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Inflammatory Signaling in a Mouse Mastitis Model using Bovine-Associated Bacterial Isolates:

A Basis for Novel Curative and Preventive Strategies?

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Turn your face toward the sun and the shadows will fall behind you.

Maori Proverb

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LIST OF ABBREVIATIONS

AP-1	Activator Protein-1
APC	Antigen-Presenting Cell
ASC	Apoptosis-Associated speCk-like protein
C/EBP	CAAT Box Enhancer Binding Protein
caspase	cysteine-aspartic protease
CCL	Chemokine (C-C motif) Ligand
CD	Cluster of Differentiation
CFU	Colony-Forming Unit
CNS	Coagulase-Negative Staphylococcus
CREB	cAMP Response Element-Binding protein
CXCL	chemokine (C-X-C motif) Ligand
CXCR	chemokine (C-X-C motif) Receptor
DAMP	Damage-Associated Molecular Pattern
DL	Detection Limit
E. coli	*Escherichia coli*
ECM	Extra-Cellular Matrix
ED	Effective Dose
ET	Endotoxin Tolerance
h	hour
i.p.	intraperitoneal
Ig	Immunoglobulin
IL	InterLeukin
IL-1R	IL-1Receptor
IM	IntraMammary
IMI	IntraMammary Infection
KC	Keratinocyte Chemoattractant
KO	Knock-Out
LIX	Lipopolysaccharide-Induced CXC chemokine
LPS	LipoPolySaccharide
LTA	LipoTeichoic Acid
MAMP	Microbe-Associated Molecular Pattern
MCP-1	Monocyte Chemoattractant Protein-1
MDP	Muramyl DiPeptide
MIC	Minimum Inhibitory Concentration
MIP	Macrophage Inflammatory Protein
MMPs	Matrix MetalloProteinase
NF-kappaB	Nuclear Factor-kappa B
NLR	NOD-like receptors
p.i.	post-induction

PD	Protective Dose
PGN	PeptidoGlycaN
PMN	PolyMorphoNuclear
PRR	Pathogen Recognition Receptor
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
S. aureus	*Staphylococcus aureus*
S. chromogenes	*Staphylococcus chromogenes*
S. fleurettii	*Staphylococcus fleurettii*
S. uberis	*Streptococcus uberis*
SCC	Somatic Cell Count
SD	Standard Deviation
TA	Teat Apex
TF	Transcription Factor
TLR	Toll-Like Receptor
TNF-alpha	Tumor Necrosis Factor-alpha
wT	wild Type

General Introduction

In part adapted from:

Breyne, K. & Meyer, E. Mammary Glands: Anatomy, Development and Diseases (2014) **6**: 141-172

I. MASTITIS, AN INFLAMMATORY DISEASE AFFECTING MAMMALS

BACKGROUND: MAMMALIAN MILK PRODUCTION RESULTS FROM A CONSERVED MAMMARY GLAND ONTOGENY

Milk production is a mammalian hallmark wherein neonates are nourished postpartum through a very effective and adaptable organ (Kuruppath, Bisana et al. 2012). Hundred millions of years ago, evolutionary reprogramming of apocrine cutaneous glands associated with either hair follicles or evolutionary merging of different skin glands, resulted in a new excretion organ to support altricial offspring (Oftedal 2002; Olsson and Oldenborg 2008). The Eutherian (Placentalia) mammary gland provided for modern mammals is a result of morphological, physiological, biochemical, ecological and behavioral adaptations (Lefevre, Sharp et al. 2010).

