA	NA
45	3	1.1	0 (0)	0 (0)	0 (0)	1 (33)	1 (25)	0 (0)	1 (33)	0 (0)	0 (0)	0 (0)	NA	NA
101	1	0.4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA	NA
22	1	0.4	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	NA	NA
30	1	0.4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	NA	NA
425	1	0.4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	NA	NA
Total	**276**		**160 (58)**	**38 (14)**	**57 (21)**	**125 (45)**	**24 (9)**	**21 (8)**	**40 (15)**	**20 (7)**	**11 (4)**	**125 (45)**		

1. Clonal Complex

2. Carriage of genes determined using pangenome data from roary.

3. Number and percentage of clinical mastitis cases per CC

4. Cluster-specific odds ratio of the isolate being cultured from CM versus SCM from a generalized linear mixed model with country as a random effect.

5. Reference class

6. Only CCs with n > 10 were included in model.

7. Significance of cluster-specific odds ratio

In addition, heatmaps of the BLAST score ratio (BSR) (24) of all *S. aureus* genes annotated as (putative) SAs or SSLs by prokka (25) demonstrated that bovine *S. aureus* CCs differ in their carriage of these immune evasion factors (**Figs. S1, S2**). Notable differences in SA carriage were the high number of SAs (up to 12 different SAs) genes carried by CC151, whereas CC398 isolates lacked SAs. Although most SSLs were detected in all *S. aureus*, several unique variants of the same gene were present and carriage of these variants was CC specific. However, the SSL-7, SSL-8, SSL-9 genes were only absent in CC479 *S. aureus*. Furthermore, two yet unnamed SSL (GenBank references: WP_143564871.1 and WP_124375191) were identified that were exclusively found among CC97 isolates (**Fig. S2**).

To screen for target genes that could differentiate between major ruminant CCs in a PCR-based assay, the presence of potential CC exclusive genes was also investigated (**Fig. S3, Table S1**). The highest number of CC-exclusive genes were found for CC479 (n = 17), followed by CC20 (n = 4), CC151 (n = 3), CC8 (n = 2) and CC133 (n = 2). For CC97, CC398, only a single unique gene was identified and no CC exclusive genes were found for CC1 isolates.

Heterogeneous spatial distribution of CCs across Europe.

There was a significant difference in the distribution of *S. aureus* CCs between different countries (Fisher's Exact Test, p < 0. 001). Whereas CC151 (10 out 11 countries) and CC97 (9 out 11) *S. aureus* were detected in almost all countries, CC398 (6 out 11) and CC479 (5 out 11) were considerably less widespread in our collection (**Table 2**). The CC398 lineage was predominantly found in isolates from Poland and Spain. Isolates belonging to CC8 originated exclusively from either Italy or Bavaria region in Germany. Also, all CC50 isolates originated from Denmark (**Table 2**).

Pangenome of bovine *Staphylococcus aureus* and phylogenetic analysis.

To investigate the phylogeny of our bovine *S. aureus* isolates, the pangenome of the entire collection was determined (n = 5720 genes) and a phylogenetic tree was constructed based on a super-alignment of the 1953 genes in the core genome (genes present in > 99% of isolates) (**Fig. 1**). In addition, a second phylogenetic tree was built based on the binary presence or absence of genes in the accessory genome (**Fig. 2**). Within both phylogenetic trees, isolates clustered within CC and two major clusters of CCs could be identified. The largest cluster, labeled as cluster A consisted of CC151, CC479, CC133, CC425, CC49 and CC50 isolates and second largest cluster B included CC97, CC8, CC1, CC20, CC9, CC7, CC5, CC30 and CC101 (**Fig. 1,2**). There was uneven distribution of CM and SCM isolates between clusters, with more CM isolates being present in Cluster A (81/148, 55%) compared to cluster B (38/103,

Table 2. Number and percentage of 276 bovine *S. aureus* isolates per clonal complex ranked per country or contributing region obtained from bovine clinical and subclinical mastitis in 11 European countries.

CC[1]	N (%)	Be	Dk	Fr	Ge (Ls[2])	Ge (By[3])	Hu	It	NL	Po	Pt	Es	UK
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
151	81 (29)	13 (43)	5 (31)	4 (17)	10 (34)	8 (27)	3 (16)	6 (20)	14 (64)	0 (0)	2 (29)	2 (17)	14 (52)
97	54 (20)	4 (13)	1 (6)	7 (30)	4 (14)	6 (20)	7 (37)	9 (30)	0 (0)	5 (16)	0 (0)	2 (17)	9 (33)
479	32 (12)	8 (27)	0 (0)	0 (0)	2 (7)	8 (27)	0 (0)	3 (10)	8 (36)	0 (0)	3 (43)	0 (0)	0 (0)
133	25 (9)	4 (13)	4 (25)	2 (9)	7 (24)	2 (7)	0 (0)	2 (7)	0 (0)	1 (3)	0 (0)	3 (25)	0 (0)
398	19 (7)	0 (0)	0 (0)	1 (4)	2 (7)	0 (0)	1 (5)	1 (3)	0 (0)	10 (32)	0 (0)	4 (33)	0 (0)
1	14 (5)	1 (3)	0 (0)	1 (4)	0 (0)	0 (0)	2 (11)	1 (3)	0 (0)	7 (23)	0 (0)	0 (0)	2 (7)
20	11 (4)	0 (0)	0 (0)	4 (17)	1 (3)	0 (0)	5 (26)	0 (0)	0 (0)	0 (0)	0 (0)	1 (8)	0 (0)
8	11 (4)	0 (0)	0 (0)	0 (0)	0 (0)	5 (17)	0 (0)	6 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
9	5 (2)	0 (0)	1 (6)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3)	0 (0)	1 (3)	2 (29)	0 (0)	0 (0)
50	5 (2)	0 (0)	5 (31)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5	4 (1)	0 (0)	0 (0)	2 (9)	0 (0)	0 (0)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)
49	4 (1)	0 (0)	0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (10)	0 (0)	0 (0)	0 (0)
7	4 (1)	0 (0)	0 (0)	0 (0)	3 (10)	0 (0)	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
45	3 (1)	0 (0)	0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (6)	0 (0)	0 (0)	0 (0)
101	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
22	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)
30	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)
425	1 (0.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)
Total	**276**	**30**	**16**	**24**	**29**	**30**	**19**	**30**	**22**	**31**	**7**	**12**	**27**

1. Clonal Complex
2. Lower Saxony region
3. Bavaria region

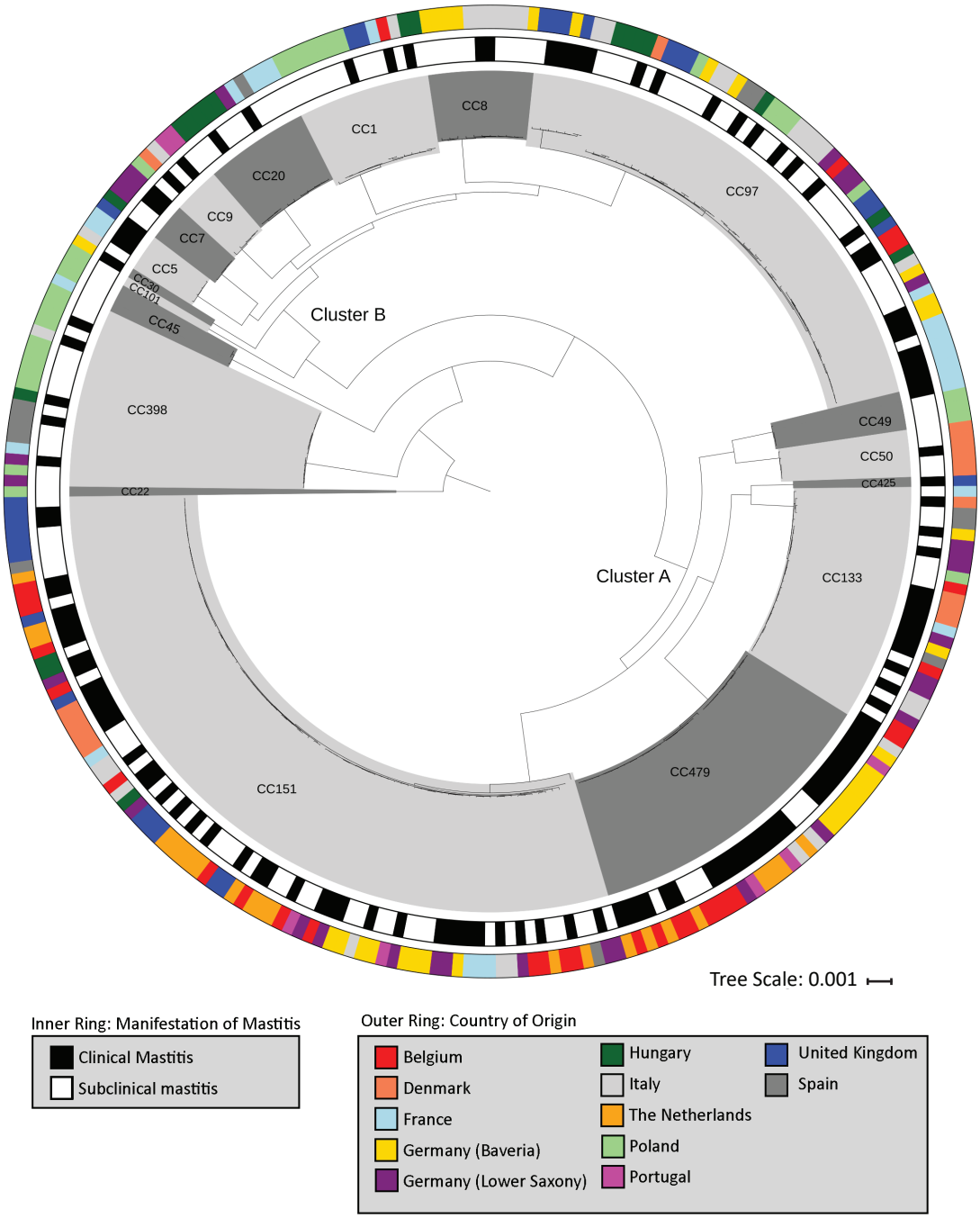
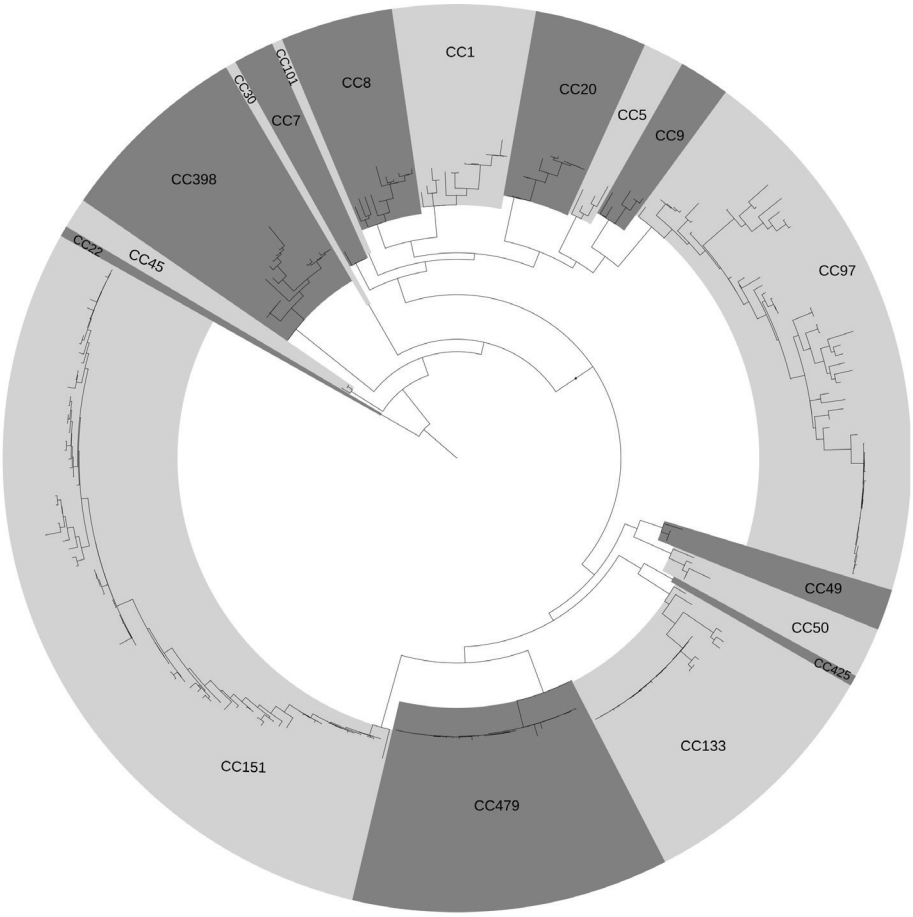


Figure 1.
Maximum-likelihood phylogenetic tree based on the alignment of core genome (genes n = 1953) performed using roary v3.12 (38) and MAFFT of 276 *S. aureus* isolates obtained from bovine clinical and subclinical mastitis cases from 11 European countries. Dark and light grey shading displays Clonal Complex (CC), the outer ring represents the country of origin and the inner ring the clinical versus subclinical mastitis of each isolate. The phylogentic tree was rooted with the CC22 clade and visualized using iTOL v3.6 (40).



Tree Scale: 0.1

Figure 2.
Maximum-likelihood phylogenetic tree based on presence or absence of genes of the accessory genome (n = 2023 genes) of 276 *S. aureus* isolates obtained from bovine clinical or subclinical mastitis from 11 European countries. Dark and light grey shading displays Clonal Complex (CC). The phylogentic tree was rooted with the CC22 clade and visualized using iTOL v3.6 (40).

36%) (Pearson's Chi-squared test $p < 0.01$).

In general, the phylogenetic tree based on the core genome was similar to the tree based on the accessory genome. The differences in quantity of horizontal branch length within CC suggests considerable variation in accessory gene content within most CCs, most notably within CC151 and CC97, but almost no variation in accessory gene carriage was observed among CC479 and CC133 *S. aureus* isolates (**Fig. 2**).

***Staphylococcus aureus* CC479 is associated with CM.**

In order to further study the association between *S. aureus* CC and clinical outcome of IMI, a generalized linear mixed model (GLMM) was built with CM versus SCM as the outcome variable, CC as predictor variable and the country of origin of isolates as a random effect. For this model, isolates belonging to CCs with $n < 10$ (CC9, CC50, CC49, CC7, CC45, CC5, CC101, CC22, CC30, and CC425) were clustered together in a single category labeled as 'other'. Including CC improved the fit over the model compared to the model with only random effects (ANOVA, $p = 0.003$) and isolates belonging to CC479 were more likely to originate from CM than from SCM (OR = 3.62; 95% CI = 1.38 - 9.50, $p < 0.01$) compared to the reference category (CC151). In addition, CC8 (OR = 0.22; 95% CI = 0.04 – 1.1; $p = 0.06$) and CC1 (OR = 0.30; 95% CI = 0.07 – 1.23; $p = 0.10$) were associated with more SCM (**Table 1**).

Clinical manifestation of mastitis is associated with a large number of differen genes

To study genetic differences that could underlay variation in pathogenicity of bovine *S. aureus* isolates, we performed a genome wide association study (GWAS) and 153 genes were associated with CM isolates. In agreement with the results from the GLMM, genes exclusively present in the CC479 isolates ($n = 59$) had the highest OR, ranging from 4.6 to 5.6 (Benjamini-Hochberg Procedure (BHP) ; p-values < 0.05). Among the CM-associated genes, a total of 20 genes matched our inclusion criteria for significant genes of interest (best pairwise comparison $p < 0.1$; BHP $p < 0.1$) and the OR of these genes ranged from 1.95 to 3.42 (**Table 3**). Among these genes, the hypothetical potentially bacteriophage derived DUF3310 domain-containing protein (OR 2.35) was the only gene associated with CM that was detected among all main lineages. Carriage of SE genes *seM*, *seN*, *seI*, *seG*, *seO* and *seU*, as well as the accessory gene regulator (agr) D type II gene was associated with CM and these genes were present in all CC479 isolates and the majority of CC151 isolates (**Table 3**). In addition, three genes from within the *S. aureus* pathogenicity island bovine 3 (SaPIbov3) were also associated with CM and were carried by all CC151, CC479 and 77% of CC97 isolates.

Table 3. Odds Ratio, carriage rate per clonal complex and GenBank references of genes associated with clinical mastitis based on a genome wide association study performed on 276 *S. aureus* isolates obtained from bovine clinical and subclinical mastitis in 11 European countries.

Gene[1]	Odds Ratio[2]	Best pairwise comparision p[3]	CC151 (%)[4]	CC97 (%)	CC479 (%)	CC133 (%)	CC398 (%)	CC1 (%)	CC20 (%)	CC8 (%)	Other CCs (%)[5]	Weighted Average (%)	GenBank Reference
DUF3310 domain-containing protein	2.35	< 0.001	82	60	97	74	95	79	80	30	50	75	WP_001624706.1
DUF2483 domain-containing phage protein	2.36	0.01	43	35	97	12	0	7	0	11	10	33	WP_001077279.1
NAD-specific glutamate dehydrogenase	2.83	0.01	100	0	97	60	0	0	0	0	30	50	SAO39934.1
phiPVL ORF050-like protein	2.23	0.02	98	77	100	0	0	14	30	0	17	59	ABD21348.1
Staphylococcal enterotoxin type C1/U	2.21	0.03	98	0	100	0	0	0	0	0	66	44	WP_109162118.1
Staphylococcal enterotoxin type G	1.95	0.04	98	0	100	0	0	0	100	0	43	49	WP_141060424.1
Staphylococcal enterotoxin type N	1.95	0.04	98	0	100	0	0	0	100	0	43	49	WP_109162119.1
Staphylococcal enterotoxin type I	1.95	0.04	98	0	100	0	0	0	100	0	43	49	QCW39073.1
Multidrug transporter protein (SaPIbov3)	2.31	0.04	99	77	100	0	0	14	30	0	17	59	WP_065315972.1
Intramembrane metalloprotease (SaPIbov3)	2.31	0.04	99	77	100	0	0	14	30	0	17	59	WP_070008570.1
Hypothetical protein (SaPIbov3)	2.31	0.04	99	77	100	0	0	14	30	0	17	59	WP_000921697.1
Staphylococcal enterotoxin type M	2.07	0.06	98	0	100	0	0	0	0	0	20	43	WP_109162116.1
SAS066 AgrD (type II)	2.52	0.06	98	0	100	0	0	0	0	0	47	46	SCU54394.1
Accessory gene regulator protein B4 (type II)	2.52	0.06	98	0	100	0	0	0	0	0	47	46	CZQ66246.1
Hypothetical protein	2.83	0.06	100	13	100	0	0	0	0	0	3	44	WP_000389772.1
TfoX/Sxy family protein	2.83	0.06	100	13	100	0	0	0	0	0	3	44	WP_000179903.1
Arsenate reductase	1.97	0.06	99	0	100	100	0	0	0	100	66	61	WP_000163240.1
trypsin-like serine protease	2.61	0.06	0	0	100	0	0	0	0	0	50	17	WP_043054986.1
SACOL0901 pathogenicity island protein	2.82	0.07	98	88	100	84	0	14	30	28	27	71	WP_109183239.1
DUF1433 domain-containing protein	3.42	0.07	98	98	100	100	0	0	100	0	63	79	WP_031900638.

1. Gene annotation by prokka and confirmed using BLAST search of gene sequence

2. Odds Ratio of causing clinical rather than subclinical mastitis from the Genome wide association study

3. Best pairwise comparison P value from GWAS performed using scoary

4. Carriage rate of gene per clonal complex

5. Includes isolates belonging to CC9, CC50, CC5, CC49, CC7, CC45, CC101, CC20, CC30 and CC425

Table 4. Odds Ratio, carriage rate per clonal complex and GenBank references of genes associated with subclinical mastitis based on a genome wide association study performed on 276 *S. aureus* isolates obtained from bovine clinical and subclinical mastitis in 11 European countries.

Gene[1]	Odds Ratio[2]	Best pairwise comparision p[3]	CC151 (%)[4]	CC97 (%)	CC479 (%)	CC133 (%)	CC398 (%)	CC1 (%)	CC20 (%)	CC8 (%)	Other CCs (%)[5]	Weighted Average (%)	GenBank Reference
putative DNA-binding protein	0.29	< 0.01	2	22	0	16	422	64	82	18	7	18	AUM57693.1
hypothetical protein	0.40	< 0.01	0	94	0	0	100	100	100	100	34	42	WP_000375476.1
hypothetical protein	0.27	0.02	0	0	0	0	100	100	100	100	34	24	WP_000070812.1
Probable antitoxin YezG	0.32	0.03	0	0	0	100	0	0	100	0	3	15	WP_000142094.1
hypothetical protein	0.38	0.03	0	0	0	0	100	100	100	100	62	27	WP_078068548.1
hypothetical protein	0.44	0.03	0	98	0	0	0	100	100	100	62	39	WP_072426418.1
hypothetical protein	0.32	0.04	0	50	0	0	100	7	0	91	52	26	WP_000431307.1
hypothetical protein	0.09	0.06	0	0	0	8	32	0	0	9	14	5	WP_000993183.1
hypothetical protein	0.26	0.06	0	0	0	0	0	100	0	100	34	13	ETO57257.1
hypothetical protein	0.43	0.06	1	0	0	100	100	100	100	100	86	39	WP_078370397.1

1. Gene annotation by prokka and confirmed using BLAST search of gene sequence
2. Odds Ratio of causing clinical rather than subclinical mastitis from the Genome wide association study
3. Best pairwise comparison P value from GWAS performed using scoary
4. Carriage rate of gene per clonal complex
5. Includes isolates belonging to CC9, CC50, CC5, CC49, CC7, CC45, CC101, CC20, CC30 and CC425

Furthermore, 61 genes were associated with SCM (i.e. an OR < 1), from which 10 genes matched our selection criteria and the OR of these genes ranged between 0.09 – 0.44 (**Table 4**). Most genes (8/10) coded for hypothetical proteins and the genes with predicted function were identified as an antitoxin YezG family protein (OR = 0.32) and a putative DNA binding protein (OR = 0.29). The SCM-associated genes were mostly carried by CC1, CC20, and CC8 *S. aureus*, but were always absent from CC479 and most CC151 isolates (**Table 4**).

Discussion

There is a large diversity of bovine *S. aureus* lineages in carriage of virulence and AMR genes (6,9), but it still unclear which genetic differences between *S. aureus* lineages are related to the observed variation in pathogenicity during bovine IMI. Therefore, this study aimed to identify genetic differences between *S. aureus* isolated from CM and SCM in dairy cattle. A secondary goal of the study was to describe the diversity of bovine *S. aureus* lineages in Europe and their carriage of immune evasion factors. We found CC479 to be strongly associated with CM rather than with SCM cases.

