STAPHYLOCOCCUS AUREUS

2.1. Impact of S. aureus mastitis

*S. aureus* is the most prevalent etiological agent causing mastitis. *S. aureus* can cause both subclinical and clinical mastitis (Barkema et al., 2006), which severity and outcome of infection depend, in part, on strain-factors and cow factors (Barkema et al., 2006). *S. aureus* express a plethora of factors which, in different manners, inhibit the host immune response against the pathogen (Bagnoli et al., 2012). *S. aureus* utilizes distinct mechanisms tailored for survival in different microenvironments encountered during host colonization or invasion (Anderson et al., 2012). Many studies have, however, confirmed its ability to invade and survive in diverse cell types, including mammary epithelial cells, neutrophils and macrophages (Mcloughlin et al., 2008).

The cure rate of antibiotic treatments for this agent is low and therefore, the disease has not been effectively eliminated and/or controlled in many herds (Pereira et al., 2011). *S. aureus* is a challenging vaccine target due to complexity of its pathogenesis involving a multitude of virulence factors. Therefore, to develop efficacious vaccines we need to understand how to avoid or compensate for the detrimental effect of evasion factors on the host immune response. The frequent incapacity of both the immune response and antibiotics to prevent infection and destroy the pathogen in the intramammary environment explains why *S. aureus* bovine mastitis constitutes a major challenge. Despite ongoing efforts over ages, no licensed *S. aureus* vaccine is currently available (Anderson et al., 2012)

In many countries, the number of mastitis cases, particularly subclinical, caused by *S. aureus* is still very high. In India, the prevalence reported for *S. aureus* mastitis was 74.04%
in 2000, 59.37% in 2009, 57.27% and 60.71% in 2012 (Sharma et al., 2012 and references within).

The most common transmission pathway occurs through transfer from an infected mammary gland to an uninfected gland via devices, such as milking equipment, common udder cloths, or the milker’s hands. Although the infected mammary gland could still be the source of these infections, it is obviously not the only reservoir of *S. aureus* on dairy farms. *S. aureus* mastitis continues to be the most important disease of the dairy industry.

### 2. 2. General Characteristics of *S. aureus*

Staphylococci belong taxonomically to the family of *Staphylococcaceae*, and are Gram-positive, catalase-positive cocci.

#### 2.2.1. Genomic content

The genome size of *S. aureus* typically varies from 2.5 to 3.1 Mb, and contains ~2,500 open reading frames. Since the first two *S. aureus* genome sequences; N315 and Mu50, were published in 2001 (Kuroda et al., 2001), other genome sequences followed rapidly: MW2 (Baba et al., 2002), MRSA252 and MSSA476 (Holden et al., 2004), COL (Gill et al., 2005), USA300-FPR3737 (Diep et al., 2006), USA300-HOU-MR (Highlander et al., 2007), NCTC8325 (Gillaspy et al.,2006)), ET3-1 (Herron-Olson et al., 2007), JH-1 and JH-9 (Mwangi et al., 2007), Newman (Baba et al.,2002) and TW20 (Holden et al.,2010).

The *S. aureus* genome consists of 1) core genes, conserved between the different lineages; 2) core variable (CV) genes, genes that vary between genomes or may even be missing; and 3) mobile genetic elements (MGEs), fragments of DNA encoding toxins, 12 virulence factors and genes involved in host adaption as well as mobilisation functions (Baba et al., 2008; Lindsay et al., 2006, 2010; Malachowa et al., 2010). The core genome contains approximately 80% of the *S. aureus* genes, including genes for surface proteins involved in adhesion, as well as genes encoding essential metabolic and regulatory properties (Gill, S. R. 2009). As a part of the core genome, the core variable (CV) genes make up 10-12% of the *S.*
aureus genome, and often encode regulators of virulence genes or surface proteins involved in host interactions, such as the surface protein staphylococcal protein A (spa). The accessory genome accounts for the remaining 20% of the S. aureus genome, consisting of MGEs containing 50% of known virulence factors in S. aureus. The MGEs include e.g. bacteriophages, pathogenicity islands, plasmids and transposons and are capable of horizontal transfer between strains (Gill, S. R. 2009). Exchange of virulence factors between strains, resulting in different virulence factor combinations, contributes to adaption of clones specialised for infection of selected hosts or environments (Herron-Olson et al., 2007; Holden et al., 2010).

2.2. Virulence factors

For the majority of diseases caused by S. aureus, pathogenesis is multifactorial, so it is difficult to determine precisely the role of any given factor. However, there are correlations between strains isolated from particular diseases and expression of particular virulence determinants, which suggests their role in a particular disease. In the last decade, the application of molecular biology has led to advances in unraveling the pathogenesis of staphylococcal diseases. Genes encoding potential virulence factors have been cloned and sequenced, and many protein toxins have been purified (Foster and Hook 1998).

S. aureus potentially produces a variety of virulence factors that contribute to persistence and pathogenicity of the organism (Haveri et al., 2008). These virulence factors are divided into two groups including surface associated factors and degradative enzymes together with exotoxins. S. aureus possess a variety of adhesin proteins such as fibronectin binding proteins, fibrinogen binding proteins and collagen binding proteins (Salasia et al., 2004). Some strains of S. aureus produce one or more additional exoproteins, which include toxic shock syndrome toxin, staphylococcal enterotoxins (SE) or SE like toxins, exfoliative toxins, and leukocidin (Dinges et al., 2000). These enterotoxins exhibit super-antigenic
activity by interacting with antigen presenting cells and T lymphocytes. Superantigens (SAgs) have ability to stimulate a large number of T cells and high level of cytokine expression. Depending on the strains, variable combinations of genes are present. Thus, accordingly, this diversity and the large variations in the presence of these genes may influence the pathogenesis of \textit{S. aureus} infection in cattle (Zecconi et al., 2006, Ote et al., 2011). Investigating distributions and the virulent factors of \textit{S. aureus} provides important information for establishing control strategies (Hwang et al., 2010).

