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Bovine *Staphylococcus aureus* mastitis: from the mammary immune response to the bacteria virulence genes

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is one of the most important bacteria in veterinary medicine. In dairy herds, it is a contagious bacterium responsible mainly for subclinical mastitis in cattle, which frequently gives rise to persistent and chronic infection. Mastitis cause considerable economic losses due to i) decreased milk production, (ii) reduced milk quality, and (iii) treatment costs. Mastitis is also a public health problem. Indeed, the strains isolated from infected glands could produce enterotoxins. Three factors interact in mastitis: the host, the pathogen and the environment. This thesis focuses on two main aspects: the host immune response and the virulence factor of *S. aureus.*

The first chapter of the thesis focused on the development of a new mammary gland model to study the innate immune response bacterial infection. The mammary gland is a complex organ, and the immune response is a consequence of the different cell population interactions. Continuous or primary epithelial cell lines have been extensively used to study the mammary gland immune response, but they are composed of a single cell population.

Previous studies explored the tissues of lactating cows, unconsidering the possibility of an already triggered immune response. To investigate the innate immune response of the bovine mammary gland, we used an explant of healthy heifer gland. This model allowed us to: i) exclude previous exposure of the udder to microorganisms, which might have damaged the cells and/or triggered an immune response, and ii) consider the interaction of the challenging microorganism with the tissue cell populations.

Our aim was to test whether this innovative model might be a valid model to investigate the innate immune response to infection. The study was carried out on 2 mm^3-sections of heifer udders, in 2 consecutive trials, using LPS or LTA in the first trial and two different concentrations of *S. aureus* in the second. Treated and untreated sections were collected after 1h, 3h and 6h incubation; in the first trial, a final time-point at 18h was considered. The mRNA

expression of TNFα, IL-1β, IL-6, IL-8 and LAP was analyzed by quantitative real-time PCR. Histological examination showed well-preserved morphology of the tissue, and apoptosis only showed a slight, not significant increase throughout the experiment. IL-1β and IL-6 were significantly up-regulated, in response to LPS or *S. aureus*, while TNF-α and IL-8 significantly increased only under LPS treatment. LAP expression showed a significant late increase when stimulated by LPS. The immunochemical staining of the sections demonstrated a higher number of T lymphocytes within the alveolar epithelium, in comparison with interstitial localization. Since the explants belonged to pubertal non-pregnant heifers, T cells may be regarded as resident cells, suggesting their participation in the regulation of mammary homeostasis. Therefore, applying our model would give new insights in the investigation of udder pathophysiology.

The second chapter of the thesis focused on *S. aureus* in bovine intrammary infections. Previous literature on the *S. aureus*-intrammamary suggested that infection might be related to a combination of *S. aureus* virulence factors beyond host factors. The present study considered 169 isolates from different Italian dairy herds that were classified into four groups based on the prevalence of *S. aureus* infection at the first testing: low prevalence (< 5 %; LP), medium–low (5.1 - 24 %; MLP), medium–high (24.1 - 40 %; MHP) and high (> 40 %; HP). We aimed to correlate the presence of virulence genes with the herd prevalence of intramammary infections in order to develop new strategies for the control of *S. aureus* mastitis. Microarray data were statistically evaluated using binary logistic regression and correspondence analysis to screen the risk factors and the relationship between prevalence group and gene. The analysis showed: (1) 24 genes at significant risk of being detected in all the herds with infection prevalence >5%, including genes belonging to microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), immune evasion and serine proteases; and (2) a significant correlation coefficient between the genes interacting with the host immune response and HP isolates against LP ones. These results support the hypothesis that virulence factors, in addition

to cow management, could be related to strain contagiousness, offering new insights into vaccine development.

ABSTRACT (in Italian)

Staphylococcus aureus (*S. aureus*) è uno dei batteri più importanti nella medicina veterinaria. Negli allevamenti da latte, è un batterio contagioso, principale agente responsabile di mastite subclinica nei bovini, che porta generalmente allo sviluppo d'infezioni persistenti e croniche. Come conseguenza, la mastite implica notevoli perdite economiche dovute alla riduzione della produzione e della qualità del latte e al costo del trattamento. La mastite deve essere considerata anche un problema di salute pubblica, non solo una perdita economica per l'allevatore e l'industria lattiero-casearia. Infatti, i ceppi di *S. aureus* possono produrre enterotossine, responsabili di tossinfezioni alimentari nell'uomo. La mastite è considerata una malattia influenzata dall'interazione di tre fattori: l'ospite, l'agente patogeno e l'ambiente. Questa tesi si concentra su due aspetti principali: la risposta immunitaria dell'ospite e i fattori di virulenza di *S. aureus*.

Il primo capitolo della tesi riguarda lo sviluppo di un nuovo modello sperimentale di ghiandola mammaria, per studiare la risposta immunitaria innata a seguito di un'infezione batterica. La ghiandola mammaria è considerata un organo complesso e la risposta immunitaria è una conseguenza delle diverse interazioni delle popolazioni cellulari. Le linee cellulari epiteliali continue o primarie sono state ampiamente utilizzate per studiare la risposta immunitaria della mammella, ma la loro composizione è di tipo monocellulare. Diversi studi hanno utilizzato i tessuti di vacche in lattazione, sottovalutando la possibilità di una risposta immunitaria già attivata. Per comprendere meglio la risposta immunitaria innata della ghiandola mammaria bovina, è stato utilizzato come modello un espianto di ghiandola mammaria di una manza sana, la quale ci ha permesso: i) di escludere un'esposizione precedente della mammella a microrganismi, che potrebbe aver danneggiato le cellule epiteliali e innescato una risposte

immunitaria innata antecedente all'esperimento, e ii) di considerare l'interazione tra le popolazioni cellulari del tessuto e il microrganismo utilizzato per l'infezione sperimentale.

Pertanto, il nostro obiettivo è stato quello di verificare se un espianto di ghiandola mammaria di manza, in cui la struttura dei tessuti sia mantenuta, potrebbe essere un modello valido per indagare la risposta immunitaria innata all'infezione. Lo studio è stato eseguito su sezioni di 2 mm^3 di ghiandola mammaria, in due prove consecutive, utilizzando LPS o LTA nel primo trial e due diverse concentrazioni di *S. aureus* nel secondo. Le sezioni trattate e non trattate sono state raccolte dopo 1h, 3h e 6h di incubazione; nella prima prova, è stato considerato un punto finale a 18h. L'espressione di mRNA di TNFα, IL-1β, IL-6, IL-8 e LAP è stata analizzata mediante real time PCR con quantificazione relativa. L'esame istologico ha dimostrato una morfologia del tessuto ben conservata, e l'apoptosi ha mostrato un lieve e non significativo aumento durante l'esperimento. IL-1β e IL-6 sono state significativamente up-regolate in risposta a LPS o *S. aureus*, mentre TNF-α e IL-8 sono aumentati significativamente solo nel trattamento di LPS. L'espressione di LAP ha mostrato un significativo aumento tardivo quando stimolato da LPS. La colorazione immunoistochimica delle sezioni ha dimostrato un maggior numero di linfociti T all'interno dell'epitelio alveolare, rispetto alla localizzazione interstiziale. Poiché gli espianti appartengono a manze mai entrate in riproduzione, i linfociti T possono essere considerati come cellule residenti, suggerendo la loro partecipazione alla regolazione dell'omeostasi mammaria. Pertanto, l'applicazione del nostro modello potrebbe dare nuove visioni nell'indagine della patofisiologia della ghiandola mammaria bovina.

Il secondo capitolo della tesi riguarda lo studio dei fattori di patogenicità di *S. aureus* nelle infezioni intramammarie bovine. La letteratura suggerisce che l'infezione sia correlata a una combinazione di fattori di virulenza del batterio, oltre ai fattori immunitari dell'ospite. Il presente studio ha preso in esame 169 isolati provenienti da diversi allevamenti italiani classificati in

quattro gruppi in base alla prevalenza dell'infezione da *S. aureus* alla prima analisi del latte: bassa prevalenza (LP), medio-bassa (MLP), medio-alta (MHP) e alta (HP). Abbiamo deciso di correlare la presenza di geni di virulenza con la prevalenza delle infezioni intramammarie per sviluppare nuove strategie per il controllo della mastite da *S. aureus*. I dati di microarray sono stati valutati usando la regressione logistica binaria e l'analisi della corrispondenza, con lo scopo di analizzare la relazione tra il gruppo di prevalenza cui appartiene l'allevamento e i geni di virulenza. L'analisi ha mostrato: (1) 24 geni a rischio significativo di essere rilevati in tutti gli allevamenti con prevalenza di infezion e> 5%, compresi i geni appartenenti alla categoria dei "microbial surface components recognizing adhesive matrix molecules (MSCRAMM)", dell'evasione immunitaria e delle serin-proteasi; e (2) un coefficiente di correlazione significativo tra i geni che interagiscono con la risposta immunitaria dell'ospite e gli isolati HP contro quelli LP. Questi risultati sostengono l'ipotesi che alcuni fattori di virulenza, oltre alla gestione dell'allevamento, siano correlati alla contagiosità del ceppo, offrendo nuove prospettive per lo sviluppo di vaccini.

1 INTRODUCTION

1.1 Bovine mastitis

Bovine mastitis is an inflammation of the mammary gland. It is considered a disease in which three factors interact: the host, the pathogen and the environment.

Mastitis represents the most important disease of dairy cattle considering its economic impact. It is hard to estimate the losses associated to clinical mastitis, because they arise from decreased milk production, the culling of chronically infected cows, the costs for veterinary treatment and penalties on milk quality (McDougall et al., 2009). The major economic consequence related to mastitis outbreak is the reduction in the quality and quantity of milk production. The reasons are both the increase of somatic cells and blood proteins against the caseins. It is also important to consider the sanitary impact related to the consumption of infected raw milk; for instance, the ingestion of pathogens or of toxins can cause food-borne diseases.

Depending on the infectious agent and the immune response of the mammary gland, three clinical presentations may occur: (i) subclinical, (ii) clinical, and (iii) chronic mastitis.

Subclinical mastitis is the most common form, in which no morphological changes are observed in either the udder or the milk. However, high numbers of microorganisms can be shed in the milk. This presentation can be demonstrated by microbiological culture of milk and somatic cell counts (in a healthy cow: < 100,000 cell/ml).

In contrast, in **clinical mastitis**, the udder and the secretions express visible changes, such as aqueous milk with the presence of solid corn, swelling and heat of the mammary gland)**.** Acute mastitis is characterized by its rapid development, with rubescence, hardening, pain of the udder, alteration of the milk, and/or complete loss of production. There are systemic signs such as fever, lack of appetite, decrease of the ruminal

functions, tachycardia, weakness, and depression. When every sign comes up more seriously and quickly, the presentation may be named peracute mastitis.

Chronic mastitis is characterized by the long duration of the disease, in which subclinical and clinical forms may alternate.

1.2 Aetiology

Bovine mastitis has an infectious aetiology, caused by many microorganisms. Around 140 different microbial species have been isolated from the mammary gland (Ranjan *et al.,* 2006), but not all of them cause mastitis. The pathogens can arise from different primary sites, they multiply in different environments and therefore the timing of the exposure of the cow to the bacteria will vary. Subsequently the acuteness and persistency of the intramammary infections (IMI) differ and also the probability of cure when therapy is given. Based on their epidemiological characteristics, the pathogens are classified as:

Contagious bacteria: the most common bacteria are *Staphylococcus aureus* and *Streptococcus agalactiae.* The primary reservoir is the udder of the cow. The diffusion of the disease takes place from cow to cow, due to poor milking management and to the inadequate sanitization of the milking equipment.

Micoplasma bovis is less common. It can cause a rapid outbreak of clinical mastitis, but can also present with a subclinical course. It is difficult to control due to the lack of therapy efficacy. The presence of this pathogen in the herd is commonly associated with the arrival of a new infected cow.

Environmental bacteria: there are 3 main groups: coliforms (*E. coli, Klebsiella spp.) Streptococcus spp* (the most important *S. uberis e S. dysgalactiae*)*, Trueperella pyogenes* and *Prototheca spp.* The primary reservoir is the environment. The frequency of the infections is related to the increase of microorganisms in the environment and to a depressed immune response of the gland. The high microbial contamination could be controlled by correct herd management and strict hygienic measures.

Opportunistic bacteria: (coagulase-negative *Staphylococcus* species, CNS). They are normally present on the healthy skin of the udder. The infection causes an inflammation with increased cellular content in milk. The disease outbreak is conditioned by a decreased immunity at teat level. The most common CNS are: *Staph. chromogenes, Staph. epidermidis, Staph. haemolyticus*.

1.3 *Staphylococcus aureus* and its pathogenesis in dairy cattle herds

Staphylococcus aureus belongs to the genus *Staphylococcus* (from the Greek: σταφυλή, staphylē, "grape" and κόκκος, kókkos, "granule"), from the family of *Stafilococcaceae*. The genus groups gram-positive cocci, with a diameter of 0.5-1 micron, non-motile and facultative anaerobes (capable of growth both aerobically and anaerobically). When grown on blood agar plates, they have large, round, white-yellow colonies, often with hemolysis; at microscope, appears as grape-like clusters.

S. aureus is considered one of the most important pathogenic *Staphylococcus* species in veterinary medicine, in ruminants, as well as in pigs, rabbits and poultry, and the major opportunistic pathogen in humans. In dairy herds, this bacterium is the most important responsible for subclinical mastitis in cattle (Bradley, 2002); it frequently gives rise to persistent and chronic infections (Degen *et al.,* 2015).

As a consequence, in both clinical and subclinical mastitis there is a substantial loss in milk production: in European countries, bulk milk with a cell concentration higher than 400,000/mL is considered unfit for human consumption (EEC directive 94/71). The average annual cost of mastitis in a herd of 100 dairy cows is estimated to be €4896 (Halasa *et al.,* 2009). Mastitis must be considered also a public health problem, not only an economic loss for the dairy industries. Indeed, the strains isolated from infected glands could produce enterotoxins. Despite the shed is generally moderate (less than 10,000 CFU/mL), food poisoning is possible in fermented raw milk products (Le Loir *et al.*, 2003; Le Marechal *et al.,* 2011) and raw milk cheese. In the herd, the principal reservoir of *S. aureus* is the infected udder; it was demonstrated that cows may harbour the bacterium on the teat skin, in the rectum or nasal cavity (Roberson *et al.,* 1994). Infected udders shed concentration of staphylococci in milk, which can vary from less than 100 CFU to more than 100,000 CFU/mL, contaminating the teat cups of the milking machine.

Therefore, transmission is possible primarily during milking from udder to udder, via the milking machine or the farmer's hands. Sakwinska *et al.* (2011) demonstrated also secondary transmission between herd animals and the farmer.

The bovine *Staphylococcus aureus* IMI pathogenesis includes different stages: entry and attachment of the bacteria to the mammary epithelium, interaction with the immune response of the host, evasion of the host immune system, bacteria survival and tissue invasion.

The development of IMI after the entry of *S. aureus* into the teat canal depends on different factors, such as the initial number of bacteria, the virulence of the strain and immunity of the host (Projan and Novick, 1997). Despite the entrance of *S. aureus* via the teat canal into the mammary gland is the start point for the development of natural IMI, a previous study showed that mastitis did not develop in all cows in experimental infection models (Schukken *et al.,* 1999). Therefore, predisposing factors, such as the genetic background of both pathogen and host, are involved in a successful colonization.

1.4 Virulence factors of Staphylococcus aureus

The differences observed in strain diffusiveness and outcome of mastitis might be related to the presence and combination of *S. aureus* virulence factors. Previous studies of *S. aureus* genome showed a chromosomal colinearity between the strains, with some genes harboured by all strains and others characterized by variable presence (Lindsay *et al.,* 2004). The bacterial genome comprises core and accessory genes (auxiliary and / or foreign genes that might be present in a given isolate, or not). In *S. aureus*, 75% of the genes correspond to the core and 25% to the accessory genome. The genes are always organized in the same order, facilitating a similarity of 98-100% at the amino acid level (Lindsay *et al.,* 2004).

The core genes are usually associated with metabolism and other housekeeping functions common to all *S. aureus* strains. They also include variable genes (not essential for growth and survival), and species-specific genes, such as some adhesion factors, surface binding proteins, exoenzymes and the capsule biosynthetic cluster. The accessory genome is the most variable genes class, consisting of genes with a horizontal transfer origin, among them pathogenicity islands, phages, plasmids, transposons and chromosomal cassettes (Lindsay *et al.,* 2004). In particular, the staphylococcal chromosomal cassette carries methicillin (SCC*mec*), fusidic acid or heavy metal resistances, and recombinase genes which facilitate the horizontal gene transfer across the genus *Staphylococcus* (Ito *et al.*, 2003). The success of *S. aureus* as a versatile pathogen is imputable to its ability to produce wide-ranging virulence factors. Specifically, modulation of virulence determinant expression and synthesis is broadly controlled by the aid of well-characterized global regulatory elements, those of **staphylococcal accessory regulator** locus (*sar*) and those of **accessory gene regulator** locus (*agr*) (Shaw *et al*. 2003). The *agr* system activates expression of extracellular proteins and represses synthesis of cell-wall associated proteins in the post exponential phase (Shaw *et al.,* 2003). The *agr* locus encodes a

quorum-sensing system, which expresses the effector molecule (RNAIII) in response to accumulation of an autoinducing peptide. The result of this reaction is the reduction of the expression of some cell-wall determinants (Oscarsson *et al.*, 2006). In particular, the *agr* system of *S. aureus* consists of 4 genes (*agr*A, B, C and D) that are co-transcribed, and of the gene RNAII. These genes interact with each other, up-regulating the expression of exotoxins and proteases, and down-regulating the expression of surface proteins. In particular, this unique regulation cascade of bacterial gene expression responds to increased bacterial cell density (Iwatsuki *et al*., 2006). The *sar* genes are a family of different homologous genes, divided in 3 groups based on genes structure (Zhu *et al.*, 2014):

1) single-domain proteins (sarA, sarR, sarT, sarV, sarX, and rot);
2) double domain proteins (sarS, sarU and sarY);
3) marR homologues (marA and sarZ).