Although eighteen different CCs were present in our isolate collection, most *S. aureus* belonged to a limited number of CCs, with the five CCs (CC151, CC97, CC479, CC133 and CC398) making up more than 75% of all isolates. All these CCs have been previously associated with bovine mastitis (6,19,26,27) and the distribution of *S. aureus* CCs differed between geographical locations. Although the isolates in our collection were not a random sample, they did originate from 254 unique herds with a maximum of one CM and one single SCM isolate from the same herd. The prevalence of *S. aureus* CCs per country from our study is in line with studies performed in Denmark (28), Germany (6) and The Netherlands (29). We must, however, note that the aim of our sampling design was to collect isolates from an equal number of clinical and subclinical cases. This, of course, does not reflect the true population of *S. aureus* isolates in dairy herds, as the prevalence of subclinical infections is substantially higher than of clinical infections.

A key finding of the current study was the association between *S. aureus* belonging to CC479 and CM. Interestingly, this corresponds with a previous study which reported that experimental infection with CC479 *S. aureus* results in more severe clinical signs and a higher bacterial load than infection with a CC151 *S. aureus* strain (30). In addition, the association between CC479 and CM was also observed in our previous study using only Dutch mastitis isolates (20). The underlaying mech-

anisms for this apparent increased virulence of CC479 remains unknown, but we have suggested a SNP in the repressor of toxins (*rot*) gene, resulting in an increased production of LukMF' by CC479 isolates as a possible cause (20). Our GWAS identified several genes associated with clinical outcome of IMI, but since *S. aureus* belonging to the same CC share a similar (virulence) gene profile, there was strong link between carriage of these genes and lineage. Therefore, it was not possible to differentiate between the effect of individual genes / sets of genes and *S. aureus* lineage on the clinical outcome of IMI. Interestingly, genes associated with CM by GWAS which were all present in CC479 isolates, were mostly also present in CC151 *S. aureus* (**Table 3**), whereas the latter CC had an approximately even distribution of isolates originating from CM (51%) and SCM (49%). Since CC151 and CC479 share most CM-associated genes, these genes are likely spuriously associated to CM, due to confounding by other factors within CC479. Differences in gene expression are more likely causes of the increased virulence of CC479, in line with our hypothesis regarding the non-functional rot gene (18). Indeed, we confirmed that this mutation was present in all CC479 isolates within our collection (results not shown). It is likely that the absence of functional rot affects the expression levels of multiple virulence genes within CC479 *S. aureus* (20), thereby likely leading to substantially increased probability of causing CM. Future research, e.g. infection studies with genetically modified CC479 isolates lacking potential virulence factors or with restored rot function, are required to identify these potential causal mechanisms.

The results from our GLMM suggest that CC8 and CC1 are less likely to cause CM in cows and most of the genes that were associated with CM were carried by CC8 and CC1 isolates. Previous work identified that CC8 predominantly make up the *S. aureus* genotype B, a highly contagious subtype of *S. aureus* (27,31). We only detected CC8 *S. aureus* among isolates from Italy and Germany, suggesting that this lineage is not widespread throughout Europe. Indeed, reports on CC8 / GTB *S. aureus* primary originate from Switzerland and Austria (27,32,33). In contrast, the CC1 lineage was detected in six different countries in study, although 50% of these CC1 isolates were collected in Poland.

Phylogenetic trees constructed based on core genome alignment and binary presence and absence of accessory genes clustered the isolates perfectly within the assigned CC. This demonstrates that MLST is an adequate genotyping technique, but still Figure 2 shows that considerable variation exists in the accessory genome among isolates within a CC. Interestingly, there was very limited variation in accessory gene carriage among CC479 and CC133 *S. aureus* isolates and this suggests a rapid clonal expansion of these specific *S. aureus* clones. The CC133 lineage is considered a ruminant-adapted lineage, but is mostly associated with small rumi-

nants (16). Therefore, it is possible that the highly similar bovine CC133 *S. aureus* isolates represent a subgroup of CC133 *S. aureus* that is adapted to bovines and this subgroup, as well as the highly similar CC479 isolates, is considerably more successful at infecting the bovine mammary gland than other CC133, CC479 *S. aureus*.

Although our study associated CM with specific genes and CCs, it is important to note that none of these genes were essential for CM in our collection. Clones that were associated with CM cases based on CC or gene carriage were also cultured from SCM and vice versa. Besides pathogen factors, the clinical outcome of an IMI depends on host factors, such as breed and stage of lactation, and environmental factors and their interaction (34,35). Nevertheless, from a diagnostic perspective, the identification of *S. aureus* isolates with an increased risk of developing into CM (e.g. CC479) or highly contagious isolates (e.g. CC8) can support farmers and veterinarians in deciding on the most appropriate intervention strategy to control *S. aureus* in a herd. For *Streptococcus uberis*, it has been demonstrated that MALDI-TOF MS performed on milk samples can identify *S. uberis* isolates with increased CM risk (36). However, it is not certain if this technique is able to distinguish between different CCs of *S. aureus*. There are several PCR based test that detect pathogen-specific genes in milk and an assay for the detection of CC8 (genotype B) *S. aureus* has already been developed (33). We identified multiple CC-specific genes that could be employed to differentiate between common ruminant-associated *S. aureus* lineages (**Table S1**).

In summary, this study identified that although only a limited number of *S. aureus* CCs is responsible for bovine mastitis in Europe, CC479 is strongly associated with CM, in both a GLMM and a GWAS, and it seems likely that differential gene expression rather than gene carriage is responsible. This demonstrates that the clinical outcome of IMI is also influenced by the characteristics of the infecting *S. aureus*. Therefore, identification of *S. aureus* CC can help predict the CM or SCM outcome of *S. aureus* IMI and highlights the potential benefit of diagnostics tools to identify *S. aureus* CC during bovine mastitis.

Methods

Bovine mastitis isolates

Twelve mastitis research groups or diagnostic labs from eleven countries (Belgium, Denmark, France, Germany, Hungary, Italy, Poland, Portugal, Spain, The Netherlands and United Kingdom) were asked to submit a convenience sample of approximately 30 *S. aureus* isolates obtained from cases of bovine mastitis. For each isolate the sampling date, farm ID, geographical location of farm (city and/or region), cow ID and clinical manifestation (CM / SCM) was reported. Groups were asked to donate an equal number of CM and SCM isolates and when possible, from different regions within their country. Isolates were recultured from their transport media onto to sheep blood agar plates, and incubated overnight at 37 °C. Next, single colonies were picked and grown in 2 mL Todd Hewitt Broth (THB) (Sigma, St. Louis, MO, USA) for 16h at 37 °C with agitation. Bacterial glycerol stocks (25% glycerol) were made by adding 0.5 mL of bacterial broth to 0.5 mL 50% glycerol solution in distilled water. Furthermore, DNA was extracted using a simple boiling protocol to confirm bacterial species by PCR targeting the *S. aureus* specific *femA* gene, as described by Hoekstra *et al.* (20). Isolates that were negative for the *femA* PCR or lacked mandatory metadata were excluded from the final collection. For each herd, a maximum of one isolate from a CM case and one from a SCM case was allowed and if multiple CM or SCM isolates were donated from the same herd, one was selected using the random number function of Excel 2015 (Microsoft, Redmond, WA, USA).

DNA extraction, genome sequencing and multilocus sequence typing

DNA for whole genome sequencing was extracted using the DNeasy UltraClean Microbial Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Purity and DNA yield were measured by spectrophotometry and whole-genome sequencing was performed using Illumina HiSeq sequencing (Illumina Inc., San Diego, CA, United States). Multilocus sequence type was determined based on genome data. Each sequence type was assignment to a CC based on eBURST analysis using PHYLOViZ Online (37).

Annotation of genomes, pan-genome analysis and phylogenetic analyses

After quality control of whole genome sequence results, genomes were annotated using prokka v1.11 (25) and the pan / core genome (genes present in 99% < of genomes) determined using roary v3.12 (38). Alignment of the core genome was performed using MAFFT v7.407 and the phylogenetic trees was built with Fasttree v2.1 (39). Subsequently, trees were visualized using iTOL v3.6 (40) and trees were rooted

using the CC22 clade. The large-scale BLAST score ratio (LS-BSR) pipeline was used to obtain matrices with BLAST score ratio of each annotated gene (24). For each isolate, the presence of the genes encoding leukocidin LukMF' (*lukM*, GenBank accession: 1262967; *lukF'*, GenBank accession: 1262954), ruminant-specific Staphylococcal complement inhibitor variant (*scn*, Genbank accession: ADN53656.1), SaPI encoded ruminant specific vWFbp variant (*SaPI vWFbp*, GenBank accession: HM234507.1), enterotoxin type I (*seI*, GenBank accession: EFC00985.1), enterotoxin L (*seL*, GenBank accession: BAO65763.1), toxic shock syndrome toxin 1 (*tsst-1*, GenBank accession: WP_001035596.1), penicillin-hydrolyzing class A beta-lactamase (*blaZ*, GenBank accession: WP_000733621.1), tetracycline resistance protein type M (*tetM*, GenBank accession: AKI94996.1) and penicillin binding protein 2A (*mecA*, GenBank accession: WP_104447100.1) was determined using LS-BSR output. Genes were identified using a threshold value of BSR of > 0.9 compared to the reference gene. In addition, a heatmap of LS-BSR score of isolates was created for genes annotated as SSL or SA by prokka (25) using the *pheatmap* package (41) of the R statistical software version v3.5.4 (42).

Statistical analysis

The GLMM analysis was performed using the lme4 package (43) of the R statistical software version v3.5.4 (42). The model used clinical manifestation of mastitis (SCM, CM) as outcome variable and CC of *S. aureus* was a fixed effect. The country of origin of isolates was used as a random effect in the model. To reduce the number of levels within the variable CC, CCs represented by < 10 isolates were grouped together into a category 'Other'. Furthermore, the association between CC and country of mastitis isolates was investigated by Fisher's exact test and association between genetic cluster and clinical manifestation was investigated using the Pearson's Chi-squared test. Both tests were performed using the R statistical software version v3.5.4 (42).

GWAS

Based on the pangenome analysis in roary, GWAS was performed using scoary v1.6.16 (44). Default settings of scoary were used during our analysis with an initial threshold of naive $p < 0.01$. Genes that matched our inclusion criteria (BHP $p < 0.1$; best pairwise comparison p value of $p < 0.1$) were considered significant results of interest.

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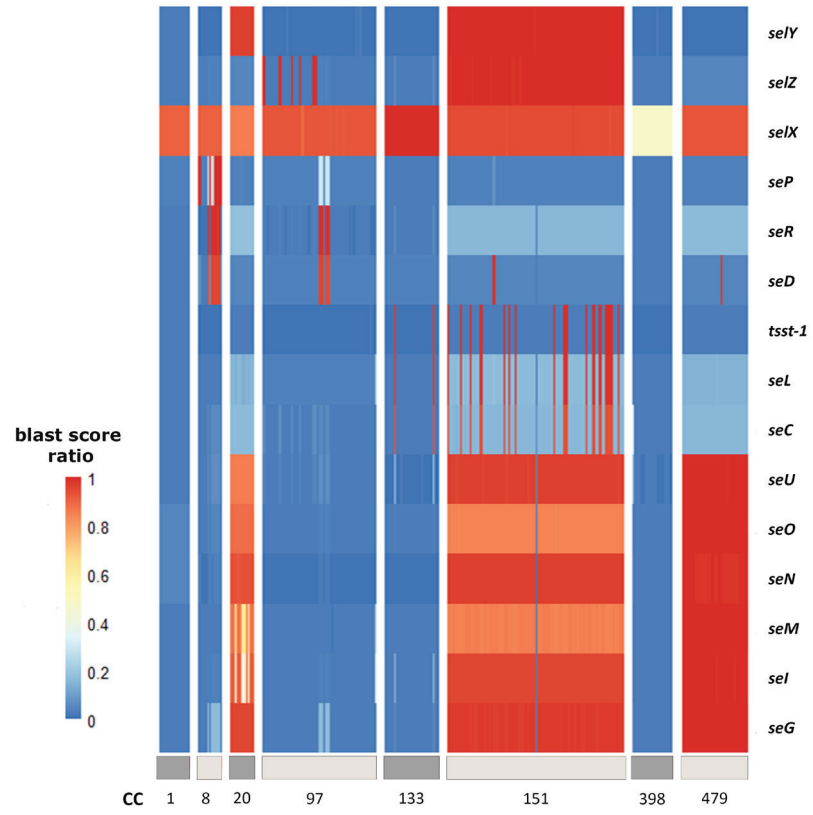
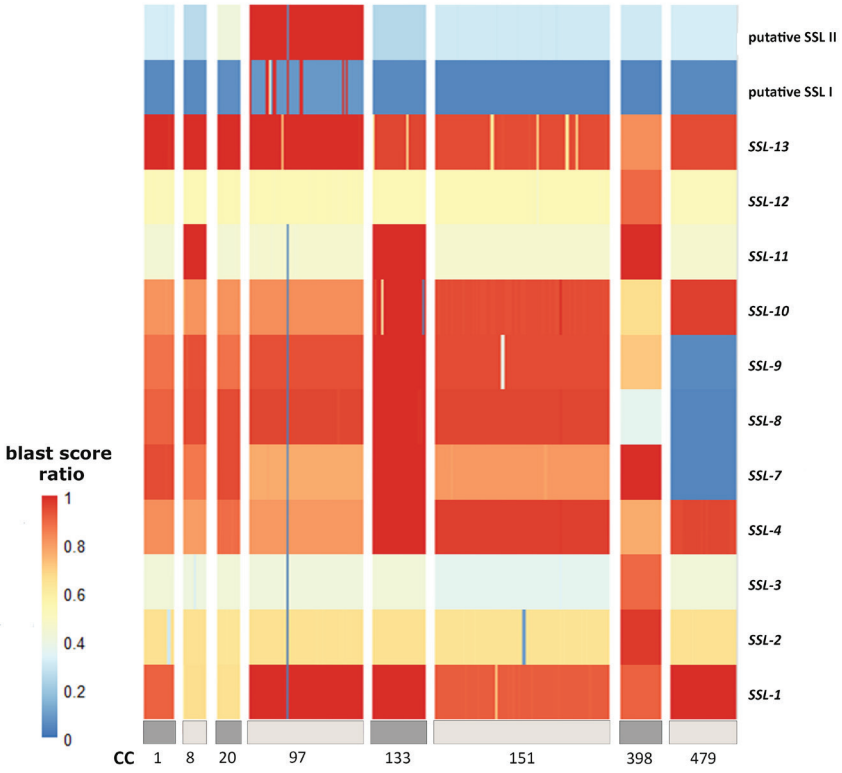
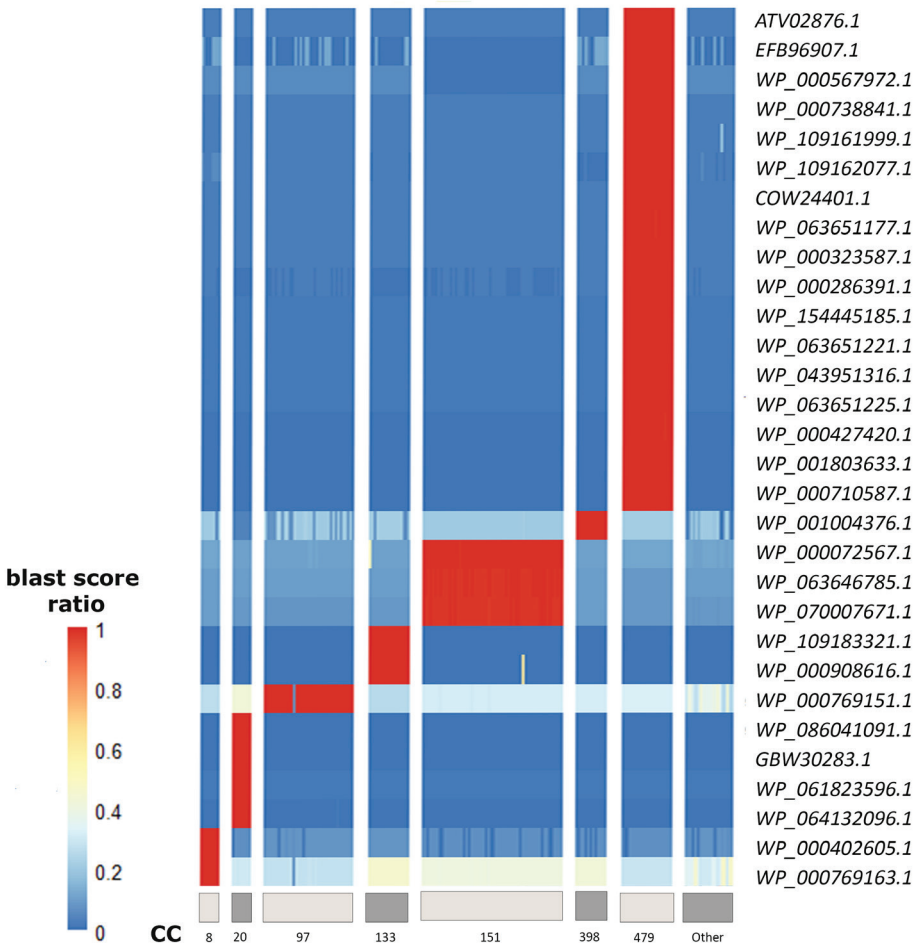
Supplementary materials



Supplementary Figure 1.
A heatmap of BLAST score ratio (24) (BSR) of genes annotated as Staphylococcal enterotoxins (SEs) by prokka (25) of bovine *S. aureus* isolates obtained from clinical and subclinical mastitis cases in 11 European countries calculated using the large-scale BLAST score ratio (LS-BSR) pipeline (24). Heatmap was visualized using the pheatmap package (41) of R statistical software version 3.5.4 (42). Dark and light grey shading displays Clonal Complex (CC) and only *S. aureus* isolates belonging to CCs with n > 10 in our collection are presented in this heatmap.



Supplementary Figure 2.
A heatmap of BLAST score ratio (24) (BSR) of genes annotated as Staphylococcal Superantigen like proteins (SSLs) by prokka (25) of bovine *S. aureus* isolates obtained from clinical and subclinical mastitis cases in 11 European countries calculated using the large-scale BLAST score ratio (LS-BSR) pipeline (24). Two yet unnamed SSLs are labeled as putative SSL I (GenBank reference: WP_143564871.1) and putative SSL II (GenBank reference WP_124375191). Heatmap was visualized using the *pheatmap* package (41) of R statistical software version 3.5.4 (42). Dark and light grey shading displays Clonal Complex (CC) and only *S. aureus* isolates belonging to CCs with n > 10 in our collection are presented in this heatmap.



Supplementary Figure 3.
A heatmap of BLAST score ratio (24) (BSR) and GenBank Reference of Clonal complex (CC) exclusive genes of bovine *S. aureus* isolates obtained from clinical and subclinical mastitis cases in 11 European countries calculated using the large-scale BLAST score ratio (LS-BSR) pipeline (24). Heatmap was visualized using the *pheatmap* package (41) of R statistical software version 3.5.4 (42). Dark and light grey shading displays Clonal Complex (CC) and isolates belonging to CCs with n > 10 (CC9, CC50, CC5, CC49, CC7, CC45, CC101, CC20, CC30 and CC425).

Table S1. Predicted function and GenBank reference of Clonal complex (CC) exclusive genes among 276 *S. aureus* isolates obtained from bovine clinical and subclinical mastitis in 11 European countries.

Associated CC	Predicted function of gene-encoded Protein	GenBank Reference
CC8	SSL-11 variant	WP_000769163.1
CC8	hypothetical protein	WP_000402605.1
CC20	site-specific DNA-methyltransferase	WP_064132096.1
CC20	hypothetical protein	WP_061823596.1
CC20	hypothetical protein	GBW30283.1
CC20	DEAD/DEAH box helicase	WP_086041091.1
CC97	hypothetical protein	WP_000769151.1
CC133	AAA family ATPase	WP_000908616.1
CC133	ATP-dependent helicase	WP_109183321.1
CC151	restriction endonuclease subunit S	WP_070007671.1
CC151	hypothetical protein	WP_063646785.1
CC151	restriction endonuclease subunit S II	WP_000072567.1
CC398	DUF5085 family protein	WP_001004376.1
CC479	Gfo/Idh/MocA family oxidoreductase	WP_000710587.1
CC479	polyketide synthase	WP_001803633.1
CC479	AMP-binding protein	WP_000427420.1
CC479	salicylate synthase	WP_063651225.1
CC479	DNA (cytosine-5-)-methyltransferase	WP_043951316.1
CC479	ABC transporter ATP-binding protein	WP_063651221.1
CC479	hypothetical protein	WP_154445185.1
CC479	hypothetical protein	WP_000286391.1
CC479	4'-phosphopantetheinyl transferase superfamily protein	WP_000323587.1
CC479	Eco47II family restriction endonuclease	WP_063651177.1
CC479	transmembrane component of ECF transporter	COW24401.1
CC479	helix-turn-helix domain-containing protein	WP_109162077.1
CC479	YqcI/YcgG family protein	WP_109161999.1
CC479	MptD family putative ECF transporter S component	WP_000738841.1
CC479	hypothetical protein	WP_000567972.1
CC479	hypothetical protein	EFB96907.1
CC479	Iron aquisition yersiniabactin synthesis enzyme	ATV02876.1

CHAPTER 4

Differences in *Staphylococcus aureus* lineages isolated from ovine and caprine mastitis, but not between isolates from clinical or subclinical mastitis

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Abstract

Staphylococcus aureus is an important mastitis pathogen, causing both clinical mastitis (CM) and subclinical mastitis (SCM) in small ruminants. In general, CM has a low incidence in sheep and goats, but can be very severe and costly. In contrast, subclinical mastitis (SCM) is common, but is associated with less costs. For both sheep and goats, *S. aureus* is the main cause of CM and associated with SCM cases with a high SCC. Recently, specific lineages of *S. aureus* have been identified that are associated with CM rather than SCM in dairy cows. It is unknown whether specific *S. aureus* lineages are associated with CM in goats and sheep. The aim of this study was to compare the clonal complex (CC), *spa*-type, leucocidin *lukM-lukF'* presence and the potential to produce LukMF' *in vitro* between CM and SCM *S. aureus* mastitis isolates obtained from sheep and goats. Also, differences between isolates from different host species were compared. Ovine (CM, n = 12; SCM, n = 29) and caprine (CM, n = 14; SCM, n = 30) isolates were obtained from eight sheep flocks and eight goat herds in The Netherlands. Overall, the isolates belonged to CC133 (85%), CC398 (7%), CC425 (5%) and CC45 (2%). Seventeen different *spa*-types were found, including six novel types, and the predominant types were t2678 (34%), t544 (18%) and t3583 (18%). Although CC133 was dominant among both sheep and goat isolates, *spa*-type CC133/t2678 was associated with ovine isolates, whereas CC133/t544 and CC133/t3583 were found mostly in goats. Presence of *lukM-lukF'* among the *S. aureus* isolates was high (87%), especially in CC133 (96%) and CC425 (100%), but the genes were absent in CC45 and CC398. *In vitro* cultured *lukM-lukF'*-positive isolates produced LukM (71 out of 74 positive isolates tested) in the range of 0.4 to 5.0 µg/mL. Interestingly, the goat-associated lineages CC133/t544 and CC133/t3583 produced more LukM *in vitro* than the sheep-associated CC133/t2678. We found no difference in LukMF' production potential between CM and SCM isolates. In sheep as well as in goats, no association was found between genotype and CM or SCM, demonstrating that the same lineages of *S. aureus* are responsible for both CM and SCM. These results suggest that subclinically infected animals in a herd or flock likely act as the reservoir of *S. aureus* causing CM. This highlights the importance of early identification and control of SCM, and suggests that controlling SCM within a herd is an effective intervention to prevent CM in small ruminants.