In addition, the staphylococcal enterotoxins are recognized agents of the staphylococcal food poisoning syndrome and may also be involved in other types of infections imposing public health threat. This emphasizes the importance of determining the toxin gene profiles of \textit{S. aureus} strains. Further, the expression of most virulence factors in \textit{S. aureus} is controlled by the accessory gene regulator (\textit{agr}) locus. Based on the amino acid sequence polymorphism of the \textit{agr} encoded auto inducing peptide and its corresponding receptor, \textit{S. aureus} can be divided into 4 major \textit{agr} groups (I-IV) (Jarraud, 2002). Determining the \textit{agr} groups might allow assessment of a possible relationship between \textit{agr} groups and the occurrence of virulence gene.

Another major area of apparent concern is the frequent report of isolation of MRSA as well as multi drug-resistant \textit{S. aureus} strains from bovine milk (Hata et al., 2010). However, testing the susceptibility to antibiotics is not a routine procedure and infections with methicillin-resistant staphylococci (MRS) in animals may go undetected occasionally. \textit{mecA}, a structural gene located on the chromosome of \textit{S. aureus}, characterizes methicillin-resistant \textit{S. aureus} (MRSA), and \textit{femA} and \textit{femB} (\textit{fem}) genes encode proteins which influence the level of methicillin resistance of \textit{S. aureus}. Again, determining the \textit{in vitro} resistance rates of staphylococci isolated from bovine mastitis to methicillin becomes necessary.
2.2.3. Cell wall components and role in the inflammatory response.

Gram-positive bacterial cell walls are composed of multiple peptidoglycan layers, wall teichoic acids linked to the Peptidoglycan and lipoteichoic acid (LTA) linked to the cytoplasmic membrane. *S. aureus* does not contain lipopolysaccharide (endotoxin), which is the main cell wall component of gram negative bacteria responsible for septic shock. However, *S. aureus* can cause septic shock and multiple organ failure (Kimpe et al., 1995). Indeed, in a canine model, infection by *S. aureus* provokes the same symptoms of septic shock as does *Escherichia coli* (Natanson et al., 1989).

The pathogenicity of *S. aureus* is postulated to depend on the expression of a wide range of cell wall-associated and secreted bacterial proteins. Although cell wall-associated and secreted proteins are keys for staphylococcal virulence, their role in innate immunity remains largely unknown. On the other hand, several cell wall components such as Peptidoglycan and lipoteichoic acids have been well studied.

Although crucial for bacterial life, purified wall teichoic acids of *S. aureus* are not very inflammatory (Majcherczyk et al., 2003). However, a number of studies suggest that the bacterial LTA of *S. aureus* may contribute to sepsis. LTA from *S. aureus* has been shown to provoke secretion of cytokines and chemoattractants (TNF-α, IL-1β, IL-10, IL-12, IL-8, leukotriene B4, complement factor 5a, MCP-1, MIP-1α and granulocyte colony-stimulating factor) from monocytes or macrophages (Cleveland et al., 1996). Complement factor 5a and leukotriene B4 are chemoattractants active on PMNs and monocytes. Thus, LTA induces an inflammatory response (Figure 2.1). However, very large amounts of LTA are necessary to induce responses of cells in vitro. Comparison of the activity of LPS versus LTA showed that staphylococcal LTA is able to promote the same strong induction of chemoattractants (IL-8, MIP-1α, MCP-1, complement factor 5a, and leukotriene B4), granulocyte colony-stimulating factor, and anti-inflammatory cytokines (IL-10) as LPS, whereas it is a weaker inducer of
TNF-α, IL-1β, and IL-6 (von Aulock et al., 2004). LTA also induces less IL-12 than LPS and subsequently IFN-γ (Kolb-Maurer et al., 2003). The cytokine pattern produced by LTA is similar to that induced by the whole bacterium (Wang et al., 2000). Furthermore, when LTA is inoculated intranasally in mice, a strong neutrophil and macrophage infiltration is observed in the lung, suggesting that LTA elicits granulocyte recruitment by producing chemoattractants. It is likely, then, that staphylococcal LTA participates in the formation of pus by recruiting neutrophils (von Aulock et al., 2004). Thus, staphylococcal LTA is a strong inducer of chemoattractant and granulocyte colonystimulating factor release, suggesting that it is not just a weak LPS-like molecule but indeed displays activities distinct from LPS.

Figure 2.1. Action of staphylococcal components to promote immune responses from immune cells (data from reference Henderson et al., 1996).
2. 3. Population structure of \textit{S. aureus}

Bacterial population structure can be interpreted by the use of different typing methods to obtain an understanding of other characteristics of the bacterial population, such as host specificity, pathogenicity, epidemic potential and the presence of virulence genes (Mathema et al., 2009). Hence, to understand \textit{S. aureus} its relation to infection of the host, the population structure needs to be defined. Several large typing studies with different methods have been performed on \textit{S. aureus}, revealing an essentially clonal population (Melles et al., 2004; Grundman et al., 2002; Takuno et al., 2012). \textit{S. aureus} population structures in different parts of the world indicate that there is a large geographical divergence in the most commonly found Clonal Complexes (Ruimy et al., 2008; Fan et al., 2009; Melles et al., 2004).

**Molecular typing**

The \textit{S. aureus} population structure has been investigated by several different methods, including multilocus enzyme electrophoresis (MLEE), pulsed field gel electrophoresis (PFGE), MLST and \textit{spa} typing.