The *sar*A gene influences the synthesis of proteases, working in an *agr*-dependent pathway (Arvidson and Tegmark, 2001). DNA binding and profiling studies indicate that *sar*A protein may directly regulate target genes via downstream effects on regulons, such as *agr* promoter, or by stabilizing mRNA during log phase (Cheung *et al*., 2007).

S. aureus can express numerous virulence factors, including **cell wall-anchored (CWA)** and **secreted proteins,** which promote bacterial escape from host immune response, as well as subsequent adhesion to and damage of host cells.

The **CWA proteins** are covalently attached to peptidoglycan and their expression varies among strains (McCarthy and Lyndsay, 2010). Recently, was proposed a classification of the CWA proteins into four groups, based on the presence of motifs defined by structure–function analysis (Foster et al., 2014):

1) **The MSCRAMM family**; these proteins have structural similarities and a common mechanism for ligand binding, which is mediated by two adjacent subdomains containing IgG-like folds in the N terminal A region. Originally the function of these proteins was associated only to the attachment to various cell surface proteins, such as collagens, elastin, fibrinogen, fibronectin, bone sialoprotein, laminin and thrombospondin, as shown by the acronym "microbial surface component recognizing adhesive matrix molecule" (Foster and Höök, 1998). Recently, the role of some of these proteins was extended to other functions, such as evasion from the host immune system. Among them, **Clumping factor A** (ClfA) **and B** (ClfB) bind immobilized fibrinogen (Ganesh *et al.,* 2008; 2011), but only ClfA promotes immune evasion by binding soluble fibrinogen and by interfering with the complement system (Hair *et al.,* 2010). **Fibronectin-binding proteins A** (FnBPA) **and B** (FnBPB) bind fibronectin and FnBPA also binds fibrinogen and contributes to immune evasion (Sinha *et al.,* 2000). While the **Serine–aspartate repeat proteins C** (SdrC), **D** (SdrD) **and E** (SdrE) seem to be crucial in the successful colonizations (Sabat *et al.,* 2006) of human tissues, their role is still unknown in bovine mastitis. SdrC and D bind the desquamated epithelial cells (Corrigan *et al.,* 2009), whereas sdrE binds the complement regulatory protein factor H (fH) to inhibit the alternative pathway of complement (Sharp *et al.,* 2012). The **collagen adhesion** (Cna) has a role in the adhesion to collagen-rich tissue (Zong *et al.,* 2005) and is involved in immune evasion, preventing the classical pathway of complement activation (Kang *et al.,* 2013).

2**) Near iron transporter (NEAT) motif family** permits the survival of bacteria in the host, promoting the heam capture of haemoglobin. Among them, the **iron-regulated surface** (*Isd*) **proteins** act in order to bind haem and acquire iron. Moreover, IsdA promotes the adhesion to desquamated epithelial cells, binding fibronecting (Clarke *et al.,* 2004) and fibrinogen, whereas isdB is involved in invasion of non-phagocytic cells, independently of haemoglobin binding.

3) **Three-helical bundle motif protein**; The **protein A** (SpA) is the representative of this group, characterized by five homologous modules at the N terminus, each consisting of a single separately folded three-helical bundles which can bind to several distinct ligands, such as immunoglobulins G, M, tumour necrosis factors, and the von Willebrand factor. In particular, the IgM bind associated with B cells by protein A induces apoptosis of host cells, such as PMN and macrophages (Goodyear and Silverman, 2004). The repeated modules are followed at the C terminus by region X, composed of Xr, a highly repetitive yet variable octapeptide and Xc, a domain of unique sequence that abuts the cell wall anchor structure of SpA (Schneewind *et al.,* 1995). 4) **The G5–E repeat family;** the proteins are classified for their identical content of G5 domains in a tandem array each one separated by 50-residue sequences that are known as E regions. The G5 domains have no known ligand-binding function. The **surface protein G** (SasG) has such a structure. Its role in bovine mastitis is still debated, even though it was demonstrated that SasG promotes the biofilm formation (Geoghegan et al., 2010) helping a successful host immune evasion.

S. aureus **secreted proteins** include: 1) enzymes (such as hyaluronidase, lipases, proteases and nucleases), 2) non-enzymatic activators (such as coagulase or staphylokinase) and 3) exotoxins (such as cytolytic toxins, leukocidins, enterotoxins, enterotoxin-like proteins, exfoliative toxins, and toxic shock syndrome toxin).

1) The **enzymes** include important factors for the persistence of infections. The **hyaluronate lyase** (hys) is a protein capable of degrading the acidic mucopolysaccharide hyaluronic acid, which composes the intercellular ground substance of human and animal connective tissue. **Lipases**, **esterases**, **fatty acid modifying enzyme** and **phospholipases** function as surfactants

against a variety of fatty acids and other lipid molecules that host cells produce in order to destroy the bacterial membrane (Chamberlain and Imanoel, 1996).

Proteases can cleave and degrade numerous important host proteins, including the heavy chain of immunoglobulins, plasma proteinase inhibitor and elastin (Karlsson and Arvison, 2002). *S. aureus* secretes four major proteolytic enzymes: a metalloprotease or aureolysin (aur), a serine glutamyl endopeptidase or serine protease (SspA), two cysteine proteases referred to as staphopain A (ScpA) and staphopain B (SspB), and six serine-like proteases that are SspA homologues (SplA, B, C, D, E, F) (Reed et al. 2001; Shaw et al. 2004). All of them are secreted as pro-enzymes and subsequently they are proteolytically cleaved to generate mature enzymes. The aur and ScpA zymogens autoactivate outside the cell; SspA and SspB activation is due to a proteolytic cascade in which aur processes SspA, which subsequently processes SspB (Mootz *et al.,* 2013).

Aureolysin is a member of the termolysin family of zinc–dependent metalloproteases. This enzyme is composed by a single chain of 301 amino acids, consisting of β-pleated-N-terminal domain and α-helical-C-terminal domain (Bambula *et al.,* 1998). Different *in vitro* studies demonstrated that aur activates prothrombin and inactivates mammalian plasma protease inhibitors by cleavage (Sabat *et al.,* 2008). Sieprawaska-lupa *et al.* (2004) suggested that this enzyme could have a role in staphylococcal resistance to the innate immune system, degrading the human antimicrobial peptide LL-37.

The SspA, also known as V8 protease, moderates adhesion of *S. aureus* to fibronectin by degrading cell surface fibronectin-binding protein. Such protein has high glutamic acid content, including a conserved motif that is essential for ligand binding. In the operon of SspA the cysteine protease SspB is also included, which in his mature form, mimics plasma serine proteases: it converts high molecular weight kininogen into heavy and light chains, hydrolyses substrate of kallikrein (another serine protease, which processes kininogen), processes the N

terminus of the fibrinogen β-chain at the same site such as plasmin and removes the N terminal domain of fibronectin with a specificity equivalent to plasminogen activator (Nickerson *et al.,* 2007). Staphopain A and B are remote members of the papain superfamily of enzymes, and show a high homology (47% amino acid identity over 174 residues). For this reason, the two cysteine proteases have been named as Staphopain A and B, respectively (Filipek *et al.,* 2003). The cysteine protease ScpA is encoded in the *scp* locus while the other cysteine protease SspB in the *ssp* locus (Filipek *et al.,* 2003). In the *scp* operon, another open reading frame codes for the inhibitor of *ScpA*, namely *ScpB*. Also, it was shown that a short open reading frame in the *ssp* operon codes for *SspC*, a small protease that inhibits *SspB* (Massimi *et al.,* 2002). Despite the homology between *ScpA* and *SspB,* and between the genes or their inhibitors *SspC* and *ScpB* (18% amino acid identity over 109 residues), the inhibitor is specific for the protease in the own operon.

2) Among the non-enzymatic activators, *S. aureus* produces two coagulases, **staphylocoagulase** and **von Willebrand factor**, which allow the formation of fibrin clots after binding to prothrombin (forming an intermediate complex called staphylothrombin) and several other plasma proteins, thereby triggering the conversion of fibrinogen to fibrin (McAdow *et al.,* 2012). In particular, coagulase is composed by three distinct regions: an N-terminus with the binding site for prothrombin, a central conserved region and a conserved C-terminus region, which comprises 2–8 tandem repeats of a 27-residue peptide. The polymorphism of the gene is used to type the different strains, both of human and of bovine origins (Schwarzkopf A. and Karch H.,1994). *S. aureus* is distinguished clinically from less pathogenic strains of staphylococci by the coagulase test (Lowy, 1998). Another non-enzymatic activator is the **staphylokinase** (sak), which destroys defensins (Jin *et al.,* 2004) and has antiopsonic activity (Rooijakkers *et al.,* 2005) in human host, whereas its role in bovine mastitis is still poorly understood. *Sak* gene is carried

by β-hemolysin-converting bacteriophages, which carry also *scn*, *chp* and *sea* genes, thereby forming an immune evasion cluster (IEC).

3) Exotoxins include cytolytic toxins which have the cytoplasmic membranes as a target. These toxins can lyse the membrane after an initial receptor interaction showing high target cell specificity, or after an interaction with a less specific fashion in an independent-receptor manner. Hemolysins and leukotoxins are the most well-known toxins affecting red blood cells or leukocytes respectively. *S. aureus* can produce four different **haemolysins** (α, β, δ e γ). Alpha-haemolysin (α-toxin) toxin was the first discovered and extensively reviewed (Bhakdi *et al,* 1991). It has a lytic function for red blood cells but not for neutrophils (Valeva *et al.,* 1997), a role in the host cell ion balace and neurotoxic function (Dinges *et al.,* 2000). Beta-haemolysin (β-toxin) is a sphingomyelinase targeting erythrocytes. Ungulate erythrocytes are more susceptible to this toxin because they have an high sphingomyelin content (Walev *et al.,* 1996). Previous *in vitro* studies in bovine mammary epithelial cells showed that β-toxin increases the damaging effect of the α-toxin, improving bacterial attachment (Cifrian *et al.,* 1995; 1996).

Delta-haemolysin (δ-toxin) is able to function as a surfactant or channel-former and also to enhance the haemolytic effect of β-toxin (Dinges *et al.,* 2000). In the last decade, it was discovered that δ-toxin belongs to the family of secreted peptides named as the phenol-soluble modulins (PSMs), which have multiple function in staphylococcal pathogenesis (Wang *et al.,* 2007).

Gamma-haemolysin (γ-toxin) is a bicomponent toxin, composed by two an S (slow) and F (fast) component, for their elution-propensity in a ion-exchange column (Woodin, 1960). In addition, *S. aureus* can produce other bi-component toxins that are structurally similar target neutrophils. They belong to the beta-barrel pore-forming toxin family: the **leukocidins** that include the Panton-Valentine leukocidin PVL (LukS-PV + LukF-PV), γ-haemolysin and leukocidin (Hlg and

Luk; HlgA + LukF and LukS + LukF), LukM/FPV, (LukM + LukF-PV), LukE/D (LukE + LukD) and lukM-lukF–PV (P83). While PVL is typical of the human isolates, the other variants have been already detected in bovine isolates, showing different potentialities against bovine PMNs (Barrio *et al.,* 2006).

Among the staphylococcal exotoxins, the large family of superantigen (Sags) includes **enterotoxins** (SEs), **enterotoxin-like proteins** (ssl), **toxic shock syndrome toxi** (TSST), and **exfoliative Toxins** (ETs). Sags act bypassing the normal antigen-presenting mechanism, binding to the outer surface of the MHC class II proteins and outside the site in T-cell receptors. As a consequence, T lymphocytes are induced to an uncontrolled proliferation and produce an excessive secretion of cytokine. Indeed, sags activate as much as 30% of the T-cells instead of the normal of 0.1‰ (Fleischer and Schrezenmeier, 1988).

1.5 The evolution of *Staphylococcus aureus* typing

The characterization of *S. aureus* is important role in the epidemiology of infectious disease. *S. aureus* typing helps to identify, track and intervene against the diseases outbreaks.

During the last decades, different molecular approaches have been used on *S. aureus* strains, in order to discriminate between isolates that are epidemiologically related from those are not. Initially, *S. aureus* was studied using the electrophoretic migration profile of plasmids on agarose gels. After the development of the polymerase chain reaction (PCR), wide typing techniques were applied, such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) techniques. However, such methods allowed discovering only random DNA sites, and the interpretation of the data obtained were scarcely appropriate for epidemiological study. The development of multilocus enzyme electrophoresis (MLEE), an electrophoretic analysis of the housekeeping enzymes, was the first major progress in the understanding of the global epidemiology of *S. aureus*. The turning point was achieved with pulsed field gel electrophoresis (PFGE) technique, where the bacterial DNA digested in agarose plugs created different strain profiles. The first *S. aureus* PFGE protocol was developed for grouping isolates in well-characterized *S. aureus* nosocomial outbreaks, demonstrating the usefulness of this technique in short time period studies (Tenover et al., 1995). Despite that, this approach is not sufficient for long-term studies or for studies of global epidemiology.

To this purpose, Enright *et al.* (2000) adopted the multi-locus sequence typing (MLST) developed in 1998 by Maiden *et al.* for *Neisseria meningitidis,* to *S. aureus*. This sequence-based approach allows discriminating bacterial isolates on the basis of the sequences of 450-bp internal fragments of seven house-keeping genes. For each gene fragment, the different sequences are assigned as distinct alleles that define the isolate. Each allelic profile is indicated as sequence type (ST). Subsequently, the bacteria with the same ST can be assigned as members of the same

clone, called “Clonal complex”.

Genotypic variants with distinct epidemiologic patterns (such as host-tropism) are now widely recognized (Peton *et al.*, 2014). The *S. aureus* genome can be grouped also based on variable region. The *agr* locus is a conserved, polymorphic and hypervariable fragment used to cluster *S. aureus* strains into one of four *agr* groups, using polymerase chain reaction (PCR) (Grinholc *et al*., 2013). Regarding bovine staphylococcal strains, Buzzola *et al*. (2007) observed that most bovine isolates belonged to *agr* group I (88%) whereas the remaining strains were evenly distributed among *agr* groups II, III and IV, hypothesizing that *agrI* strains better survive in intracellular environment. The hypervariable region of the protein A is also extensively used for *S. aureus* typing. This technique “spa typing” is a single-locus typing technique in which the base composition of the polymorphic repeat found in a strain is assigned a unique repeat code −the spa type.

1.6 Bovine mammary gland innate immunity

The immune system is characterized by its ability to recognize and discriminate between foreign invading agents and their own molecules. The immune system can be conceived as having two arms: innate immunity and acquired immunity. These two systems interact closely to provide protection against mastitis microorganisms.

While innate immunity is quickly activated at the site of infection by numerous stimuli, it does not increase by repeated exposures to the same agent. When non-specific defence mechanisms work adequately, most of the pathogens are eliminated within a short period of time and before the specific immune system activates. Indeed, mammary gland cell populations can produce a wide range of antimicrobial peptides (AMP), which are oligopeptides with a broad spectrum of targeted organisms (Bahar & Ren, 2013).

The innate immunity can be divided into two arms: the sensing and the effector arm. The sensing arm is about "how the host perceives infection", while the effector arm concerns "how the organism fights infection". Each arm can be split into humoral and cellular components.

Acquired immunity is also called specific immunity because it occurs as a result of prior exposure to a specific antigen previously encountered (active immunity), or of passive transfer of antibody (passive immunity). Because a memory is formed, subsequent responses to a previously encountered antigen are more effective. The lymphocytes are the white blood cells responsible for acquired immunity.

1.6.1 Anatomical defences

The **teat canal** (figure 1.) is the first line of defence against mastitis acting as a physical barrier against the invading microorganisms; in the distal part of the teat, sphincter muscles maintains tight closure between milkings and hinder bacterial penetration (Sudhan and Sharma, 2005; Paulrud, 2005). Increased opening of the sphincter is directly correlated to increased incidence of mastitis (Rainard *et al.*, 2005). This canal is locked between milkings and in the dry period by a keratin plug derived from the stratified epithelial lining of the canal. The plug creates a physical barrier that prevents the penetration of bacteria into the gland cistern. Keratin is able to bind and immobilized most strains of non-encapsulated mastitis-causing bacteria (Rainard *et al.,* 2005).

Some components of keratin have microbicidal activity: esterified and not esterified fatty acids, such as myristic acid, palmitoleic acid and linoleic acid, are bacteriostatic. Keratin cationic proteins can bind electrostatically to the mastitis-causing bacteria, altering their cell wall and affecting their resistance to osmotic pressure (Hogan *et al.,* 1987; Paulrud, 2005). The bactericidal efficiency of keratin may be limited by different factors, above all inadequate milking procedures. Moreover, a high accumulation of fluid occurs within the udder, close to parturition, causing increased intramammary pressure. Thus, the leakage of mammary secretions and dilatation of the teat canal increase susceptibility to mammary infections.