Proteome analysis of different mammalian genomes confirms the conserved ontogeny of the mammary gland (Lemay, Lynn et al. 2009). The mammary gland is rudimental at birth and develops further into a post-pubertal quiescent gland in a process called mammogenesis (Lombardi 1998). At the onset of lactation, the slumber state of the exocrine gland differentiates with parturition due to female sex hormonal signaling (Lamote, Meyer et al. 2004). Morphologically, the mammary gland generally enlarges due to extensive branching of ducts and alveolar formation. Once fully functional, lactogenesis is initiated and sustained in the galactopoietic stage. Alveolar secretion starts with the highly protein- and fat-rich colostrum leading to the production of copious mature milk with a relative stable constitution under the influence of prolactin (Lacasse, Lollivier et al. 2012). Mammary secretions contain a diversity of constituents synthesized and secreted by several cellular pathways. Milk proteins such as casein are produced in the endoplasmic reticulum and are guided by the Golgi system to the cellular membrane. Budding of this membrane results in micelles loaded with proteins encoded in the mammary epithelial cells and is perceived as a mildly invasive apocrine secretion method. These membrane remnants, together with both *de novo* mammary triglyceride formation and *ex novo* liver or diet lipids locally released, render milk its characteristic fatty nature (Neville 2009). Milk saccharides such as lactose are also produced by the mammary cell compartment, while minerals such as calcium ions can be released through transepithelial transport from the bloodstream. The latter have important implications creating a hormonally-regulated osmotic gradient and eventually fluid excretion (Anantamongkol, Ao et al. 2012). In lactating animals that lose their milking stimuli, regression of the complete mammary gland to its state prior to pregnancy is introduced either through weaning of the litter or through entering the dry-off period. This involution process involves programmed cell death programs of specific epithelial cell populations and is reversible at its onset. Dead mammary cells are first discarded from their originating epithelial layer (anoikis) and subsequently released in the alveolar lumen (Watson and Kreuzaler 2011). In each state (embryonic, prepregnant, fully functional gestational and involutional) the mammary gland is compartmentalized in a parenchymal and a stromal portion. The ductal parenchym of pseudo-stratified cuboidal architecture consists of contractile myoepithelial cells and encircles another layer of epithelial cells that encloses

the lumen. The alveolar parenchyma concentrated in lobuli has a similar structure consisting of the same cell types. These different cell layers can be identified by means of their specific cytokeratin expression (Sleeman, Kendrick et al. 2006). The luminal mammary epithelial cells have a secretory potential which depends on the mammary stage. When alveoli are full of secretion, the myoepithelial cells contract and guide the milk through the ducts which collects in sinuses towards the orifice, where it gets released. Embedded in both layers of epithelia from this alveo-lobular structure, reside stem cells responsible for the morphological dynamicity of the post-natal mammary gland. These two layers contain several functionally distinct subpopulations, including stem, progenitor and differentiated cells (Smith, Welm et al. 2012). This complex cellular framework is supported by an elaborate stromal compartment called the Extra-Cellular Matrix (ECM). Based on its position in the gland, the ECM is subdivided in basal lamina, intra- and inter-lobular stroma and fibrous connective tissue. These ECM molecules actively participate in the organization of the complex mammary gland architecture and furthermore in cell functions including adhesion, survival, apoptosis, proliferation and differentiation of the mammary gland (Brownfield, Venugopalan et al. 2013). Although they are not yet fully understood, alterations in ECM constituents likely have implications in mammary gland diseases due to their influence on internalization of bacteria in hosts' epithelia following a mammary infection (Dziewanowska, Patti et al. 1999; Dziewanowska, Carson et al. 2000), on infiltration and activation of immune cells and on tumor development (Lochter and Bissell 1995; Van Laere, Van den Eynden et al. 2006).

I.1. What is Mastitis?

Although milk production by the mammary gland is essential for all mammals, cow milk is of key importance for the dairy industry. Dairy cow milk differs in composition of milk derived from other Mammalian species (Lemay, Lynn et al. 2009) but has no fixed composition *per se*. Cow milk composition depends on breed, stages of lactation, and composition of the ration. Similar as other mammals, the composition of cow milk stays relatively constant during mid-lactation, with most significant changes occurring in early and late lactation (Ilves, Harzia et al. 2012).

The interest for cow milk dates back from human civilizations that finally developed agriculture. Men selected dairy animals characterized by relatively dilute milks containing modest levels of fat, caseins, and whey proteins, and relatively high levels of lactose. The reason why men specifically selected cows or other dairy species is still a matter of speculation. Some people claim that dairy milk resembles human milk, in being dilute, or perhaps such species were easier to milk because their dilute milk accumulated in substantial storage cisterns between bouts of suckling (Oftedal 2012). Nevertheless, improvements in efficiencies of production and technological advances in milk removal still continues today with lower number of dairy herds and ever higher total milk production (Thompson-Crispi, Atalla et al. 2014). In the 80'ies, the US needed around 350,000 herds to produce 60,000 million kg/year while in 2010 less than 100,000 herds were able to produce 90,000 million kg/year (von Keyserlingk, Martin et al. 2013). These yields far surpass the one needed to nourish the newborn calf and not surprisingly, such a high production is associated either directly or indirectly with

an increased rate of reproduction-related health problems, i.e. mainly mastitis but also metritis (Roche 2006).