By the use of molecular typing techniques, the spread of clones in community can be identified and kept under surveillance. In outbreak situations, epidemiological typing can be used to find the transmission modes of the epidemic clones, and to monitor the reservoir of the infectious agent. For epidemiological surveillance, typing systems reveal the prevalence of pandemic, endemic or epidemic clones in the population and in different geographical areas (Struelens, M. J. 1998). Different applications may have different requirements, but in general, a typing regime requires proper typeability, reproducibility, discriminatory power and stability, and it should be easy to interpret and use (Struelens, M. J. 1996). Today, a range of techniques are in use for typing of staphylococci, with different strengths and weaknesses (Table 2.1).
Table 2.1. Examples of current typing methods for *S. aureus*. Based on (Guttman et al., 2010; Stefani et al., 2012; Struelens et al., 2009).

<table>
<thead>
<tr>
<th>Method</th>
<th>Target</th>
<th>Strengths</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>spa</em> typing</td>
<td>Sequence polymorphism in the variable X region of the gene encoding <em>S. aureus Protein A</em></td>
<td>Rapid, high throughput, standard nomenclature, interlaboratory reproducibility</td>
<td>Misclassification of particular lineages due to recombination/homoplasy</td>
</tr>
<tr>
<td>Pulsed-field gel electrophoresis (PFGE)</td>
<td>Polymorphisms in restriction sites on the chromosome</td>
<td>High discriminatory power</td>
<td>Technically demanding, time-consuming, limited interlaboratory reproducibility, multiple nomenclatures</td>
</tr>
<tr>
<td>Multilocus sequence typing (MLST)</td>
<td>Sequence determination of allelic variants of seven housekeeping genes</td>
<td>Interlaboratory reproducibility, standard nomenclature</td>
<td>Low throughput, high cost</td>
</tr>
<tr>
<td>Whole genome sequencing (WGS)</td>
<td>Whole genome</td>
<td>Extremely high discriminatory power</td>
<td>Demanding data interpretation</td>
</tr>
</tbody>
</table>

From the early bacteriophage typing studies in the 1950s, based on the ability of bacteriophages to lyse different staphylococci, ‘epidemic types’ of staphylococci were first identified, giving us hints on the staphylococcal population structure (Wentworth, B. B. 1963). PFGE, AFLP, multiple locus variable number tandem repeat analysis (MLVA), repetitive element PCR, and random amplified polymorphic DNA (RAPD) are all methods where variation in the nucleotide sequence is detected indirectly by primer-binding and/or restriction sites, and are often referred to as band-based methods or molecular fingerprinting (Smyth and Robinson., 2010). In PFGE, chromosomal DNA is digested with a restriction enzyme that cleaves infrequently, to obtain large fragments which after being exposed to a switching electric field on an agarose gel produce a banding pattern or fingerprint (Bannerman et al., 1995). As a highly discriminatory method, it has been widely used for typing of staphylococci, and has been considered to be the gold standard in typing of *S.*
* aureus* outbreak investigations (Tenover et al., 1995). However, the comparison of data from fingerprints run in different laboratories can be challenging, and in addition the interpretation of the results is still quite subjective.

*spa* typing is a sequence-based method, where the variable number tandem repeat (VNTR) region of Staphylococcal Protein A is analysed, and a *spa* type is assigned based on the order and number of the short, typically 24 bp repeats. *spa* typing has discriminatory power comparable to that of PFGE, and can be used both for outbreak investigations as well as population studies due to the slow accumulation of point mutations and relatively fast changes in repeat numbers (Koreen et al., 2004). However, recombination may disturb the congruence between *spa* types and sequence types/clonal complexes on some occasions (Robinson and Enright 2004).

A study by Xie et al (2011) conducted in China, showed toxin gene typing combined with PFGE or MLST could increase the discriminatory power of genotyping *S. aureus* isolates. Their overall genotyping results showed high genetic diversity of the strains regardless of their geographical distributions, and no strong correlation between genetic background and toxin genotypes of the strains. Another study from Japan (Hata et al., 2010) in genetic analysis of *S. aureus* isolates from bovine mastitis showed MLST to have a strong correlation to the results of PFGE, *coa* PCR RFLP, *spa* typing and coagulase serotyping methods. Ote et al 2011 in their study showed a high number of genotypic subtypes demonstrating further the large variation in the presence of virulence genes in *S. aureus* isolates and the considerable diversity of strains populations that are able to cause mastitis in cows. In Indian context, Kumar et al., 2011, Padmaja & Halami., 2013, Abimanyu et al., 2013 performed toxin gene profiling of *S. aureus and . Sanjiv et al., 2008; Upadhyay, et al., 2012; Kumar et al., 2011 performed RFLP studies of *S. aureus. Padmaja & Halami, 2013 performed RAPD with *S. aureus* isolate. Kumar etal., (2011) in their study on
molecular surveillance of putative virulence factors and antibiotic resistance in *Staphylococcus aureus* isolates recovered from intra-mammary infections of river buffaloes showed significantly resistance to tetracycline, macrolides, lincosamides and aminoglycosides. MecA gene was detected in 5.4% of isolates of *S. aureus*. The isolates showed variation in expression of pathogenic factors. Distribution of virulent genes between methicillin-susceptible and resistant isolates was uneven. The distribution of high pathogenic characteristic in antibiotic-susceptible isolates indicated that these were equally responsible in maintaining the intra-mammary infections in animals and cannot be overlooked. The surveillance of pathogenic and antibiotics resistance factors of isolates revealed that certain genetic elements were over producing in mastitic *S. aureus* isolates especially from clinical cases.

Sanjiv et al., (2008) in their study on epidemiological typing of *Staphylococcus aureus* by DNA restriction fragment length polymorphism of *coa* gene showed out of twenty one staphylococcus aureus isolates of bovine mastitic milk origin from herds at different locations, one of the isolates was *coa* gene deficient whereas the rest revealed polymorphism in the *coa* gene. The isolates revealed three different types of *coa* gene products (600, 680 or 850 bp) and three distinct RFLP patterns were obtained with aluI digests of PCR products. It was concluded that not all the *S. aureus* isolates possessed the *coa* gene, the *coa* genotype was location-specific and this character of the isolates can be used in epidemiological investigations. Due to lack of structured epidemiology investigation, a clear epidemiological picture is missing in Inian context.