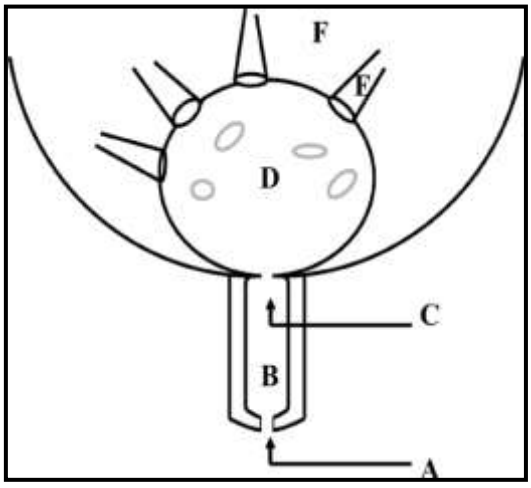


Figure 1 Schematic view of the anatomical structures of the lower part of the bovine mammary gland. A = teat canal, B = teat cistern, C = plica annularis mucosae (Fürstenberg rosette), D = gland cistern (cistern lactiferous), E = large milk duct, and F = alveolar parenchyma (Vangroenweghe *et al.,* 2006).

1.6.2 Humoral defences

The **complement system** plays important roles in immune response. It participates in control of inflammation, opsonisation of bacteria, recruitment of neuthrophils, and –whether directly or in cooperation with phagocytes− recognition and ingestion of bacteria (Barrio *et al.,* 2003; Rainard, 2003). The proteins of the complement complex are synthetized mainly by hepatocytes but also by monocytes and tissue macrophages (Sordillo and Streicher, 2002). Complement (C) is present in the milk of healthy glands at low but significant concentrations. The overall function of complement in mammary gland defence has yet to be fully determined, but available knowledge supports its predominant role as pro-inflammatory mediator in coliform mastitis (Riollet *et al.,* 2000). For the lack of C1q, the classical pathway is impossible, but the alternative pathway can operate with a double outcome: deposition of opsonic C3b and iC3b on bacteria, and generation of the pro-inflammatory fragment C5a. The chemotactic fragment C5a has been shown to induce the migration of neutrophils through the mammary epithelium. The fragment C5a is also a potent stimulator of the phagocytic function of neutrophils (Alluwaimi, 2004).

Lactoferrin (Lf) is a glycoprotein mainly produced by the secretory epithelium and to lesser extent by PMNs, such as leukocytes (Persson *et al.,* 2002). It is first known for its iron-binding functions, that lead to bacteriostasis and protection against oxygen radicals catalysed by free iron. Moreover, it activates the complement system via the alternative pathway (Oseas *et al.,* 1981). Additionally, lactoferrin enhances Natural Killer cells action (Clare and Swaisgood, 2000), increases the inflammation and stimulates macrophages in their phagocytic and cytotoxic activities (Broxmeyer and Platzer, 1984).

Bovine milk contains small amount of Lf (20-200 μg/mL) in comparison to bovine colostrum (2-5 mg/mL) and to the secretions of non-lactating mammary gland, which can contain very high concentration of Lf (20-100 mg/mL). The expression of Lf is inversely related to alveolar

development and its bacteriostatic and bactericidal activities are likely to be more important when the mammary gland is fully involuted (Molenaar *et al.,* 1996). Moreover, mammary epithelial cells (MEC) secrete citrate in milk, creating an ideal buffer that chelates iron and makes it available to bacteria.

Transferrin (Tf) is similar to lactoferrin for its iron-chelating functions, and is found in milk. This protein comes from blood, by transcytosis in the normal gland and through exudation of plasma during mastitis. Bovine milk contains low amounts of transferrin (20-40 µg /mL), higher amounts are present in colostrum (1mg/mL). Transferrin may allow a first iron-chelating bacteriostatic effect, before the increase of Lf concentration (Alluwaimi, 2004).

Lysozyme (Lz; N-acetylmuramyl hydrolase**)** is one of the most extensively studied antibacterial milk proteins produced by neutrophils, monocytes, macrophages and epithelial cells. It has a well-recognized bactericidal effect against both Gram-positive and Gram-negative bacteria. Its bactericidal effect is due to the cleavage of the peptidoglycan of Gram-positive and Gram-negative cell wall. Lysozyme is 14 kDa enzyme directed against the 1-4 glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid residues of peptidoglycan. Lz is highly active against many Gram-positive species and it can synergize with antibodies, complement or lactoferrin, allowing the disruption of the outer membrane of Gram-negative bacteria and the access to peptidoglycan (Ganz, 2004). Due to the small concentration of IgA in ruminant milk and to the low amount of lysozyme, it is unknown whether this mechanism is active in the bovine mammary gland (Sordillo and Streicher, 2002). Despite the low Lz concentration, the enzyme seems to represent one of the fundamental components of mammary gland innate immune defense in preventing pathogen invasion. Together with lactoferrin, Lz is one of the most abundant proteins contained in neutrophils-specific granules (Ganz, 2004). Lz helps to kills

ingested bacteria by promotingphagolysosome function and preventing colonization through exocytosis of polymorphonuclear leucocyte (PMN) secondary granules (Zecconi and Smith, 2003).

NAGase (N.acetyl-beta-D-glucosaminidase) is an intracellular enzyme which is mainly released from the polymorphonuclear leucocytes during phagocytosis and cell lysis (Akerstedt et al., 2012). In a previous study, normal levels of NAGase were reached five days after calving in healthy mammary glands, while a slight increase was observed at the end of lactation (Mattila et al., 1986). During subclinical mastitis, the enzyme activity was shown to increase in the milk, also in relation to the pathogen isolated (Akerstedt *et al.,* 2012). Recently, mammary epithelial cells of a continuous cell line, BME-UV1, were shown to produce NAG when infected with *S. aureus* strains, as well as in the absence of stimuli (Mazzilli and Zecconi, 2010).

Xanthine oxidase is an enzyme of the membrane of milk fat globules. It catalyses the formation of nitric oxide from inorganic nitrite, which under aerobic condition leads to generation of peroxynitrite, a powerful bactericidal agent (Alluwaimi, 2004).

With xanthine oxidase, **lactoperoxidase** is the most abundant enzyme in the bovine milk, constituting the 0.5% of the whey proteins (30 mg/L−1) (Fox and Kelly, 2006). In the presence of thiocyanate with hepatic origin and hydrogen peroxide with either bacterial or endogenous possible origins, this enzyme can produce activated oxygen products, as hypothiocyanate, a reactive metabolite which promotes bactericidal activity of phagocytes (Sordillo *et al.,* 1997).

Also **myeloperoxidase** is a lysosomal enzyme, produced in the primary granules of leukocytes. This enzyme has an important role in the neutrophil oxygen-dependent antimicrobial system

(Fox and Kelly, 2006; Cooray, 1994) and catalyses oxidase reactions (such as the oxidation of chloride) providing an additionally bactericidal activity (Sordillo and Streicher, 2002).

Innate immunity also comprises **natural antibodies of** the serum. Rather low concentrations (less than 1%) opsonize efficiently *S. aureus* and *E. coli.* The recurring type of antibodies in adult serum and milk of cow are IgM. These antibodies are produced in absence of external antigenic stimulation.They are polyreactive and can be directed against bacterial antigens, such as lipopolysaccharide, lipoteichoic acid and peptidoglycan (Wijga et al., 2013), and also against self-antigens (Baumgarth et al. 2005).

Among the AMP, **Lingual antimicrobial peptide** (LAP) −a member of the β-defensin family− exhibits antibacterial activity against a broad spectrum of microorganisms (Ganz, 2003; Swanson *et al.,* 2004). LAP is accumulated in leucocytes and epithelial cells, being secreted after a direct stimulation (Huang *et al.,* 2012).

Cytokines are a wide and uneven group of small proteins (less than 50kDa) playing an important role in cell signalling. They comprise interleukins, chemokines, colony stimulating factors, interferons and tumor necrosis factors but not hormones or growth factors. They play an important role in the balance between humoral and cell-based immune responses in the mammary gland. Their high affinity for their receptors gives them a high power in the immune response modulation even at femto- or nano-molar concentrations. Among them, **chemokines** are a large family of cytokines with a small molecular weight ranging from 7 to 15 kDa. They are subdivided into two families based on the first cysteine residue: the family called CC chemokines, also known as beta-chemokines and the family CXC, known as alpha-chemokines, which have an intervening amino acid between the first two cysteines. In both families have

immune pro-inflammatory and homeostatic functions. Their essential physiologic role is to recruitwell-defined leukocyte subsets. Some cytokines clearly promote inflammation, while others suppress the production of inflammatory cytokines (Table 1).

- **Interleukin-1 (IL-1)** is an important pro-inflammatory cytokine, which exists both in cytoplasmatic and secreted forms, known as IL-1α and IL-1β, respectively. This cytokine is produced by different mammary cells, mainly by macrophages and epithelial cells and also by lymphocytes. It is a key mediator of local and systemic immune responses, regulating cells proliferation and apoptosis, and the expression of IL1 itself and other cytokines, such as IL-6, IL-8, IL-12, and TNF-*α*., enzymes for the eicosanoid synthesis and acute phase proteins (Schukken *et al.,* 2011). In *E. coli* mastitis, the influx of neutrophils has been associated with a sharp elevation of IL-1 level, being postulated that IL-1 is indirectly involved in chemo-attraction of neutrophils (Schuster *et al,* 1997). Incontrast, the role of IL-1 is negligible or transient in *S. aureus* mastitis (Riollet *et al.,* 2001). The immunotherapeutic properties of IL-1 are prevented by its pro-inflammatory nature. Some studies have shown that IL-1 (as well as other pro-inflammatory cytokines) mediates acute septic shock, and may aggravate its prognosis (Ohtsuka et al., 2001; Shuster and Kehrli, 1995).

- **Interleukin-2 (IL-2)** induces growth, differentiation, and/or activation of of B lymphocytes, NK cells and CD8+ lymphocytes. Primarily produced by T (CD4+) lymphocytes, it has also been detected in epithelial cells of both normal and mastitic mammary glands (Alluwaimi and Cullor, 2002). IL-2 has a therapeutic role in the treatment of *S. aureus* mastitis (Erskine *et al.,* 1998). The infusion of IL-2 in infected quarters enhances the recruitment of lymphocytes, neutrophils, macrophages and

eosinophils, up-regulates MHC class-II expression, and increases antibody titres in milk and serum.

- **Interleukin-6 (IL-6)** is a pleiotropic cytokine with pro- and anti-inflammatory properties produced by macrophages, T lymphocytes and epithelial cells (Okada *et al.,* 1997). This cytokine regulates the acute phase protein synthesis, producing a febrile response. Moreover, it promotes the transition of the inflammatory process, decreasing neutrophil and favouring monocyte recruitment into the mammary gland (Kaplanski *et al.,* 2003). It is expressed in healthy mammary glands and in infected ones. In cooperation with IL-10, IL-6 can also upregulate IL-1 receptor antagonist and soluble TNF receptors, decreasing the action of the pro-inflammatory cytokines IL-1 and TNF-α, respectively (Bannerman, 2009).

- **Interleukin-8 (IL-8)** is a chemokine produced by monocytes, T lymphocytes, and macrophages, as well as epithelial and endothelial cells. It plays an important role in neutrophil recruitment to the mammary gland (Boyso et al., 2006) and it is actively produced in *E. coli* mastitis, whereas it is present in lower concentration in mastitis caused by *S. aureus* (Hagiwara et al., 2001). The increase of IL-8 concentration is associated with higher somatic cell count, highlighting the crucial role of IL-8 in the recruitment of leukocytes into the mammary gland, which is essential for the elimination of invading pathogens. Barber and Yang (1998) demonstrated the biological role of IL-8 in attracting neutrophils to infected bovine udder, showing lower neutrophil chemotactic activity in the presence of anti-IL-8 antibodies in mastitic mammary secretion. Moreover its fundamental role in mastitis has been shown in several *in vitro* and *in vivo* studies (Strandberg *et al.,* 2005; Griesbeck-Zilch *et al.,* 2008; Zbinden *et al.,* 2014).

- **Interferon-γ (IFN-γ)** is produced by CD4+ and CD8+ lymphocytes, and Natural Killer cells. IFN-γ is an important mediator of inflammation. It activates and recruits neutrophils, enhancing their phagocytic activity. Furthermore, IFN-γ induces production of IL-12, which regulates lymphocyte cellular dichotomy (T helper-1 and T helper-2) and is an important mediator linking innate with acquired immune response in mammary gland (Oviedo-Boyso *et al.,* 2007).

- **Tumor necrosis factor-α (TNF-α)** is an adipokine involved in systemic inflammation and a member of pro-inflammatory cytokines that stimulates the acute phase reaction. The overwhelming inflammatory response is the cause of severe symptoms occurring during coliform mastitis and is accompanied by elevation of TNF-α levels. Such cytokine has been detected not only in infected bovine mammary gland but also in normal quarters (Alluwaimi, 2004).

  Previous studies have shown that high levels of TNF-α seem to regulate physiological changes of the mammary gland as well as immunological functions (Alluwaimi, 2004). In coliform mastitis, TNF-α induces plasma haptoglobin, promotes recruitment and activation of neutrophils and raises intra-mammary and systemic nitrites and nitrates. In contrast to the continuous release of TNF-α in coliform mastitis, in *S. aureus* infected glands, TNF-α transcriptional activity has only a short episodic elevation at 24 h post infection (Alluwaimi, 2004). Incontrast, *in vitro* studies using bMEC have shown a significant increase of TNF-α one hour after experimental challenge with *S. aureus*, demonstrating increased production of this cytokine in the early phase of infection (Standberg et al, 2005). In addition to IL-2 and IFN-γ increases, T cells have shown increased TNF-α release after stimulation with *S. aureus* enterotoxin (Yokomizo et al., 1995).

Table 1 Most important bovine mammary gland cytokine functions.

Cytokine	Functions
IL-1	Mediates acute phase inflammatory response Increases neutrophil numbers Enhances neutrophil phagocytosis and bactericidal activity Triggers neutrophil migration into infected mammary gland
IL-2	Enhances mammary mononuclear cell proliferation Enhances cytoxic and bactericidal activities of lymphocytes Increases plasma cells numbers Activates NK cells
IL-6	Mediates acute phase inflammatory response Decreases neutrophil and favours monocyte recruitment influx into the udder Upregulates IL-1 receptor antagonist and soluble TNF receptors, decreasing the action of IL-1 and TNF-α
IL-8	Indices inflammation Mediates IL-1 induced neutrophil migration Potent chemoattractant
IFN-γ	Enhances neutrophil phagocytosis and bactericidal activity Reverses suppressive effects of mammary gland secretions
TNF-α	Enhances acute phase inflammatory response Enhances neutrophil phagocytosis and bactericidal activity Enhances endothelial adhesion molecule expression

1.6.3 Cellular defences

In the mammary gland, leucocytes, including neutrophils, macrophages and lymphocytes, are most representative cells of innate immunity. Other cell types should be added to leucocytes: dendritic cells and mammary epithelial cells. The latters are at the interface between body and invading microorganisms and represent an important component of an efficient immune response.

In a healthy bovine mammary gland (and depending on lactation stage or dry period), the distribution of leukocytes varies. In the early and late stage of lactation, lymphocytes decrease and PMNs increase (Miller *et al.,* 1993). In contrast, in the early postpartum period, macrophages seem to achieve their highest concentration (68%) (Park *et al.,* 1992).

The milk of a healthy bovine mammary gland contains a few leukocytes and epithelial cells, even though the amount of these cells depends on the lactation phase. As lactation progresses, cell concentration can slightly increase and after drying-off, udder tissue undergoes several physiological changes. In the early dry period, somatic cell counts increase to 2-5 x 10^6/mL, then they decrease and stabilise at 1-3 x 10^6/mL during most of the period. At parturition, cell counts are usually higher than 1 x 10^6/mL, and decrease to 10^5/mL in 7-10 days after calving (Sharma *et al.,* 2011).

- **Macrophages** represent the dominant cell type in the secretion of involute udder and in the tissues of a healthy mammary gland, but not in the milk (Pilla *et al.,* 2013). These cells are characterized by a large horseshoe-shaped nucleus, which exhibit lower ability to migrate between endothelial cells than neutrophils (Paape *et al.,* 2000). This population of cells is involved both in innate and acquired immune response. They phagocytize bacteria, which are destroyed with proteases and reactive oxygen species. Macrophages are also involved in the involution of the mammary gland, ingesting

cellular debris and accumulated milk components (Outteridge and Lee, 1981). The phagocytic activity of these cells can be increased in the presence of opsonic antibodies. Furthermore, they can secrete substances that promote both migration and bactericidal activity of neutrophils. Activated macrophages are triggered to release prostaglandins, leukotrienes, and cytokines that can augment local inflammatory processes.

- **Neutrophils** (PMN) represent another important cell type of innate immune responses, which can be considered as the second line of the innate immune system of the bovine mammary gland. In low numbers in healthy mammary glands, neutrophils represent up to 90% of all mammary cells in mastitic quarter milks (Sordillo and Streicher, 2002).

  Their multilobulated nucleus structure allows them to migrate by diapedesis through the endothelial junctions and rapidly reach the infection sites (Paape *et al.,* 2000). During infection, the neutrophils are recruited by chemotactic stimuli into the milk, where they and they phagocytose pathogens, exploiting their crucial activity in the elimination of invading bacteria. They phagocytose using an oxygen-dependent system, which includes the production of hydroxyl and ROS and an oxygen-indipendent system that produces peroxidases, lysozyme, hydrolytic enzymes and lactoferrin. PMNs contain several antimicrobial peptides, such as defensins, myeloperoxidase and different proteases (Bank and Ansorge, 2001; Selsted *et al.,* 1993). In severe intramammary infections, such a plethora of degrading molecules can also affect the epithelium of the mammary gland, decreasing the synthetic activity of the organ (Prin-Mathieu *et al.,* 2002).