Introduction

Mastitis is a common disease among dairy goats and sheep that is responsible for economic losses due to reduced milk production (1,2), decreased milk quality (3,4), increased lamb mortality (5), costs of treatment, and culling (2,6). Furthermore, behavioral differences are observed in animals suffering from mastitis and this, together with clinical symptoms, indicates a reduction in animal welfare (7). Clinical mastitis (CM) is characterized by the classical signs of inflammation (pain, swelling, heat, erythema and loss of function), causing visible abnormalities in milk and/or udder (8). In the present study subclinical mastitis (SCM) is defined by a positive bacteriological culture from normal looking milk, in the absence of clinical symptoms (2). Since the use of elevated SCC as an indicator for SCM in small ruminants is debated (9,10), we chose not to consider it to define SCM. Although SCM is common in goats and sheep (11,12), it only results in a limited decrease in milk yield (1,4,10) and relatively low costs for the farmer (2). Many- SCM cases are caused by coagulase-negative staphylococci (CNS), but also by *Staphylococcus aureus* (12,13). In contrast, CM has a low incidence, but is responsible for more economic losses than SCM (e.g. higher reduction in milk yield and quality, increased lamb mortality and the culling of animals), highlighting the importance of CM control to reduce the costs of mastitis on farms (2,4,5,14). Most CM cases in sheep and goats are caused by *S. aureus* (14) and the dominant lineages of *S. aureus* isolated from these animals are clonal complex (CC) 133, CC130 and CC522 (15,16). Although several studies consider *S. aureus* isolates from goats and sheep to be part of the same population based on CC, typing techniques with higher resolution, such as *spa*-typing, do detect different *S. aureus* lineages associated with the two species (17–19). Among CC133 isolates obtained from small ruminants, certain *spa*-types were predominantly found in goats (t1166, t7304) and other types (t2678, t9088) in sheep (18,19).

The severity of infectious diseases is determined by the pathogen, the host, and their interaction, but it is largely unclear to what extent the clinical severity of mastitis is driven by host or pathogen factors (20,21). Recently, we identified a bovine-associated lineage of *S. aureus* (sequence-type (ST) 479) that was associated with CM rather than SCM in cattle (22). High production of leukocidin LukMF', a ruminant-associated virulence factor that is a potent killer of ruminant neutrophils *in vitro* (23), by ST479 *S. aureus* was the likely explanation for this association with CM. To our knowledge, associations between *S. aureus* lineages and CM or SCM has not previously been investigated in sheep and goats.

The aim of this study was to describe the genetic diversity of *S. aureus* isolates obtained from cases of ovine and caprine mastitis in the Netherlands, and to determine to what extent this variation is associated with CM or SCM and with host species. Multilocus sequence typing (MLST) and staphylococcal protein A (*spa*) genotyping were performed and the proportion of isolates carrying *lukM-lukF'* and their LukMF' production potential were determined.

Materials and Methods

Sample Collection

A convenience sample of 18 Dutch dairy goat farmers were asked to aseptically collect milk samples from any goat suffering from CM. For the present study, the definition of CM was visible abnormalities in the udder or milk or both. For every case of CM, farmers were asked to fill out a form to record goat ID, date of CM, date of kidding, parity, affected udder half, type of mastitis (gangrenous mastitis or non-gangrenous mastitis), clinical symptoms, appearance of the milk, and treatment. The milk samples from CM were frozen at -20 °C by the farmer. At the end of the sampling period, the samples were shipped to the Veterinary Microbiological Diagnostic Center (VMDC) at Utrecht University for bacteriological culture and speciated according to NMC guidelines (24), resulting in 14 *S. aureus* CM isolates from eight different herds. SCM isolates (n = 30), originating from five herds that also contributed caprine CM isolates, were used from a previous study on SCM in dairy goats (10).

Ovine mastitis isolates were obtained from milk samples from 238 meat sheep from 15 different herds. During sampling, udder halves showing abnormalities in appearance of the milk or the udder or both were classified as having CM. All milk samples were cultured on blood agar plates according to the NMC guidelines (24) and bacterial species were determined by use of MALDI-TOF (25). A positive bacterial culture result in the absence of reported abnormalities in milk and/or udder was classified as SCM. A total of 41 *S. aureus* isolates were found, originating from eight different herds, including 12 samples originating from cases classified as CM and 29 as SCM.

Genotyping of isolates

DNA extraction from *S. aureus* isolates was performed using a boiling protocol, as described by Hoekstra *et al.* (2018). The polymorphic X-region of the Staphylococcal Protein A (spa) genes of the isolates were amplified according to the Ridom StaphType standard protocol (www.ridom.org). When standard *spa*-typing primers failed to yield a PCR product in an isolate, an alternative set of *spa*-primers (26) was used. Amplicons were purified using ExoSAP-IT PCR Cleanup Reagent (Affymetrix, Santa Clara, CA, USA) following manufacturer's instructions and sequenced using Sanger sequencing (Baseclear, Leiden, The Netherlands). Based on sequenced PCR amplicons, *spa*-types were assigned using BioNumerics v7.5 (Applied Maths, Sint-Martens-Latem, Belgium) and the *spa*-typing plugin. A minimum spanning tree (MST) of *spa*-types was created in BioNumerics v7.5 by the *spa* clustering methods of the *spa*-typing plugin, using the same settings as described by Mekonnen *et al.* (2018).

For MLST, a subset of ovine isolates (n = 10) was selected, representing the diversity of *spa*-types among ovine isolates. Typing was performed according to the protocol described on the MLST website, http://saureus.mlst.net. The MLST of caprine isolates was obtained from whole genome sequence (WGS) data. For WGS, DNA was extracted using MasterPure Gram-positive DNA purification kit (Cambio, Cambridge, United Kingdom), and HiSeq sequencing was performed according to the manufacturer's protocol (Illumina Inc., San Diego, CA, United States) and genotyping was done based on the assembled genomes of these isolates.

Detection of *lukM-lukF'* and *in vitro* LukM production.

PCR Amplification of *femA*, *lukM*, *lukF'* and measurement of *in vitro* LukMF' production potential was performed as previously described by Hoekstra *et al.,* 2018. Briefly, to measure the LukMF' production potential, isolates were grown in broth under controlled conditions for 8 hours and the LukM concentration in broth was measured using ELISA (22,23).

Presence of *lukM-lukF'* in WGS of *S. aureus* isolates was investigated by BLASTN using reference sequences for *lukM* (GenBank accession: 1262967) and *lukF'* (GenBank accession: 1262954). Identity scores higher than 95% were considered positive for *lukM* or *lukF'*.

Statistical Analysis

For the LukM ELISA, LukM values were extrapolated from a LukM standard curve using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA). Differences in LukMF' production potential between different lineages were tested by Kruskal-Wallis test followed by Dunn's Test in GraphPad Prism 7. To compensate for a possible farm effect on LukMF' production potential, a subset of the LukMF' production potential dataset with only a single *spa*-type value per farm was also used. In case of multiple isolates with the same *spa*-type on a farm, the averaged LukM production of isolates belonging to this *spa*-type was used.

The Mann-Whitney test was used to compare differences in LukMF' production potential between *S. aureus* isolated from sheep / goats and CM / SCM (for all isolates and within ovine / caprine isolates). Differences in LukMF' production potential between *spa*-types was only tested among types with n > 10 using the Kruskal-Wallis test followed by Dunn's test. Fisher's exact tests were performed to study association between clinical severity and CC, *spa*-type and lukM-lukF'. Using the same technique, the association between host species and CC, *spa*-type was also investigated. All tests were performed using IBM SPSS Statistics v.24 (IBM Corp, Armonk, NY, USA).

Results

The results of MLST, *spa*-typing and presence of *lukM-lukF'* among *S. aureus* isolates are reported in **Table 1** and an overview of the distribution of CM/SCM over different *spa*-types is shown in **Figure 1**. Most isolates (85%) obtained from goats and sheep belonged to CC133 and this lineage was present on 15 out of 16 farms (**Supplementary Table 1**). Significant differences (Fisher's exact test, $p = 0.001$) were observed between the CC of caprine and ovine mastitis isolates, with CC398 being exclusively found in goats and CC425, CC45 only in sheep (**Figure 1**). Multiple *spa*-types were present among CC133 isolates, the dominant types being t2678 (40% of CC133 isolates), t544 (21%) and t3583 (21%). The distribution of the dominant CC133 *spa*-types differed between hosts (Fisher's exact test, $p < 0.001$). Isolates belonging to CC133/t544 (ovine: 0%, caprine: 34%, $p < 0.001$) and CC133/t3583 (ovine: 5%, caprine: 30%, $p = 0.004$) were associated with caprine mastitis and those designated CC133/t2678 (ovine: 56%, caprine: 14%, $p < 0.001$) with ovine mastitis (**Table 1**). *Spa*-types t544, t3583 and t2678 were genetically related, as is illustrated by the fact that they cluster together in the MST (**Figure 1**).

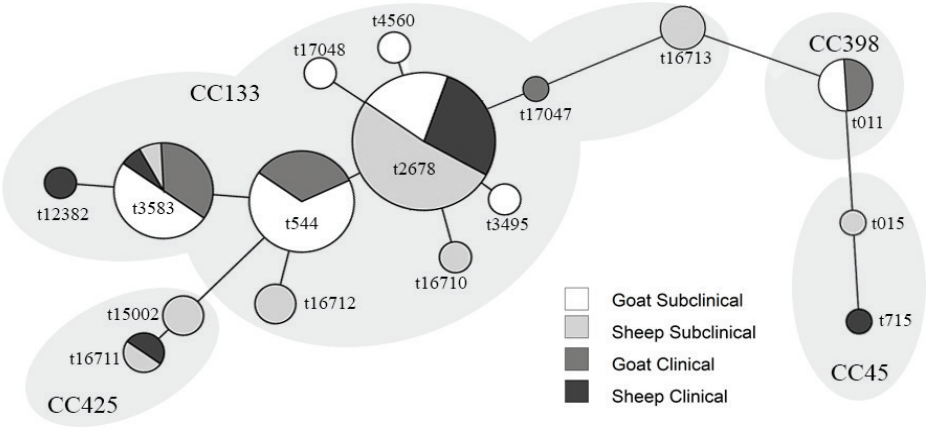

Figure 1.
Minimum spanning tree (MST) of *spa*-types of ovine (n = 41) and caprine (n = 44) *S. aureus* mastitis isolates cultured from 16 Dutch farms. Each *spa*-type is depicted by a single node, with the size of the node representing the number of isolates associated with this spa type and colors representing host species (goat, sheep) and clinical or subclinical mastitis. Associated clonal complex (CC) is displayed in shading.

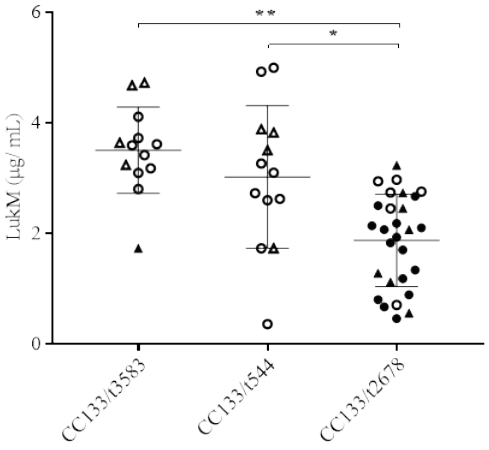

Figure 2.
LukM levels *in vitro* after eight hours of culture of *lukM-lukF'*-positive *S. aureus* CM (triangles) and SCM (circles) mastitis isolates obtained from sheep (black) and goats (white) belonging to the three dominant genotypes CC133/t3583 (n =13), CC133/t544 (n = 13) and CC133/t2678 (n = 28), tested by ELISA. Production potential of isolates (n =17) belonging to ten other *spa*-types was measured but is not shown in this figure. Statistical significance was analyzed by Kruskal-Wallis test followed by Dunn's test. Asterisks indicate a significant difference at **: $P < 0.01$, *: $P < 0.05$.

Table 1.
Clonal complex (CC), *spa*-type and *lukM-lukF'* carriage of 85 *S. aureus* isolates obtained from cases of ovine and caprine mastitis from eight goat herds and eight sheep flocks in The Netherlands.

				Ovine			Caprine		
CC[1]	**n (%)**	***spa*-type**	***spa*-repeats[2]**	**n (%)**	***lukM-lukF'*[3] n (%)**	**CM n (%)**	**n (%)**	***lukm-lukF'*[3] n (%)**	**CM n (%)**
133	73 (85)	t2678	03-16-12-21-17-23-13-17-17-17-23-24	23 (56)	23 (100)	8 (35)	6 (14)	6 (100)	0 (0)
		t3583	03-16-21-17-23-13-17-17-17-17-23-24	2 (5)	2 (100)	2 (100)	13 (30)	13 (100)	5 (38)
		t544	03-16-12-21-17-23-13-17-17-17-17-23-24				15 (34)	13 (87)	5 (33)
		t16713[4]	03-16-12-21	4 (8)	4 (100)	0 (0)			
		t16712[4]	03-12-12-12-21-17-23-13-17-17-17-17-23-24	2 (5)	2 (100)	0 (0)			
		t1671[4]	03-16-12-21-22-17-23-13-17-17-17- 23-24	1 (2)	1 (100)	0 (0)			
		t12382	03-21-17-23-13-17-17-17-17-23-24	1(2)	0 (0)	0 (0)			
		t3495	03-16-12-21-17-23-13-17-17-23-24				1 (2)	1 (100)	0 (0)
		t4560	03-16-12-21-17-23-13-17-17-17-17-17-23-24				1 (2)	1 (100)	0 (0)
		t17048[4]	03-16-12-21-23-13-17-17-17-23-24				1 (2)	1 (100)	0 (0)
		t17047[4]	03-16-12-21-17-16-17-17- 23-24				1 (2)	1 (100)	1 (100)
		NA[5]	NA	2 (5)	2 (100)	0 (0)			
398	6 (7)	t011	08-16-02-25-34-24-25				6 (14)	0 (0)	3 (50)
425	4 (5)	t15002	14-44-12-12-17-23-18-17-17-17-17-23-24	2 (5)	2 (100)	0 (0)			
		t16711[4]	14-44-12-12-17-23-18-17-17-17-17-17-23-24	2 (5)	2 (100)	1 (50)			
45	2 (2)	t015	08-16-02-16-34-13-17-34-16-34	1 (3)	0 (0)	0 (0)			
		t715	09-02-16-34-13-17-34	1 (3)	0 (0)	1 (100)			
Total	**85 (100)**			**41 (100)**	**38 (93)**	**12 (29)**	**44 (100)**	**36 (82)**	**14 (32)**

1. Clonal Complex
2. Numerical code of spa repeats chosen by Ridom StaphType (www.ridom.org).
3. Number and percentage of *lukM-lukF'*-positive isolates.
4. Novel *spa*-types.
5. Isolates not typeable using any of the *spa* primer sets used in our study.

Presence of *lukM-lukF'* was common in CC133 and CC425, but absent in CC45 and CC398 (**Table 1**). The LukMF' production potential was measured in 71 *lukM-lukF'*-positive isolates, with production levels after eight hours of culture ranging from 0.4 to 5.0 µg/mL. CM and SCM isolates produced similar levels of LukM, both in ovine (CM: 2.0 ± 0.9 µg/mL, SCM: 2.0 ± 0.8 µg/mL; Mann-Whitney test: p = 0.95) and caprine (CM: 3.6 ± 0.9 µg/mL; SCM: 3.0 ± 1.0 µg/mL; Mann-Whitney test: p = 0.07) isolates separately, and also when comparing all isolates (CM: 2.8 ± 1.2 µg/mL, SCM: 2.5 ± 1.0 µg/mL, Mann-Whitney test: p = 0.28). Among the isolates belonging to the three dominant *spa*-types (representing 76% of the total isolate collection), the ovine-associated CC133/t2678 isolates produced LukM at significantly lower levels (1.9 ± 0.8 µg/mL) than the dominant caprine CC133/t3583 (3.5 ± 0.7 µg/mL, Dunn's test, p < 0.001) and CC133/t544 isolates (3.0 ± 1.3 µg/mL, Dunn's test, p = 0.007) (**Figure 2**). A similar but not significant trend was seen in a subset of data using an averaged LukM value per *spa*-type per farm (Supplementary figure 1). Fisher's exact tests on all isolates revealed no association between SCM or CM and CC (p = 0.52), *spa*-type (p = 0.65) or presence of *lukM-lukF'* (p = 0.25). Similar results were seen when the analysis was performed for ovine and caprine isolates separately (Results not shown).

Discussion

In this study, we compared the genotype and LukMF' production potential of *S. aureus* isolates originating from CM and SCM in both goats and sheep. Although most of both caprine and ovine isolates belonged to CC133, the dominant *spa*-type within CC133 found in sheep differed from the ones in goats. Interestingly, isolates from these caprine-associated *spa*-types produced higher levels of LukM *in vitro* than isolates from the sheep-associated *spa*-type.

The majority of small ruminant mastitis *S. aureus* isolates in this study belonged to CC133, a lineage with a broad host range (27,28), although it has been primarily associated with ruminants, and in particular with goats and sheep (15,16,28). Similar observations were made in Denmark, where CC133 was also the dominant lineage found in sheep and goats (18). Besides CC133, CC522 and CC130 lineages are considered to be dominant small ruminant-associated lineages (16), but these types were not found in our study. Although *S. aureus* lineages found among sheep and goats are considered to be highly similar (15), systematic differences in *spa*-types between isolates from the two host species were observed in this study, with CC133/t2678 being associated with ovine and CC133/t3583 and CC133/t544 with caprine mastitis. In addition, there were differences in LukMF' production potential,

with CC133/t544 and CC133/t3583 producing on average around 1.5 times more LukM than CC133/t2678. A possible explanation for variation in LukMF' production potential are differences in expression levels of genes involved in the regulation of leukocidin production (Agr quorum-sensing system, SaeRS two-component systems, Rot) (29). Differences in *spa*-repeats between the three dominant *spa*-types were small, and CC133/t2678 and CC133/t3583 were not exclusively associated with one host species. Furthermore, previous studies have also described CC133/t544 isolates in sheep (18,19). Differences in *spa*-type between CC133 *S. aureus* obtained from sheep and goats have been observed in multiple studies, but the dominant *spa*-type per host species differed per country (17–19). It is unclear whether differences in *spa*-type between sheep and goats reflect functional adaptations of the *S. aureus* lineage to the host.

Lineages other than CC133 (CC398, CC425 and CC45) only made up 14% of mastitis isolates in this study, and all of these lineages have previously been isolated from goats and sheep (30,31). We found the CC398/t011 lineage only in goats and CC398 is the predominant lineage of livestock associated-MRSA in Europe (32). The CC425 lineage is predominantly associated with *S. aureus* from wildlife origins (28) , and was only found in a single sheep flock.

The virulence genes *lukM-lukF'* were present among most isolates and we found no relationship between presence of *lukM-lukF'* or LukMF' production potential and clinical severity of mastitis in sheep or goats. Previously, *lukM-lukF'*-positive *S. aureus* were associated with CM in cattle (33), and we also found that high production of LukMF' was linked with bovine CM (22). Presence of *lukM-lukF'* and production potential of LukMF' are associated with CC (22,34). Most of our isolates (85%) belong to a single CC and this could explain why we found no differences in presence and production potential of LukMF' between CM and SCM isolates.

The association between *lukM-lukF'* and CC133 has previously been reported in bovine, ovine and caprine isolates (27,34). However, two CC133/t544 isolates, obtained from the same goat herd, lacked lukM-lukF'. Genome analysis revealed that the prophage associated with *lukM-lukF'* (phiPV-83) (35) was still present in these isolates, but lacked the region containing the actual leukocidin genes. All CC425 and the majority of CC133 isolates found in our study carried *lukM-lukF'*, whereas CC425 and CC133 obtained from wildlife ruminant hosts (red deer, roe deer, chamois) rarely harbored *lukM-lukF'* (28). This could suggest that harboring *lukM-lukF'* gives *S. aureus* increased fitness within domesticated ruminants, but not within wildlife species.

We found no differences between CM and SCM isolates in sheep nor goats. This suggests that the clinical manifestation of mastitis is driven by host factors rather than by the pathogen *S. aureus* . Still, it is possible that differences between *S. aureus* isolates beyond the resolution of the typing methods used in our study determine the outcome of intramammary infections. However, a recent infection study in goats using two different lineages of *S. aureus* showed that host factors determined the clinical manifestation of mastitis, since animals infected with the same *S. aureus* strain had different clinical outcomes (36).

Mastitis isolates were collected from eight sheep flocks and eight goat herds across The Netherlands, but there was an uneven distribution of the number of isolates per farm (**Supplementary table 1**). As a consequence, farm is a potential confounder for observed differences in LukMF' production potential. To compensate for this, a subset using the averaged LukM value per *spa*-type per farm was formed and similar trends in differences in LukMF' production potential were still present in this subset. The uneven distribution of isolates can also result in over- or under representation of *S. aureus* lineages in our dataset, especially in the caprine isolates, because one farm contributed a large number (43%) of the caprine isolates (**Supplementary table 1**). However, the *spa*-types found within that goat farm were similar to *spa*-types found on other farms and the overall variation in *spa*-types between caprine isolates was low. Therefore, we do not expect that this sampling bias substantially affected our main finding that there was no association between genotype and clinical outcome of infection.

Because persistent SCM cases can develop into CM at a later point in time (37), the same strain may be associated with both CM and SCM, depending on the time of sampling. Additionally, the clinical outcome of an infection is at least in part determined by host factors (36). This illustrates that the CM or SCM phenotype classification of an isolate based on a single mastitis case is imperfect. However, other studies using the same classification did identify strain variation in pathogenicity of bovine mastitis (22,33,38). This demonstrates that despite misclassification of strains and the resulting reduction in power, our study approach should in principle be able to identify pathogen related factors that contribute to severity of an intramammary *S. aureus* infection.

In conclusion, although most mastitis isolates obtained from both sheep and goats belonged to CC133, the CC133/t2678 lineage was associated with ovine mastitis and CC133/t544, CC133/t3583 with caprine mastitis. We found no significant differences between *S. aureus* isolated from CM or SCM cases originating from small ruminants, implying that animals with SCM are a reservoir of *S. aureus* responsible for CM. This finding suggests that controlling SCM within a herd is an effective intervention to prevent CM in small ruminants.