In essence mastitis (*mast* = breast; *itis* = inflammation), can be defined as an inflammatory disease of the (lactating) udder in ruminant species (Rauw, Kanis et al. 1998; Contreras and Rodriguez 2011). Mastitis is characterized by typical changes which can be either directly observed i.e. clinical symptoms of udder tissue inflammation and organoleptic loss of milk quality, or lack visual symptoms i.e. subclinical mastitis. Alongside with other parameters, the latter can be detected by the measurement of Somatic Cell Count (SCC). Milk SCC levels of individual quarters above a well-defined threshold (200,000 - 250,000 cells/ml) give an indication of udder inflammation (Smith, Hillerton et al. 2001). From an economical perspective, the consequences of mastitis are not only limited to the compromised milk quality (e.g. lower production and inadequate composition) and health traits of the animal (lameness, metabolic or reproductive disorders), but also to the lower economic value of milk, increased veterinary and labour costs, and an increased need for cow replacement (Asaf, Leitner et al. 2014; Ducrocq and Wiggans 2014).

Because of its substantial economic and animal welfare impact, mastitis was mainly recognized as a relevant pathological process for cows and other dairy animals. However, an increasing number of researchers from veterinary and human medicine have recently intensified the sharing of their knowledge on mammary gland inflammation. This interdisciplinary communication resulted in a major added value for the field of mastitis research. Consequently, the now accepted concept is that not only ruminants but probably all mammals and certainly also humans suffer from mammary gland inflammation (Contreras and Rodriguez 2011).

I.2. Major versus Minor Mastitis Udder Pathogens

The movement of leukocytes and serum proteins from the blood to the affected udder can be due to amalgam of causes. Indeed, mastitis is a complex disease associated with a variable origin, severity and outcome depending on the environment, pathogen or host (Schukken, Gunther et al. 2011; De Vliegher, Fox et al. 2012). Mammary gland inflammation can be elicited by infectious (bacteria, viruses, mycoplasmas, algae, yeast, …) and non-infectious agents (chemical and physical factors, trauma, obstruction lymph vessels, ...). The most common etiological agent of mastitis in dairy cattle are bacteria and these will therefore be the main focus of this PhD thesis (Pyorala 2003).

Mastitis-causing bacteria can be discriminated based on the severity of their induced symptoms (Viguier, Arora et al. 2009). Major mastitis pathogens such as *Staphylococcus aureus* (*S. aureus*), *Streptococcus uberis* (*S. uberis*), and the coliforms such as *Escherichia coli* (*E. coli*) are usually considered more damaging to the udder of dairy cows than minor mastitis pathogens such as coagulase-negative staphylococci (CNS) (Reyher, Haine et al. 2012; Watts 1988). In addition to the virulence feature, mastitis pathogens can also be categorized based on their epidemiology i.e. as being either contagious or environmental. The host is the main source of contagious mastitis pathogens and transmission occurs primarily from one dairy cow to another. On the other hand,

environmental mastitis pathogens are more likely to be transmitted from the habitat to the dairy cow (e.g. bedding in the stable).

Staphylococcus aureus is a Gram-positive pathogen that can be isolated from quarters of dairy cows with either clinical or subclinical mastitis. In Flanders, a Belgian region with about 280,000 dairy cows, *S. aureus* could be isolated in 25% of the infected milk samples that had a composite SCC of 250,000 cells/ml in three consecutive years (Piepers, De Meulemeester et al. 2007). In addition, *S. aureus* was the most frequently isolated pathogen from quarters that had visible signs of clinical mastitis in a nationwide study conducted in Canada (Olde Riekerink, Barkema et al. 2008; Sol, Sampimon et al. 2000). Besides *S. aureus*, that is generally recognized as a contagious major pathogen, the coliform *E. coli* is often considered as an environmental major pathogen. In Flanders, *E. coli* is isolated in about 15% of the mastitis cases and the likelihood of isolation of this pathogen has been associated with the increase in severity of the mastitis symptoms (Verbeke, Piepers et al. 2014). Nevertheless, reports have emerged on *E. coli* isolates that are capable of inducing chronic mastitis due to internalization into the mammary epithelial cells (Almeida, Dogan et al. 2011; Döpfer, Barkema et al. 1999). These major mastitis pathogens may infect the udder despite the presence of antibacterial mechanisms in the milk environment (Barrio, Rainard et al. 2003; Rainard 2003; Benkerroum 2010). It has been increasingly reported that a number of mastitis-associated isolates may have actively adapted to thrive in milk. For example, mastitis-associated *E. coli* grown in milk are known to up- and down-regulate hundreds of proteins in contrast to the same strains grown in conventional culture media (Lippolis, Bayles et al. 2009). This observation indicates that bacteria that cause mastitis have a higher fitness in this environment. Fitness of bacteria is defined as the ability of a germ to adjust its metabolism to environmental conditions, in order to survive and grow (Laurent, Lelievre et al. 2001). Although proper confirmation of this hypothesis is still lacking and thus warrants in-depth research, the term “Mammary Pathogenic *E. coli* (MPEC)” was introduced suggesting distinct *E. coli* with specific mammary pathogenicity and inherent local inflammatory potential (Shpigel, Elazar et al. 2008). Interestingly, similar indications are also accumulating for *S. aureus* mastitis-associated isolates. It is known that the mastitis-associated *S. aureus*-suppressing activity of antibiotics in milk cultures is lower than in broth cultures (Louhi, Inkinen et al. 1992).