- **Lymphocytes** are specialized cells of the immune system, characterized by membrane receptors that recognize antigens. These specific structures give them the immunological characteristics of specificity, diversity, memory, and self/non-self recognition. Among

the lymphocyte subsets in the mammary gland, the T lymphocytes, which include **CD4+ (T helper)** and **CD8+ (T cytotoxic or T suppressor)**, are the most numerous. B lymphocytes are in very low concentrations and do not play an important role in the immune response of mammary gland. During mastitis, the CD4+ T lymphocytes are the prevalent cell population, producing immunoregulatory cytokines that activate CD8+ lymphocytes and macrophages. B cells produce antibodies against invading pathogens, with specific cell surface receptors. Their differentiation can be stimulated by bacterial substances, such as lypopolysaccarides (LPS) (Paape *et al.,* 2000).

Suppressor T lymphocytes are thought to modulate immune responses during bacterial infections, as observed in the early stage of lactation (Sordillo and Streicher, 2002). Together with major histocompatibility complex (MHC) class I molecules, CD8+ lymphocytes carry out cytotoxic activitities. During *S. aureus* intramammary infections, the presence of CD8+ cells can affect the total lymphocyte function and −in some cases− predispose the animal to chronical forms (Park *et al.,* 1992). Another lymphocyte subpopulation are the **Natural killer (NK)** cells, which use the Fc receptors in an antibody-dependent cytotoxic activity without the involvement of MHC. NK cells are large granular nonimmune cells produced and differentiated in bone marrow, lymph nodes, spleen, and tonsils. These cells remove damaged epithelial cells and intracellular pathogens.

- Bovine **mammary epithelial cells** (MEC) are able to release a variety of inflammatory mediators from the apical membrane (e.g., cytokines, antimicrobial peptides, and arachidonic acid metabolites), which indicate that they are involved in the immune response (Sprong *et al.,* 2001). Despite the role of these messenger molecules is not completely understood, it is known that they can express toll like receptors (TLRs)

(Akira *et al.,* 2006) and may recruit neutrophils and lymphocytes into milk (Günther *et al.,* 2010). The epithelial cells have both a sentinel and an effector role and they are well suited to exert these functions, due to their position to invading microorganisms (Günther *et al.,* 2010). A burst of cytokines synthesis follows the contact between MEC and pathogens: the reaction is stronger after *E. coli* challenge in comparison with *S. aureus* experimental infection (Günther et *al.,* 2010). These cells are the first to come in contact with invading pathogens, thus activation of immune response of MEC is involved in the evolution of infection. *In vitro* studies using a mammary epithelial cell line (MAC-T) showed that the cells secreted IL-8 under stimulation with LPS, in a time- and dose-dependent manner (Boudjellab *et al.* 1998). Analogously, when stimulated by LPS, primary cultures of MEC show enhanced expression of chemokines, (Standberg et al, 2005).

1.6.4 In vitro studies of bovine mammary gland

The bovine mammary gland immune response has been studied with *in vitro* models which are less costly and more stable than *in vivo* models. In order to understand the molecular mechanisms of the ruminant mammary gland functions, the last decades, different *in vitro* models have been developed, including established cell lines and primary bovine epithelial cell cultures (PbMEC).

Because they derive from a monoclonal population, established cell lines are considered to be relatively homogeneous. They are usually used to investigate the bovine mammary epithelium. Because they are characterized by a low biological variability, the use of the same cell lines in different laboratories promotes inter-laboratory data comparability (Matitashvili *et al*., 1997). Two clonal cell lines derived from bovine mammary epithelium are commercially available: MAC-T cells and BME-UV cells. Both of them are established from primary bovine mammary epithelial cells by a stable transfection with a plasmid, which prevents the senescence even after numerous passages.

However, the comparison of publication is problematic: changes in cell morphology and metabolism may occur even in cells maintained in *in vitro* systems for prolonged time periods (Buhering et al. 2004).

Primary MEC cultures are also utilized in many studies regarding mammary gland patho-physiology. The cells are obtained from mammary parenchymal tissue collected from lactating animals (German and Barash, 2002). The difficulty of this model relates with the cytological identification of epithelial cells, when a culture is set up. Mammary epithelial cells are not necessarily a homogeneous population: they can contain a small percentage of cells ofmesenchymal origin, such as fibroblasts (Matitashvili et al., 1997).

Both models are considered as reliable tools to investigate the functions of mammary epithelial cells, but the mammary tissue consists of different cell types, including fibrocytes, resident

macrophages, endothelial cells, and adipocytes. Notably, all these cells produce cytokines and other immune-modulatory molecules, orchestrating a most complex play. Therefore, to consider interactions among different cell populations, few models have been developed from explants of the mammary gland. Two of them addressed the physiology or the immune response of the udder (Baumrucker and Stemberger, 1989; Rabot et. al., 2006), and one more recent paper investigated the location-specific expression of chemokines in the teat canal and Fürstenberg's rosette (Lind *et al.,* 2014).

2 AIM

Though considerable efforts have been made to improve bovine mastitis diagnosis and therapy, bovine mastitis still causes huge economic losses in the dairy farms. This pathology is the result of the interaction between the pathogen, the host response and the environment. Therefore, the research on different aspects is needed to better understand the mechanisms involved in the mammary gland immune response induced by an infection.

This thesis focuses on two main aspects: i) the *in vitro* immune response of the dairy cow mammary gland when challenged with a pathogen, and ii) the virulence factor of *S. aureus*, that is one of major agents of mastitis and cause of economical losses to the dairy farm.

Previus studies of mammary immune response showed the need for a model the could bridge the gap between primary or continuos cell lines and the mammary tissue, taking into account the interactions among all the different cell populations. Also, knowing for sure that the tissue had not previously been exposed to pathogens plays a fundamental role in the investigation of the immune response.

Therefore, the first aim of the study was to set up an explant of the mammary gland, using a healthy heifer gland, in order to investigate the whole tissue immune response - not only the epithelial cell response - to infection. To that end, two experiments were performed: (i) with synthetic molecules, lipopolysaccharide (LPS) or lipoteichoic acid (LTA), and (ii) using different concentrations of a *S. aures* field strain. In both trials, the composition, the vitality, the proliferation and the innate immune response were evaluated.

The aim of the second part of the thesis was to deepen the knowledge regarding the attitude of *S. aureus* toward higher diffusion within the herd, in order to develop new strategies for the control of *S. aureus* mastitis; among them the possible identification of new vaccine targets. To that end, the genes carried by the *S. aureus* strains collected in Italian herds with higher and lower mastitis

prevalence, were characterized using DNA-microarrays analysis. The association between virulence factors and strain prevalence at herd level was investigated the using Binary logistic regression and Correspondence analysis.

STUDY 1: An explant of heifer mammary gland to study the immune response of the organ.

The data reported in this chapter refer to the already published paper:

Magro, G., Brevini, T. A. L., De Maglie, M., Minozzi, G., Scanziani, E., Piccinini, R. (2017). An explant of heifer mammary gland to study the immune response of the organ. *Research in Veterinary Science*, 6 (114) 44-50.

3 MATERIAL AND METHODS

The study was carried out in 2 consecutive trials, using the udder of two different heifers, both sent to the abattoir for infertility reasons. While different treatments were applied, sampling and culture conditions were identical.

3.1 Optimization of the samples collection

Several heifer mammary glands were used in order to standardize the collection methodology.

After slaughter, each udder was transferred to the laboratory. For each udder, sections were collected in 4 replicates for a few weights: small 0, 01 g, medium 0, 02 g and large 0, 05 g. All the samples were briefly washed with a solution of phosphate-buffered saline (PBS) with 1:100 antibiotic-antimycotic solution containing 100 units penicillin, 0.1 mg streptomycin and 0.25 µg amphotericin B for mL.

One of the repetitions of each weight was fixed with 10% formaldehyde, at room temperature embedded in paraffin. Subsequently, sections were stained with hematoxylin and eosin and observed under an Eclipse E600 microscope (Nikon, Japan), in order to test the morphological condition and vitality of the sample. Remaining sample repetitions were stored in RNA later (Sigma-Aldrich) at −20 °C before RNA extraction. Samples of different weights were tested with two types of lysis tissue techniques: (i) with a Rotar stator for 30 sec twice, or (ii) with 50 mg of acid-washed glass beads using Retsch MM301(Retsch GmbH &co.) for 20 min. RNA concentrations were quantified by use of the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The purity of RNA (A 260 /A 280) of ~2 was the criteria for the best lysis techniques.

3.2 Sampling and culture conditions

In both trials, the udder of a heifer was taken at the slaughterhouse and immediately transported to the laboratory. The skin and fat were removed and a section of mammary parenchyma was aseptically taken and washed with Hanks' Balanced Salts Solution (HBSS) added with 1:100 antibiotic-antimycotic solution containing 100 units penicillin, 0.1 mg streptomycin and 0.25 µg amphotericin B for mL. The tissue was then trimmed into smaller sections of 2 mm^3 weighing 0.02 g. The udder sections were cultured at 37 °C with 5% CO2 in 96-well plates in the following medium, supplemented with 10% fetal calf serum (FCS): 50% Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F12), 30% Roswell Park Memorial Institute (RPMI) 1640 medium, 20% NCTC-135 medium, containing 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 mM glutathione, 10 µg/mL L-ascorbic acid, 1 µg/mL hydrocortisone, 1 µg/mL insulin, 5 µg/mL transferrin, and 0.5 µg/mL progesterone. The media were purchased from Thermo Fisher Scientific (Waltham, USA), HBSS and supplements from Sigma-Aldrich (St. Louis, USA).

3.3 Treatments

Seven replicates of each treatment and control were used in both experiments. In the first trial, explants were treated with 1µg/mL of lipopolysaccharide (LPS) or 0,5 µg/mL of lipoteichoic acid (LTA, both from Sigma-Aldrich, USA); in the second trial, two different concentrations of *Staphylococcus aureus* (*Staph. aureus*) strain MI 390 from our collection were used. After overnight incubation on blood agar plate at 37 °C, the bacteria were suspended in the culture medium and inoculated into the wells to a final concentration of 10^2 CFU/mL or 10^4 CFU/mL. Optical density was measured spectrophotometrically at 600 nm and was confirmed the following day with the plate count method. Lipopolysaccharide was used as a positive control in

the second trial. Negative control wells were treated similarly, but no synthetic molecules or bacteria were added. In both trials, treated and untreated sections were collected after 1 h, 3 h and 6 h incubation; in the first trial, a final time-point was at 18 h post-incubation. The sections of 6 out of the 7 repetitions, taken throughout the experiment, were immediately stored in RNAlater (Sigma-Aldrich, USA) at −20 °C until RNA extraction. The remaining sections were fixed in 10% neutral buffered formalin for at least 48 h at room temperature, routinely processed for paraffin embedding, and sectioned at 4-μm thickness.

3.4 Morphological analysis and inflammatory cells detection

To study the morphological aspects of the tissue, histological sections were stained with Hematoxylin-eosin (HE). In the second trial, they were also evaluated by immunohistochemistry (IHC). Sections of formalin-fixed paraffin-embedded tissues were simultaneously dewaxed and unmasked by heat induced epitope retrieval and Buffer H (Bio-optica, Italy). Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 and slides were rinsed and treated with phosphate buffer saline (PBS, Sigma-Aldrich, USA) containing serum to reduce nonspecific background staining. To detect ether histiocytes/macrophages or T cells, rabbit polyclonal antibody anti-Iba1 (Ionized Calcium-binding Adapter molecule-1, Wako chemicals, Germany) or rabbit polyclonal antibody anti-CD3 (Dako, Denmark) were used as primary antibodies, , respectively. Sections were then incubated with biotinylated secondary antibody (goat anti-rabbit) and labelled by the avidin-biotin-peroxidase (ABC) procedure with a commercial immunoperoxidase kit (VECTASTAIN® Elite ABC-Peroxidase Kit Standard). The immunoreaction was visualized with 3,3-diaminobenzidine (Peroxidase DAB Substrate Kit). All reagents were purchased from Vector Laboratories (USA). Substrate and sections were counterstained with Mayer's haematoxylin (Merck, USA). Known positive control sections were included in each immunolabelling assay. Sections were blind evaluated with a light microscopy

by a veterinary pathologist and 3 different anatomical structures (alveolar, ductular and interstitial) semi-quantitatively scored as follows: - = absent, + = rare cells, ++ = some cells; +++ = numerous cells.

3.5 Proliferation and apoptosis

Both cell proliferation and apoptosis of the *ex vivo* sections were evaluated using an immunofluorescence-based assay. Before the optimization of the sample collection, we performed preliminary tests on different *ex* vivo sections without treatment, in which we evaluated the cell proliferation and the apoptosis at 0h and 16h. Sections were boiled for 5 min in antigen unmasking solution (Vector Laboratories, USA). Non-specific sites were blocked with PBS containing 5% Bovine Serum Albumin (BSA, Sigma-Aldrich, USA) and 10% non-immune serum (Thermo Fisher Scientific, USA). Samples were incubated overnight at 4°C with a primary rabbit polyclonal antibody specific for the Ki67 marker of cell proliferation (1:50 dilution, Abcam, UK). Sections were washed with PBS and incubated with the secondary antibody (Alex Fluor® 488 goat anti-rabbit, Thermo Fisher Scientific, USA) for 1 h in darkness. Nuclei were stained with 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA). The percentage of apoptotic cells was evaluated using a commercial kit (In situ Cell Death Detection Kit, TMR red, Roche, Basel, Switzerland) following the manufacturer's instructions. The test is based on the Terminal deoxynucleotidyltransferase-mediated dUTP Nick-end Labeling (TUNEL) assay. For each slide, a minimum of 4 pictures were taken and blind observed under an Eclipse E600 microscope (Nikon, Japan). Pictures were acquired with Nis Elements Software (Version 4.0) with constant exposure parameters, then analyzed with the image analysis software ImageJ (http://rsbweb.nih.gov/ij/index.html). Threshold adjustments were applied to generate a black and white image and marker expression was normalized by DAPI fluorescence. Cell

proliferation and apoptosis were evaluated by cell count, considering every cell type, and expressed as a percentage.

3.6 RNA extraction and quantitative real-time PCR

Tissue samples were lysed using 50 mg of acid-washed glass beads on a mixer mill Retsch MM301 (Retsch GmbH &co., Germany) and total RNA was extracted using TRIzol reagent (Sigma-Aldrich, USA), according to the manufacturer's recommendations. Total RNA yield and purity were determined by the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Germany). Genomic DNA removal and reverse transcription was performed using the QuantiTect Reverse Transcription kit (Qiagen, Netherlands) and 1 µg RNA as a template.

The quantitative real-time PCR (qPCR) was performed following MIQE guidelines (Bustin *et al.,* 2009) and qbase+ software (Biogazelle, Belgium) was used for the gene expression analysis. All the samples were normalized to the negative control, in order to compare the results in the different time-points and experiments. For the qPCR reaction, 2 µL of cDNA was amplified in a 15-mL reaction volume with the Eco Real Time PCR Detection System (Illumina Inc., USA), using Eva Green Supermix (BioRad, USA) and different concentrations of primers. Three reference genes (Ubiquitously Expressed Transcript, UXT; Eukaryotic Translation Initiation Factor 3K, EIF3K; Ribosomal Protein Large P0, RPLP0) validated with geNORM analysis, were selected as the best combination of internal standard for mammary gland tissue (Bonnet *et al.,* 2013). Primers sequences of the target genes TNF-α and LAP were from Tomasinsig *et al.* (2010), and those of IL-1β, IL-6 and IL-8 from Griesbeck-Zilch *et al.* (2008). All the primers are listed in the table 2.

Table 2 PCR primers for reference genes (UXT, RPLP0, EIF3K) and for target genes (TNF-α, IL-1 β, IL-6, IL-8 and LAP).

Target Genes	Genbank Accession Number	Primers Sequences (5'→3')		Product Lenght	[Primer]	Efficiency and Regression Coefficient (R^2)
UXT	NM_001037471	Fw: Rev:	TGTGGCCCTTGGATATGGTT GGTTGTCGCTGAGCTCTGTG	101	300 nM	106.58% 0.99199
EIF3K	NM_001034489	Fw: Rev:	CCAGGCCCACCAAGAAGAA TTATACCTTCCAGGAGGTCCATGT	125	200 nM	103.95% 0.99017
RPLP0	NM_001012682	Fw: Rev:	CAACCCTGAAGTGCTTGACAT AGGCAGATGGATCAGCCA	227	300 nM	103.52% 0.996733
TNF-α	AF_011926	Fw: Rev:	CTGGTTCAGACACTCAGGTCCT GAGGTAAAGCCCGTCAGCA	183	200 nM	93.37% 0.993431
IL-1 β	M_37211	Fw: Rev:	AGTGCCTACGCACATGTCTTC TGCGTCACACAGAAACTCGTC	114	300 nM	107.1% 0.990783
IL-6	NM_173923	Fw: Rev:	GCTGAATCTTCCAAAAATGGAGG GCTTCAGGATCTGGATCAGTG	215	200 nM	105.22% 0.990968
IL-8	AF232704	Fw: Rev:	ACACATTCCACACCTTTCCAC ACCTTCTGCACCCACTTTTC	149	200 nM	104.72% 0.989868
LAP	S_76279.1	Fw: Rev:	AATTCTCAAAGCTGCCGT CACAGTTTCTGACTCCGC	164	200 nM	108.72% 0.987092

Each sample was tested in duplicate and repeated in case of a standard deviation > 0,5. The following thermal protocol was applied: Taq polymerase activation at 98 °C for 1 min, 40 cycles of denaturation at 95 °C for 10 s, and annealing/extension at 60 °C for 45 s. Non-reverse transcribed controls and no template controls were included in each run. Melt curves were obtained collecting fluorescence data every 0.3 °C during melting. The qPCR efficiencies were determined using three-fold serial dilutions of cDNA prepared from heifer mammary tissue, in triplicate. In order, to obtain the best standard curve, we tested all the primers at different

concentration: 200 nM, 300 nM and 400 nM. The final qPCR efficiencies ranged 93.37-108.72%, with a regression coefficient (r^2) of 0.99. The relative quantification of gene expression was performed using the comparative Ct method (ΔΔCt) and the geometric mean of reference gene abundance was used for normalization purposes.