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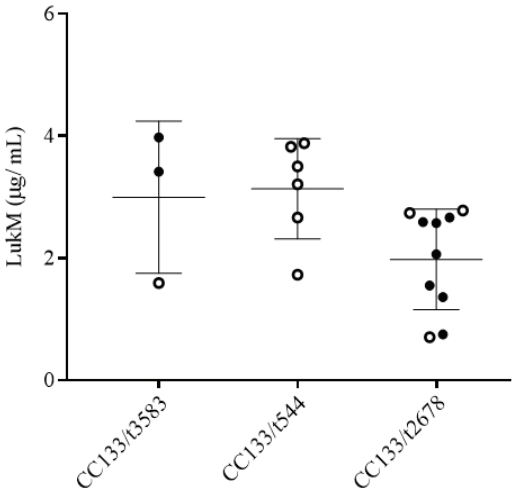
Supplementary Figures



Supplementary Figure 1.
LukM levels *in vitro* after eight hours of culture of *lukM-lukF'*-positive *S. aureus* mastitis isolates obtained from sheep (black) and goats (white) in subset of single isolate of the same *spa*-type per farm. When multiple isolates of the same *spa*-type were present within a single farm, the average lukM production of these isolates was used. Isolates belong to genotypes CC133/t3583 (n = 3), CC133/t544 (n = 6) and CC133/t2678 (n = 10), tested by ELISA. Statistical significance was analyzed by Kruskal-Wallis test followed by Dunn's test.

Supplementary Tables

Supplementary Table 1. Goat herd and sheep flock distribution of 85 *S. aureus* isolates obtained from cases of ovine and caprine mastitis in The Netherlands

		Goat herds								Sheep flocks								
CC[1]	***spa*-type**	**A**	**B**	**C**	**D**	**E**	**F**	**I**	**J**	**A**	**C**	**D**	**G**	**I**	**J**	**M**	**O**	**Total**
133	t2678		1	4	1					3	6	3	5	1	2	3		29
	t3583	7	6									2						15
	t544	6			2	2	1	1	3									15
	t16713												3		1			4
	t16712												2					2
	t16710											1						1
	t12382												1					1
	t3495			1														1
	t4560				1													1
	t17048			1														1
	t17047	1																1
	NA[2]									1						1		2
398	t011	5	1															6
425	t15002													2				2
	t16711													2				2
45	t015									1								1
	t715																1	1
Total		19	8	6	3	3	1	1	3	5	6	6	11	5	3	4	1	85

1. Clonal Complex
2. Isolates not typeable using any of the *spa* primer sets used in our study.

CHAPTER 5

Activation of a bovine mammary epithelial cell line by ruminant-associated *Staphylococcus aureus* is lineage dependent

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Abstract

Bovine mastitis is a costly disease to the dairy industry and intramammary infections (IMI) with *Staphylococcus aureus* are a major cause of mastitis. *Staphylococcus aureus* strains responsible for mastitis in cattle predominantly belong to ruminant-associated clonal complexes (CCs). Recognition of pathogens by bovine mammary epithelial cells (bMEC) plays a key role in activation of immune responsiveness during IMI. However, it is still largely unknown to what extent the bMEC response differs according to *S. aureus* CC. The aim of this study was to determine whether ruminant-associated *S. aureus* CCs differentially activate bMEC. For this purpose, the immortalized bMEC line PS was stimulated with *S. aureus* mastitis isolates belonging to four different clonal complexes (CCs; CC133, CC479, CC151 and CC425) and interleukin 8 (IL-8) release was measured as indicator of activation. To validate our bMEC model, we first stimulated PS cells with genetically modified *S. aureus* strains lacking (protein A, wall teichoic acid (WTA) synthesis) or expressing (capsular polysaccharide (CP) type 5 or type 8) factors expected to affect *S. aureus* recognition by bMEC. The absence of functional WTA synthesis increased IL-8 release by bMEC in response to bacterial stimulation compared to wildtype. In addition, bMEC released more IL-8 after stimulation with *S. aureus* expressing CP type 5 compared to CP type 8 or a strain lacking CP expression. Among the *S. aureus* lineages, isolates belonging to CC133 induced a significantly stronger IL-8 release from bMEC than isolates from the other CCs, and the IL-8 response to CC479 was higher compared to CC151 and CC425. Transcription levels of IL-8, tumor necrosis factor alpha (TNFα), serum amyloid A3 (SAA3), Toll-like receptor (TLR)-2 and nuclear factor κB (NF-κB) in bMEC after bacterial stimulation tended to follow a similar pattern as IL-8 release, but there were no significant differences between the CCs. This study demonstrates a differential activation of bMEC by ruminant-associated CCs of *S. aureus*, which may have implications for the severity of mastitis during IMI by *S. aureus* belonging to these lineages.

Introduction

Mastitis is a major economic problem for the dairy industry and the predominant causes of bovine mastitis are intramammary infections (IMI) with bacteria (1). The mammary gland has a well-developed innate immune system that responds to incoming pathogens during IMI, and bovine mammary epithelial cells (bMECs) play an important role in activating the early innate immune response (2). Pattern recognition receptors (PRRs), such as Toll-like receptor (TLR) 1, TLR-2, TLR-6, nucleotide-binding oligomerization domain-like receptors (NLR) 1, NLR-2, expressed by bMEC are activated by pathogen-associated molecular patterns (PAMPs) (3), initiating the activation of intracellular signaling pathways that increase expression and release of chemokines, cytokines, and antimicrobial peptides (2). Proinflammatory cytokines, such as interleukin 8 (IL-8) and tumor necrosis factor alpha (TNFα), initiate the inflammatory response by recruiting and activating leukocytes (4). Furthermore, antimicrobial peptides, like serum amyloid A3 (SAA3), β-defensins and cathelicidins, directly attack bacteria in the lumen of the mammary gland (5–7).

The innate immune response of the bovine mammary gland to different bacterial species varies, most notably between *Staphylococcus aureus* and *Escherichia coli* (8). This differential response is dictated by a pathogen-specific activation of bMEC, since challenge of bMEC, both *in vitro* and *in vivo*, with *S. aureus* results in a weak transcription response compared to challenge with *E. coli* (9,10). This might be explained by the large number of immune evasion molecules produced by *S. aureus*, several of which affect host recognition of the bacteria (11). For instance, the exo-protein Staphylococcal superantigen-like protein (SSL) 3 binds to the extracellular part of TLR-2 without activating the cell (12). Moreover, *S. aureus* reduces contact between PAMPs and host PRRs by covering its surface with capsular polysaccharides (CPs) (13) and wall teichoic acids (WTAs) (14). Experimental overexpression or deletion of these immune evasion factors affects the virulence of *S. aureus* (12,14).

Staphylococcus aureus responsible for IMI in cattle predominantly belong to ruminant-associated clonal complexes (CCs), such as CC151, CC97, CC479, and CC133 (15,16) and recently, it was reported that the *in vitro* bMEC response to *S. aureus* differs between some of these CCs (17,18). Strains belonging to CC151 induce a lower release of proinflammatory cytokines from bMEC than CC97, CC71 and sequence type (ST) 136 *S. aureus* (17,18). Whereas most CCs used in these *in vitro* studies were bovine-associated, little is known about CCs of *S. aureus* associated with mastitis in small ruminants, such as CC133, CC130 and CC425 (19,20). It is known that *S. aureus* can jump species barriers, and cause disease in a new host species

(21). It is important to understand the processes that enable *S. aureus* to switch hosts in order to find new targets to control infections (22). Small ruminant associated *S. aureus* have been shown to occasionally infect cattle (16), but it is unknown to what extent the immune response to these lineages differs from infections with bovine associated *S. aureus* lineages. Clonal complex 479 has been associated with severe bovine mastitis cases (16,23), hence, the effects of CC479 strains on bMEC, which have not been studied thus far, may yield valuable insights into the pathogenesis of this lineage. Since the strength of proinflammatory responsiveness of bMEC towards invading pathogens is expected to influence the course and outcome of IMI, increased understanding of the interaction between *S. aureus* belonging to different CC and bMEC during IMI can give insight in the variable pathogenicity of *S. aureus* lineages in bovine mastitis.

In the present study, the potential of four *S. aureus* CCs, associated with bovine and small ruminant mastitis, to activate bMEC was investigated. The spontaneously immortalized bMEC line PS was used as a model for bMEC. This cell line expresses the same PRRs as primary bMEC, has a stable cell morphology and responds to artificial TLR-2 ligands Pam2, Pam3 and control PAMPs (lipopolysaccharides, lipoteichoic acid), making it a useful surrogate for primary bMEC (24). To validate our assay, we used genetically modified *S. aureus* lacking factors (WTA, CP and protein A) expected to affect recognition of *S. aureus* by bMEC.

Materials and Methods

PS Cell Culture Conditions

The PS cell line, a spontaneously immortalized cell line originating from cultured primary bMEC that was kindly donated by Dr Pascal Rainard and Dr Pierre Germon of the French National Institute for Agricultural Research, was used as a model for bMEC (24). PS cells were cultured in growth medium (GM) consisting of DMEM/F12 medium (Thermofisher, Waltham, MA, USA) containing 1 µg/mL hydrocortisone (Merck, Kenilworth, NY, USA), 10 ng/mL insulin-like growth factor 1 (IGF-1) (Peprotech, London, UK), 5 ng/mL fibroblast growth factor (FGF) (Peprotech), 5 ng/mL epidermal growth factor (EGF) (Merck), 20 mM HEPES (Merck) and 2 mM Glutamax (Thermofisher).

Bacteriological Culture

A panel of *S. aureus* isolates (n = 35) obtained from cases of ruminant mastitis in the Netherlands was used. Isolates were randomly selected from an in-house *S. aureus* collection, previously described by Hoekstra *et al*. (20,21), to represent the bovine-associated CCs 151 (n = 7), 479 (n = 9) and small ruminant-associated CCs 133 (n = 15) and 425 (n = 4). In addition, a set of genetically modified and corresponding wildtype (wt) *S. aureus* strains (**Table 1**) were selected to validate the PS cell system. Strain RN4220 Δ*tarO* (belonging to CC8) lacks an essential gene for WTA synthesis, resulting in the absence of WTA, and RN 4220 Δ*tarS*/Δ*tarM* lacks genes required for glycosylation of WTA (25). These isolates were kindly donated by Prof. Andreas Peschel, University Tubingen, Germany. The wt Reynolds strain (belonging to CC25) carries the CP type 5 (*cap5*) gene, which is replaced by CP type 8 gene (*cap8*) in the genetically modified Reynolds *cap8* strain and deleted in the Reynolds Δ*cap5* strain (26). These isolates were kindly donated by Jean C. Lee, Division of Infectious Diseases, Brigham and Women's Hospital, Boston. In the Newman Δ*spa* strain (belonging to CC8), the gene coding for surface protein A (*spa*) is deleted (27), and these strains were kindly donated by Prof. Jan Maarten van Dijl, University Medical Center Groningen, The Netherlands.

Bacteria, from glycerol stocks, were cultured overnight at 37 °C on blood agar plates. Several colonies were picked and washed in 50 mL PBS. After centrifuge (4000× g for 10 min), bacteria were resuspended in 5 mL of stimulation medium (SM), consisting from DMEM/F12 medium (Thermofisher) containing 20 mM HEPES (Merck)

Table 1. List of genetically modified *S. aureus* strains and their corresponding wildtype (wt) used in this study.

Strain Name	CC[1]	Relevant Characteristics	Reference
Reynolds *cap5*	25	wt Reynolds strain, expressing *cap5*	(26)
Reynolds *cap8*	25	Substitution of *cap5* region with the cap8 region	(26)
Reynolds Δ*cap5*	25	Deletion of *cap5*, capsular polysaccharide negative strain	(26)
Newman wt	8	wt Newman strain	(27)
Newman Δ*spa*	8	Deletion of *spa* gene	(27)
RN4220 wt	8	wt RN4220 strain	(25)
RN 4220 Δ*tarO*	8	Deletion of *tarO*, essential for wall teichoic acid (WTA) synthesis	(25)
RN 4220 Δ*tarS* / Δ*tarM*	8	Deletion of *tarS* and *tarM*, responsible for WTA glycosylation	(25)

1. Clonal Complex

and 2 mM Glutamax (Thermofisher). The number of colony forming units (CFU)/mL was estimated using the optical density (OD) at 660 nm, based on the conversion OD660 of 0.5 equals 5×10^8 CFU/mL, which was confirmed by plating and counting bacterial suspensions prior to performing the assays. Bacterial suspensions were diluted further to the appropriate CFU/mL for stimulation experiments.

PS Cell Responses to *Staphylococcus aureus*

To study the bMEC responses towards *S. aureus*, we used 10^5 PS cells at passage number 13, which was the earliest passage we could use to obtain enough cells to perform all experiments. These cells were seeded in a volume of 1 mL per well in a 24-well plate and cultured in GM until they formed an approximate 80% confluent monolayer. They were then cultured overnight in SM. During these periods, the cells formed a monolayer, but did not show growth in number of cells, so we assumed the numbers of cells to have remained 10^5 per well. After two washing steps with phosphate-buffered saline (PBS) (Merck), PS cell monolayers were exposed to wt or genetically modified *S. aureus* in SM at a multiplicity of infection (MOI) of 160, by adding 1.6×10^7 bacteria. The MOI was based on pilot experiments that demonstrated that at this multiplicity, PS cells have the strongest IL-8 response to *S. aureus* in the absence of cytotoxic effects, as assessed by trypan blue staining and microscopy examination. The same maximum MOI for stimulating PS cells with *S. aureus* was also found by Deplanche *et al*. (28). After three hours, bacteria were removed by washing cells two times with PBS, followed by incubation in 0.5 mL SM containing 100 µg/mL streptomycin and 100 I.U./mL penicillin to prevent growth of bacteria. After five hours, the supernatant of each well was collected and stored at −20 °C. Supernatant of unstimulated cells was used as negative control. As a positive control, cells were exposed to 100 ng/mL of the synthetic TLR2 agonist Pam2-CSK4 (InvivoGen, San Diego, CA, USA). For each isolate, the stimulation assay was performed in triplicate.

IL-8 ELISA

IL-8 production by PS cells was measured by ELISA designed using the bovine IL-8 development kit (MabTech, Nacka Strand, Sweden). ELISA 96 wells microplates (Corning Inc., Corning, NY, USA) were coated overnight at 4 °C using 50 µL of 0.5 µg/mL monoclonal antibody MT8H6 diluted in PBS. After a washing step with 300 µL 0.25% Tween20 in PBS, plates were blocked using 200 µL blocking reagent (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Next, 100 µL of supernatant or IL-8 standards were added to 96 well plates and were incubated under agitation at room temperature for 1 h. Following a washing step with 300 µL 0.25% Tween20 in PBS, 50 µL of biotinylated monoclonal antibody 26E5 (0.1 µg/mL diluted in blocking re-

agent) was added. Following 1 h of incubation at room temperature, the plate was washed again and 100 µL of Streptavidin-HRP (1:2000 dilution in blocking reagent) (BD Biosciences, Franklin Lakes, USA) was added. After 1 h of incubation and a final washing step, 100 µL of HRP substrate 3,3',5,5'-tetramethylbenzidine (Merck) was added and the reaction was stopped after 15 min using H_2SO_4. Extinctions (450 nm) were measured on a Microplate Reader. Using a standard curve, the IL-8 levels in supernatant were extrapolated using GraphPad Prism version 7 (GraphPad Software, La Jolla, CA, USA). The detection range of the bovine IL-8 ELISA was 2–250 pg/mL.

Total RNA Extraction and Reverse Transcription

Transcription levels of *TNFα*, *SAA3*, *NF-κB* and *TLR-2* genes of PS in response to randomly selected CC151 (n = 4), CC479 (n = 4) and CC133 (n = 4) isolates were measured using quantitative real-time PCR (qPCR). For these experiments, 2×10^5 cells per well were seeded in a 12-well plate and cultured until 80–90% confluence. Stimulation with bacteria was performed using the same protocol and volumes as described in Section 2.3. Five hours after removal of bacteria, RNA was extracted directly from monolayers within the 12-well plates using the RNeasy Micro Kit (QIAGEN, Venlo, The Netherlands) according to the manufacturer's instructions and converted to cDNA using the iSCRIPT cDNA Synthesis Kit (BioRad, Hercules, CA, USA). Primers for genes of interest (*IL-8*, *TNFα*, *SAA3*, *NF-κB*, *TLR-2*) were taken from literature and are displayed in **Table 2**. Quantitative real-time PCR (qPCR) Master-mix was prepared as follows: 5 µL Iq Sybr green Master-mix (Biorad), 1 µL of forward and reverse primers each (final concentration 100 nM), 1 µL of demineralized water and 2 µL of 1:20 diluted cDNA. For qPCR, a three-step PCR protocol was used: an initial 10 min denaturation at 95 °C, followed by 40 cycles with 15 s of denaturation at 95 °C, 30 s annealing at primer specific temperature, and 30 s of elongation at 72 °C. Fluorescence was detected after each cycle. At the end of the first PCR the melting curve of the PCR product was determined for each primer set to verify the specificity of the PCR reaction, but not in subsequent PCRs using the same primer set. A 10-fold serial dilution of a positive control sample was run in each reaction to estimate the efficiency of the qPCR and the relative gene transcription was calculated using the comparative Ct method.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7 software. Each isolate was tested in triplicate and genetically modified strains and their wt counterpart in quadruplicate. Supernatant of each replicate was measured twice by ELISA. For statistical testing, averaged IL-8 levels in supernatant were log10 transformed and analyzed by ANOVA followed by Tukey's Multiple comparison (TMC) test. When

only two groups were available, the Student's T test was performed. Correlations between IL-8 protein production levels and IL-8 gene transcription, and between log-transformed *IL-8*, *TNFα*, *NF-κB* and *TLR-2* gene transcription were analyzed using Pearson's correlation coefficient. A significance level of 0.05 was used for all tests.

Table 1. Sequences, product size and annealing temperature of primers for genes representing activation of bMEC

Gene	Primer Sequence	Product Size (bp)	Annealing Temperature (°C)	Reference
IL-8	Fwd: ATGACTTCCAAGCTGGCTGTTG	149	60	(29)
	Rev: TTGATAAATTTGGGGTGGAAAG			
TNF-α	Fwd: CCACGTTGTAGCCGACATC	155	60	(29)
	Rev: CCCTGAAGAGGACCTGTGAG			
SAA3	Fwd: CTTTCCACGGGCATCATTTT	188	60	(30)
	Rev: CTTCGGGCAGCGTCATAGTT			
NF-κβ	Fwd: CTGGAAGCACGAATGACAGA	179	60	(31)
	Rev: GCTGTAAACATGAGCCGTACC			
TLR-2	Fwd: CATTCCCTGGCAAGTGGATTATC	201	62	(29)
	Rev: GGAATGGCCTTCTTGTCAATGG			
Ubiquitin	Fwd: AGATCCAGGATAAGGAAGGCAT	198	62	(29)
	Rev: GCTCCACCTCCAGGGTGAT			

Results

WTA and CP, but not Protein A, Modulate Activation of PS Cells

PS cells released IL-8 (37.7 ± 14.9 pg/mL; n = 8) in response to incubation with the synthetic TLR-2 agonist Pam2. The wt strains RN4220 (5.9 ± 4.9 pg/mL), Newman (5.6 ± 1.8 pg/mL) and Reynolds *cap5* (8.0 ± 4.0 pg/mL) induced a comparable IL-8 release from PS cells (**Figure 1**). The IL-8 release from PS cells after stimulation with Newman Δ*spa* strain (8.4 ± 6.4 pg/mL) and its wt counterpart (**Figure 1A**) did not differ. Stimulation with the genetically modified Reynolds *cap8* (3.0 ± 1.2 pg/mL) and Reynolds Δ*cap5* (2.2 ± 0.4 pg/mL) induced weaker IL-8 releases (**Figure 1B**) compared to Reynolds *cap5* wt (ANOVA; p = 0.004). Stimulation with the WTA deficient RN4220 Δ*tarO* (122.6 ± 27.3 pg/mL) and RN 4220 *tarS*/*tarM* (36.8 ± 10.7 pg/mL) strains resulted in higher IL-8 production by PS cells than the RN4220 wt (ANOVA; p < 0.001, **Figure 1C**).

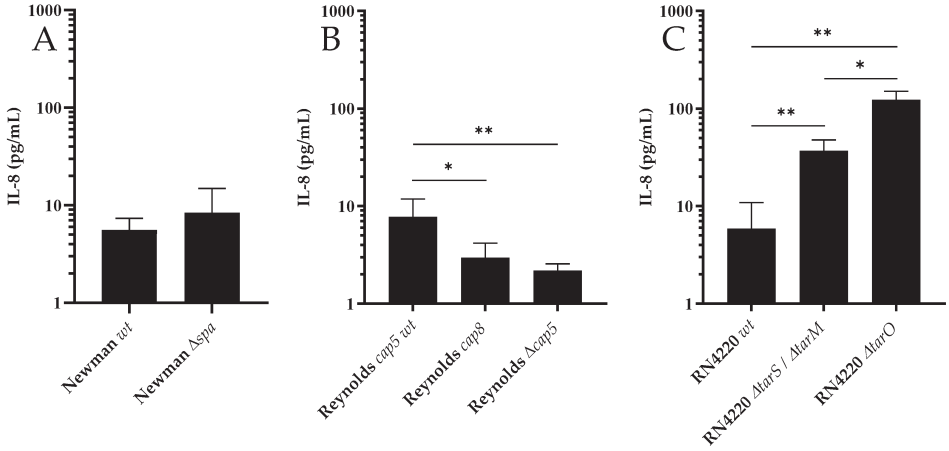

Figure 1.
Interleukin 8 (IL-8) production by PS cells after stimulation with wt and genetically modified strains to investigate the effect of protein A (**A**), capsular polysaccharides (**B**) and wall teichoic acid (WTA) (**C)** on bovine mammary epithelial cells (bMEC) activation. Supernatant was collected 5 h after the end of bacterial stimulation. For each bacterial strain, stimulation was performed four times and each supernatant was measured twice by ELISA. Bars show average IL-8 production ± SD. Levels of IL-8 were log10 transformed and analyzed using one-way ANOVA followed by Tukey's multiple comparisons test or the Student's T test (** $p < 0.01$, * $p < 0.05$).

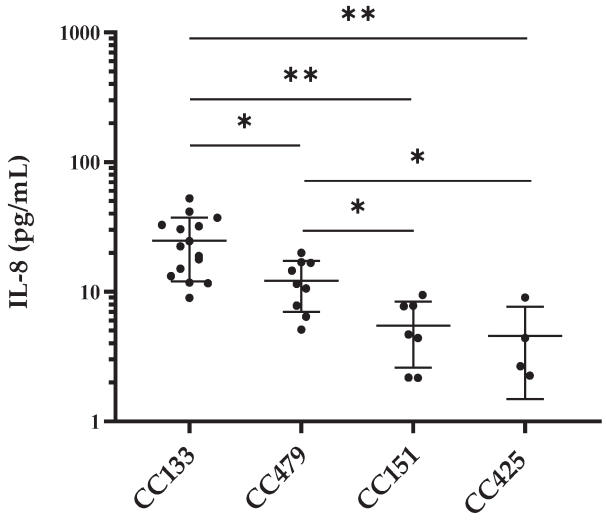

Figure 2.
IL-8 production by PS cells in response to stimulation with ruminant-associated *S. aureus* isolates, belonging to CC133 (n = 15), CC479 (n = 9), CC151 (n = 7) and CC425 (n = 4). Each dot provides average production of three culture replicates. Each replicate was measured twice by ELISA. Bars show average IL-8 production ± SD. Levels of IL-8 were log10 transformed and analyzed using one-way ANOVA followed by Tukey's multiple comparisons test (** $p < 0.01$, * $p < 0.05$).