A variety of CNS species have been isolated from milk samples of bovine mastitis cases, the most commonly being *S. chromogenes*, *S. haemolyticus*, *S. simulans* or *S. epidermidis* (Capurro, Artursson et al. 2009; Piessens, Van Coillie et al. 2011; Taponen, Koort et al. 2007). CNS species can be retreived in either milk samples of dairy cows with clinical or subclinical symptoms but, contrary to the symptoms inflicted by major mastitis pathogens, CNS mastitis is usually associated with much milder symptoms (Jarp 1991). Besides the difference in virulence, the fitness of a CNS species may also differ from major mastitis pathogens. The challenge dose of *S. chromogenes* to experimentally induce mastitis in dairy cows has been reported to be 100 to 1000 times higher than those generally used to induce *S. aureus* or *E. coli* mastitis (Simojoki, H., T. Orro, et al. 2009). Once CNS colonization is established in the udder, the infection may either be transient and disappear spontaneously or persist in the mammary gland. Studies on CNS species are complicated by the fact that they are abundantly

present and are not always implicated in an udder infection. It was demonstrated that *S. chromogenes* may colonize the teat apex pre-partum without being associated with an intramammary infection (De Vliegher, Laevens et al., 2003). Furthermore, several CNS species such as *S. fleurettii, S. sciuri*, and *S. equorum* were retrieved in the environment but rarely in mastitis samples (Piessens, Van Coillie et al. 2011). Furthermore, this seemingly contradictory result is probably due to the fact that CNS are most commonly referred to as one group of bacterial species, while in reality a high diversity exists between species and even between strains or isolates within one species (Thorberg, Danielsson-Tham et al., 2009). Lastly, it should be remarked that emerging reports also attribute beneficial features of CNS isolates which gains increasing interest. CNS isolates may inhibit major mastitis pathogens either by competitive exclusion and/or the production of antibacterial substances (Braem, Stijlemans et al. 2014).

The reason why pathogens with low fitness or low virulence features still can induce inflammation in the udder of dairy cows is a subject for ongoing research. Interestingly, many minor or major pathogens share Microbe-Associated Molecular Patterns (MAMPs) that can each on an individual basis induce sterile mastitis. For example, peptidoglycan (PGN) and lipoteichoic acid (LTA) (see section II.1 of the general introduction) both elicit an inflammatory response when exposed to mammary epithelia. Although MAMPs are present on both CNS and *S. aureus* mastitis-associated isolates, they induce different mastitis symptoms. It is suggested that subtle variations of these MAMPs may be responsible for the observed different degrees in pathogenicity. Next to the MAMPs that may be similar between minor an major pathogens, some features are restricted to pathogens such as invasion in the host tissue, secretion of toxins, etc. and have to be detected by the host differently (see section II.3. of the general introduction). Although this latter host response is not yet characterized during mastitis, mastitis in the udder will be more complex and could be explained by a specific combination rather than a synergy of MAMPs (Shpigel, Elazar et al. 2008).