3.7 Statistical analysis

The statistical analysis of the data was performed using the software SPSS Statistics (version 21.0; IBM, USA). Mean values were compared by Two-way ANOVA for gene expression or by One-way ANOVA for apoptosis and then by Scheffè *post-hoc* test. Time and treatment variables were used as main effects, and target gene expression or apoptosis percentage as the dependent variables. Results were considered as statistically significant at *P* values <0.05.

4 RESULTS

4.1 Morphological analysis and inflammatory cells detection

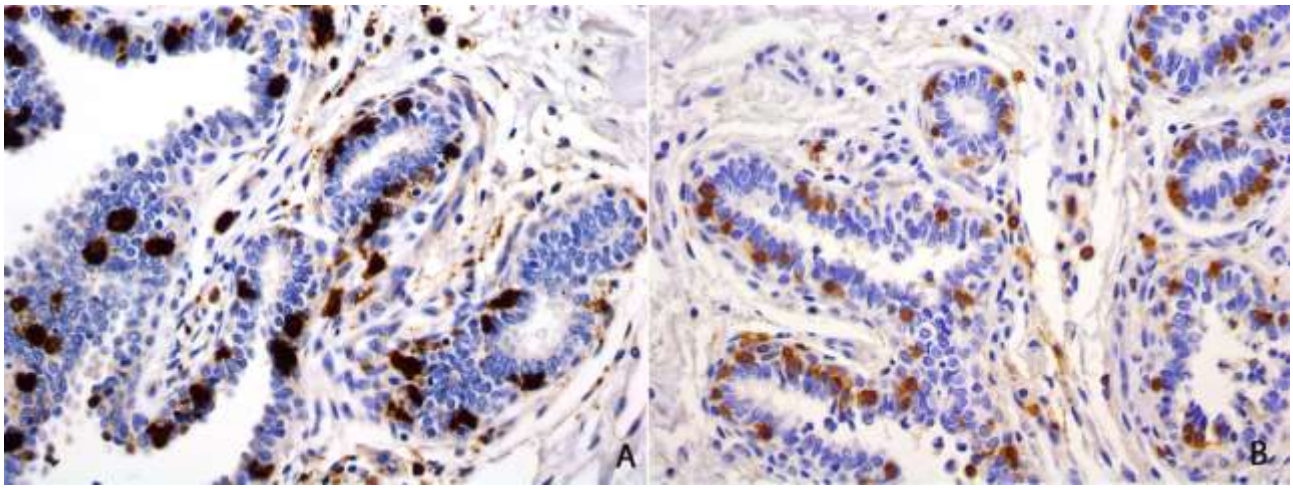
At histological examination, all samples showed well-preserved morphology and the presence of normal mammary tissue consisting in alveolar and ductular structures surrounded by interstitial stromal tissue, indicating that the procedure used to sample the udder caused minimal tissue damage. In the samples of the second trial, IHC highlighted the presence of CD3 positive T lymphocytes and Iba1 positive histiocytes/macrophages mainly in alveoli and ductular structures and in 3 samples mononuclear cells also infiltrated the interstitium (Table 3 and Fig. 2).

Table 3 Inflammatory cell detection by immunohistochemical staining in 3 different anatomical structures (alveolar, ductular and interstitial).

TIME	TREATMENT	IMMUNOSTAINING	
		Iba1	**CD3**
	Untreated sample	alveolar structures: ++* ductular structures: ++ interstitium: -	alveolar structures: + ductular structures: - interstitium: -
1h	10^2 CFU/mL *Staph. aureus*	alveolar structures: + ductular structures: ++ interstitium: -	alveolar structures: + ductular structures: + interstitium: -
	10^3 CFU/mL *Staph. aureus*	alveolar structures: + ductular structures: ++ interstitium: +	alveolar structures: + ductular structures: - interstitium: +
	Untreated sample	alveolar structures: + ductular structures: + interstitium: +	alveolar structures: ++ ductular structures: + interstitium: +
3h	10^2 CFU/mL *Staph. aureus*	alveolar structures: + ductular structures: + interstitium: -	alveolar structures: + ductular structures: + interstitium: -
	10^3 CFU/mL *Staph. aureus*	alveolar structures: + ductular structures: + interstitium: -	alveolar structures: + ductular structures: + interstitium: -
	Untreated sample	alveolar structures: + ductular structures: + interstitium: +	alveolar structures: + ductular structures: + interstitium: +
6h	10^2 CFU/mL *Staph. aureus*	alveolar structures: + ductular structures: + interstitium: -	alveolar structures: + ductular structures: + interstitium: -
	10^3 CFU/mL *Staph. aureus*	alveolar structures: + ductular structures: + interstitium: -	alveolar structures: + ductular structures: + interstitium: +

* Semi-quantitative scoring of the immunoreaction: - = absent, + = rare cells, ++ = some cells, +++ = numerous cells.

Figure 2 Immunohistochemistry detection of inflammatory cells (400x). 1) Numerous intraepithelial macrophages/histiocytes (Iba1positive cells) are recognized in alveolar and ductular structures, while positive cells are barely detected in the interstitial stroma. 2) Numerous, mostly intraepithelial T lymphocytes (CD3 positive cells) are located in alveolar and ductular structures.



4.2 Proliferation and apoptosis assay

The results of preliminary test before the optimization of the sample collection, showed that proliferation of the section at sampling was 3.32 % and apoptosis was 13.35% (Figure 3), whereas after 16 h the values were 0.07% and 13.38%, respectively (Figure 4).

The Ki-67 assay performed in the first trial showed very low and comparable proliferation both in treated sections and in the controls throughout the experiment. Therefore, the assay was not repeated in the second trial.

Figure 3 Image outputs of preliminary tests on the explant at time 0 using the ImageJ software. A) Proliferation assay: DAPI (Blue), Nuclear marker; Ki67 (Green), cell proliferation marker; with the respectively threshold adjustment images. B) Apoptosis assay: DAPI (Blue), Nuclear marker; TUNEL (Red), apoptosis marker; with the respectively threshold adjustment images.

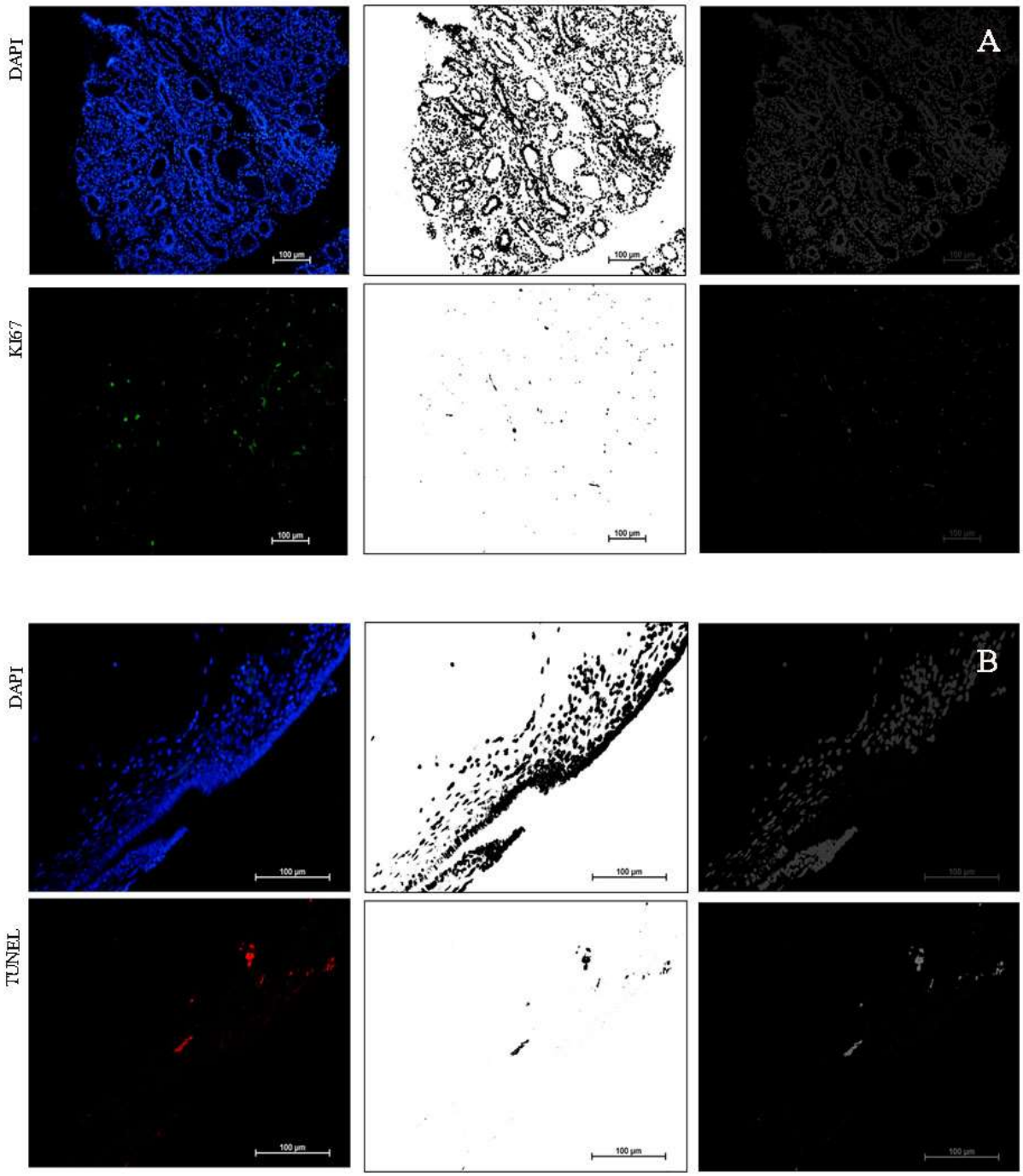
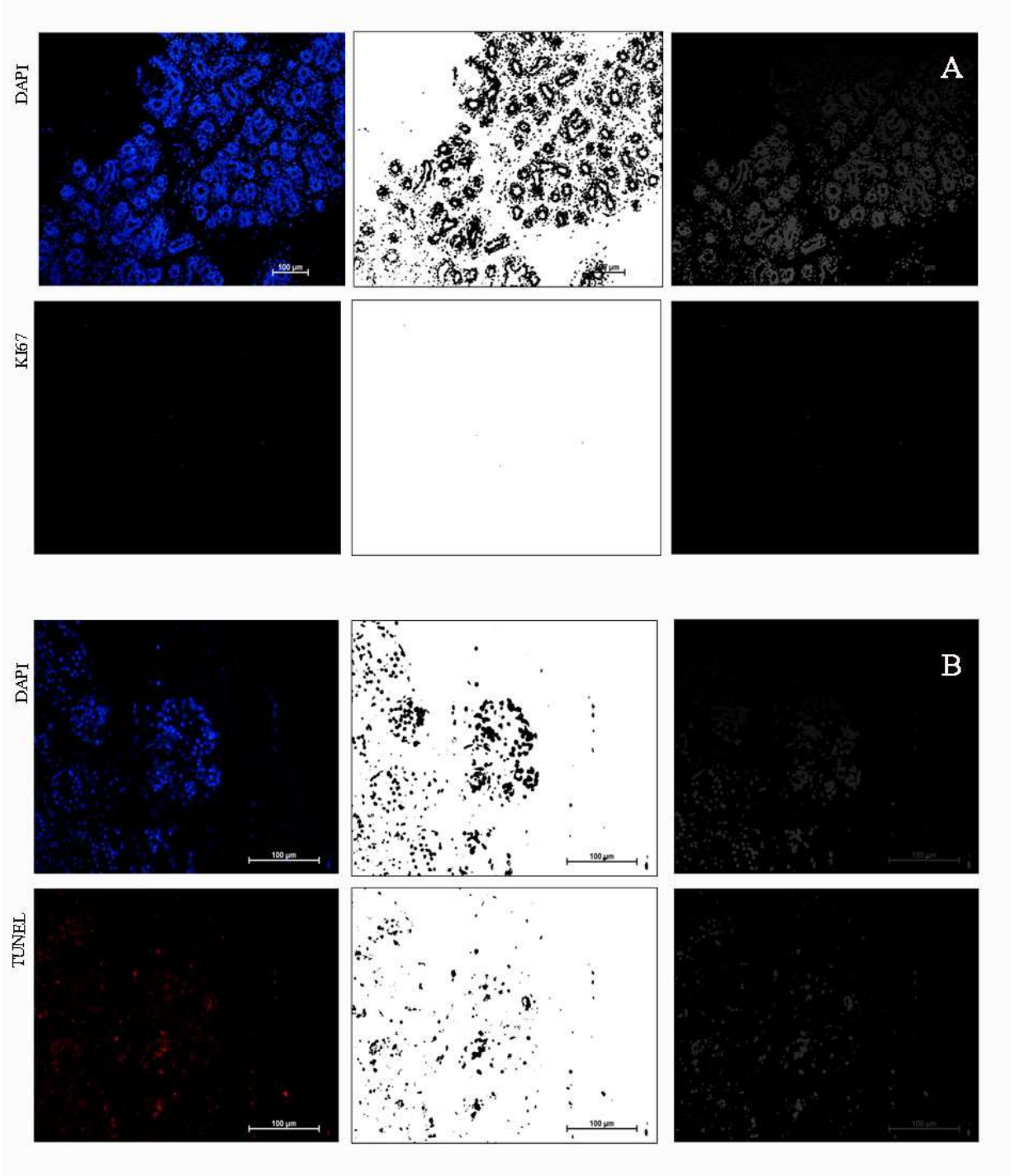
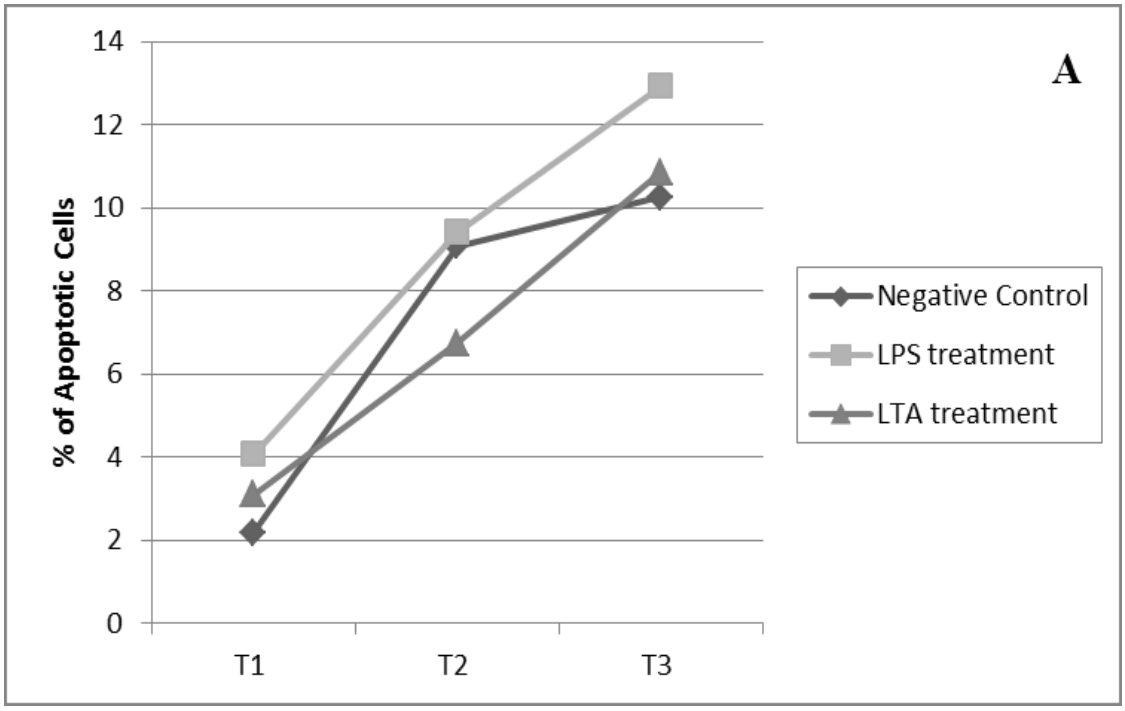


Figure 4 Image outputs of preliminary tests on the explant after 16 hours of culture using the ImageJ software. A) Proliferation assay: DAPI (Blue), Nuclear marker; Ki67 (Green), cell proliferation marker; with the respectively threshold adjustment images. B) Apoptosis assay: DAPI (Blue), Nuclear marker; TUNEL (Red), apoptosis marker; with the respectively threshold adjustment images.