IL-8 Production by PS Cells Stimulated with *S. aureus* Isolates

Significant differences in IL-8 release were observed after stimulation of PS cells with *S. aureus* isolates belonging to CC133 (n = 15), CC479 (n = 9), CC151 (n = 7) and CC425 (n = 4) (ANOVA; p <0.0001). CC133 isolates induced a stronger IL-8 release (24.7 ± 12.7 pg/mL) than CC479 (12.2 ± 5.1 pg/mL), CC151 (5.5 ± 2.9 pg/mL) and CC425 (4.6 ± 3.1 mL) (**Figure 2**). Furthermore, the release of IL-8 was higher after stimulation of PS cells with CC479 compared to CC151 and CC425.

Transcription of Genes by PS Cells Following Stimulation with *S. aureus* Isolates

IL-8 production was used as the primary read out parameter for bMEC activation. To further investigate whether IL-8 production after activation of PS cells by *S. aureus* is representative for bMEC activation and the ensuing proinflammatory response in general, the gene transcription levels of *IL-8* and *TNFα*, *SAA3*, *NF-κB* and *TLR-2* genes were measured after exposure to 12 *S. aureus* isolates as representatives of the three CCs most relevant in ruminants (CC133, CC479, CC151). The association between mRNA transcription levels and IL-8 protein release was quantified. Indeed, release of IL-8 was significantly correlated with relative transcription of *IL-8* (Pearson r = 0.81; p = 0.001), *SAA3* (r = 0.86; p < 0.001) and *NF-κB* (r = 0.78; p = 0.02). Furthermore, the transcription of *NF-κB* was correlated with the transcription of *IL-8* (r = 0.83; p < 0.001) and *SAA3* (r = 0.82; p = 0.001). The release of IL-8 protein by PS cells in response to CC133 (36.3 ± 23.5 pg/mL) was slightly higher, although not significantly, than CC151 (6.4 ± 3.2 pg/mL) (ANOVA; p = 0.055) (Figure 3A). Relative transcription levels were not significantly different between PS cells stimulated with isolates of different CCs for *IL-8* (ANOVA; p = 0.34), *TNFα* (ANOVA; p = 0.46), *SAA3* (ANOVA; p = 0.10), *NF-κB* (ANOVA; p = 0.41) and *TLR-2* (ANOVA; p = 0.75) (**Figure 3**).

Discussion

During IMI, bMEC are amongst the first cells to come in contact with invading microorganisms. They are crucial for activation of the early innate immune response of the mammary gland and upon recognition of pathogens, bMEC upregulate the expression and release of proinflammatory cytokines (2). We investigated the potency of four *S. aureus* CCs, commonly associated with ruminant mastitis, to activate the bMEC cell line PS.

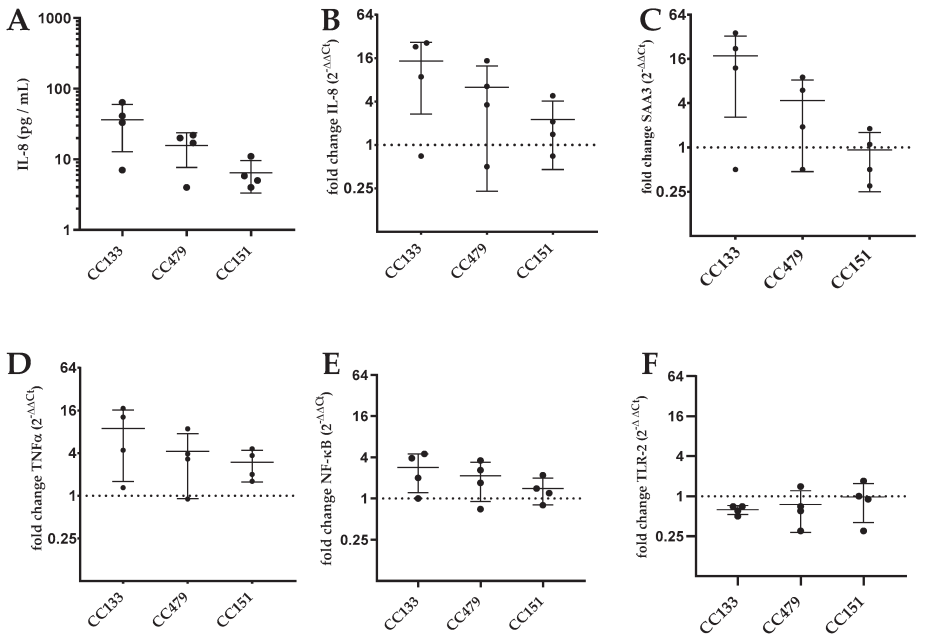

Figure 3.
IL-8 production (**A**) and relative transcription of *IL-8* (**B**), serum amyloid A3 (*SAA3*) (**C**), tumor necrosis factor alpha (*TNFα*) (**D**), *NF-κB* (**E**) and Toll-like receptor (*TLR*)-2 (**F**) after stimulation of PS cells with isolates belonging to CC133 (n = 4), CC479 (n = 4) and CC151 (n = 4). For each isolate, the assay was performed in triplicate. IL-8 protein was measured in duplicate by ELISA and transcription of genes by quantitative real-time PCR (qPCR) in triplicate. Relative transcription ($2^{-\Delta\Delta Ct}$) was normalized to negative controls and corrected for the reference gene ubiquitin. Log10 transformed IL-8 concentrations and relative transcription were analyzed using one-way ANOVA.

Using wt and genetically modified *S. aureus* strains, we first validated that our bMEC model could detect variation in *S. aureus* PAMPs. The bMEC activation by *S. aureus* belonging to CC133, CC479, CC151 and CC425 was investigated and there were significant differences in PS cell response between *S. aureus* CCs, most notably CC133 inducing stronger IL-8 release than other CCs.

The first step in the activation of bMEC during IMI is detection of bacterial PAMPs by PRRs (2). The immortalized bMEC line PS expresses TLR-1,2,6 and NLR-1,2 (24), which recognize Staphylococcal PAMPs, such as peptidoglycan, lipoteichoic acid, lipoproteins and phenol-soluble modulins (PSMs) (3). As expected, stimulation with the synthetic TLR-2 agonist Pam2 triggered IL-8 release by PS cells. Since intracellular signaling pathways that are activated by PRRs (mitogen-activated protein kinase (MAPK), NF-κB pathway) increase expression of multiple proinflammatory cytokines (32), we used IL-8 production as a proxy reflecting general activation of PS cells.

Using genetically modified *S. aureus* strains, we found that absence of WTA synthesis (RN4220 Δ*tarO*) and glycosylation (RN4220 Δ*tarS*/Δ*tarM*) in *S. aureus* increases IL-8 release after exposure of PS cells to the pathogen. Similar to peptidoglycan and lipoteichoic acid, WTA is part of the *S. aureus* cell wall but it does not trigger a proinflammatory response from monocytes, and likely also not from bMEC (33). A possible explanation for increased bMEC activation in response to WTA deficient strains is that the lack of WTA in the outer layer of the cell wall makes the underlying cell wall components more easily accessible for recognition by PRRs. The Reynolds *cap5* wt strain triggered a higher IL-8 response from PS cells than the Reynolds *cap8* or Δ*cap5* strains. Binding of both Staphylococcal CP5 and CP8 to MEC triggers IL-8 release (34), which could explain the decreased IL-8 response to Δ*cap5* compared to *cap5* wt. However, this does not explain the differential response of PS cells to *cap5* and *cap8* strains. Previous work demonstrated that the Reynolds *cap5* strain is more resistant to neutrophil killing than the Reynolds *cap8* strain (26), suggesting differences in host recognition of CP type 5 and CP type 8. We did not find an effect of deletion of *spa* in *S. aureus* on PS cell activation. In an assay employing HEK293 cells transfected with TLR2, Hilmi *et al.* (35) reported a reduced TLR-2 activity after challenge with a *spa* deletion mutant compared to the wildtype strain. However, they also report that factors other than *spa* also control the TLR2 activity. Since the decrease in IL-8 release in this study was relatively small and many factors can affect bMEC activation (36), it is possible that our PS assay was not sensitive enough to detect an effect of *spa* deletion on bMEC activation. Alternatively, protein A may not affect PRR recognition in bMEC. Overall, our experiments using genetically modified strains demonstrated that the PS cell assay is capable of detecting differences in PAMP expression and/or factors that mask PAMPs, and therefore is a suitable model to investigate bMEC activation by ruminant-associated *S. aureus* lineages.

Stimulation with CC133 isolates resulted in a higher IL-8 release than all other CCs tested and the IL-8 release triggered by CC479 isolates was higher than that by CC151 or CC425 isolates. Of these selected CCs, only bMEC activation by *S. aureus* belonging to CC151 has been subject of previous studies (17,18). Similar to our results, CC151 induced a weak cytokine response by both primary bMEC (17) and the immortalized MEC line MAC-T (18) compared to CCs included in those studies, CC71, CC97 and ST136. Variation in bMEC responsiveness to *S. aureus* may depend on either the host MEC or on genetic differences between bacteria. Therefore, we used the bMEC model PS to quantify the bMEC response induced by *S. aureus*. Thus, the observed differential bMEC response depends on variation in gene carriage and/or expression levels between tested *S. aureus* lineages. Although the presence or absence of several genes can affect bMEC recognition, the exact mechanisms for the differential activation of bMEC by *S. aureus* CCs are unknown.

Clearly, our experiments showed that the absence of WTA in the bacterial cell wall of *S. aureus* increases bMEC activation and that bMEC responds differently to CP type 5 and CP type 8. The presence of these factors alone cannot explain the differential bMEC response towards CCs, since WTA is present in all *S. aureus* (37) and CCs tested in this study all have CP type 8 (19). Expression levels of CP and WTA in *S. aureus* is highly variable and are tightly controlled by multiple systems, including repressor of toxins (*rot*) and the accessory gene regulator (*agr*) system, which is divided into four separate groups (I-IV) based on polymorphisms of *agr* genes (37). In addition, these regulatory systems also control expression and release of PAMPs, such as PSMs (38), and immune evasion factors that inhibit TLR-2 activation (12). Since the CCs tested in this study belong to different *agr* types (CC133 to *agr* type I and CC151, CC479, CC425 to type II) (16,39) and CC479 *S. aureus* have been reported to carry a non-functional copy of the *rot* gene (23), differences in expression of these *agr*/*rot* controlled genes are likely and could be a possible explanation for the differential bMEC response towards ruminant-associated CCs. In addition, a previous study associated reduced bMEC activation by *S. aureus* with a negative Staphaurex latex agglutination test (SLAT) phenotype (29). Our results agree with this observation, since CC151 is SLAT negative and CC133 and CC479 are SLAT positive *S. aureus* (19,40). The SLAT is based on functionality of bacterial surface proteins that mediate adherence to host cells (40), but it is unclear if this is also related to bMEC activation.

This study focuses on differences between *S. aureus* CCs rather than differences in the host that can affect the bMEC response but, nevertheless, it is likely that host factors also affect the recognition of *S. aureus* by bMEC. Single nucleotide polymorphisms in the PRRs TLR-1 and 2 have been associated with increased risk of mastitis in cattle (41), and it is expected that SNPs in TLRs affect the recognition of pathogens. Therefore, it is a possibility that individual animals respond differently to the same bacterial strain of *S. aureus* and thus, results from this study should be confirmed in different bMEC models. Besides variation between individual animals, variation in MEC response to pathogens between different ruminant species is also expected, since TLR-2 receptors of cattle and goats share 92.5% amino acid identity, and between 85.3–95.3% for other TLR (1–10) receptors (42). We expected that bovine CCs would be more adapted to bovine PPRs, resulting in less activation of bMEC by *S. aureus* belonging to these CCs compared to *S. aureus* of small ruminant-associated CCs. Indeed, CC133 stimulation resulted in a stronger IL-8 release than the bovine-associated CCs, but surprisingly, CC425 induced only a weak response of PS cells. Therefore, we cannot conclude that small ruminant-associated *S. aureus*, in general, induces a stronger immune response in bMEC than bovine associated lineages. In a follow-up study, we will compare the response of bovine

MEC and caprine MEC to small ruminant and bovine-associated CCs of *S. aureus* to investigate the effect of the interaction between host species and *S. aureus* lineage on MEC activation.

Release of Il-8 by PS cells was used as a proxy for bMEC activation in this study, and indeed there was a correlation between IL-8 protein release and transcription levels of *IL-8*, *TNFα* and *SAA3*, genes that are all regulated by the NF-κB pathway (2). However, there were no significant differences in gene transcription levels of PS cells stimulated with a subset of CC133, CC479 and CC151 isolates. This could be attributed to the relatively small number of experiments in which expression could be measured. Due to the strong link between IL-8 protein release and gene expression, we consider the differential bMEC activation by ruminant-associated CCs from our IL-8 protein-based assay as representative of the activation of bMEC.

Since bMEC activation during IMI plays a key role in initiating the immune response of the mammary gland, the level of bMEC activation by pathogens is expected to be of major influence on the course of mastitis (8). Infections with *E. coli* trigger a strong proinflammatory response in bMEC and often result in more severe cases of mastitis than infections with *S. aureus* (8), and therefore we hypothesize that increased bMEC activation may also increase the severity of IMI by *S. aureus*. Elevated bMEC activity results in a stronger innate immune response, recruiting a higher number of neutrophils to the site of infection and increasing the clearance rate of bacteria from the mammary gland (40). Although reduced bacterial survival of *S. aureus* can be considered positive for the host, the high influx of neutrophils can also potentially harm the mammary gland due to released reactive oxygen metabolites, matrix metalloproteinase and prolonged periods of diapedesis of leukocytes through mammary parenchymal tissue (43). When the induced immune response is not effective in clearing the pathogen, the duration of the mastitis will increase and influx of leukocytes will continue, resulting in more tissue damage, and potentially a more severe mastitis. Although the level of bMEC activation by *S. aureus* likely contributes to the severity of an IMI, it is important to emphasize that the clinical severity of IMI depends on several factors, such host conditions and production of bacterial virulence factors (44). Recently, we observed that *S. aureus* belonging to CC479 are associated with clinical rather than subclinical mastitis cases in cattle (23). In the current study, CC479 isolates induced a stronger reaction from bMEC than the other bovine-associated lineage CC151, which could potentially contribute to the severity of mastitis. Future *in vivo* studies are needed to identify the contribution of bMEC activation on the pathogenesis of Staphylococcal mastitis.

In conclusion, the current study investigated bMEC activation by *S. aureus* isolates belonging to four ruminant mastitis-associated CCs (133, 479, 151 and 425) and showed that these CCs differ in their ability to activate bMEC, which may influence the outcome of IMI. Future studies are required to elucidate how the *in vitro* activation of MEC translates to the *in vivo* immune response of the mammary gland and how this affects the pathogenesis and clinical outcome of a *S. aureus* IMI.

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Conflicts of Interest: The authors declare no conflicts of interest

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CHAPTER 6

The interaction of a host-associated and non-host-associated *Staphylococcus aureus* strain with primary bovine and caprine mammary epithelial cells

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Abstract

Intramammary infections with *Staphylococcus aureus* are a common cause of mastitis in both dairy cows and goats. In general, clinical mastitis caused by *S. aureus* in goats occurs less frequent in cows, but the disease is often much more severe in goats. Although strains of *S. aureus* responsible for mastitis in cows and goats tend to belong to different clonal complexes (CC), it is unclear whether the difference in disease severity between these species depends on the bacterial strain, host species or their interaction. A critical step in the interaction between host and pathogen during intramammary infection is the sensing of bacteria by mammary epithelial cells (MEC) and the subsequent release of proinflammatory cytokines. We hypothesized that host-adapted *S. aureus* trigger a weaker immune response than other lineages, because a weaker immune response favors survival of the host adapted strain. Previously, we demonstrated that the small ruminant-associated CC133 triggered a stronger response from bovine MEC than the bovine-associated CC151 and CC479. However, since CC425, also primarily associated with small ruminants and wildlife, induced a weaker response of bovine MEC than CC133, it is unclear whether host-associated *S. aureus* are weaker activators of MEC of their natural host. Therefore, we compared the response of three primary bovine MEC and three caprine MEC lines towards a bovine (CC479-t543) and caprine-associated (CC133-t544) *S. aureus* strain to investigate if there is a differential response of MEC to a host-associated and non-host-associated *S. aureus* strain. Cells were stimulated with live bacteria for three hours and the relative transcription levels of *IL-8*, *TNFα*, *SAA3, NF-κβ* and *TLR-2* relative to those in unstimulated cells were measured by qPCR at three hours and twenty-four hours after the end of stimulation. There were no significant differences in transcription between the four experimental groups (bMEC-CC133, bMEC-CC479, cMEC-CC133 and cMEC-CC479) at both timepoints and a principal component analysis performed on the designated transcription profile of the four groups at three hours after exposure showed no differences between groups. However, after twenty-four hours, the transcriptional profile of cMEC-CC133 was separated from the other groups in the PCA and this response of cMEC-CC133 was weaker than of the other groups. This suggests that this goat-associated *S. aureus* strain elicits a weaker response from cMEC than the cow-associated CC151 strain, whereas bMEC responded similarly to goat- and cow-associated strains. As only two *S. aureus* strains were tested on a limited number of primary MEC lines, further research is needed to substantiate our findings.

Introduction

Mastitis, inflammation of the mammary gland, is a major problem for the dairy industry since it impacts animal health and welfare, and is responsible for economic losses due to reduced milk production and quality, costs of treatment and the culling of animals (1,2). Intramammary infections (IMI) with bacteria are the predominant cause of mastitis (3) and *Staphylococcus aureus* is a common causative pathogen in both dairy cows and goats (4,5). These infections are often subclinical and chronic in cows, but can also result in clinical cases of mastitis (6). In contrast, *S. aureus* is mostly associated with clinical mastitis rather than subclinical mastitis in goats and these clinical cases are often more severe than in cows (3). Strains of *S. aureus* responsible for IMI in cows and goats generally belong to different clonal complexes (CCs), with CC151, CC97 and CC479 being associated with mastitis in cows and CC133, CC130 and CC522 with mastitis in goats (7,8). It is unclear whether the difference in disease severity between goats and cows depends on the infecting *S. aureus* strain, the host species, or their interaction (3).

A critical phase in host-pathogen interaction during IMI is the sensing of bacteria by mammary epithelial cells (MEC) (9). These cells detect pathogen-associated molecular patterns (PAMPs) using pathogen recognition receptors (PRRs) and upon activation of these receptors, MEC release proinflammatory cytokines (IL-8, TNFα), chemokines and antimicrobial proteins (Serum amyloid A3 (SAA3)) under the control of the NF-κβ signaling pathway (6). The PRRs Toll-like receptor (TLR)1, TLR-2, TLR-6 and nucleotide-binding oligomerization domain-like receptor (NLR) 2 are activated by Staphylococcal PAMPs (10). The strength of the proinflammatory response of MEC induced by *S. aureus* is weak compared to other bacterial species (5,11) and in addition, it has been identified that *S. aureus* CCs differentially activate bovine MEC (12,13). Previously, we reported that CC133 *S. aureus* strains triggered a stronger IL-8 release from the immortalized bovine MEC line PS compared to CC151, CC479 and CC425 (14). Whilst CC151 and CC479 are considered cow-associated CCs (7), CC133 and CC425 are primarily associated with small ruminants (7,15) and wildlife species (16). These findings suggest that the MEC response to *S. aureus* depends on CC, but we did not show that host-associated CCs elicit a weaker response in general than CCs associated with a different host species. However, our previous study was performed using a single immortalized bovine MEC line (14), which does not reflect variation between individuals in their response. Neither was the potential species difference, both the origin of *S. aureus* isolates and the responding host, addressed. In this study, we use primary MEC derived from goats and cows to study the effect of the interaction of host-associated and non-host-associated *S. aureus* with caprine and bovine MEC on transcription levels of *IL-8*, *TNFα*, *NF-κβ*, *SAA3* and *TLR-2*.

Materials and Methods

Isolation of primary mammary epithelial cells

Mammary tissue was obtained from three freshly euthanized female goats housed at Wageningen Bioveterinary Research, Lelystad, The Netherlands that were sacrificed for study of Baliu-Piqué *et al.*, 2018 (17). Tissue samples were transported to our lab on ice in Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich, St. Louis, MO, USA). Upon arrival, samples were briefly submerged in 70% ethanol to disinfect the tissue. After removal of fatty tissue, samples were minced into small cubes of < 5 mm and incubated under agitation for 10 minutes in HBSS containing 100 I.U./mL penicillin and 100 µg/ mL streptomycin. After washing twice with HBSS, tissue cubes were centrifuged at 300g for 5 minutes and resuspended in HBSS containing 200 U/ mL collagenase type IV (Thermofisher Scientific, Waltham, MA, USA). Digestion was performed at 37 °C with agitation, and single cells were collected after 60 and 120 minutes using a 70-µm cell strainer (Corning, Corning, NY, USA). The obtained cell suspensions were centrifuged at 300g for 5 minutes and, after two washing steps in HBSS, resuspend in 6 mL of growth medium (GM), consisting of Advanced DMEM/ F12 medium (Thermofisher Scientific) containing 1 µg/mL hydrocortisone (Merck, Kenilworth, NY, USA), 10 ng/mL insulin-like growth factor 1 (IGF-1) (Peprotech, London, UK), 5 ng/mL fibroblast growth factor (FGF) (Peprotech), 5 ng/mL epidermal growth factor (EGF) (Merck), 100 µg/mL of streptomycin, 100 I.U./mL of penicillin and 20 mM HEPES (Merck) and 2mM Glutamax (Thermofisher Scientific). Cells were incubated in six-well plate systems at 37°C in a humidified atmosphere containing 5% CO2. After 2-4 days of culture, MEC were enriched by selective detachment of fibroblasts with 0.25% trypsin for 3-4 minutes (Thermofisher Scientific). Fresh medium was added to MEC still attached to the plate surface and were allowed grow further. Selective trypsinization was performed three times. Cells were frozen at passage 1,2 and 4 in freezing medium, that consisted out of 60% Advanced DMEM/F12, 30% fetal bovine serum and 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich), after being detached from the plate surface using 0.25% trypsin for 4-6 minutes. Aliquots of frozen cells were stored at -140 °C before being used in further experiments. The three cMEC lines were labeled as cMEC 0703, cMEC G2709 and cMEC G2909.

For the bovine MEC lines, mammary tissue originating from three cows was obtained from a slaughterhouse in The Netherlands, quickly after exsanguination. After transport on ice in HBBS, tissue was cleaned with 70% ethanol and outer layers of sample were cut to reduce chance of contamination. The sample was then fully submerged into 70% ethanol, after which mincing, primary cell isolation and culturing was performed as described above. The bovine MEC lines were labeled as bMEC B1, bMEC B2 and bMEC B3.