I.3. Insights in the Current Curative and Preventive Mastitis Strategies

For almost 50 years the use of antibiotics against mastitis has been standard practice as dictated by the 10-point management program of the National Mastitis Council (NMC_Guidelines 2014). Routinely, these antibiotics are infused in the udder rather than parenterally and the panel of antimicrobial agents is limited to a few classes (Table 1). The intramammary antibiotics are delivered intracisternally through the teat canal and can be given during the dry period as well as in the milking period. During the dry period, higher doses can be used than in a milking cow. Furthermore, antibiotics remain in the udder for longer during dry cow therapy as a lot of antibiotic is lost at each milking with lactating cow therapy. At last, dry cow therapy uses long lasting antimicrobial compounds (Gruet, Maincent et al. 2001). In the UK, the use of intramammary administered antibiotics was about 0.95 g/infected dairy cow in 2013 (Pyorala, Taponen et al. 2014). The intensive use of antibiotics is predominantly due to their activity against subclinical mastitis pathogens (such as *S. aureus*), while it sometimes has limited benefits in shortening the duration of a clinical mastitis infection (typically caused by *E. coli*). Hence, if recovery is not really expected from treatment with antibiotics it rather is from anti-inflammatory drugs (Hogan and Larry Smith 2003).

Such use of antibiotics may lead to antibiotic resistance in bacterial isolates of mastitis cases. A Canada-wide study has determined that the prevalence of antibiotic resistance in bovine mastitis pathogens isolated from milk samples is low (Saini, McClure et al. 2012). Nevertheless, multi-resistance strains (e.g. livestock-associated multi-resistant *S. aureus*) have started to emerge in mastitic udders and may cause significant problems for the human health (Vanderhaeghen, Cerpentier et al. 2010; Vanderhaeghen, Hermans et al. 2010). Moreover, the prevalence of antibiotic-resistant mastitis-associated isolates may peak after selective culling or segregation of persistent infected cows from the herd. This can be explained as follows: major mastitis pathogens have more severe symptoms compared to the mild CNS-induced mastitis. Elimination of the animals suffering from a major mastitis pathogen such as *S. aureus* due to culling, may positively select for animals with minor pathogens such as CNS (Barlow 2011). Consequently, CNS species that inherently contain a antimicrobial resistance panel far exceeding the one of *S. aureus* mastitis isolates, will become the main infectious species in that herd (Frey, Rodriguez et al. 2013; Owens and Watts 1988; Piessens, De Vliegher et al. 2012; Pyorala and Taponen 2009).

Complementary to curative antibiotic treatment, increasing efforts have been done to develop vaccines for mastitis over the recent years. These vaccines can systemically be administered during the prepartus and aim to enhance the cows' immunologic resistance to mastitis-causing bacteria. The main commercially available vaccines against *E. coli*-induced mastitis are J-5 Bacterin (Zoetis), Endovac-Bovi (IMMVAC) and J Vac (Merial). J-5 based vaccines (J-5 and J Vac) contain an inactivated mutant strain of *E. coli* 0111:B4 strain while Endovac-Bovi contains a killed re-17 mutant *Salmonella typhimurium* (Talbot and Lacasse 2005). The former bacterins induce a non-specific immune response in the host and claim reduction in severity of symptoms, LPS opsonisation and enhanced bacterial clearance (Dosogne, Vangroenweghe et al. 2002). Two commercial vaccines are on the market against *S. aureus*. While Lysingin (Boehringer Ingelheim Vetmedica) contains lysates of different *S. aureus* strains, Startvac (Hipra) contains a mixture of inactivated *E. coli* J5 strain and the *S. aureus* SP 140 strain expressing a Slime Associated Antigenic Complex. However, field trials or experimental challenge carried out with Lysigin in adult cows showed only limited effectiveness in reducing infection duration and severity (Camussone, Veaute et al. 2014). Startvac efficacy was estimated in two herds and was associated with a moderate reduction in incidence and duration of new *S. aureus* and CNS infections (Schukken, Bronzo et al. 2014). Next to the antibacterial effect, the vaccine was suggested to reduce the severe inflammatory reaction after inoculation compared to non-vaccinated dairy cows (Deberdt, Piepers et al. 2012).

Despite the development of various vaccines, few have claimed satisfactory outcomes. The development for anti-mastitis vaccines seems much more difficult compared to anti-viral vaccines. It is suggested that the immunogenic antigens of mastitis pathogens are capsular polysaccharides that can vary between strains (Talbot & Lacasse 2005). As such, it is preferable to consider multiple pathogens at once and the host-associated inflammatory response they induce (Tiwari, Babra et al. 2013).

Table 1: Antimicrobial agents commonly used as curative therapy for bovine mastitis.