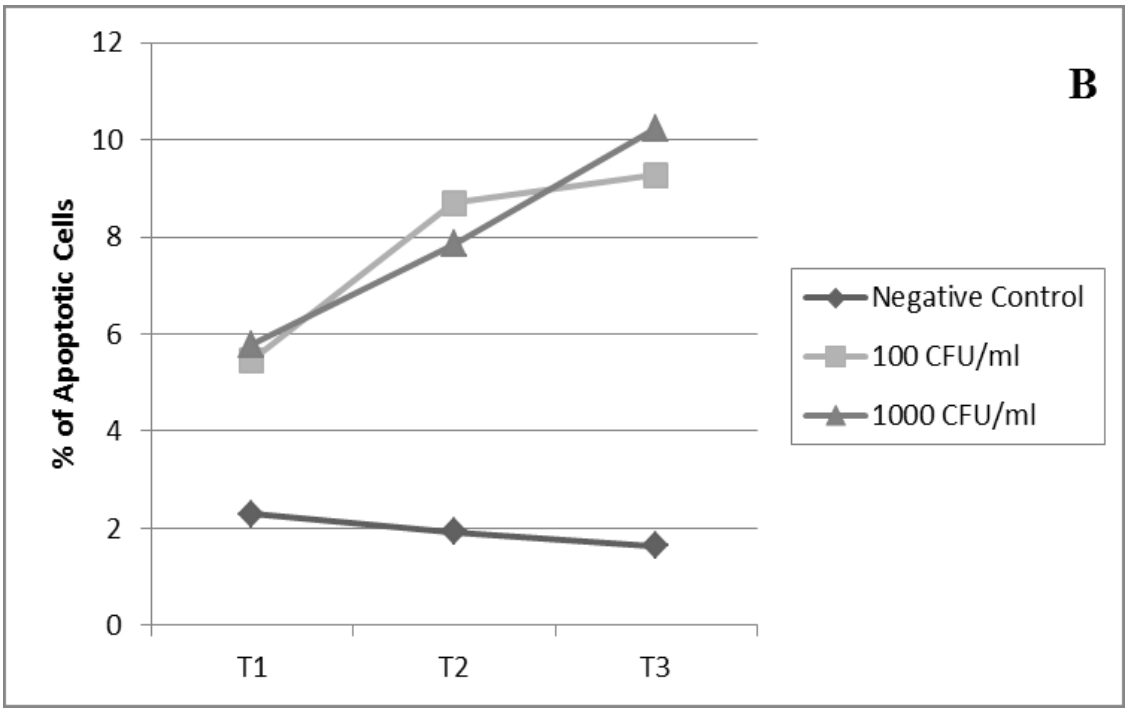
In the TUNEL assay, positive cells were detected at each time point in all experimental groups, but without significant differences between treatments and controls. In the first trial, the percentage of apoptotic cells ranged between 2.19% - 10.25% in the negative controls and 4.07 % - 12.9 % in the LPS treated samples (Figure 5A).

Figure 5 Results of the TUNEL assay expressed as the percentage of apoptotic cells: A) First trial, with the untreated sections of parenchymal mammary tissue of heifer (dark grey, negative control), treated with LPS (light grey), or LTA (grey).



In the second experiment, the levels ranged between 1.62% - 2.28 (in the controls) and 5.45% - 10.22% (in bacteria challenged samples, Figure 5B).

Figure 5 Results of the TUNEL assay expressed as the percentage of apoptotic cells:B) Second trial, with the untreated sections of parenchymal mammary tissue of heifer (dark grey, negative control), treated with 10^2 CFU/mL (light grey), or with 10^3CFU/mL (grey). Data are shown as means of all measures throughout the 4 time points of the experiment.



4.3 qPCR

The gene expression results of trial 1 are reported in Figure 6, and those of trial 2 are expressed in Figure 7. Effect of LPS treatment was significant for TNF-α, IL-1β and IL-8 mRNA expression ($P < 0.001$), but no significant differences were demonstrated between the time points. For IL-6 mRNA expression, effects of treatment, time, and time x treatment were $P < 0.001$, $P = 0.042$ and $P = 0.017$. LAP mRNA expression was significantly increased only by LPS treatment ($P = 0.007$). Effect of the treatment with *S. aureus* (10^3 UFC/mL) was significant for IL-1β and IL-6 mRNA expression ($P < 0.001$); time was also significant for both cytokine expression ($P < 0.001$ and $P = 0.007$, respectively), while time x treatment was significant only

for IL-1β ($P = 0.003$). Regarding specifically this last cytokine, the first time point was higher in comparison with the second and the third ones. In both trials, IL-6 expression was higher at 1 h than at 6 h. Bacterial treatment did not induce any effect on TNF-α, IL-8 and LAP mRNA expression. Effect of LTA treatment was not significant for cytokines, or LAP expression.

Figure 6 Cytokine expression in untreated sections of parenchymal mammary tissue of heifer (negative control, white bar), treated with LPS (dark grey bar), or LTA (grey bar). A) TNF-α, B) IL-1β, C) IL-6, D) IL-8, E) LAP. Data are shown as means ± standard deviations (SD) of all measures throughout the 4 time points of the experiment.

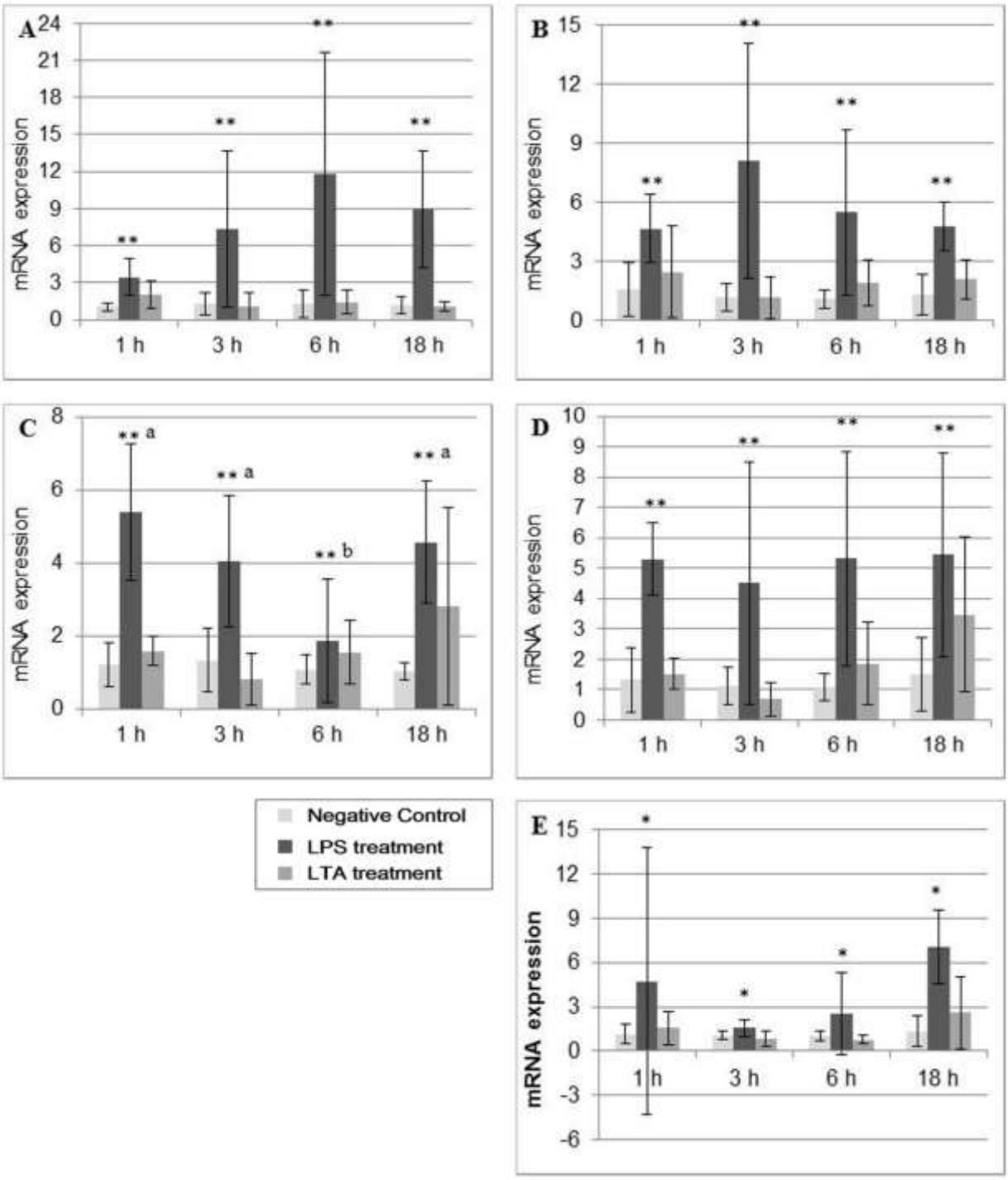
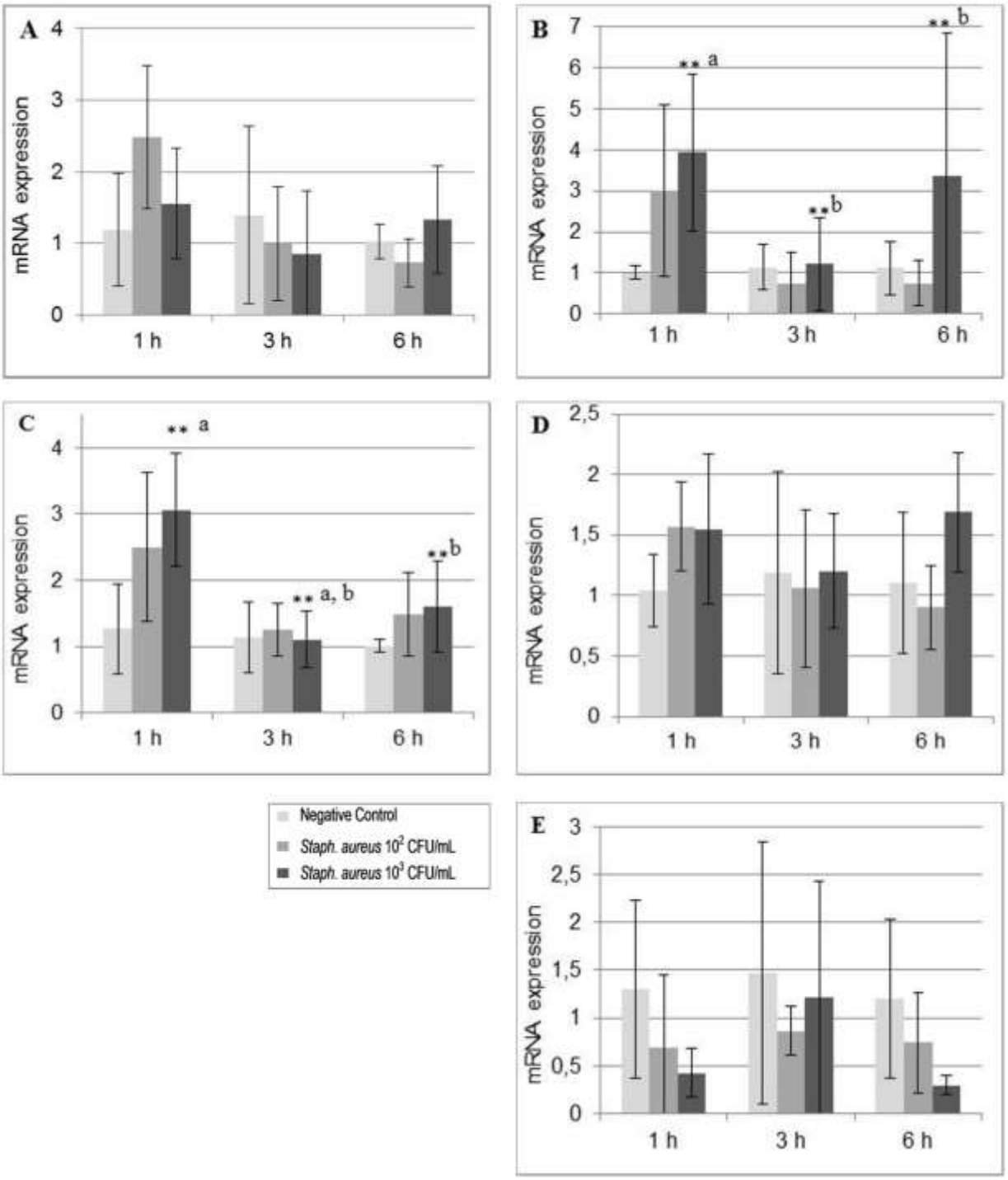


Figure 7 Cytokine expression in untreated sections of parenchymal mammary tissue of heifer (negative control, white bar), in sections treated with *S. aureus* 10^2 UFC/mL (grey bar), or 10^3 UFC/mL (dark grey bar). A) TNF-α, B) IL-1β, C) IL-6, D) IL-8, E) LAP. Data are shown as means ± standard deviations (SD) of all measures throughout the 3 time points of the experiment.



5 DISCUSSION

The development of explants as models to investigate the morpho-pathology of organs is a promising approach, especially used in human medicine (Drew *et al.,* 2015; Vadstrup *et al.,* 2016). To our knowledge, only a few studies have been conducted and published in veterinary medicine, in which this model is applied to investigate the bovine mammary gland. Two of them investigated the physiology and immune response of the udder (Baumrucker and Stemberger, 1989; Rabot *et. al.,* 2007). One recent paper investigated the expression of chemokines in the teat canal and Fürstenberg's rosette (Lind *et al.,* 2015). While all of these studies explored the tissues of lactating cows, our model focused on heifers. Because it explored animals before they were lactating, our experimental design helped investigate the pathogenesis of intramammary infections in the dairy cow. Compared to those of lactating or involuting tissues, tissues collected from clinically healthy heifers have not yet been exposed to microorganisms, which might have damaged cells and/or triggered immune responses that prevent from measuring innate immunity. Increasing evidence has emerged to suggest that mammalian innate immunity also exhibits an immunological memory of past threats (Netea *et al.,* 2011). In addition, the progressive decline of epithelial cell number during lactation and the extensive cell turnover (Capuco *et al.,* 2001) account for the difficulty to develop a reliable explant model of lactating mammary gland. As demonstrated by HE staining the morphology of explants was unaltered in our study. Data regarding mammary cell proliferation in healthy heifers are extremely scarce in literature: in one paper, the values dropped from 6.8% in 2-month old animals, to 2.3% in 8-month animals (Ellis and Capuco, 2002), while pubertal heifers have never been investigated. At the beginning of lactation, a proliferation index of 0.3% was observed (Capuco *et al.,* 2001). On the other hand, ovariectomy was demonstrated to indirectly stop mammary cell proliferation (Velayudhan *et al.,* 2015), what might explain the negligible proliferation observed in our study, since the heifers used had been slaughtered as a consequence

of infertility. Taken together, these results enabled us to focus on the innate immune response of the tissue, testing the mRNA expression of cytokines and antibacterial peptides such as LAP, after challenge with different stimuli.

In our first trial, we treated the explant with 1µg/mL of LPS, as suggested by the literature (Im *et al.,* 2014). The decision regarding the amount of LTA was much more uncertain, due to huge discrepancies in the LTA concentrations reported in published papers (Strandberg *et al.,* 2005; Yu *et al.,* 2010; Im *et al.,* 2014). Because LPS, but not LTA, induced mRNA expression of pro-inflammatory cytokines, we hypothesized that such a result could be due to a weak stimulus, rather than a hypo-responsiveness of the explant. To check our hypothesis, we then treated the model with a *S. aureus* strain isolated from subclinical bovine mastitis and belonging to Clonal Complex 8 − a broadly diffused group that causes bovine intramammary infections.

The significant up-regulation of TNF-α and IL-8 mRNA expressions by LPS stimulation is in accordance with previous studies using primary MEC cultures (Strandberg *et al.,* 2005; Yang et al., 2008), while in mammary parenchymal explants IL-8 (but not TNF-α) expression was elicited during LPS treatment (Rabot et al., 2007). The lack of mRNA expression for these cytokines following stimulation with LTA or *S. aureus* is similar tothat observed in *in vivo* experimental mastitis induced with LTA or *S. aureus* (Yang et al., 2008). When using primary MEC cultures and inactivated *S. aureus*, epithelial cell expression of only TNF-α (Griesbeck-Zilch et al., 2008) or both cytokines (Yang et al., 2008) was demonstrated. However, the response elicited by inactivated *S. aureus* was 20 to 50-fold lower (for TNF-α or IL-8, respectively), in comparison with the one induced by *E. coli*. Moreover, given that *S. aureus* mastitis is characterized by very different clinical outcomes, we hypothesized that this result might also depend on the mildness of the strain used to challenge the model. Future experiments using higher bacterial load and different *S. aureus* strains are needed to check that hypothesis.

Our explant showed a significant up-regulation of IL-1β and IL-6 expression, in response to LPS

or *S. aureus*. In comparison with previous studies based on primary MEC cultures (Griesbeck-Zilch et al., 2008), we registered an earlier expression, reaching the highest levels within 3 hours post challenge. This result might be due to the use of the whole tissue, rather than a single cell population, therefore our model might be more suitable to study the mammary immune response. The increasing LAP mRNA expression throughout the experiment, when the model was treated with LPS might be explained by the storage of the molecule in epithelial cells and its release following a direct stimulation (Huang *et al.,* 2012). The lack of response to *S. aureus* challenge might be due to a low bacterial count, which could be ineffective to drive LAP expression. Unfortunately, no previous study is available in the literature, to compare the results.

Immunohistochemical results gave new insights into the localization of lymphocytes in the mammary tissue, demonstrating a higher number of apparently resident T cells (intraepithelial cells, IEL) within the alveolar epithelium, in comparison with interstitial localization. A previous study reported the presence of lymphocytes and macrophages within epithelial lining and subepithelial stroma in healthy lactating mammary quarters (Sordillo *et al.,* 1989), while neutrophils were located mainly in the alveolar lumen. In another study, $CD8^+$ T lymphocytes were predominant over $CD4^+$ and were preferentially distributed in the epithelial area, rather than in the inter-alveolar connective tissue, indicating their possible role in the immunological surveillance of mammary epithelia (Yamaguchi *et al.,* 1999). In both cases, the udders had been taken from slaughtered multiparous cows, therefore lymphocytes might have migrated into the epithelium during lactation, as a consequence of previous bacterial stimulation and macrophage signaling. In our explants, neutrophils were not detected at histology, indicating the uninfected status of the tissue and the lack of previous exposure to microorganisms. Given that the tissue belonged to non-pregnant heifers, epithelial T lymphocytes may be regarded as resident IELs. To date, no information is available about IEL role in the mammary gland, while their function in the gut immune response is being extensively studied. They play a crucial role in the regulation

of intestinal homeostasis and in maintaining epithelial barrier function. The localization close to the external environment allows for rapid responses against infections, facilitating a timely switch of the innate immune response to the adaptive one (Sheridan and Lefrançois, 2010). A previous paper reported the phenotypical similarity between predominant T cells in milk during lactation and IELs in the intestine (Asai *et al.,* 2000). Based on our findings, IELs appear to reside in the mammary gland long before parturition, hence their role might be as fundamental as the one played by intestinal IELs. As a consequence, pathophysiological studies of the organ should not leave aside the interaction between epithelial cells and resident lymphocytes, when exploring the response of bovine udder to bacterial or environmental/managerial stimuli.