The various typing methods differ when it comes to discriminatory power, accuracy and reproducibility, costs and technical challenges. Which method is the most appropriate to study *S. aureus*, depends on the study question asked. For local studies of population structure and short-term outbreaks, it is advantageous to use a method based on hyper
variable loci, such as *(spa)* typing, whereas for global population studies and long-term studies, methods based on stable housekeeping genes (such as MLST) are preferred.

### 2.4. Clinical Significance

*S. aureus* is a prolific mastitis causing bacterium that resides naturally in the environment of the dairy cow. The cure rate of antimicrobial treatments for this agent is low and, therefore, the disease has not been effectively eliminated and/or controlled in many herds (Barkema et al., 2006). Staphylococcal infections are characterized by an ability to colonize the mammary tissue and survival of the bacterial inside epithelial cells, macrophages and even neutrophils. It is commonly assumed that most IMI are result of cow-to-cow transmission, however other sources of *S. aureus* bacteria in the environment of dairy cow have been described. Presumably, contagious strain of *S. aureus* co-exists with a large collection of noncontagious strains (Zadoks et al., 2001). Haveri et al., (2008) compared bacterial genomics of strains from persistent infections and form transient infections and found that genetic elements such as clonal type and penicillin resistance were over-represented in *S. aureus* isolated from persistent IMI. This microorganism is characterized by dynamic fluctuations and cyclic bacterial shedding in milk, which leads to fluctuations in milk SCC that normally fluctuate depending on organism’s number and viability (Schukken et al., 2011).

### 2.5. Clinical manifestation and outcome

Clinical manifestation of bovine mastitis caused by *S. aureus* following IMI can vary from subclinical to a peracute, gangrenous form (Barkema et al., 2006). Subclinical mastitis is the most common and likely the most problematic. The only detectable response is an increased milk SCC but the pathological changes; reduced secretory cell and luminal areas, lead to lowered quality and quantity of milk production. Unfortunately, milk SCC may
remain under threshold limits and infection is often chronic when detected. *S. aureus* mastitis generally responds poorly to treatment (Barkema et al., 2006), relapses are common, and the infection is easily transmitted to unaffected quarters of the same cow. Virulence of the infecting strain and host factors, including the efficacy of the immune response of the cow, which may be related to age and lactation stage, are assumed to affect the severity of the clinical signs and persistence of infection. Older cows are more often infected with *S. aureus* compared with primiparous cows and develop more severe inflammation (Pyörälä and Pyörälä, 1997; McDougall et al., 2007). High SCC in the affected quarter and multiple infected quarters in the same cow impair the prognosis (Sol et al., 2000; Zadoks et al., 2001).

**2.6. Pathogenesis of bovine *S. aureus* intramammary infection**

An infection like IMI can be considered a condition where adverse colonisation by a microbe has occurred in the host animal. Inflammation, the host’s response to the microbe, is seen as swelling, redness, pain, heat and interference in the normal function of the affected organ. The general term for inflammation of the mammary gland is mastitis, which can occur with or without infection.

Development of *S. aureus* infection in the bovine mammary gland can be divided into the following stages: entry of *S. aureus* bacteria in the mammary gland, interaction between the bovine immune system, evasion of immune defence, survival and tissue invasion.

Entry of *S. aureus* into the teat canal can lead to IMI, but this depends on certain conditions as for any infection: the initial number of bacteria, access of the microbe to the target tissue, virulence of the strain and immunity of the host (Projan and Novick, 1997).

**2.6.1. The anatomical-physical barrier of the teat canal**

Entry of a sufficient number of *S. aureus* bacteria via the teat canal into the mammary gland is required for the development of natural IMI. The infective dose of *S. aureus* in bovine IMI is not known precisely, but quantities as small as 10 cfu have caused infection in
an experimental model where bacteria were infused directly into the teat duct (Reiter et al., 1970; Postle et al., 1978). However, infection did not develop in all cows in experimental infection models (Schukken et al., 1999), which suggests that *S. aureus* does not always multiply sufficiently in the mammary gland, but other predisposing factors need to be present. First line defence mechanisms against invasion of *S. aureus* are the physical barriers of the teat canal. A tightly constricted teat canal rapidly closes the end of the teat apex between milkings. Keratin material covering intact teat canal epithelium inhibits bacterial growth, although heifers have sometimes harboured *S. aureus* in this tissue (Trinidad et al., 1990). Attachment of *S. aureus* to host cells or extracellular matrix molecules is a critical step for colonisation and intramammary infection. Adherence to these substances is thought to occur by non-specific physicochemical mechanisms and by specific bacterial host-cell binding (Kluytmans et al., 1997). The bacteria must then resist flushing of milk until adherence to the epithelial cells lining the ductules and alveoli of the mammary gland is accomplished (Frost et al., 1977). *S. aureus* adheres to bovine mammary epithelial cells better than most other bacterial species (Frost et al., 1977) and the presence of milk enhances adherence (Mamo and Fröman, 1994). Adherence to teat canal cells depends on the origin of the cell type (Sutra and Poutrel, 1994). *S. aureus* adheres particularly well to the cells of the upper part of the mammary gland (Frost et al., 1977). *S. aureus* is also able to adhere to fat globules, which allows dissemination to the upper part of the gland by floating (Sandholm et al., 1989).