Mechanism of action	Antibiotic	Antibiotic class
DNA gyrase		
	Novobiocin	aminocoumarins
dihydropteroate synthetase		
	Sulfamethoxazole	sulfonamide
	Trimethoprim/sulfatrimethoprim	sulfonamide
30S subunit of the ribosome		
	Gentamicin	aminoglycoside
	Neomycin	aminoglycoside
	Streptomycin	aminoglycoside
	Oxytetracycline	tetracycline
	Tetracycline	tetracycline
50S subunit of the ribosome		
	Chloramphenicol	amphenicol
	Florfenicol	amphenicol
	Clindamycin	lincosamide
	Erythromycin	macrolide
	Lincomycin	lincosamide
	Pirlimycin	lincosamide
	Spiramycin	macrolide
	Tylosin	macrolide
beta-lactamase		
	Amoxicillin	aminopenicillin
	Ampicillin	aminopenicillin
	Cefalexin	cephalosporin (1^{th} gen)
	Cefalonium	cephalosporin (1^{th} gen)
	Cefapirin	cephalosporin (1^{th} gen)
	Cefazolin	cephalosporin (1^{th} gen)
	Cefquinome	cephalosporin (4^{th} gen)
	Ceftiofur	cephalosporin (3^{th} gen)
	Cefuroxime	cephalosporin (2^{th} gen)
	Cephalothin	cephalosporin (1^{th} gen)
	Cephaparin	cephalosporin (1^{th} gen)
	Cloxacillin	aminopenicillin
	Hetacillin	aminopenicillin
	Oxacillin	aminopenicillin
peptidoglican synthesis		
	Vancomycin	glycopeptide
topoisomerase ligase inhibitor		
	Enrofloxacin	fluoroquinolone

Table adapted from (Guerin-Faublee, Carret et al. 2003; Sawant, Sordillo et al. 2005; Marshall and Levy 2011; Ruegg 2012)

Although it is hypothesized that the extreme heterogeneity of pathogens that are able to induce mastitis limit the efficacy of a single-bacterin-based vaccine in the field, alternative strategies to protect the udder against severe mastitis have been proposed that exploit the richness of bovine-associated bacterial isolates to circumvent severe clinical mastitis (Lohuis, Kremer et al. 1990). These kind of protective strategies are based on recurrent infections of the dairy cows' udder. Experimental and observational studies involve the inoculation of the dairy cow with minor or major pathogens or utilize natural infection prior to an infection with major pathogens. It is suggested that colonization of the first infectious agent prevents the colonization and virulence of the second infection (Suojala, Orro et al. 2008). Furthermore, it is proposed that the colonization of the first infectious agent not only has an effect on the local macrophages, but also on the epithelia cells in the udder. This would subsequently result in a change of the immune response towards the second infectious agent that enters the udder (Gunther, Petzl et al. 2012; Petzl, Gunther et al. 2012). Ideally, the first infectious agent would be an commensal or minor mastitis pathogen that could circumvent the infection of a major mastitis pathogen (Rainard and Poutrel 1988). In that respect, CNS species have been suggested as colonizing agent as they exhibit a wide range of different bacterial species that contain properties such as milk promoting factors, production of anti-bacterial molecules and mild virulence (Piepers, Opsomer et al. 2010; Piessens, De Vliegher et al. 2012; Vanderhaeghen, Piepers et al. 2014).

Overall, this research area is characterized by an intense debate due to the high diversity of results among studies (especially between experimental and observatory studies) and the relatively low number of reports. The difficulty to study this subject has been suggested to be due to confounding dairy cow factors such as breed, season of calving, age, and stage of lactation but also herd-level factors such as overall microbiome in the herd or the environment (Reyher, Haine et al. 2012).

II. HOST INNATE IMMUNE SIGNALING OF INTEREST FOR MASTITIS

BACKGROUND: THE UNIQUE AND COMPLEX IMMUNOBIOLOGY OF THE MAMMARY GLAND

Milk is an unique liquid with a dual role in nourishment and in immunological protection of the newborn (Ward and German 2004). Based on phylogenetic tree data, scientists discovered the mammary gland as an organ that has evolved from antimicrobial glands into milk-producing entities (Fig.1). Many milk nutrients are now considered as an evolutionary bypass of these original antimicrobial molecules. To illustrate this remarkable modification the documented transition of lysozyme into α-lactalbumin is a nice example. Whereas α-lactalbumin is a prototypical mammalian protein that enables lactose production in the mammary gland, lysozyme is an anti-bacterial peptide producing enzyme that dates back already to non-vertebrate species. Despite their functional differences, both proteins share a 40% amino acid identity and have a nearly identical three-dimensional structure (Irwin, Biegel et al. 2011). Moreover, xanthine oxido-reductase (XOR) is crucial for the formation and secretion of milk fat from the lactating epithelium. It has an antimicrobial function through the production of both Reactive Oxygen Species (ROS) and Reactive Nitrogen species (RNS). The remnants of the innate immune system in the mammary gland are not only restricted to lysozyme and XOR but are also represented