Staphylococcus aureus culture conditions

The two *S. aureus* strains used in this study were obtained from a case of bovine (CC479-t543) and caprine (CC133-t544) clinical mastitis in The Netherlands (15,18). Bacterial culture and preparation for stimulation experiments were performed as described by Hoekstra *et al.* 2019 (14). Briefly, bacteria, from glycerol stock, were cultured overnight on blood agar plates and several colonies were picked and washed in phosphate-buffered saline (PBS) buffer (Thermofisher Scientific). After centrifugation, the bacteria were resuspended in stimulation medium (SM), Advanced DMEM/F12 medium (Thermofisher Scientific) containing 20 mM HEPES (Merck) and 2mM Glutamax (Thermofisher Scientific), and the number of colony forming units (CFU)/mL) was estimated using the optical density at 660 nm (14). Suspensions were diluted in SM to the appropriate CFU/mL for stimulation experiments.

Primary mammary epithelial cell stimulation experiments

Stimulation experiments using the two *S. aureus* strains were always performed in parallel within the same batch. Bovine (n = 3) or caprine (n = 3) MEC of passage 4 or 5 were seeded in wells of a 12 well plate systems, 2 x 10^5 cells per well in a volume of 1 mL, and cultured in GM for 24-48 hours at 37 °C with 5% CO2 until cells formed a monolayer of 80-90% confluence, corresponding to approximately 5 x 105 cells per well. This monolayer was washed 2x with PBS and incubated overnight in SM with 100 µg/mL of streptomycin, 100 I.U./mL of penicillin. The following day, SM was removed, and cells were again washed 2x with HBSS. For each individual MEC line, six wells were challenged with 1 mL of CC133 *S. aureus* suspension (approximately 8 x 10^7 CFU/mL) in SM, which corresponds to a multiplicity of infection of 160 (14)) and six well with 1 mL of CC479 *S. aureus* suspension (approximately 8 x 10^7 CFU/mL). As a negative control, MEC in four wells were incubated in SM and as a positive control, MEC in two wells were stimulated with the synthetic TLR-2 agonist Pam2 (InvivoGen, San Diego, CA, USA) in a concentration of 100 pg/mL. After 3h of stimulation, cells were washed 2 x by PBS and incubated in 1 mL of SM containing 100 µg/mL streptomycin and 100 I.U./mL penicillin to prevent growth of bacteria. After 3h and 24h, RNA was extracted for further analyses.

Total RNA extraction, reverse transcription and qPCR

For each experiment, RNA was extracted using the RNeasy Micro Kit (QIAGEN, Venlo, The Netherlands) according to the manufacturer's instructions and converted to cDNA using the iSCRIPT cDNA Synthesis Kit (BioRad, Hercules, CA, USA). Primers for the IL-8, TNFα, NF-κβ, SAA3, and TLR-2 genes were taken from literature, as shown

in **Table 1**. In view of sequence variation in the bovine and caprine TNFα gene, different primer sets were used to measure TNFα transcription by caprine and bovine MEC. The qPCRs were performed in triplicate as described by Hoekstra et al. 2019 (14) and relative transcription ($2^{-\Delta\Delta Ct}$) levels were normalized to negative controls using the comparative Ct method (19). Relative transcription was corrected for the reference gene ubiquitin and transcription levels were 2log transformed using Microsoft Excel (Microsoft, Richmond, WA, USA).

Data analysis

All data analysis was performed using R version 3.5.4 (20) and visualized using the R package *ggplot2* (21). Gene transcription levels at 3h and 24h were analyzed separately. Correlations between transcription levels of different genes were analyzed using Pearson's correlation coefficient. Transcription levels at 3h and 24h were compared using a paired t-test and relative gene transcription between groups (bMEC-CC133, bMEC-CC479, cMEC-CC133 and cMEC-CC479) was compared using ANOVA. The relative transcription levels of *IL-8*, *TNFα*, *NF-κβ*, *SAA3* and *TLR-2* were analyzed using a principal component analysis (PCA) in R and separate PCAs were performed on the transcription profile at 3h and 24h after bacterial stimulation. Plots of the first two principal components (PCs) were visualized using the R package ggbiplot (22). A significance level of 0.05 was used for all statistical tests.

Table 1. Primer Sequences, product sizes and annealing temperatures for PCR of genes representing activation of bovine and caprine MEC.

Gene	Primer Sequence	Product Size (bp)	Annealing Temperature (°C)	Reference
IL-8	Fwd: ATGACTTCCAAGCTGGCTGTTG	149	60	(23)
	Rev: TTGATAAATTTGGGGTGGAAAG			
TNF-α (bovine)	Fwd: CCACGTTGTAGCCGACATC	155	60	(23)
	Rev: CCCTGAAGAGGACCTGTGAG			
TNF-α (caprine)	Fwd: GCACTTCGGGGTAATCGGC	192	60	(24)
	Rev: GCCTTGAGGGCATTGGCAT			
SAA3	Fwd: CTTTCCACGGGCATCATTTT	188	60	(25)
	Rev: CTTCGGGCAGCGTCATAGTT			
NF-κβ	Fwd: CTGGAAGCACGAATGACAGA	179	60	(24)
	Rev: GCTGTAAACATGAGCCGTACC			
TLR-2	Fwd: CATTCCCTGGCAAGTGGATTATC	201	62	(23)
	Rev: GGAATGGCCTTCTTGTCAATGG			
Ubiquitin	Fwd: AGATCCAGGATAAGGAAGGCAT	198	62	(23)
	Rev: GCTCCACCTCCAGGGTGAT			

Results

The relative transcription of genes by MEC of the four different experimental groups at both timepoints are shown in **Figure 1A-E**. The average relative transcription of *SAA3* was higher at 24h (6.26 ± 1.32 2log $2^{-\Delta\Delta Ct}$) than at 3h (4.19 ± 1.71 2log $2^{-\Delta\Delta Ct}$; Paired t-test; p < 0. 001), but the transcription levels of *NF-κβ* decreased over time (3h: 0.45 ± 1.06 2log $2^{-\Delta\Delta Ct}$; 24h: 1.14 ± 0.45 2log $2^{-\Delta\Delta Ct}$; Paired t-test; p < 0. 05) and the transcription levels of *IL-8*, *TNFα* and *TLR-2* did not differ between timepoints. There were no significant differences in transcription levels of any of the genes between the four experimental groups (**Figure 1A-E**). The transcription levels at 3h and 24h of *IL-8*, *NF-κβ*, *TNFα*, and *SAA3* were correlated to the transcription levels of one or more other genes, but *TLR-2* transcription was not related to any of the other genes (**Supplementary Table 1**). The response of MEC lines to stimulation with Pam2 is shown in **Supplementary Table 2.**

Next, the transcription profile of the four groups was further analyzed using PCA. The first PC on the transcription profile of MEC at 3h after bacterial stimulation explained 51.4% of variation in gene transcription levels and the second PC explained 18.1% (**Figure 2A**). Transcription of *TNFα* and *SAA3* contributed most to the first PC, whereas the second PC was mostly loaded by *NF-κβ* and *TLR-2* at 3h, the experimental groups formed no separated clusters within the PCA, since all clusters overlapped (**Figure 2A**). There was more variation in the transcriptional profile of bMEC lines compared to cMEC lines, as the cMEC lines formed considerable smaller clusters than bMEC lines. At 24h after stimulation, the PCA of gene transcription levels explained 81.5% (PC 1: 60.3%; PC 2: 21.2%) of the variation gene transcription (**Figure 2B**). Transcription of *TNFα*, *NF-κβ*, *SAA3* and *IL-8* had an approximately equal contribution to the first PC and the second PC was almost entirely loaded by *TLR-2*. In this PCA, the cMEC-CC133 experimental group formed a separate cluster and had on average lower scores on PC1 and PC2 than the other groups (**Figure 2B**), corresponding to lower transcription levels in this group of the variables that loaded these PCs.

Discussion

Although *S. aureus* is a common pathogen responsible for IMI in both cows and goats, the severity of mastitis and the genetic profile of causative *S. aureus* strains differ between the two species (3). Hence, it is unclear if the differences in severity of mastitis following *S. aureus* infection between goats and cows depends on characteristics of the causative *S. aureus* strain, the host or the interaction be-

tween the two. In this study, we investigated if the MEC response towards a bovine (CC479-t543) and caprine -associated *S. aureus* (CC133-t544) strain differs between primary bovine and caprine MEC. Although there was no evidence of a specific interaction between host species and host-associated *S. aureus* at three hours after stimulation, the transcription response of cMEC stimulated with CC133 was lower than that of the other groups at twenty-four hours.

The early cytokine response of the mammary gland plays a major role in activation of the innate immune responses, since these cytokines are essential for leukocyte recruitment and activation (6). As expected, proinflammatory cytokine transcription by MEC increased after stimulation with *S. aureus* (**Figure 1A-E**). The transcription levels of *SAA3*, *IL-8* and *TNFα* were correlated (**Sup. Table 1**), in line with the fact that transcription of these genes is regulated by the *NF-κβ* activation pathway (25,26). Similar to previous work (23,27), the transcription levels of *SAA3* increased over time, whereas the transcription levels of *IL-8* and *TNFα* remained stable between 3h and 24h after *S. aureus* stimulation. This could be related to the different roles of SAA3, IL-8 and TNFα in the immune response of the mammary gland, since SAA3 acts primarily as an acute phase protein and IL-8, TNFα as proinflammatory cytokines (9,28).

Although multiple studies investigated the response towards *S. aureus* or *S. aureus* PAMPS of caprine MEC (24,29) and bovine MEC (13,23,30), this study was the first to our knowledge to directly compare the response of MEC of different ruminants towards the same *S. aureus* strains. Interestingly, our data suggest that in caprine MEC, the host-associated *S. aureus* strain used induced a lower transcription response, than the non host-associated strain. Although differences between pathogen-host interaction groups were non-significant for each of the individual transcription levels of the 5 genes used in our work, the PCA revealed that the overall transcription profile was different for the interaction between the goat-associated *S. aureus* and the goat MEC after the stimulated period. The goat MEC produced a weaker response when exposed to a goat-associated strain, which would *in vivo* likely lead to a smaller inflammatory response. This may partly explain the fact that clinical mastitis by *S. aureus* in goats is less common than in cows. The weaker response is possibly sufficient to control the infection, and this mild inflammation may not lead to clinical signs of mastitis apart from perhaps an elevated somatic cell count, as is commonly seen in goats infected with *S. aureus* (31). Occasionally, however, this mechanism may not control the infection sufficiently. In that situation, the infection may grow to large numbers of bacteria and consequently production of toxins will increase (32,33), responsible for tissue damage and therefore a much more severe clinical picture. Nevertheless, we should interpret our data with

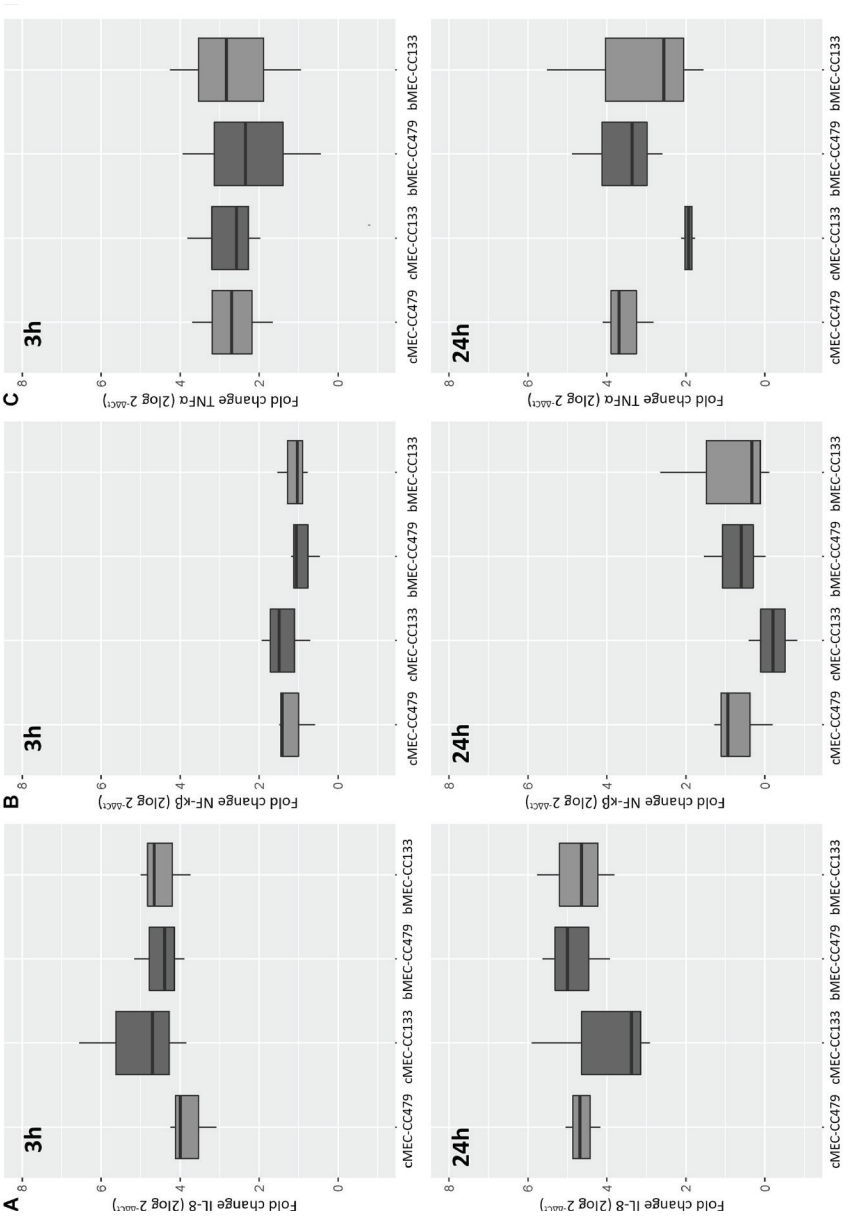
A
B
C
3h
24h
Fold change IL-8 (2log 2-ΔΔCt)
Fold change NF-κβ (2log 2-ΔΔCt)
Fold change TNFα (2log 2-ΔΔCt)
cMEC-CC479
cMEC-CC133
bMEC-CC479
bMEC-CC133
0
2
4
6
8

Figure 1.
Relative transcription levels (2log $2^{-\Delta\Delta Ct}$) of *IL-8* (A), *NF-κB* (B), *TNFα* (C), *TLR-2* (D) and *SAA3* (E) of MEC at 3h and 24h after end of bacterial stimulation. Each caprine MEC (n = 3) and bovine MEC (n = 3) line was stimulated in triplicate with 2 different *S. aureus* strains (belonging to CC133 or CC479) and RNA was extracted at 3h and 24h after stimulation. For each individual repeat, the transcription of genes was determined by qPCR in triplicate. Relative transcription was normalized to negative controls and corrected for the reference gene ubiquitin. Averaged relative transcription levels of each MEC were 2log transformed. Based on experimental conditions, samples were grouped into four different experimental groups: bMEC-CC133 (n = 3), bMEC-CC479 (n = 3), cMEC-CC133 (n = 3), and cMEC-CC479 (n = 3). Relative transcription levels between groups were analyzed using one-way ANOVA.

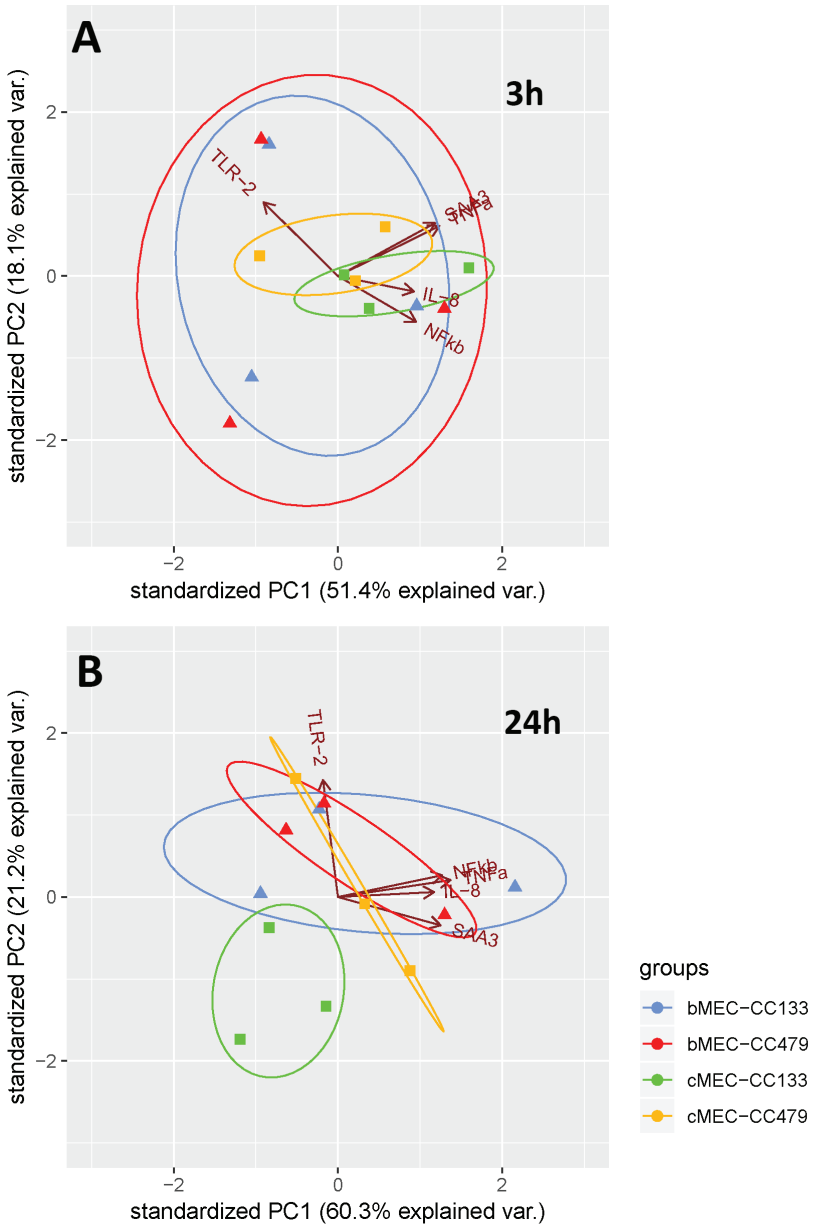

Figure 2.
Principal component analysis (PCA) of the relative transcription levels of 5 genes from four experimental groups stimulated with *S. aureus* at 3h (A) and 24h (B) after end of bacterial stimulation. Each cMEC (n = 3) and bMEC (n = 3) line was stimulated in triplicate with 2 different *S. aureus* strains (CC133, CC479) and RNA was extracted at 3h and 24h after stimulation. For each individual repeat, the transcription of genes was determined by qPCR in triplicate. Relative transcription ($2^{-\Delta\Delta Ct}$) was normalized to negative controls and corrected for the reference gene ubiquitin. Averaged relative transcription of each MEC was 2log transformed. Based on experimental conditions, samples were assigned to four different experimental groups: bMEC-CC133 (blue), bMEC-CC479 (red), cMEC-CC133 (green) and cMEC-CC479 (yellow). Figures display the first 2 PCs and percentage of explained variation per PC.

caution, as the number of cell lines used (n = 6) and the number of *S. aureus* strains tested (n = 2) were limited. Previous studies have demonstrated that differences in the cytokine response of bovine MEC to *S. aureus* CCs are relatively small and that there is considerable variation in MEC response to the same bacterial strain (12,13). Furthermore, we used multiple primary MEC lines obtained from different animals and there was considerable variation between MEC lines within the same experimental group (**Figure 1** and **Figure 2**). This could be attributed to variation in the processing and culture of MEC, but genetic differences between individual animals may also play a role. Multiple studies have demonstrated sequence variation in TLR-2 genes between individual goats and cows, with specific TLR polymorphisms being associated with increased susceptibility to bacterial infections in cows (34,35) and with SCC in goats (36). Therefore, it is likely that PRR function and consequently pathogen recognition differs between individual animals. Although the use of primary MEC is likely a better reflection of the interaction between MEC and bacteria in vivo than immortalized cell lines, it also leads to more variation and this makes it more difficult to detect differences in MEC response. Altogether, the usage of multiple primary cell lines and a low number of strains tested, lowered the statistical power of this study to detect differences between the groups. Similarly, the finding that the CC133-t544 *S. aureus* evoked a smaller response in the goat MEC, may not be reproducible in larger studies.

Follow-up studies should investigate an effect of host-adaptation on MEC activation, by comparing MEC activation by *S. aureus* strains belonging to a larger selection of cow (e.g. CC151, CC97, CC479) and goat (e.g. CC130, CC522, CC133) -associated CCs, in addition to non-ruminant-associated CCs (37). By using multiple associated CCs per host species, it may be possible to differentiate between the effect of the individual *S. aureus* CC and the host-origin of the CC on the response of MEC.

In summary, this study has shown that caprine MEC, tend to have a weaker transcription response to a goat-associated *S. aureus* strain compared a non host -associated strain, whereas bovine MEC did not differ in their response. However, due to limitations in our study design, additional studies are required to assess whether if host-associated *S. aureus* CCs differentially activate MEC of different host species.

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Supplementary Figures

Supplementary Table 1.
Pearson's Correlation Coefficients of the relative gene transcription (2log $2^{-\Delta\Delta Ct}$) of *IL-8*, *NF-κβ*, *TNFα*, *TLR-2* and *SAA3* by three caprine and three bovine MEC lines stimulated with two *S. aureus* strains and measured at 3h and 24h after stimulation

	***IL-8*[1]**	***NF-κβ***	***TNFα***	***TLR-2***	***SAA3***
IL-8		ns	0.55**	ns	0.40*
NF-κβ	ns[2]		0.53**	ns	ns
TNFα	0.55**	0.53**			0.71**
TLR-2	ns	ns	ns		ns
SAA3	0.40*	ns	0.71**	ns	

1 = Relative gene transcription (2log $2^{-\Delta\Delta Ct}$) normalized to negative controls and corrected for the reference gene ubiquitin

* = Pearson's Correlation Coefficients p value < 0.05

** = Pearson's Correlation Coefficients p value < 0.01

ns = Not significant

Supplementary Table 2.
Relative transcription (2log $2^{-\Delta\Delta Ct}$) of *IL-8*, *NF-κβ, TNFα*, *TLR-2* and *SAA3* by three caprine and three bovine MEC lines in response to 3h incubation with Pam2 at 3h and 24h after end of stimulation

MEC line[1]	Timepoint[2]	Relative transcription (2log $^{2-\Delta\Delta Ct}$)				
		IL-8[3]	*NF-κβ*	*TNFα*	*TLR-2*	*SAA3*
cMEC 0703	3h	4.25	1.59	3.09	1.36	6.58
	24h	3.36	0.73	2.46	-0.13	6.28
cMEC 2703	3h	3.69	0.50	2.09	1.20	6.58
	24h	2.49	0.50	1.49	-0.17	5.22
cMEC 2903	3h	3.82	-0.60	1.36	-1.10	6.44
	24h	4.29	NA[4]	NA[4]	NA[4]	NA[4]
bMEC 1	3h	7.84	3.62	9.04	1.16	10.07
	24h	7.24	3.55	7.18	0.37	12.96
bMEC 2	3h	5.22	0.83	5.71	3.32	6.98
	24h	3.59	-0.10	2.62	1.49	6.94
bMEC 3	3h	4.32	0.76	-0.13	-0.76	1.03
	24h	1.36	NA[4]	NA[4]	1.46	1.63

1. Mammary epithial cell obtained from primary goat (cMEC) or cow (bMEC) mammary tissue
2. Timepoint of RNA extraction after stimulation of MEC with Pam2
3. Relative gene transcription (2log $2^{-\Delta\Delta Ct}$) normalized to negative controls and corrected for the reference gene ubiquitin
4. Relative transcription not available due to technical problems with qPCR

CHAPTER 7

Summarizing Discussion

Introduction

Intramammary infections (IMI) with *Staphylococcus aureus* are a common cause of ruminant mastitis and in cattle, these infections mostly result in subclinical mastitis (SCM) but can also cause clinical mastitis (CM) (1,2). The outcome of infection is determined by environmental, host and pathogen factors, and the interaction between them (2). Since there is considerable variation in the carriage of virulence factors, and in particular those involved in immune evasion, between *S. aureus* strains (3), these strains might differ in their pathogenicity and host-pathogen interactions during ruminant mastitis. The studies described in this thesis explored the molecular epidemiology of ruminant *S. aureus* and the aim of the thesis was to assess if genetic characteristics and immune evasion strategies of *S. aureus* affect the clinical outcome of IMI. In addition, interactions between ruminant mammary epithelial cells (MEC) and *S. aureus* of different clonal complexes (CC) were also examined.