Teat traumas increase the risk of colonisation of the teats by *S. aureus*. Epithelial damage reveals the underlying subepithelial components, e.g. fibrinogen and collagen, which allows staphylococcal surface proteins to mediate adherence to the host cell matrix (Patti et al., 1994). Exotoxins secreted by *S. aureus* can also be involved in the epithelial damage (Mamo et al., 1988). Even minor traumas, such as those from milking, can facilitate entry of
S. aureus into the teat. Callusing of the teat canal prevents tight closing and extremely callused teats were shown to be more susceptible to S. aureus mastitis (Zadoks et al., 2001). A study by Myllys et al., (1994b) demonstrated that teat canal colonisation, teat canal infection and IMI were far more common in quarters where the teat orifice epithelia were experimentally abraded compared with control quarters.

2.6.2. Innate immune response of the mammary gland

The second line of defence comprises the innate immune system that includes defence cells (neutrophils, macrophages, natural killer cells, and dendritic cells) and humoral factors (lactoferrin, lysozyme, lactoperoxidase, protein A, and complement) present in the teat duct and in the milk (Rainard, 2003; Rainard and Riollet, 2003). Bacterial colonisation leads to influx of somatic cells, primarily PMN, as response to cytokine production triggered by the bacteria. Phagocytosis and killing by leukocytes play the main roles in host defence against S. aureus infection. Numerous exoproteins, like Sags secreted by S. aureus, can interfere with phagocytosis. Phagocytosing cells expressing ingested staphylococci or their products in association with MHC II molecules on their surface in order to present them to T cells. Accumulation of neutrophils and capsule formation often surrounds gland areas infected by S. aureus and the bacteria remain sheltered within furuncles or abscesses.

S. aureus may avoid intracellular death and survive within the host cells (Mullarky et al., 2001), where it is protected from the host defence mechanisms and effects of antimicrobials. S. aureus is not regarded as an intracellular microorganisms but replication within bovine mammary epithelial cells has been demonstrated (Almeida et al., 1996). In the intracellular environment, S. aureus can fall into nutritional stress but still survive.

S. aureus mastitis is characterized by a more moderate and delayed SCC increase, due in part, to limited cytokine response (Bannerman et al., 2004). Congruently, Rainard et al., (2003) also described no detection of IL-1β, TNF-α, IL-8, bovine serum albumin, in milk.
whey from *S. aureus* infected animals. Rainard et al., (2008) also showed that LTA from *S. aureus* induced an increase in chemokine and IL-1β, but little TNF-α in the milk.

Although, *S. aureus* is regarded as a gram-positive bacteria, the expression of TLR2 were correlated with TLR4, indicating coordinating regulation of these two PRRs (Goldammer et al., 2004), although the expression of TLR9 was not increased in mastitis (Goldammer et al., 2004). Cytokine gene expression in mammary epithelial cells induced by *S. aureus* infection was delayed and less than 5% of the cytokine expression observed in experiment of *E. coli* (Yang et al., 2008; Mount et al 2009). This impaired proinflammatory activation is paralleled by a complete lack of NF-κB activation in primary bovine mammary epithelial cells by *S. aureus* or LTA. In contrast to *E. coli* and LPS that activates strongly NF-κB in these cells. A large proportion of this activation is attributable to TLR-mediated signalling (Figure 2.2), since dual transdominant negative DN-MyD88-DN-TRIF factor blocks more than 80% of the pathogen-related NF-κB activation in primary bovine mammary epithelial cells. These facts may contribute to well-known ability of this bacterium to establish chronic intramammary infections (Yang et al., 2008; Guntler et al., 2011). For example, the Interleukin (IL)-8 and TNF-α were not detected in milk from quarters experimentally infected with *S. aureus* (Bannerman et al., 2004), although, the mRNA expression of TNF-α in mammary cells increases during infection (Alluwaimi et al., 2003). Expression of IL-8 by milk somatic cells was also increased in *S. aureus* challenged mammary glands, but in lower magnitude than *E. coli* challenged mammary glands. Although, the expression of IFN-γ was not increased in milk somatic cells from *S. aureus* challenged quarters. *In vitro*, mammary epithelial cells demonstrated greater mRNA expression of IL-1β, IL-8 and TNF-α 24 h after infection with *E. coli* than *S. aureus* (Lahouassa et al., 2007). Wellnitz et al., (2011) also found that infusion of *E. coli* LPS induced an increased TNF-α in milk from glands given LPS, but not by *S. aureus* LTA. The
levels of lactate dehydrogenase, an enzyme released by degenerating cells, was greater in milk from glands instilled with LPS than with LTA. LPS was also a stronger induced of IL-8 and IL-1β. Conversely, the ability of to induce clinical or subclinical mastitis was dependent on the dose used. LTA proved to induce strongly the secretion of the chemokines CXCL1, CXCL2, CXCL3 and CXCL8, which induced neutrophils recruitment. The complement-derived chemoattractant C5a was generated in milk only with the highest dose of LTA used. Furthermore, the pro-inflammatory cytokine IL-1β has been induced in milk, but there is few amount of TNF-α and no IFN-γ (Rainard et al., 2008). The Muramyl Peptide (MDP), an elementary constituent of the bacterial peptidoglycan, induce a prompt influx of neutrophils mediated by chemoattractants for these leukocytes (CXCL1, CXCL2, CXCL3, CXCL8 and C5a) and the highest concentrations of these chemoattractants were followed after challenge in combination with LTA, which signal transduction is mediated by TLR2, although they not contribute significantly to pro-inflammatory cytokines. Thus, TLR2 and NOD2, a major sensor for MDP, pathways could cooperate to trigger an innate immune response to *S. aureus* mastitis (Bougarn et al., 2010). Induction of immune functions in mammary epithelial cells is accomplished via the activation of the relevant TLR and their downstream signaling pathways (Figure 2.2). Induction of these genes by *S. aureus* is reduced, due to, in part, impairment of MyD88 signaling, immediately downstream from trans-membrane TLR (e.g., TLR2, TLR4). *S. aureus* apparently prevents the formation of so-called Myddosome around TIR domain of the TLR forming the structural platform for the attachment of further downstream acting factors (Schukken et al., 2011). As a consequence, *S. aureus* elicits an immune response in these cells dominantly by IL-6, while *E. coli* also activates IL-1β and TNF-α (Gunther et al., 2010; 2011; Schukken et al., 2011). The upregulation of IL-6 by both bacteria may be due to a MyD88 independent mechanism (Gunther et al., 2010; 2011), which as cited above is associated with latephase NF-κB response. It has also been suggested that *S.
Impaired NF-κB activation in mammary epithelial cells resulting in very low cytokine expression (Lara-Zarate et al., 2011). These authors reported that bovine prolactin stimulates *S. aureus* internalization in bovine mammary gland by regulating several innate immune elements, which is often modulated by NF-κB (Lutzow et al., 2008). On the other hand, prolactin induced NF-κB activation in bovine mammary epithelial cells; however, it was inhibited by *S. aureus* in presence of this hormone. When, these authors blocked NF-κB activation with acetylsalicylic acid, an inhibition of *S. aureus* internalization was found (48%) in prolactin stimulated cells. The infection of bovine mammary epithelial cells with *S. aureus* induced inhibition of NF-κB activation in the presence of prolactin that correlates with down regulation in prolactin-mediated TNF-α (27%) and nitric oxide production in mammary epithelial cells. Curiously, Griesbeck-Zilch et al., (2008) encountered differences in expression of TLR2 and TLR4 by mammary epithelial cells in *S. aureus* and *E. coli* infections only after 24 h, when *S. aureus*-induced expression was significant lower. In contrast, after 1 h *S. aureus* induced a significantly higher expression level of TNF-α and IL-1β, but after 6 and 24 h the transcription activity in *E. coli* treated cells was higher. In contrast, *E. coli* induced a significant increase expression of IL-8 after 1h, but *S. aureus* caused no alteration in this chemokine. The regulated upon activation, Normal TCell expressed and secreted (RANTES) increased in *S. aureus* and *E. coli* treated bovine mammary epithelial cells after 1 h, whereas after 6 and 24 h the expression was significantly higher in *E. coli* treated cells. Lactoferrin showed a deviating expression pattern to pathogen stimulation, in which at 1 h *E. coli* induced a higher mRNA expression, whereas the highest level was reached after 24 h of *S. aureus* stimulation. The complement factor 3 was the only factor that responded equally to both microorganisms. Genini et al., (2011) described that mastitis induced a prominence of metabolic and stress signals in the early stage and of the immune response and lipid metabolism in the late stage, both mechanisms apparently...
modulated by few genes. Comparison of *E. coli* and *S. aureus* infections in cattle revealed
that affected genes showing opposite regulation had the same altered biological functions and
provided evidence that *E. coli* caused a stronger host response. The majority of genes with
opposed regulation associated with immune response belong to antigen presentation,
inflammatory response, cell-to-cell signaling and interaction network. Both cell death and
lipid metabolism were among the most significant molecular functions altered in proteins of
cows infected with either *E. coli* or *S. aureus*. After 48 h post-challenged with *S. aureus*
TLR1 was significantly expressed in ductal, gland cistern and teat canal, TLR3 showed a
moderate increase in teat canal tissue, TLR6 and TLR7 presented a moderate increased in
gland cistern tissue, TLR5 and TLR7 were also significantly increased in alveolar in alveolar
tissue.