6 Conclusion

Our explant demonstrated to preserve the intact structure of the mammary gland, with an interesting infiltration of mononuclear cells, mainly T lymphocytes that can be regarded as resident cells. Analogously to the intestinal IELs, these T lymphocytes may participate in organ homeostasis. Therefore, applying our model would give new insights in the investigation of udder pathophysiology. Due to the large request to limit the use of animals for experimental purposes, our explant would represent a valid alternative to cell lines or 2D primary cultures. Further studies are required to investigate the response of the model to different bacterial strains and/or stress-related stimuli.

STUDY 2: Virulence genes of *S. aureus* from dairy cow mastitis and contagiousness risk

The data shows in this chapter refer to the already published paper:

Magro, G., Biffani, S., Minozzi, G., Ehricht, R., Monecke, S., Luini, M., Piccinini, R. (2017). Virulence Genes of *S. aureus* from Dairy Cow Mastitis and Contagiousness Risk. *Toxins*, 9(6).

4 MATERIAL AND METHODS

4.1 Herds, sampling and microarray analysis

The study considered 169 *S. aureus* isolates, collected in 60 dairy herds located in different Italian regions between 2006 and 2014. The herds had a size range of 35 - 285 milking cows and were undergoing a control program for *S. aureus* mastitis. Quarter milk samples were aseptically taken from all lactating cows and delivered to the laboratory. Most cows did not show signs of clinical mastitis. Somatic cells (SCC) were counted on a Bentley Somacount 150 (Bentley Instruments, Chaska, MN, USA), and bacteriological analysis was performed (Hogan *et al.,* 1999). Coagulase-positive staphylococcal strains were confirmed as *S. aureus* using a duplex real-time PCR assay (Pilla et al., 2013) and then frozen at -80°C in Microbank bacterial preservation system (Thermo Fisher Scientific Inc, Waltham, MA, USA). The prevalence of *S. aureus* infections at herd level was calculated and 1 to 4 isolates per herd were included in the study, depending on the prevalence and on colony morphology on blood agar plate.

Bacterial DNA was extracted using DNeasy kit (QIAgen, Hilden, Germany), with the addition of lysostaphin (5 mg/mL; Sigma-Aldrich, St. Luis, MO, USA) for bacterial lysis. Amount and quality of DNA samples was measured on a NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA).

A DNA microarray (*S. aureus* Genotyping Kit 2.0; Alere Technologies GmbH, Jena, Germany) was used to genetically characterize the *S. aureus* strains. The tool detects a total of 330 different sequences, including accessory gene regulator alleles, genes coding for virulence factors and for microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), capsule type-specific genes, and numerous antimicrobial resistance genes. Probes for the methicillin-resistance genes *mecA* and *mecC* are also included. The overall pattern was analyzed automatically for the presence or absence of specific genes and compared to a database of strain

profiles allowing the assignment to Clonal Complexes (CC). The genotyping service was performed at Alere Technologies (Jena, Germany).

4.2 Statistical analysis

Four classes were defined a priori, based on the prevalence of intramammary infections by *S. aureus* found in the first sampling of all lactating cows in each herd: low prevalence (LP) when <5% cows tested positive, medium-low (MLP) or medium-high (MHP) when the infection ranged 5.1-24% or 24.1-40%, respectively, and high prevalence (HP) when >40% cows had at least one quarter infected by *S. aureus*. Prior to the statistical analyses, the genes which did not show any variation (i.e. only positive or negative results) were excluded, eventually retaining 169 genes.

4.2.1 Binary logistic regression (BLR) and risk factors calculation

Binary logistic regression analysis was conducted with the SPSS software (SPSS Inc., Chicago, IL).

This type of statistical test allows using dummy variable or dichotomous variable as the microarray data. In particular, each gene was analyzed separately/independently. The dependent variable (dichotomous) was the presence or absence of the specific gene, while the prevalence classes were treated as categorical. The LP class was used as reference for the analysis. *P*-values lower than 0.05 were considered significant. Frequencies of genes within each class of prevalence were also estimated with the SPSS software as shown in Table 4. For each variable, the regression coefficient (*B*) and the Wald test result (used to test significance) are shown. Further Odds ratio (for each variable category) were estimated (Exp (b)) within the SPSS software environment.

4.2.2 Data editing and correspondence analysis (CA)

The use of contingency tables is a widespread method, usefull to analyse cross-tabulations results in order to detect and measure the strength of the patterns of association between the observed variables. A number of statistical approaches are used for these purposes, encompassing hypothesis testing, logistic regression, and log-linear modelling. Besides these approaches, Correspondence Analysis (hereafter CA) is an exploratory statistical technique which falls into the classical multivariate statistical methods of dimension reduction based on the singular value decomposition (SVD), e.g. principal component analysis. Originally, CA was developed to analyze contingency tables in which a sample of observations is described by two nominal variables, but it was rapidly extended to the analysis of high dimensional data. The method is particularly helpful in analyzing cross tabular data in the form of numerical frequencies, and results in an elegant but simple graphical display which permits more rapid interpretation and understanding of the data. Multiple correspondence analysis (MCA) is an extension of correspondence analysis (CA) and allows investigating the pattern of relationships of several categorical dependent variables. It is the multivariate extension of CA to analyze tables containing three or more variables. The usual purpose in using CA is to graphically represent these relative frequencies in terms of the distance between individual row and column profiles and the distance to the average row and column profile, respectively, in a low-dimensional space. In this study, a contingency table was created counting the number of genes (row) per each prevalence herd class (column). The final contingency table is represented by the $I \times J$ matrix $\mathbf{X}$, whose generic element $x_{i,j}$ gives the number of observations that belong to the ***i*th** level of the first nominal variables (169 genes) and the ***j*th** level of the second nominal variable (4 herd prevalence classes). The grand total of the table is noted **N**. The goal of CA is to transform this contingency table into two sets

of factor scores (one for the rows and one for the columns), which give the best representation of the similarity structure of the rows and the columns of the table. In order to calculate the factor scores, the contingency table is firstly transformed into a probability matrix **Z**, computed as $\boldsymbol{Z = N^{-1}X}$. Then, following Abdi Béra (2014) and Nenadić and Greenagre (2007), the factor scores are obtained from the following *generalized* singular value decomposition (**GSVD**):

$$\boldsymbol{(Z - rc^T) = P\Delta Q^T \; with \; P^T D_r^{-1} P = Q^T D_c^{-1} Q = I}$$

Where $\boldsymbol{r}$ and $\boldsymbol{c}$ denote the vectors of the row and column totals of **Z**, respectively. The subtraction of $\boldsymbol{rc^T}$ from $\boldsymbol{Z}$ centers the matrix while $\boldsymbol{P, \Delta}$ e $\mathbf{Q}$ are the left and right singular vectors, and the diagonal matrix of singular values, respectively. From the **GSVD** the factor scores are obtained as:

$$\boldsymbol{F = D_r^{-1} P\Delta} \; and \; \boldsymbol{G = D_c^{-1} Q\Delta}$$

An important statistic in CA is the total variance of the data matrix or *inertia* (Healey, 2009) which is calculated on relative observed and expected frequencies:

$$\boldsymbol{Inertia = \emptyset^2 = \sum_{i=1}^{I} \sum_{j=1}^{J} \frac{(p_{ij} + r_i c_j)^2}{r_i c_j}}$$

CA was implemented using the *ca* (Nenadić and Greenagre, 2007), *FactorMineR* (Lê *et al.,* 2008), *vcd* (Meyer *et al.,* 2006) and *CAinterprTools* (Alberti, 2015) libraries of R (R Development Core Team, 2008).

5 RESULTS

Out of 169 *S. aureus* strains tested, 157 (92.9%) were MSSA and 12 (7.1%) were MRSA. The isolates were distributed in the four classes of prevalence of *S. aureus* mastitis as follows: 45 (26.63%) were in the Low Prevalence (LP) herds, 44 (26.03%) in Medium–Low Prevalence (MLP), 33 (19.53%) in Medium–High Prevalence (MHP) and 47 (27.81%) in High Prevalence (HP). The herds were similar in the extensive animal husbandry, while the average number of lactating cows was not homogeneous among prevalence classes. The main characteristics of the herds in the four groups are reported in table 6.

Table 4 Main characteristics of the herds in the four prevalence classes of S. aureus intramammary infections. LP, low prevalence herds; MLP, medium-low prevalence; MHP, medium-high; HP, high prevalence.

Prevalence Class	Cubicle Houses (No.)	Stanchion Barns (No.)	Lactating Cows, Average (min.–max.)
LP	20	-	96.2 (15–245)
MLP	15	1	67.6 (40–130)
MHP	10	1	52.0 (15–120)
HP	13	-	70.6 (15–195)

The results of the microarray analysis performed on *S. aureus* isolates and the diffusion of the different Clonal Complexes (CCs) throughout the classes of prevalence are summarized in Table 5.

Table 5 Overall distribution of the different Clonal Complexes (CCs) of *S. aureus* and CC distribution in the classes of prevalence of mastitis by *S. aureus* at herd level (low prevalence, LP; medium–low, MLP; medium–high, MHP; high prevalence, HP).CC8 was the most frequently isolated group of *S. aureus* and mostly related with the three classes of medium and high prevalence, whereas CC398 was typical of LP herds. The other most commonly represented *S. aureus* groups were CC97 (12.4%) and ST126 (8.3%).

CC	Number of Strains	Overall Distribution of the CC (%)	Distribution of CC in the Groups of Prevalence (%)			
			LP	MLP	MHP	HP
CC1	7	4.14	4.54	2.22	0	8.51
CC5	4	2.37	0	0	12.12	0
CC8	70	**41.42**	11.36	**46.67**	**42.42**	**63.83**
CC20	4	2.37	6.82	0	3.03	0
CC97	21	12.43	9.09	22.22	6.06	10.64
CC101	1	0.59	0	0	0	2.13
CC133	3	1.77	6.82	0	0	0
CC398	24	14.20	**36.36**	8.89	0	8.51
CC479	5	2.96	6.82	4.44	0	0
CC522	2	1.18	0	4.44	0	0
CC705	12	7.10	9.09	11.11	9.09	0
ST126	14	8.28	4.54	0	27.27	6.38
ST72	1	0.59	2.27	0	0	0
agr IV, undef. CC	1	0.59	2.27	0	0	0

Twenty-six genes were detected in all the strains: among them, we found several important virulence factors, such as leukocidin/γ-haemolysin genes *lukF/S* and the homologue *lukX/Y* (=*lukA/B* or *lukG/H*)/*hlgA*; the genes encoding the proteases aureolysin (*aur*), glutamyl endopeptidase (*sspA*) and staphopain B (*sspB*); the genes encoding staphylococcal exotoxin-like

proteins *setC* (*selX*) and *setB*; and the hyaluronate lyase genes (*hys*). MSCRAMMs such as clumping factor A (*clfA*) and B (*clfB*), the cell surface elastin-binding protein (*ebpS*), the enolase enzyme (*eno*), and the van Willebrand factor binding protein (*vwb*) were also detected in all isolates. Some genes were not detected: most of them encoded antibiotic resistance, exfoliative toxins, or one of the capsule type 1 locus genes (*capK1*). Overall, 75% of the strains were assigned to *agr* group *I*, 20% to *agr II*, and the remaining 5% to *agr III*. Different clonal complexes carried *agr I* (CC101, CC133, CC20, CC398, CC522, CC8, CC97, ST71 and ST72) or *II* (CC479, CC5, CC705 and ST126), respectively. Only CC1 harbored *agr III*. The binary logistic regression analysis (BLR) performed on each gene separately, using LP as the reference class, identified the following genes as related to the herds with *S. aureus* infection prevalence >5%: three enterotoxins with the same plasmid origin (*sed*, *ser*, *sej*); a leukocidin (*lukD/E*); the disrupted β-haemolysin (*hlb*) and the genes inserted by the truncating phage, i.e., staphylokinase (*sak*) and the staphylococcal complement inhibitor (*scn*); proteases (*splA*, *splB* and *splE* and an allelic variant of aureolysin); and MSCRAMMs, such as the fibrinogen-binding protein (*fib*), elastin-binding protein (*ebpS*) and allelic variants of clumping factor B (*clfB*), fibronectin-binding protein A and B (*fnbA* and *fnbB*), *S. aureus* surface protein G (*sasG*), serine–aspartate repeat protein D (*sdrD*) and the van Willebrand factor-binding protein (*vwb*). These genes are listed in Table 6.

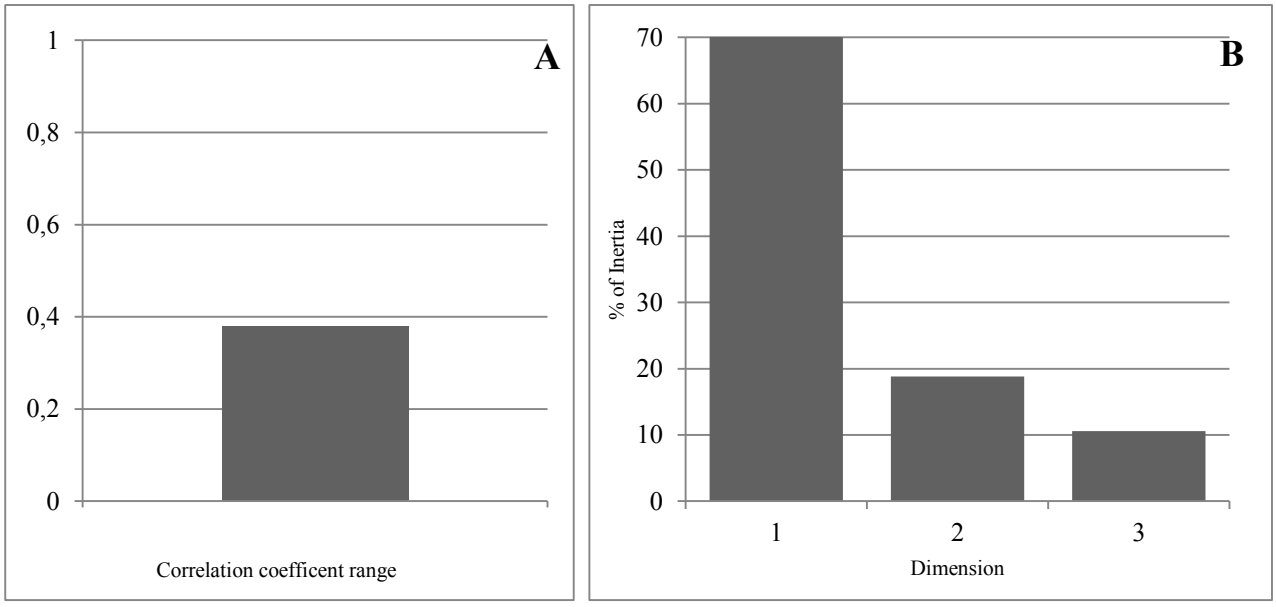
Table 6 Relative risk of detection of the genes with a significant different distribution in the four classes of prevalence of intramammary infections by *S. aureus*, using the lower frequency class as reference. MLP, herds with prevalence 5.1–24%; MHP, prevalence 24.1–40%; HP, prevalence >40.1%.

Genes	Relative Risk to the LP Class				
	Sign.	MLP	MHP	HP	
sea	0.034	1.61	3.41	5.53	enterotoxin A
sed	0.001	1.91	4.84	7.97	enterotoxin D
ser	0.001	1.64	4.92	8.05	enterotoxin R
sej	0.001	1.64	4.92	8.05	enterotoxin J
lukD	0.001	5.53	16.12	6.76	leukocidin D component
lukE	0.010	3.50	>25.00	6.76	leukocidin E component
hlb probe 3	0.007	4.66	4.126	6.09	haemolysin beta
sak	0.020	1.50	3.69	5.78	staphylokinase
scn	0.020	1.50	3.69	5.78	staphylococcal complement inhibitor
splA	0.036	3.65	>25.00	5.71	serin–protease A
splB	0.050	3.56	>25.00	4.90	serin–protease B
splE	0.000	5.04	7.25	10.47	serin–protease E
aur ★	0.050	3.56	>25.00	4.90	aureolysin
fib	0.050	3.56	>25.00	4.90	fibrinogen-binding protein
ebpS probe 612	0.004	5.48	>25.00	6.75	cell surface elastin-binding protein
clfB ▲	0.000	8.60	11.74	17.40	clumping factor B
fnbA ✦	0.000	2.94	7.11	7.21	fibronectin-binding protein A
fnbB ✦	0.000	16.99	13.93	31.81	fibronectin-binding protein B
sasG ▲	0.000	5.82	3.69	6.19	*S. aureus* surface protein G
sasG *	0.000	5.26	2.23	6.54	
sdrC ✦	0.002	3.97	2.29	5.68	Serine–aspartate repeat protein C
sdrC *	0.000	3.28	12.75	15.22	
sdrD ※	0.002	4.27	3.25	7.52	Serine–aspartate repeat protein D
vwb ※	0.000	4.55	3.38	12.78	van Willebrand factor-binding protein

* Indicates other allelic variants than MRSA252 (CC30). ▲ indicates the allelic variant shared by COL (CC8) and Mu50 (CC5).✦ indicates the allelic variant of COL (CC8). ★ indicates other allelic variants than MRSA252 (CC30) and RF122 (CC151/CC705). ※ indicates the allelic variant shared by COL (CC8) and MW2 (CC1).