In this summarizing discussion, the following topics will be further explored. In Part 1, I will discuss the most common *S. aureus* CCs responsible for ruminant mastitis to create insight in their distribution, evolution, host species and pathogenicity. Part 2 discusses key differences in immune evasion strategies between these *S. aureus* CCs. Finally, the practical applications of these findings and directions for future research are discussed. This chapter starts off with a brief summary of the main findings of the studies described in this thesis.

Main findings of this thesis

- *Staphylococcus aureus* isolates belonging to CC479 were associated with CM rather than SCM in both a Dutch (**Chapter 2**) and European (**Chapter 3**) bovine mastitis isolate collection. A likely explanation for the increased virulence of CC479 *S. aureus* is their high production of the bovine immune evasion factor LukMF' (**Chapter 2**).

- Ruminant-associated *S. aureus* CCs differed in their carriage of immune evasion factors and had a heterogeneous spatial distribution in Europe (**Chapter 3**).

- *Staphylococcus aureus* isolated from ovine and caprine CM and SCM cases in The Netherlands belonged to similar CCs and were comparable in both LukMF' carriage and *in vitro* LukMF' production (**Chapter 4**). Infec-

tions with CC133 *S. aureus* were the dominant cause of mastitis in both sheep and goats, but different CC133 *spa*-types were associated with ovine (t2678) and caprine (t544, t3583) mastitis.

- Ruminant *S. aureus* CCs differed in their activation of the bovine MEC line PS, with CC133 inducing a stronger release of IL-8 than CC151, CC479 and CC425 (**Chapter 5**).
- A goat-associated CC133 *S. aureus* strain elicits a weaker response from primary caprine MEC than a cow-associated CC479 strain, whereas primary bovine MEC responded similarly to both these strains. (**Chapter 6**).

Part 1

Ruminant-associated *Staphylococcus aureus* clonal complexes

Staphylococcus aureus is known to colonize and infect a wide range of host species and has a clonal population structure, with certain *S. aureus* CCs being associated with specific hosts (4,5). Most animal-associated clones presumably originated from humans, and host jumps of *S. aureus* between species are followed by a subsequent host adaptive evolution (6–8). Adaptation of the bacterium to a new host occurs through multiple genetic mechanisms, such as allelic diversification, core genome mutations and the uptake of specific mobile genetic elements (MGE) (4,5). These MGEs are assumed to be present in the host's microbiota and acquisition of these elements is likely beneficial for survival of *S. aureus* (4,8). There are multiple *S. aureus* CCs that are associated with, and likely adapted to, ruminants (7,9). In the collection of European bovine mastitis isolates described in **Chapter 3**, CC151, CC97, CC133, CC479, and CC398 were the five most common CCs, together representing more than 75% of the collection. In addition, bovine CC8 isolates, representation the highly contagious genotype B (GTB) (10,11), were also present in this collection (4% of European isolates). The following section discusses the geographical spread, evolution, virulence and other key features of these six ruminant associated CCs.

CC151 and CC97 were the two most prevalent CCs in the European bovine isolate collection described in **Chapter 3**, with almost 50% of isolates belonging either to CC151 or CC97. These CCs are considered the dominant bovine *S. aureus* CCs (12), since they been cultured from mastitis cases throughout Europe (7,13,14), North and South America (15–17), Asia (18), Australia (19) and Africa (20,21). Whereas CC151 *S. aureus* have almost exclusively been isolated from cattle (22), a human-specific

CC97 clone has also been identified that resulted from a cattle-to-human host jump (23). Although CC151 and CC97 *S. aureus* share the same host specificity, phylogenetic analysis of both the core genome and accessory genome demonstrated that the genetic profile of CC151 and CC97 differs substantially (**Chapter 3**). The host switch of CC151 is estimated to have taken place around 5500 years ago (Bayesian credible intervals 95%: 3082 - 8981 years), coinciding with the expansion of cattle domestication throughout the Old World, whereas for CC97, this host switch was estimated around 1800 years ago (Bayesian credible interval 95%: 879 - 3108 years) (24). This would make CC151 the "oldest" ruminant-associated *S. aureus* CC, contributing to their wide global spread. Furthermore, this can also explain the considerable genetic diversity within these CCs, since CC151 and CC97 isolates differed substantially in their accessory genome (**Chapter 3**) (13). The studies described in **Chapter 2** and **Chapter 3** showed that the proportion of CM of SCM isolates was approximate even for both CC151 (51% CM isolates) and CC97 (42% CM isolates). However, there are indications that clones of CC151, CC97 differ in their virulence during mastitis. A recent infection study in cattle reported that animals infected with a CC151 (field) strain were more likely to develop CM compared to animals infected with CC97 *S. aureus*, that only developed SCM (25). In addition, the commonly used model *S. aureus* strain N305, belonging to CC97, is associated with mild and chronic mastitis (26), whereas the model strain RF122 (CC151) reproducibly induces severe mastitis (26,27). A likely explanation for this discrepancy between the results of our field studies and these *in vivo* infection studies, is the considerable genetic variation among CC151 and CC97 *S. aureus* isolates in our field collection. Using a single strain of CC151, CC97 might misrepresent the virulence and the outcome of infection of these CCs, and further work using a larger collection of field strains is required to determine if infections with CC151, CC97 *S. aureus* differ in their clinical outcome in cattle.

The ruminant-associated *S. aureus* CC479 is less widespread compared to CC151, CC97 since it is mostly isolated from bovine mastitis cases in North-West Europe (Netherlands, Germany, Denmark) (14,28–30). *Staphylococcus aureus* belonging to CC479 share many accessory genes with CC151 *S. aureus* (**Chapter 3**) and it is therefore likely that these CCs share a common ancestor that was already adapted to ruminants. Interestingly, there was little variation in the accessory genome of CC479 strains collected throughout Europe compared to the genetic variation within other CCs (**Chapter 3**). This, together with the limited geographical spread of this CC, suggest a rapid clonal expansion of CC479. There was an association between CC479 *S. aureus* and CM rather than SCM in both a Dutch and European collection of bovine mastitis isolates (**Chapter 2, 3**). This finding is supported by a study in cattle that reported higher clinical scores and bacterial count in animals infected with

a CC479 strain compared to CC151 *S. aureus* strains (31). Together, these findings suggest that infections with CC479 *S. aureus* are more likely to result in CM in cattle compared to other ruminant-associated *S. aureus*.

The *S. aureus* CC133 is, in contrast to CC151, CC97 and CC479, associated with multiple ruminant species, such as goats, sheep, cattle and wild ruminants, but is predominantly isolated from sheep and goats (32–34). Indeed, the study described in **Chapter 4** shows that CC133 is the dominant CC among *S. aureus* isolates collected from caprine and ovine mastitis, with more than 85% of *S. aureus* belonging to CC133. In the European bovine isolates collection, most CC133 isolates originated from Northern-Germany and Denmark, in agreement with previous studies that reported high prevalence of CC133 *S. aureus* in these areas (35,36). Although CC133 isolates from collected from cattle, goats and sheep, typing techniques with a higher resolution than multi locus sequence typing (*spa*-typing, whole genome sequencing (WGS)), demonstrated differences between CC133 isolates obtained from different host species (**Chapter 3, 4**). Among CC133 *S. aureus* obtained from Dutch goats and sheep, *spa*-type t544 was associated with caprine mastitis and t2679 with ovine mastitis (**Chapter 4**). The *spa*-type t1403 was dominant among CC133 strains collected from cattle (35). A phylogenetic tree constructed based on accessory genome of CC133 *S. aureus* obtained from bovine mastitis cases throughout Europe (**Chapter 3**) and caprine mastitis CC133 strains originating from The Netherlands (**Chapter 4**), shows that bovine and caprine CC133 form separate clusters (Sup Fig. 1). Interestingly, there was less genetic variation among European bovine CC133 isolates than among Dutch caprine CC133 isolates. This suggests that this specific bovine clone of CC133 *S. aureus* had a rapid clonal expansion among cattle. The proportion of CM of SCM isolates belonging to CC133 was even in both the bovine, caprine and ovine collection of mastitis isolates (**Chapter 3, 4**).

The CC398 has a wide host range since it has been isolated from goats, sheep, dairy cattle and veal calves but is mostly linked to pigs (37,38), and this CC is strongly associated with antimicrobial resistance (AMR), in particular to methicillin resistance (37). Therefore, CC398 is commonly referred to as livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA). Although the study described in **Chapter 3** indeed found a strong association between AMR gene carriage and CC398, only a proportion (58%) CC398 isolates were positive for the *mecA* gene and therefore not all CC398 isolates can be considered LA-MRSA. Although CC398 *S. aureus* are reported to colonize humans with close contact with animals, these colonization are often not persistent and therefore are not considered a major human health concern (39,40). The prevalence of CC398 *S. aureus* among bovine mastitis isolates was generally low but differed greatly between countries (7,30).

Whereas 7% of *S. aureus* strains in our European collection belonged to CC398, the CC was overrepresented in Poland (33% of Polish strains) and Spain (33% of Spanish strains) and together these countries contributed almost 80% of CC398 strains (**Chapter 3**). This CC is also associated with a low-within herd of prevalence of mastitis, suggesting that CC398 *S. aureus* have a low transmission rate (41), and no association was found between CC398 and clinical outcome of IMI in both dairy goats and cattle (**Chapter 2, 3, 4**).

Genotype B *S. aureus* are a ruminant-associated clone of the predominantly human-associated CC8 (8,10) and this clone is estimated to have only recently switched host from humans to cattle (8). Likely due to this recent host-switch, GTB have a limited geographical spread and are mostly isolated from bovine mastitis cases in Switzerland (42,43). The prevalence of CC8 *S. aureus* within the European isolate collection was low (4%), presumably because it contained no Swiss isolates and all CC8 isolates originated from either Germany or Italy (**Chapter 3**). Interestingly, GTB *S. aureus* are associated with high within-herd prevalence of bovine subclinical mastitis (41,44) and are therefore considered more contagious than other *S. aureus* CCs. Within the European isolate collection, CC8 tended to be more likely to originate from SCM than CM cases of bovine mastitis (**Chapter 3**).

Part 2

Immune evasion strategies of ruminant-associated *Staphylococcus aureus*

As Part 1 described, there are multiple ruminant-associated CCs of *S. aureus* and some CCs are associated with a specific clinical outcome of IMI. Variation in genetic makeup, be it in gene carriage or gene regulation, must underlay these differences in pathogenicity between *S. aureus* CCs. *Staphylococcus aureus* has access to large number of immune evasion factors, that manipulate immune defenses using different strategies (45,46). The carriage of these immune evasion factors varies greatly between *S. aureus* strains but is strongly associated with CC (47,48). Therefore, ruminant-associated CCs vary in their immune evasion strategies and in the following section, I describe how these CCs differ in their ability to 1) induce a cytokine response from MEC, 2) kill neutrophils using leukocidin LukMF', 3) form bacterial biofilms, 4) modulate the immune response by Superantigens (SAs) and Staphylococcal superantigen-like proteins (SSLs) and how this influences the pathogenicity of the bacterium during mastitis.

Avoiding detection: differential MEC activation by ruminant-associated *Staphylococcus aureus*

One of the first steps in activation of the immune responses of the mammary gland is the detection of microorganisms by MEC (49). When activated by pathogen associated molecular patterns (PAMPs), these cells release proinflammatory cytokines that active and recruit leukocytes to the mammary gland (49). This cytokine release by MEC in response to *S. aureus* is generally weak compared to release due to other mastitis pathogens, such as *Escherichia coli* (50,51). Ruminant-associated *S. aureus* also differ in their ability to trigger a response from MEC (52,53). The study described in **Chapter 5** reported that activation of the bovine MEC model PS was CC-dependent, with CC133 *S. aureus* inducing a higher release of IL-8 from CC479, CC151 and CC425 isolates. Although the differences in induced cytokine release between CCs were small and a relative low number of strains was tested, a weak bovine MEC response towards CC151 *S. aureus* is in line with previous studies (52,53). CC97 *S. aureus* have been reported to induce a stronger cytokine response from bovine MEC than CC151 strains (52,53). These studies also demonstrated that this strong MEC activation by CC97 resulted in higher recruitment of neutrophils in conditioned media compared to MEC stimulation with CC151 *S. aureus* (53). Interestingly, CC151 is a weak activator of MEC but is linked with CM, whereas CC97 induce a strong response of MEC but are more associated with SCM (25). It is possible that weak MEC activation increases the pathogenicity of CC151 *S. aureus* since reduced release of cytokines likely increase the time it takes for the immune system to detect and respond properly to *S. aureus*. This gives the bacteria more time to establish itself within the mammary gland in the early stages of infection, giving it more opportunity to multiply, produce immune evasion factors or form biofilms. These bacterial defenses reduce the effectiveness of the immune response of the mammary gland, increasing bacterial survival and likely contributing to a more severe inflammatory response.

The functional mechanisms behind the differential MEC activation by ruminant *S. aureus* are not fully understood. The absence of wall-teichoic acid (WTA) from the bacterial cell surface increased the release IL-8 by MEC following stimulation with *S. aureus* (**Chapter 5**), demonstrating that the composition of the cell surface influences immune recognition of *S. aureus* by MEC. In addition, the species of MEC can also influence differential MEC activation, since the study described in **Chapter 6** shows that a CC133 strain induced a weaker transcription response from caprine MEC than the CC479 strain, whereas bovine MEC responded similarly to both strains (**Chapter 6**). Due the exploratory nature of this study, more research is needed to investigate the effect of host species on differential MEC activation by *S. aureus* CCs.

In summary, ruminant *S. aureus* CCs differ in their activation of bovine MEC but is unclear how this differential MEC activation affects the pathogenesis of mastitis.

Killing of neutrophils by leukocidin LukMF'

Proinflammatory cytokines released by MEC recruit immune cells to the mammary gland, and the key recruited effector cells that target *S. aureus* are neutrophils (54). These cells phagocytize *S. aureus* and release reactive oxygen species, defensins and antimicrobials peptides to kill the invading bacteria (54). Ruminant-associated *S. aureus* strains can defend themselves from neutrophils by releasing leukocidin LukMF', a potent and specific killer of ruminant neutrophils (55). The highest concentration of LukMF' is present in close proximity to the bacterium, forming a protective zone which likely increases its survival (31). Carriage of genes coding for LukMF' is common among ruminant-associated *S. aureus* CCs, since almost all *S. aureus* belonging to CC151, CC479 and CC133 and a proportion of CC97 *S. aureus* possess the LukMF' encoding genes. Genes encoding LukMF' are lacking in CC8 and CC398 *S. aureus* (**Chapter 2, 3, 4**). In addition to variation in gene carriage, production levels of LukMF' differ greatly between CCs, since CC479 *S. aureus* produce considerably higher levels of LukMF' compared to CC151 and CC133 *S. aureus* (**Chapter 2**) (56). A mutation in the start codon of the repressor of toxins (rot) gene exclusively present in CC479 *S. aureus* is the plausible cause of this increased production, since the absence of rot is known to elevate leukocidin production by *S. aureus* (57). LukMF' carriage was higher among CM strains than among SCM strains in the European bovine mastitis strain collection (**Chapter 3**), but not within the Dutch collection (**Chapter 2**). This could be explained by the high carriage rate (96%) of *lukM-lukF'* among Dutch strains and the relatively low number of available isolates (n = 55). However, the high LukMF' producing CC479 was associated with CM in both the Dutch and European isolate collection (**Chapter 2, 3**). Leukocidin LukMF' can increase the virulence of *S. aureus* during IMI through multiple possible mechanisms. Since neutrophils are unable to kill *S. aureus* because of LukMF', the bacterial stimulus of neutrophil recruitment is not removed, and the influx of neutrophils continues. This increases the period of diapedesis of neutrophils through the mammary parenchymal tissue, damaging the tissue of the mammary gland (54,58). Furthermore, lysed neutrophils by LukMF' will release their harmful content, such as reactive oxygen metabolites and matrix metalloproteinase, into the mammary gland, resulting in further tissue damage (58). Altogether, the studies described in this thesis demonstrate that the production of LukMF' contributes to the pathogenicity of *S. aureus* during IMI.

Shielding from neutrophils by biofilm production

An alternative strategy of *S. aureus* is to protect itself from neutrophils and other immune cells by the formation of bacterial biofilms: aggregates of bacteria surrounded by a matrix of exopolymers (59). Within these biofilms, *S. aureus* are more difficult to eliminate by neutrophils (60). In addition, biofilms can increase bacterial transmission as *S. aureus* can safely multiply within biofilms and then shed bacterial cells to different sites in the mammary gland or possibly, new hosts (59). Increased biofilm production has been linked to increased persistence of *S. aureus* during bovine SCM (12,61). The studies described in this thesis did not investigate biofilm formation of *S. aureus* but other studies did demonstrate that CCs differ in their (*in vitro*) ability to produce biofilms (52,62,63). CC97 and CC8 produce high amounts of biofilm, whereas CC151 and CC398 are weak producers of biofilm *in vitro* (52,62,63). Biofilms likely increase the survival of *S. aureus* within the bovine mammary gland (59) and this is supported by the observation that within-host evolution of an CC97 *S. aureus* during chronic SCM over the course of three months selected for increased (*in vitro*) biofilm production (64). Interestingly, these adapted strains caused lower mortality in a mouse infection model compared to non-adapted *S. aureus* strains (64), suggesting that increased biofilm production reduces the virulence of *S. aureus*. It is however, still unclear how *in vitro* biofilm production potential translates to the ability to produce biofilm during IMI (63) and therefore, further research is needed to fully uncover the contribution of differential biofilm production to the pathogenicity of ruminant *S. aureus*.

Manipulation of the immune response by SAs and SSLs

When the initial innate immune response of the mammary gland is unable to clear *S. aureus* from the mammary gland, the adaptive immune response mediated by T and B lymphocytes is initiated (65). *Staphylococcus aureus* can manipulate this response by the production of SAs and SSLs (45). This first group represents a diverse class of bacterial toxins that induce a Vβ-specific proliferation of T lymphocytes, resulting in uncontrolled release of proinflammatory cytokines such as IL-1, IL-2, IL-6 and TNF-α (66). This hyper-reaction of the immune system lowers the activation and recruitment rate of immune cells that are most effective against *S. aureus*, increasing the survival of the bacteria despite the strong inflammatory response (67). In addition, SAs can cause severe damage to the mammary gland due to the constant influx of immune cells and SA-induced apoptosis of MEC (68,69). The majority of SA genes are located on MGEs, and the most notable of these elements are the enterotoxin gene cluster (EGC) within the *S. aureus* pathogenicity island (SaPi) vSAβ, containing up to 7 different SA genes (*seG*, *seI*, *selM*, *selN*, *seO*, *selU*, *selV*),

and the SaPibov1, containing *tsst-1*, *seC* and *selL* (66). Among ruminant-associated CCs present in the European isolate collection, carriage of the EGC was associated with CC151 and CC479 *S. aureus*, whereas the SaPibov1 was only found in a proportion of CC151 (23%) and CC133 (8%) *S. aureus* (**Chapter 3**). The SA genes *seP*, *seR* and *seD* were exclusivey found in CC8 strains. Two SAs with an increased potency of bovine T lymphocyte activation, *selZ* and *selY*, (66) were present in all CC151 and a proportion of CC97 strains (**Chapter 3**). This demonstrates that there is a large variation in SA carriage among ruminant CCs, with CC151 encoding more SAs than any other ruminant CCs and CC97, CC398 have access to the least amount of SAs. As demonstrat-ed by the study described in **Chapter 3**, carriage of the EGC was associated with CM rather than SCM. Together with a previous study that demonstrated that genetically modified RF122 (CC151) *S. aureus* lacking the EGC caused less CM than their wildtype counterparts (66), this strongly suggest that EGC-encoded SAs contribute to the pathogenicity of *S. aureus* during IMI. It is however, still unclear by which mechanisms these SAs influence the bovine immune response since the expression levels of EGC-encoded SAs are relatively low (66) and is seems therefore unlikely that they would trigger a hyperinflammatory response. However, it is suggested that low levels of these SAs induce immunosuppressive regulatory CD4+ T lymphocytes, lowering the magnitude of the immune response and therefore increasing the survival of *S. aureus* within the mammary gland (66,67). This can contribute to a more severe mastitis case because the bacteria have more opportunity to produce virulence factors or form biofilms that are harmful to the host. In addition, since the immune defenses are unable to clear the bacteria, the inflammatory response of the host is expected to continue, resulting in further tissue damage to the mammary gland by the ongoing influx of leukocytes.

The SSL family of proteins are structurally related to SAs but lack superantigen capacities and in-stead, interfere with a wide range of innate immune defenses (46,67). As reported in the study described in **Chapter 3**, bovine *S. aureus* strains carried a large number of different SSL genes and several CC-specific variations of SSL-1, SSL-3, SSL-4, SSL-7 and SSL-10 were identified. It is yet unclear if there are functional differences between these SSL variants. Of all CCs studied, only CC479 lacked the genes coding for SSL-7, SSL-8 and SSL-9 (**Chapter 3**). These SSLs differ in their function, since SSL-7 blocks the formation of com-pletent C5, an important chemoattractant and activator of neutrophils (70,71) and SSL-8 inhibits the inter-action between fibronectin and extracellular matrix, reducing the motility of keratinocytes (72). The function of SSL-9, however, is still unknown. The lack of these SSLs likely reduces the ability of CC479 *S. aureus* to suppress the immune response of the mammary gland compared to other ruminant-associated CC but it unclear how this relates to the pathogenicity of CC479 *S. aureus*. It possible that in absence of

these immunosuppressive factors, the inflammation reaction of mammary gland is increased, resulting in a higher in-flux of neutrophils into the mammary gland. However, since CC479 are very capable in killing in neutrophils because of their high production levels of LukMF', this increased neutrophil recruitment will not result in a more effective elimination of *S. aureus*. Instead, the increased diapedesis of neutrophils and the release of harmful proteins from lysed neutrophils can result in more tissue damage in the mammary gland. Overall, the studies described in this thesis demonstrated that SAs encoded by the EGC likely influence the clinical outcome of IMI but the contribution of SSLs to the pathogenicity of *S. aureus* during IMI is still unclear.