Conversely, the genes encoding TLR4, NOD1 and NOD2 were significantly
decreased in teat canal tissue, TLR6 in ductal tissue and TLR8 in gland cistern tissue. TLR2,
TLR9 and TLR10 showed no differential expression across these four tissues regions. Of the
regions examined, chemokine and effector molecule expression was most significantly
stimulated in alveolar tissue, in particular the expression of serum amyloid A and
haptoglobin, two acute phase proteins and defensins-β 4 and 5 (Whelehan et al., 2011).
Thus, *S. aureus* appears to mostly circumvent the host immune response and IMI typically
result in a very moderate host response with minimal observable innate immune response.
(Bannerman et al., 2004; Bannerman, 2009; Petzl et al., 2008; Schukken et al., 2011).
Figure 2.2. Different signaling pathways of TLR2, Nod, and TNFR1 in response to ligand, resulting in activation of NFkB, the nuclear transcriptional factor responsible for regulation of the immune response genes. RIP1, receptor-interacting protein 1; FADD, Fas-associated death domain protein; TRAF2, TNF receptor-associated factor 2; PI3K, phosphatidylinositol 3-kinase composed of two subunits (p85 and p110).

In many cases, however, the pathogen can evade the host immune response, resulting in its survival and propagation in infected BMECs. This persistence can lead to a lengthy non-shedding subclinical phase in which *S. aureus* proliferates in the gland, ultimately resulting in the development of immunopathology that enables the dissemination of infection.
to other tissues and shedding from the host. The survival of the pathogen in the host cells is believed to be achieved through a diverse range of mechanisms including the inhibition of phagosome maturation and the suppression of key immune-regulatory pathways that mediate the host immune response to infection. Therefore, analysis of the BMECs transcriptome in response to *S. aureus* infection may offer a deeper understanding of the cellular processes governing pathogen-epithelial cell interactions and how modulation of these cellular pathways can result in pathology. Furthermore, identification of transcriptional markers of infection may enable novel diagnostics for mastitis, providing new tools for disease management. On-going developments in mammalian genome resources and high-throughput deep-sequencing technologies continually provide improved methodologies for analysis of the gene expression changes induced in mastitis caused by *S. aureus* in vivo. Sequencing-based methods measure absolute gene expression and avoid many inherent limitations of earlier microarray-based assays (Wang et al., 2013).