In the correspondence analysis (CA), the association between rows (genes) and columns (prevalence class) was 0.36, confirmed by a Pearson's Chi-squared test (chi-square = 1612.4, df = 750, $p < 0.0001$). The results of the magnitude of correlation are shown in Figure 8A: the threshold of 0.20 is considered as an indicator of correlation (Bendixen, 1995; Healey, 2009). Regarding the observed variability (*inertia*) and its decomposition in the dimensions, the first, second and third dimension explained 70.6%, 18.8% and 10.6% of the observed variance, respectively (Figure 8B.).

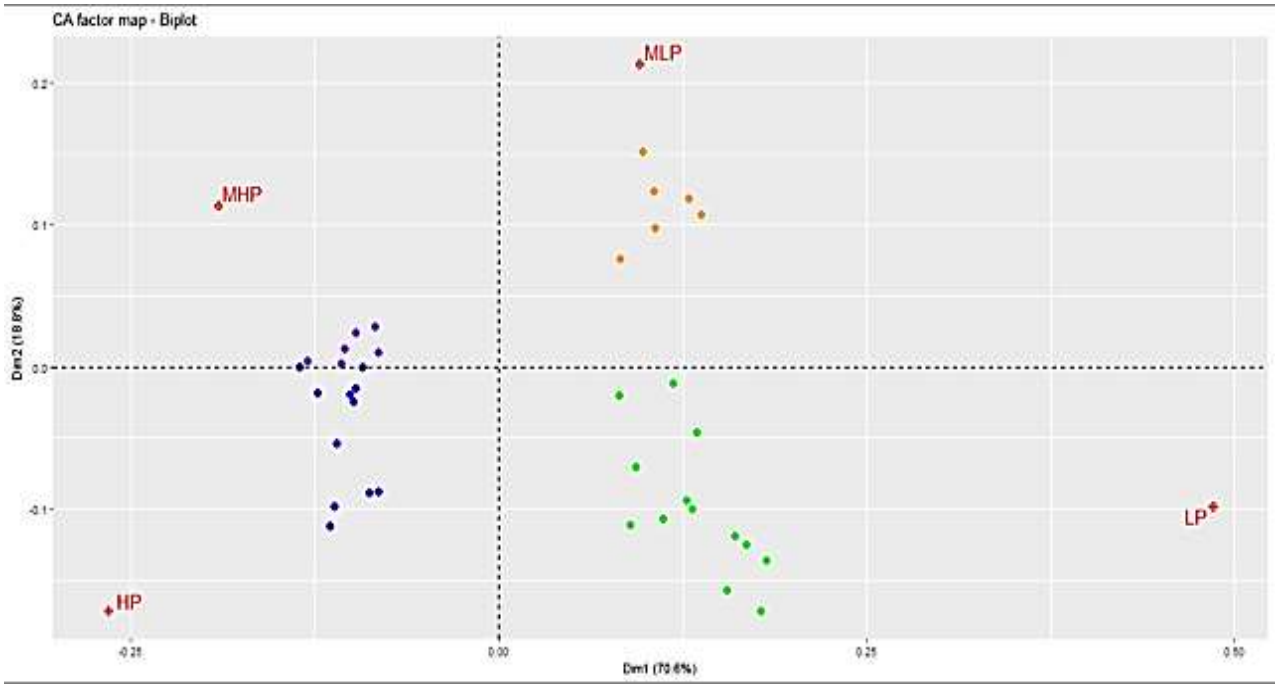
Figure 8 Correlation coefficient between 169 genes and four prevalence classes of intramammary infections by *S. aureus*. (A) Correlation coefficient: a value above 0.20 suggests a moderate to strong correlation; (B) proportion of variances retained by the first three dimensions.



The Malinvaud's test (1987) was applied to identify the optimal number of dimensions to retain, and showed that the first two dimensions were significant ($p < 0$ and $p < 0.0001$, respectively). Following Greenacre (2007), these dimensions were then used to produce a *biplot* (Figure 6), displaying the relative position of the row points (i.e., the genes) in the space (i.e., the prevalence

classes). The relative distance between points of different type is the *correspondence* between the categories that made up the table. The distance between each class and 0, such as between data points and 0, indicates the degree of similarity: HP and LP are much more distant from 0, when compared to MHP and MLP. For this reason, we considered only the two extreme classes.

Figure 9 Relative position of the genes (points) in the four prevalence classes (space: low prevalence, LP; medium–low, MLP; medium–high, MHP; high prevalence, HP).



According to Greenacre (2007), a rule of thumb to select the most important row variables that are related to each column variable is to use a threshold based on the average contribution, defined as 100number of rows*10. Applying this formula to our data set, the threshold was = 6, meaning that 10 genes were to be considered for LP, as well as for HP (Table 7).

Table 7 Genes significantly correlated with the extreme classes of intramammary infection prevalence (low prevalence, LP; high prevalence, HP).

Gene	Prevalence Class	
ebpS ♦	LP	cell surface elastin-binding protein
tetM	LP	tetracycline resistance
aur Y	LP	aureolysin
fib Y	LP	fibrinogen-binding protein
vga †	LP	ATP-binding protein, streptogramin A resistance
cna	LP	collagen-binding adhesin
dfrS1	LP	dihydrofolate reductase type 1
clfB ◈	LP	clumping factor B
capJ1	LP	O antigen polymerase
fexA	LP	chloramphenicol/florfenicol exporter
fnbB ♦	HP	fibronectin-binding protein B
sej	HP	enterotoxin J
ser	HP	enterotoxin R
sed	HP	enterotoxin D
vwb ※	HP	van Willebrand factor-binding protein
sdrD ※	HP	serine-aspartate repeat protein D
fnbA ♦	HP	fibronectin-binding protein A
sak	HP	staphylokinase
scn	HP	staphylococcal complement inhibitor
sea	HP	enterotoxin A

♦ indicates the allelic variant of Sequence Type 45. Y indicates the allelic variant of MRSA252 (CC30). † indicates the allelic variant of BM3327. ◈ indicates the allelic variant of MW2 (CC1). ※indicates the allelic variant shared by COL (CC8) and MW2 (CC1). ♦ indicates the allelic variant of COL (CC8).

6 DISCUSSION

The variability in the virulence of *S. aureus* strains plays a central role in the development of intramammary infections of the dairy cow and in the subsequent spread to other animals. In order to identify the genes that might be mostly implicated in the virulence of the strains, we used the microarray technology (Monecke *et al.,* 2007) to characterize, on the molecular level, 169 isolates from dairy cow mastitis, collected in 60 herds located in different Italian regions. The results were then associated with the prevalence of *S. aureus* intramammary infections at herd level and statistically analyzed using two different approaches, the BLR and the CA. The former detected and measured the strength of the patterns of association between each gene and the LP class of prevalence; the latter investigated the pattern of relationships of several categorical dependent variables, showing which gene was dominant across each prevalence group and graphically representing these relative frequencies in a low-dimensional space. In comparison with previous studies (Schlotter et al., 2012; Clark & Foster, 2006; Speziale et al., 2014; Kuroda et al., 2008; Sabat et al., 2006; Rice et al., 2001; Monecke et al., 2007; Piccinini *et al.,* 2010), the present one considered a higher number of isolates and, especially, the ability of the strain to spread within the herd. We decided to use the array technology because it is the best method for cost-benefit relation: proper bioinformatics was performed with consensus probes for all targets and, even if such a method cannot differentiate between functional and non-functional genes, it is very difficult to elucidate this topic. Indeed, a gene might be active when the infection process starts, and later become inactive once the infection progresses. On the other hand, NGS technology also has many disadvantages, including the analysis of time, the loss of short repeats, and the scarcity of standards. While the presence of a gene is not always correlated with its expression, its absence always means lack of expression. Therefore, the analysis of

presence/absence of genes coding for virulence factors in cows affected by *S. aureus* mastitis represents the first step toward functionality studies.

Regarding the distribution in CCs, a notable result was the finding of 8.3% ST126. This represents a cow-associated lineage that has been found in the Americas and in Southern Europe (see MLST database) (Snel *et al.,* 2015) while studies from Central and Northern Europe failed to detect it (Monecke *et al.,* 2007; Piccinini *et al.,* 2010; Snel *et al.,* 2015; Artursson et al., 2016). Isolates of this lineage were observed in LP, but also in MHP and HP herds.

Both MSSA and MRSA strains grouping in CC398 belonged to the LP class of prevalence. This is in accordance with another recent Italian study demonstrating that CC398-MRSA was associated with low prevalence infections in dairy herds (Luini *et al.,* 2015). Since MSSA as well as MRSA from this lineage are common in a variety of other livestock animals (especially poultry and pigs), it might be assumed that these isolates represent spillovers from other farm animals, whereas humans as well as rodents and flies might have served as vectors.

We found a high prevalence of CC8-MSSA in the herds studied in the present study. CC8-MSSA is a common strain in humans, but it was already reported as a strain frequently causing bovine mastitis in Western Switzerland, suggesting a recent host shift from humans to cows concurrent with a loss of the ability to colonize humans (Sakwinska *et al.,* 2011)

The distribution of the CCs in the classes of infection prevalence was not uniform, reflecting an higher risk of contagiousness for certain lineages in comparison to others. For this reason, the statistical association of some virulence factors with HP herds is of interest from a mastitis control perspective, despite the possible bias due to CC distribution.

The CA clearly distinguished LP and HP as extreme prevalence classes. Interestingly, LP genes were mostly allelic variants only found in MRSA strains: this finding is in accordance with a recent paper (Luini *et al.,* 2015) demonstrating that livestock MRSA are typically not diffusive. Regarding the carriage of *cna* by LP strains, the gene has been suggested not to play an

important role in S. aureus intramammary infections (Klein *et al.,* 2012). Different genes were significantly related to the strains isolated in those herds, where the prevalence of *S. aureus* mastitis was above 5%. They were involved in the evasion of host immune response (*sak* and *scn*), in the killing of phagocytes (lukD/E) or displayed superantigenic activity (*sed*, *ser* and *sej*); some genes were involved in tissue adhesion (fib) and invasion (*splA*, *splB*, *splE*). This result was strengthened by the CA, which highlighted a significant correlation in the distribution of *sed, ser, sej, sak* and *scn* with prevalence of *S. aureus* intramammary infections exceeding 40%. *Sed, ser,* and sej belong to a cluster harbored by different CCs, out of them CC151 and CC479 were indicated as the most frequent ones (Schlotter *et al.,* 2012). The genes sea, sak and scn, which are carried by β-hemolysin-converting bacteriophages, were present uniquely in some CC8 strains; their prevalence was higher than in other studies on bovine isolates, although lower than in studies on isolates from humans (Monecke *et al.,* 2007; Piccinini *et al.,* 2010; Snel *et al.,* 2015; Artursson et al., 2016; Luini *et al.,* 2015; Sakwinska *et al.,* 2011; Klein *et al.,* 2012; Luedicke *et al.,* 2010). Accordingly, a recent paper reported that CC8 strains of bovine or human origin differed for the mobile genetic elements, among them the β-hemolysin-converting prophages: all bovine-only isolates were devoid of such prophages (Resch *et al.,* 2013), probably because the untruncated hlb is necessary in ungulates for the different structure of erythrocyte membranes. This also supports the concept of a recent transmission from humans into cows (Sakwinska *et al.,* 2011). Nevertheless, a cleary contagious trait of CC8 strains has not yet been identified.

While the role of enterotoxins in bovine mastitis is not completely elucidated, they are suspected to promote *S. aureus* infections in cattle. Leukocidins target PMNs, weakening the host immune response. Both variants lukD/lukE and lukF-PV(P83)/lukM have been associated with bovine mastitis (Yamada et al., 2005; Schlotter *et al.,* 2012). While one or both variants were detected in most CCs, they were absent in CC398. Even though lukF-PV(P83)/lukM was

suggested to play an essential role in the etiology of bovine mastitis, our results showed a higher frequency of lukD/E and a significant correlation with the risk of being detected in MP and HP. This result could possibly be explained by an over-expression of this leucocidin variant, in the absence of lukF-PV(P83)/lukM. However, it also could be interpreted as accidental circumstance related to an ongoing epidemic of lukD/E-positive, lukF-PV(P83)/lukM-negative CC8 clone, assuming that its current spread was linked to factors other than leukocidin activity.

Altogether, the genetic array demonstrated in the HP S. aureus strains could counteract the efficacy of mammary immune responses, enabling the microorganism to promptly infect the herd. The MSCRAMM family includes different adhesins, which are essential for initial stages of infection. Among them, the fibrinogen-binding protein (fib) demonstrated a high risk of being detected in herds with *S. aureus* prevalence >5%, suggesting an involvement in strain contagiousness. The result differs from what was reported in a previous study, which described the gene as not significantly associated with mastitis (Larsen *et al.,* 2000). Other adhesins such as clfA and clfB, epbS and vwb were harbored by all strains, contrarily to previous studies, reporting variable frequencies (Luedicke *et al.,* 2010; Resch *et al.,* 2013; Kot *et al.,* 2016; Xue *et al.,* 2011). Interestingly, an allelic variant of clfB, epbS and vwb was prevalence-related, as it was detected in more contagious strains. The significantly different distributions of MSCRAMM allelic variants in the four groups of S. aureus prevalence and the correlation with HP herds for some of them might be the result of selective pressure. Indeed, different environments and management practices could amplify differences between strain virulence patterns (Piccinini *et al.,* 2010). A sdrD allelic variant was also included in the group of the genes at risk and correlated with HP; however, the role of this protein in bovine mastitis is still unclear, even though a significant association between sdrD and clinical mastitis was demonstrated (Xue *et al.,* 2011). Proteases promote invasion through degrading some of the cell surface components, such as fibronectin, fibrinogen and elastin (Kolar *et al.,* 2013). The literature regarding the role of

proteases in dairy cow mastitis is scarce: one study highlighted the high frequency of splA and sspA, but the variability of splE (Xue *et al.,* 2011). In human medicine, an association between the presence of splA/splB and S. aureus invasive endocarditis was found in hospitalized patients (Rasmussen *et al.,* 2013). Our data seem to support those results, since a significant difference in the frequency of spls was detected among the groups of mastitis prevalence, with a higher risk of carriage by more contagious strains.

7 CONCLUSION

The control of S. aureus mastitis is mostly based on the separation of infected cows; since two vaccines are available on the market, and the level of protection offered is not the same, because of the important role played by herd factors (Scali *et al.,* 2015). Considering the results of the present study, strain contagiousness appears to be related to an entire pattern of virulence factors, which promote both adhesion/invasion of mammary tissue and the immune response of the gland. These results offer new insights applicable in the development of vaccines against S. aureus mastitis. To determine repeatability a multi-centre study across several countries is recommended.

8 GENERAL CONCLUSION OF THE THESIS

Mastitis is well known to be influenced by the interaction of the host, the pathogen and the environment. Among responsible bacteria, *S. aureus* is one of the most common causes of bovine intramammary infections. It is a contagious bacterium, easily transmissible and the infected host poorly responds to treatment. The welfare of dairy cows is adversely affected by mastitis; therefore, understanding in depth the pathogenesis of the disease is an interesting field of study.

With this thesis we focused on different aspects involved in *S. aureus* mastitis outcome, starting from the development of a model to study the immune response of the udder, to the virulence genes of the bacteria.

Because the mammary gland is a complex organ, we developed a new multi-cellular and three-dimensional tissue explant which was used in two trials with: i) sintethic molecules (LPS and LTA), and ii) two different concentration of a *S. aureus* field isolate. The use of an explant of healthy heifer gland, differently to previus studies based on lactating mammary glands, allowed us to exclude previous exposure of the udder to any microorganism and to be more close to *in vivo* condition.

During the first study, we faced difficulties in setting up a reliable model, since the mammary gland is characterized by a high variability in tissue composition. For this reason, most studies have been conducted on stabilized cell lines or primary bovine epithelial cell cultures. Despite that, the positives results of the tests regarding morphology, proliferation and apoptosis enabled us to investigate the innate immune response of the tissue, using the mRNA expression of cytokines and antibacterial peptides such as LAP, after challenge with different stimuli. The results of the first trial showed significant increased expression for LPS but not for LTA in challenged sections. We hypothesized that such result might be due to a weak stimulus, rather than to a hypo-responsiveness of the explant. The significant results of the second trial, in which

the model was treated with a *S. aureus* strain, cofirmed our hypothesis. Using our model, we found an interesting infiltration of mononuclear cells, mainly T lymphocytes that can be regarded as resident cells. Mononuclear cells are responsible for the innate immune response, therefore not considering their interaction with epithelial cells in order to have less variability, could cause a loss of information.

Thereafter, we considered the different patterns of virulence genes of *S. aureus*, to understand outcome and diffusion of mastitis. Indeed, the prevalence in the bovine herds can be really different from herd to herd. Most of *in vivo* and *in vitro* studies used control strains, scarcely considering the genome variability inside the bacterial population. The results of our study showed that strain contagiousness could not be related to a single virulence factor but to an entire pattern of virulence factors, responsible for both adhesion/invasion of mammary tissue and the evasion of the gland immune response.

In conclusion, this thesis showed that multiple variables should be taken into account when studying bovine mastitis, starting from a deepened knowledge of the bovine mammary gland innate immune response, to the virulence pattern of the microbial strain causing the infection.

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10 LIST OF PUBLICATIONS

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