Expression of immune evasion factors

As discussed above, bovine *S. aureus* CCs differ greatly in their carriage of immune evasion factors but the expression of many of these factors are regulated by the same systems. The accessory genome regulator (agr) and rot systems are involved in the regulation of leucocidins (73), SAs (67,74), SSLs (75), WTA/CP (76) and the formation of biofilms (73). Since ruminant-associated lineages belong to different agr types (CC133, CC97, CC398, CC8 to agr type I and CC151, CC479 to type II) (41) and a rot mutation was present in CC479 *S. aureus* (56), differences in expression profile of immune evasion factors can be expected between CCs. A transcriptomic comparison between CC398 and bovine CC8 *S. aureus* strains identified that contagious CC8 *S. aureus* have higher transcription levels of several genes involved in adhesion, complement inhibition and agr activation than CC398 strains (77), despite that both CC8 and CC398 belong to agr type I. As illustrated in **Chapter 3**, CC151 and CC479 *S. aureus* are quite similar in their carriage of virulence genes but differ in their expression of virulence factor LukMF' (and presumably other virulence factors regulated by rot) and their virulence. Altogether, this illustrates that expression levels of virulence factors by ruminant-associated *S. aureus* CCs play a significant role in determining the course of disease during IMI.

Directions for future research and concluding remarks

The studies described in this thesis show that ruminant-associated CCs of *S. aureus* differ in their strategies of immune evasion, activation of MEC and virulence during ruminant mastitis. These findings provide new directions for future research on the pathogenicity of *S. aureus* during mastitis. A key knowledge gap is the molecular mechanism of the increased virulence of CC479 *S. aureus*. Increased production levels of leukocidin LukMF' by CC479 are a likely causal mechanism, but it is un-

known if the production levels of other virulence factors (e.g. SAs, SSLs) are also increased in CC479 *S. aureus*. The mutation in the start codon of the rot gene is the likely mechanism for the increased LukMF' production, and it would be of interest to investigate if rescuing the rot gene (by inserting a functional rot copy in CC479) reduces LukMF' production potential. In addition, experiments using isogenic Δ*rot* mutant of CC151 or CC133 are required to demonstrate the role of rot in regulation of LukMF' production. As demonstrated by this thesis, future studies on the pathogenicity of ruminant *S. aureus* should also take expression levels of virulence factors into account rather than focusing only on gene carriage of these factors. The observation that different *spa*-types of CC133 are associated with different ruminant host species also raises interesting questions surrounding this CC and host-adaption of *S. aureus*. It possible that these *spa*-types reflect host-adapted clones within CC133 or are a consequence of a rapid clonal expansion of CC133 within certain animal populations. Since CC133 *S. aureus* are associated with horses and wildlife species, further comparison of the genomes of CC133 of different host species could give valuable insight in the molecular mechanisms of host-adaption of *S. aureus*.

The findings of this thesis also have several practical implications for the control of ruminant mas-titis on dairy farms. Currently, *S. aureus* mastitis is treated at a species level, but the studies described in this thesis demonstrate that the biological properties of *S. aureus* CCs differ and are associated with differ-ent clinical manifestations of mastitis In addition, these CCs differ in their contagiousness (9,41). A clinical, but non-contagious mastitis case would require a different intervention strategy than contagious SCM case and therefore, it might be beneficial to develop *S. aureus* CC-specific intervention strategies for mastitis to increase the efficiency of mastitis control within herds. Although identification of *S. aureus* CC is technically possible (e.g. using a PCR for CC-specific genes (**Chapter 3**)), the economical relevance of *S. aureus* CC-specific intervention strategies is still unclear as there are still key knowledge gaps surrounding the epidemiological properties of ruminant *S. aureus* CCs. For instance, it is unclear how persistent and contagious infections with CC479 *S. aureus* are. When these infections have a low transmission rate and are unlikely to spread within the herd, a single CC479 CM case is of lower economic consequences than less severe but more contagious *S. aureus* infections. In addition, identification of the CC of the causative *S. aureus* strain during SCM could potentially be used as prognostic marker for severity of mastitis. In order for this to be feasible in practice, more knowledge of the pathogenesis of mastitis caused by CC479 *S. aureus* is required. If these infections rapidly develop from SCM into CM, there is only a short time period to identity *S. aureus* CC and implement the proper intervention strategy, which would limit the practical benefit of screening for CC479.

Finally, for CC-specific intervention strategies to be efficient, more knowledge is required about the ruminant *S. aureus* populations in Europe. The study described in **Chapter 3**, in addition to previous work (7,42), demonstrates that the distribution of ruminant CCs differs between European countries. Since the high virulent (e.g. CC479) or contagious (e.g. CC8) *S. aureus* were both associated with specific countries and regions in Europe, the optimal strategy of intervention and treatment of mastitis likely varies between countries. As demonstrated in **Chapter 2** and **Chapter 3**, the genetic background of CM and SCM isolates differs. Since these clinical manifestations of mastitis also differ in their incidence in dairy herds, using a combination of isolates obtained from both CM and SCM will not give a proper reflection of the overall population of ruminant *S. aureus* per country. Therefore, future studies on the population structure of ruminant *S. aureus* should describe the genetic diversity separately for isolates obtained from CM and SCM to get a more complete representation of the ruminant *S. aureus* population.

In conclusion, the studies described in this thesis have increased our understanding of the molecular epidemiology of ruminant *S. aureus* and identified differences in immune evasion strategies, host-pathogen interactions and pathogenicity between ruminant-associated CCs. This demonstrates that the type of infection *S. aureus* influences the clinical outcome of IMI. Therefore, it might be beneficial to implement specific interventions based on the infecting *S. aureus* strain rather than treating *S. aureus* mastitis on a species level. These findings are early but important steps towards strain-specific treatment and intervention strategies of *S. aureus* mastitis and this could represent the next step in the ongoing battle against mastitis on dairy farms.

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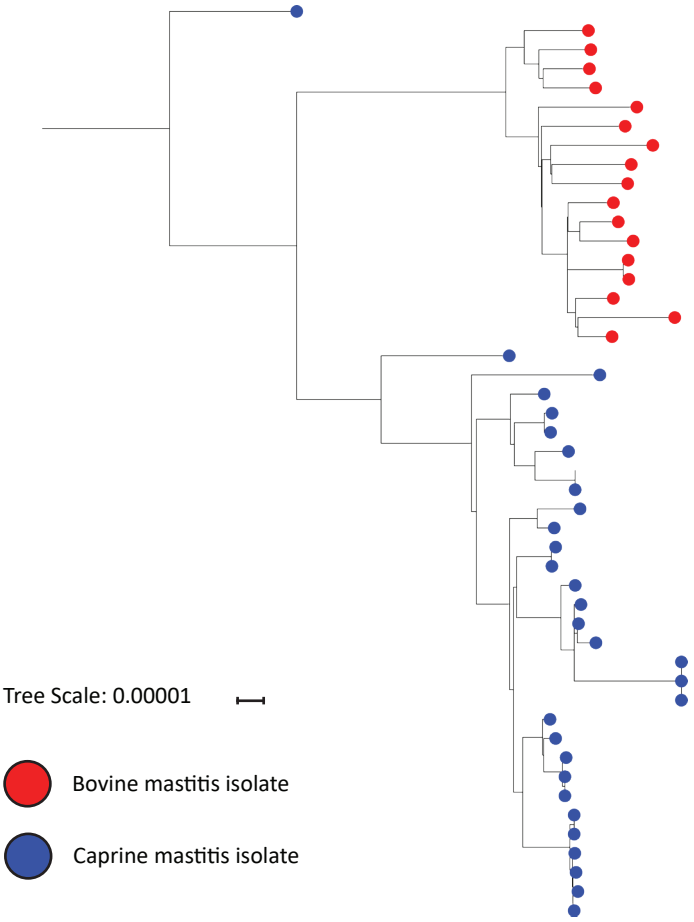
Supplementary Figures



Supplementary Figure 1
Maximum-likelihood phylogenetic tree based on presence or absence of genes of the accessory genome (n = 975 genes) of a collection of bovine CC133 *Staphylococcus aureus* (red, n= 17) collected from mastitis cases in Europe and caprine CC133 (blue, n = 31) isolates caprine mastitis cases in The Netherlands. Phylogenetic tree was visualized using iTOL v3.6

Appendix

Nederlandse Samenvatting
Dankwoord
Curriculum Vitae
List of Publications

Nederlandse Samenvatting

Introductie

Mastitis is een ontsteking van het uierweefsel en is een veel voorkomend probleem in de melkveehouderij. De ziekte veroorzaakt een vermindering in melkproductie en kwaliteit, brengt kosten voor medicatie met zich mee en kan resulteren in het voortijdig afvoeren van dieren. Infecties met de *Staphylococcus aureus* bacterie zijn een veel voorkomende oorzaak van mastitis. Deze infecties kunnen zowel heftige klinisch, als milde subklinische ontstekingen veroorzaken. De *S. aureus* bacterie is een opportunistische pathogeen met een groot gastheer bereik, maar er zijn duidelijke genetische en functionele verschillen tussen *S. aureus* stammen afkomstig van verschillende diersoorten. Om goed onderscheid te kunnen maken tussen deze subtypes van de bacterie, worden *S. aureus* stammen vaak ingedeeld in verschillende groepen (o.a. Clonal Complexes (CCs)) op basis van hun genetische achtergrond, wat ook wel genotyperen wordt genoemd.

Staphylococcus aureus is in staat om het immuunsysteem van de gastheer te omzeilen door de productie van verschillende stoffen die onderdelen van de immuunreactie kunnen uitschakelen. De genen die coderen voor deze immuun evasie factoren zijn vaak onderdeel van het variabel genoom van de bacterie. Hierdoor beschikt niet elke *S. aureus* stam over dezelfde set van immuun evasie factoren, wat van invloed kan zijn op de pathogeniteit van de bacterie. Omdat *S. aureus* stammen genetisch van elkaar kunnen verschillen, is het mogelijk dat verschillende *S. aureus* types verantwoordelijk zijn voor klinische en subklinische ontstekingen van het uierweefsel. In dit proefschrift is de moleculaire epidemiologie van *S. aureus* verantwoordelijk voor mastitis in herkauwers onderzocht, met als doel om te bepalen of specifieke genetische factoren (genotype, aanwezigheid van bepaalde immuun evasie factoren) geassocieerd zijn met de klinische uitkomst van intramammaire infecties met *S. aureus*.

Moleculaire epidemiologie van *Staphylococcus aureus* verantwoordelijk voor mastitis

In runderen zijn infecties met *S. aureus* voornamelijk geassocieerd met subklinische en vaak chronische ontstekingen maar *S. aureus* infecties kunnen soms ook resulteren in heftige, klinische mastitis gevallen. In **Hoofdstuk 2** werden *S. aureus* isolaten vergeleken die afkomstig waren van klinische en subklinische mastitis gevallen bij Nederlandse runderen. Hierbij werd specifiek gekeken naar de rol van leukocidine LukMF': een cytotoxine met een sterk effect op runder immuuncellen, en met name

op neutrofielen. Aangezien deze cellen een belangrijke rol spelen in de immuunreactie tegen *S. aureus*, was de verwachting dat de productie van LukMF' bijdraagt aan de klinische ernst van de ontsteking. Alhoewel bijna alle *S. aureus* isolaten positief waren voor de genen die coderen voor LukMF', waren er wel duidelijke verschillen in hun *in vitro* productie van het toxine. Bepaalde *S. aureus* isolaten hadden namelijk een verhoogde productie van LukMF' *in vitro* en er was een sterke correlatie tussen hoge *in vitro* productie en meetbare LukMF' waardes in de melk afkomstig van het mastitis geval. De *S. aureus* isolaten behoorde voornamelijk tot de types CC151 (73%) en CC479 (22%), en alle hoog LukMF' producerende stammen waren van het genotype CC479-t543. Er was een sterke associatie tussen klinische mastitis, hoge LukMF' productie en het type CC479-t543. Een mutatie in het startcodon van het repressor of toxins (Rot) gen was de vermoedelijke oorzaak van de verhoogde toxine productie door CC479-t543 *S. aureus*, aangezien Rot een negatieve regulator is van toxine productie door *S. aureus*.

Als een vervolg op deze studie, werd er in **Hoofdstuk 3** een grotere collectie van klinische en subklinische *S. aureus* isolaten vergeleken afkomstig uit 11 Europese landen. Van al deze bacteriën werd het volledige genoom bepaald, waardoor we een compleet inzicht kregen in de genetische variatie van deze *S. aureus* collectie. De isolaten behoorde tot een relatief laag aantal verschillende CCs, aangezien 75% van de collectie tot slechts 5 verschillende CCs behoorde. De meest voorkomende CCs waren CC151, CC97, CC479, CC133 en CC398. Er waren grote verschillen in de aanwezigheid van immuun evasie en antibioticaresistentie genen tussen deze *S. aureus* CCs. Zo beschikten CC151 en CC479 *S. aureus* over een grote hoeveelheid verschillende immuun evasie en toxine genen maar beschikten juist amper over antibioticaresistentie genen. Dit in tegenstelling tot CC398 *S. aureus*, die juist veel resistentie genen bij zich droegen maar geen andere immuun evasie factoren. Er waren duidelijke verschillen in de distributie van deze CCs tussen landen. Sommige CCs waren aanwezig in bijna elk land (CC151, CC97) maar sommige werden juist alleen aangetroffen in bepaalde landen of regio's, zoals CC479 (Nederland, België, Duitsland) en CC398 (Spanje, Polen). Net als in **Hoofdstuk 2**, was er ook in deze collectie een associatie tussen CC479 *S. aureus* en klinische gevallen van mastitis. Meerdere genen waren ook geassocieerd met klinische mastitis (waaronder enkele superantigen genen) maar omdat deze genen zelf ook sterk geassocieerd waren met bepaalde CCs (CC151, CC479), was het niet mogelijk om onderscheid te maken tussen het effect van de genen en het effect van CC op de klinische uitkomst van infectie.

Naast infecties in runderen, is *S. aureus* ook een veel voorkomende veroorzaker van mastitis in schapen en geiten. Anders dan bij runderen, zijn infecties met *S. aureus* bij deze dieren voornamelijk geassocieerd met klinische mastitis. Ook zijn er genetische verschillen tussen *S. aureus* stammen afkomstig van runderen en kleine herkauwers. In **Hoofdstuk 4** werd daarom specifiek onderzoek gedaan naar de moleculaire epidemiologie van *S. aureus* verantwoordelijk van klinische en subklinische mastitis gevallen in schapen en geiten. De grote meerderheid van deze isolaten (>85%) behoorde tot CC133. Binnen deze CC133 *S. aureus* isolaten waren er wel verschillende *spa*-types aanwezig. Behalve CC133, werden er ook CC398 (7% van isolaten) en CC425 (5% van isolaten) *S. aureus* aangetroffen. Bijna alle CC133 en CC425 *S. aureus* waren positief voor de LukMF' genen, maar deze waren afwezig in de CC398 isolaten. Er waren geen genetische verschillen tussen isolaten afkomstig van klinische en subklinische ontstekingen. Wel waren er kleine verschillen tussen geiten en schapen stammen, aangezien CC133-t2678 het meest voorkomende *S. aureus* type was bij schapen en CC133-t544 en CC133-t3583 de dominante types waren onder de geiten isolaten. Alhoewel deze types licht verschilde in hun *in vitro* productie van LukMF', is het nog onduidelijk of er verdere functionele verschillen zijn tussen CC133 *S. aureus* afkomstig van geiten en schapen.

Interacties tussen *Staphylococcus aureus* en gastheer epitheelcellen

De interacties tussen de pathogeen en de gastheer spelen een belangrijke rol in het activeren van de immuun reactie tijdens intramammaire infecties. Een belangrijke schakel in het opstarten van de immuunreactie van de uier is de activatie van uier epitheelcellen door bacteriële eiwitten. Deze cellen produceren cytokines die immuuncellen, waaronder neutrofielen, aantrekken naar de uier om daar de bacterie aan te vallen. De reactie van epitheelcellen op stimulatie met *S. aureus* is over het algemeen vrij zwak in vergelijking met andere bacterie soorten maar het was nog onduidelijk of epitheelcellen anders reageren op verschillende *S. aureus* types. In **Hoofdstuk 5** werd daarom de response van uier epitheelcellen op verschillende *S. aureus* CCs onderzocht. Hiervoor werd gebruik gemaakt van de runder epitheelcellijn PS. Om de bruikbaarheid van deze cellijn te testen, werd deze eerst gestimuleerd met verschillende mutant *S. aureus* stammen die factoren misten of over-produceerden die de activatie van epitheelcellen mogelijk konden beinvloeden. Deze experimenten toonden aan dat de productie van teichonzuren, een onderdeel van de celwand van *S. aureus*, de herkenning van de bacterie door epitheelcellen sterk verminderd. Vervolgens werden verschillende *S. aureus* isolaten getest. Stimulatie met CC133 *S. aureus* zorgde voor een grotere afgifte van cytokine IL-8 door de PS cellen dan stimulatie met CC151, CC479 en CC425 *S. aureus*. Ook reageerde de PS cellen sterker op CC479 *S. aureus* dan op CC151

en CC425 isolaten. Het is echter nog onduidelijk wat de mechanismes zijn achter deze differentiële activatie van runder epitheel door verschillende *S. aureus* CCs. Vervolgens werd er in **Hoofdstuk 6** gekeken of geiten en runder epitheelcellen anders reageren op een geiten-geassocieerde *S. aureus* stam (CC133) en een runder-geassocieerd *S. aureu*s stam (CC479). Waar de runder epitheelcellen hetzelfde reageerde op beide stammen, reageerden de geiten epitheelcellen sterker op stimulatie met CC479. De opzet van deze experimenten was beperkt, en daarom is er meer onderzoek nodig naar het effect van de gastheer op de differentiële activatie van MEC op *S. aureus* CCs.

Conclusie

De studies beschreven in dit proefschrift demonsteren dat niet elke *S. aureus* die mastitis veroorzaakt in herkauwers hetzelfde is. Ze behoren tot verschillende genotypes, dragen andere immuun evasie en antibioticaresistentie genen met zich mee en verschillen in hun productie van leukocidine LukMF' en activatie van gastheer epitheel cellen. Dit toont aan dat *S. aureus* types er verschillende strategieën op na houden om het immuunsysteem van de gastheer te ontwijken. De sterke associatie tussen klinische mastitis en CC479 toont aan *S. aureus* types ook verschillen in hun pathogeniteit tijdens infecties. Momenteel wordt *S. aureus* mastitis voornamelijk behandeld op een soort niveau maar deze thesis toont aan dat kennis van het genotype van de infecterende bacterie mogelijk bij kan dragen aan de behandeling en preventie van *S. aureus* mastitis op boerderijen.

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Curriculum Vitae

Jurriaan Hoekstra was born on 7 March 1990 in Rhenen, The Netherlands and grew up in Veenendaal. In 2008, he started his university education at Utrecht University and in 2011 he obtained his BSc degree in Biology. In the same year, he started the master program Cancer, Stem Cells and Developmental Biology at Utrecht University. After a year, he switched his master program to the Toxicology and Environmental Health program, organized by the Institute of Risk Assessment Sciences and Utrecht University. During this MSc, he conducted an internship at the Veterinary Public Health department of the Institute of Risk Assessment Sciences of Utrecht University under the guidance of Dr. Heike Schmidt on the reduction antibiotic resistant bacteria on pig farms. In 2014, he performed his second internship at KWR Water Research Institute on the presence of antibiotic resistant bacteria in Dutch surface water and drinking water treatment plants under the supervision of Dr. Luc Hornstra. His final literature thesis was written under the guidance of Dr. Nico van den Brink of Wageningen University on the immunotoxicology of cadmium. In addition to his academic activities, he was an active member of the International Student Organization AEGEE-Utrecht and the Utrecht Student Floorball Club Jungle-Speed. After completing his MSc, Jurriaan performed a six-month traineeship at the European Chemical Agency in Helsinki, Finland.

In October 2015, he started as a PhD candidate at Veterinary Faculty of Utrecht University. The candidacy was under the supervision of Dr. Gerrit Koop, Prof. Dr. Arjan Stegeman and Prof. Dr. Victor Rutten. During his candidacy, Jurriaan studied the molecular epidemiology of *Staphylococcus aureus* responsible for intramammary infections in dairy cows, goats and sheep. The research conducted during his PhD is described in this thesis. Since April 2020, Jurriaan works at the Centre for Sustainability, Environment and Health at the National Institute for Public Health and the Environment in Bilthoven, The Netherlands.

List of Publications

Genomic analysis of bovine *Staphylococcus aureu*s from clinical versus subclinical mastitis in eleven European countries. **J. Hoekstra**, A. Zomer, V. Rutten, L. Benedictus, A.S. Stegeman, M.P. Spaninks, T. Bennedsgaard, A. Biggs, S. De Vliegher, D. Herrera Mateo, R. Huber-Schlenstedt, J. Katholm, P. Kovács, V. Krömker, G. Lequeux, P. Moroni, L. Pinho, S. Smulski, K. Supré, J. Swinkels, M.A. Holmes, T.J.M.G. Lam and G. Koop. **To be submitted**

The interaction of a host-associated and non-host-associated *Staphylococcus aureus* strain with primary bovine and caprine mammary epithelial cells. **J. Hoekstra**, V. P. M. G. Rutten, L. Benedictus, T.J.G.M. Lam, M. P. Spaninks, M.A.M. Oosterveer-van der Doelen, J.A Stegeman and G. Koop. **To be submitted**

Activation of a bovine mammary epithelial cell line by ruminant-associated *Staphylococcus aureus* is lineage dependent. **J. Hoekstra**, V. P. M. G. Rutten, T. J. G. M. Lam, K. P. M. van Kessel, M. P. Spaninks, J. A. Stegeman, L. Benedictus and G. Koop, **Microorganisms, 2019 7(12), p688**

Differences between *Staphylococcus aureus* lineages isolated from ovine and caprine mastitis but not between isolates from clinical or subclinical mastitis. **J. Hoekstra,** V.P.M.G.Rutten, M. van den Hout, M.P. Spaninks, L. Benedictus and G. Koop. **J Dairy Sci. 2019, 102(6):5430-5437**

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High Production of LukMF' in *Staphylococcus aureus* Field Strains Is Associated with Clinical Bovine Mastitis. **J. Hoekstra**, V.P.M.G. Rutten, L.S. Sommeling, T. van Werven, M.P. Spaninks, B. Duim, L. Benedictus and G. Koop. **Toxins 2018 May 15;10(5)**