2.7. Regulation of Immune Response

2.7.1. Histone modifications during bacterial infection

The long coexistence of bacterial pathogens with their eukaryotic hosts, and their coevolution, have provided pathogens with an amazing capacity to exploit host cell functions for survival, replication inside or outside cells, and escape from early innate immune responses.

It has increasingly become clear that histone modifications and chromatin structure are key regulators of eukaryotic transcription and, thus, good targets for pathogens during an infection (Paschos and Allday 2010). In fact, viruses have long been described as able to manipulate the host chromatin to impose a transcriptional program beneficial for the maintenance of infection. Recent reports show that bacterial pathogens are also able to induce chromatin remodeling, thereby imposing a specific transcriptional profile.
Similar to viruses, bacteria provoke histone modifications and chromatin remodeling in infected cells, thereby altering the host’s transcriptional program and in most cases dampening the host innate immune response (Hamon and Cossart 2008). Various bacterial products or secreted factors may induce chromatin/histone modifications, involving different host signalling cascades.

Histone tail modification is a key control point of chromatin structure and therefore gene regulation (Starhl and Allis 2000). However, many recent studies show that any given modification has the potential to both activate or repress transcription, and it is the context in which they are found—i.e., the surrounding modifications—that is important for determining their function. The addition or removal of certain tail modifications thus present an additional mean for regulating gene expression effectuated by the enzymes responsible for the post translational modifications. Writer enzymes include histone methyl transferases (HMTs) and histone acetyl transferases (HATs), responsible for methylation and acetylation, respectively. The removal of modifications are effectuated by erase enzymes like histone demethyl transferases (HDMTs) and histone deacetylases (HDACs). The modification of certain DNA regions have classically been attributed to interactions between DNA, regulating transcription factors and such writers or eraser enzymes (Shilatifard 2006).

These modifications are dynamic and reversible. In general, acetylation, phosphorylation, and ubiquitination of histone tails are associated with active transcription, while de-acetylation and biotinylation result in gene repression. Methylation, which can occur on either a lysine or an arginine residue, is the best-characterized modification to date. Methylation is associated with either transcriptional activation or repression depending on the lysyl residue modified and whether this residue is mono-, di-, or trimethylated.

The other well-described mark, acetylation, has been mostly shown associated with active transcription. It is thought that acetylation renders chromosomal domains more
accessible to the transcription machinery. Acetylation is one of the most studied post translational modifications in histones. This post translational modification is regulated by specific enzymes, like histone acetyltransferases (HAT) or histone deacetylases (HDAC) that modulated the balance of acetylated histones (Peserico et al., 2011).

**TLR-Induced Chromatin Modification**

Innate immunity is the first line of defense against a bacterial infection, and most organisms are able to mount an efficient early, nonspecific response leading to the recruitment of cellular effectors and inflammation. Microbial components that elicit an inflammatory response have been called microbial associated molecular patterns (MAMPs) and include LPS, bacterial flagellin, lipoteichoic acid, peptidoglycan, and nucleic acids. Host cells recognize MAMPs through PRRs present either at the cell surface and/or on endosomes, for TLRs, or in the cytoplasm, for nucleotide- binding oligomerization domain proteins (NODs) and NODlike receptors (NLRs). These receptors activate signaling cascades leading to transcriptional activation of immunity genes such as cytokine genes (reviewed in Akira and Takeda, 2004). LPS is the major component of the outer membrane of Gram negative bacteria and is one of the best-characterized agonist of host inflammatory signaling responses. LPS is recognized by TLR4, and downstream signaling includes activation of the nuclear factor-kB signaling cascade (NF-kB), activation of all three MAPK cascades, and increased transcription of genes for proinflammatory cytokines such as interleukin-12 (IL-12), IL-6, and tumor-necrosis factor (TNF) (Akira and Takeda, 2004). The first link between LPS stimulation and chromatin remodelling has been established through the study of a gene activated by LPS, the IL-12 cytokine gene. This cytokine is produced by activated macrophages and dendritic cells and is required for the activation of T cells. It has been shown that a nucleosome spans the promoter of the IL-12 gene, which is displaced upon LPS stimulation, thereby allowing transcription to occur. Nucleosome repositioning by LPS
occurred in a TLR4-dependent manner and correlated with histone H3 and H4 acetylation at the IL-12 promoter. From these data, it was first suggested that LPS stimulation of TLR4 induces histone acetylation and nucleosome remodeling, allowing for NF-kB to gain access to the IL-12 promoter. One year later, another group elucidated the mechanism underlying chromatin remodeling and NF-kB accessibility at the IL-12 promoter and other NF-kB-dependent promoters (Saccani et al., 2002). The authors showed that activation of the p38 MAPK pathway upon LPS stimulation of TLR4 induced phosphorylation of H3S10 and phosphorylation/acetylation (S10/ K14) of H3 (phosphorylation at serine 10 and acetylation on lysine 14 of the same histone tail), which were crucial modifications for recruiting NF-kB to the promoter of certain genes, such as IL-12. Therefore, the current model is that some NF-kB-activated genes require phosphorylation of H3S10 and phosphoacetylation of H3S10K14 via the p38 MAPK pathway so that their promoters become accessible to NF-kB, allowing transcription to occur. However, it also identified MAPK-independent mechanisms of phosphorylation of H3S10 and p38- and phosphorylated H3S10-independent mechanisms of gene activation, suggesting that there are at least two other modes of NF-kB-dependent gene activation. LPS induction of TLR4 and expression of inflammatory cytokines and chemokines is of great importance for the host to clear

Modulation of host transcription by pathogens is a well-accepted concept but how specific programs are controlled by pathogens remains elusive. As detailed above, the fact that, histones can be modified at specific promoters during infection starts to shed light on this important issue. Indeed, to date, many studies remain at the correlative level. Whether changes in histone modification are specifically induced by the bacterium to subvert normal host responses or are the normal host responses to this pathogen will have to be determined. The likely scenario is that multiple signalling cascades converge on the promoters of the affected genes, and the contribution of each one of them will need to be determined in future
studies. Moreover, of particular focus, how *S. aureus* induced histone modification regulates host immune response is an area yet to be extensively ventured.

### 2.7.2. miRNA mediated regulation

Much has been learned about the molecular, cellular, and organismal components of the immune system and the functions of these components in immune responses and tolerance. At the molecular level, the focus of most studies has been on protein-coding genes. For decades, control of cellular behavior was thought to be the exclusive purview of protein-based regulators. The recent discovery of small RNAs (sRNAs) as a universal class of powerful RNA-based regulatory biomolecules has the potential to revolutionize our understanding of gene regulation in practically all biological functions. In general, sRNAs regulate gene expression by base-pairing with multiple downstream target mRNAs to prevent translation of mRNA into protein. Illuminating the functional roles of sRNAs in virulence and host immunity can provide the fundamental knowledge for development of next-generation antibiotics using sRNAs as novel targets.

The discovery of miRNA genes revealed an unexpected layer of genetic programs that regulate the immune response at the post-transcriptional levels (Ambros et al., 2008, Ruvkun et al., 2008). Emerging evidence indicates that miRNA play important roles in diverse biological processes and a number of recent reviews have summarized the growing body of knowledge regarding miRNA functions in immune cells (Xiao et al., 2007; O’Connell et al., 2010) (Figure 2.3).

They act as post-transcriptional regulators of gene expression, altering mRNA stability and translation efficiency by hybridizing to the 39 untranslated regions (UTRs) of certain subsets of mRNAs (collectively as many as 60% of all mRNA transcripts) BiY et al., 2009). Since their initial discovery in Caenorhabditis elegans in 1993 (Lee et al., 1993), researchers have gained much insight into the prevalence of miRNA in other species. The
The latest miRBase database (release 19) contains 21,264 precursor miRNAs, expressing 25,141 mature miRNA products, in 193 species (Kozomara and Jones 2011). miRNAs have been shown to play key roles in the regulation of innate and adaptive immunity in humans and mice (O’Connell et al., 2010). miR-146a, for example, regulates the innate immune response to bacterial infection, targeting TNF receptor-associated factor 6 (TRAF6) and Interleukin-1 receptor-associated kinase 1 (IRAK1) (Williams et al., 2008). Studies elucidating the regulatory roles of miRNAs in bovine infection and immunity, however, are more limited. Bovine miRNAs have been shown to be expressed in a wide range of tissues, including immune-related ones (Xu et al 2009), but only a handful of studies have investigated how the expression of bovine miRNAs are altered in response to infection. A recent RT-qPCR study, for example, highlighted the differential expression of five inflammation related miRNAs (miR-9, miR-125b, miR-155, miR-146a and miR-223) in response to E. coli lipopolysaccharide (LPS) and S. aureus enterotoxin B stimulation of bovine monocytes (Dilda et al., 2011). Two other recent studies have used a similar approach to identify several miRNAs that were differentially expressed in the mammary gland tissue of cattle with mastitis (Naeem et al., 2012; Hou et al., 2011). These and other studies suggest roles for individual miRNAs in regulating bovine immunity, however, according to Ensembl v66 there are over 1,300 annotated miRNAs in the bovine genome.
Figure 2.3. Panoramic view of miRNAs converged with the NF-κB network.

Recent investigations implicated miRNAs in the regulation of development, differentiation, and disease. miR-146a limits Toll-like receptor signaling by blocking the signaling molecule TRAF6 (Taganov et al., 2007), and miR-155 targets the lipid phosphatase SHIP1 (O’Connell et al., 2009), an important signal for macrophage activation. miR-132 has anti-inflammatory effects by binding acetylcholine (ACH) mRNA, a critical inhibitor of peripheral inflammation (Shaked et al., 2009). The exposure of cultured macrophages to lipopolysaccharide (LPS) leads to up-regulation of miR-155, which targets the mRNA for CCAAT/enhancer binding protein Beta (C/EBP Beta), implicated in the regulation of pro-inflammatory cytokines during macrophage activation and the acute phase response. Studies by Liu et al., (2009) demonstrated that the induction of miR-147 by TLR prevents excessive inflammatory response through a negative-feedback loop mechanism. TLR stimulation
induces miR-147 and requires activation of both NF-κB and IRF3. Furthermore, miR-147 attenuates the TLR-induced inflammatory response in macrophages (Liu et al., 2009). In another study, miR-105 was shown to modulate TLR-2 translation in human gingival keratinocytes. Dysregulated pro-inflammatory cytokine production is a hallmark of several infection and miRNAs are likely to play significant, but until now vaguely characterized, roles in aberrant cytokine regulation. mir146 which was induced by LPS in human monocytes, acted as negative feedback regulator for TLR induced inflammatory response through targeting IRAK-1 and TRAF-6. Similarly Liu et al 2009 had reported that miRNA-147 was another microRNA induced by TLR stimulation in murine macrophages, functions as a negative regulator of TLR induced inflammatory response, although the molecular target was not identified. Qi et al., 2012 demonstrated that miR-210 targets NF-κB inhibited the expression of p50 and subsequent NF-kB activation. miRNA-21 inhibits TLR-2 agonist induced lung inflammation in mice.

It has been recently suggested that the TLR dependent reprogramming of inflammatory genes is mediated by two distinct levels of regulation (Glazar and McCall 2010). The first level is transcriptional control mediated by epigenetic modifications, and the second level is regulated by the TLR-4 dependent differential expression of miRNAs (miR-221, miR-579 and miR-125b). Although several studies have been carried out in this emerging area of miRNA with respect to numerous infections, its role in mastitis is in incipient stage and yet to be unravelled.