by a vast array of antibacterial proteins of lactoperoxidase, lactoferrins and beta-defensins (Huang, Morimoto et al. 2012), oligosaccharides (Moon, Kim et al. 2007), fatty acids (Batovska, Todorova et al. 2009), mucins (Sando, Pearson et al. 2009) and secreted milk antibodies such as Immunoglobulins (Igs) (Boumahrou, Chevaleyre et al. 2012).

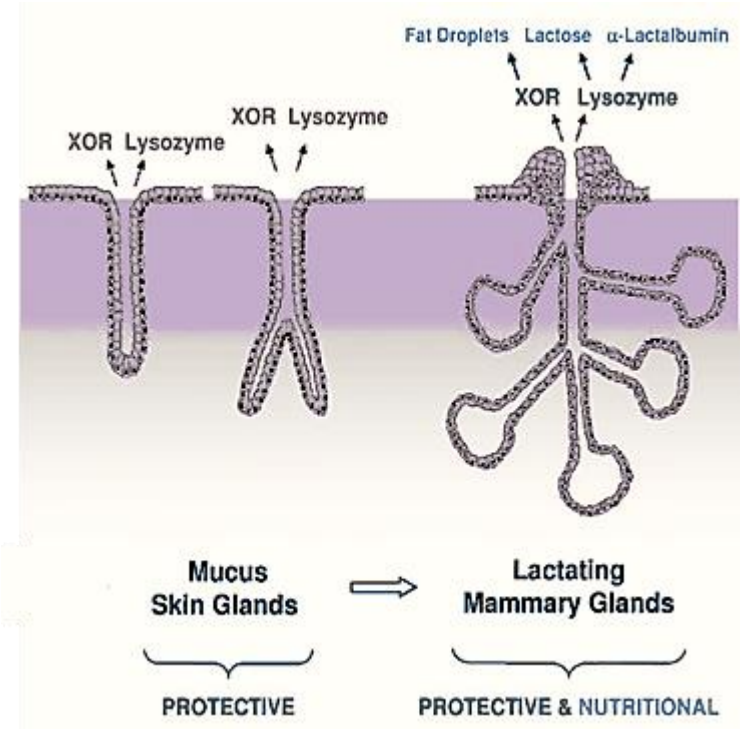

Figure 1: Initially, the mammalian skin enhanced its surface and produced mucus as protection against infectious diseases. This mucosal skin gland evolved into a mammary gland organ with an additional nutritional function beside its more ancient innate immune function. The relics from its innate immune history are still inherently present in Xanthine OxidoReductase (XOR) and Lysozyme. Adapted from (Vorbach, Capecchi et al. 2006).

Complementary to this large group of secreted molecules, the immunobiology of the mammary gland also contains an extensive cellular component that aids the host to fight intramammary infections. In general, the prevailing leukocyte populations of a healthy mammary gland sensing infectious bacteria consist of both innate and acquired immune cells i.e. macrophages and lymphocytes, respectively. During mammary gland infection, macrophages function as Antigen-Presenting Cells (APC) but also act as professional phagocytes against invading microorganisms. They trigger the innate immune response through prostaglandin-, leukotriene- and cytokine-mediated signaling (Elazar, Gonen et al. 2010). Moreover, these macrophage-derived chemokines partly guide polymorphonuclear leucocytes (PMN) from blood into the infected udder, which either nullify or at least weaken bouts of microbial agents (Sordillo and Streicher 2002). This is supported by the possibility of inducing mammary inflammation with a major cell wall component derived from Gram-negative bacteria, i.e. lipopolysaccharide (LPS). In the latter sterile mastitis model, macrophages were shown to be the key

cell type responsible for efficient neutrophil infiltration (Elazar, Gonen et al. 2010). Upon an inflammatory insult, phagocytic macrophages also neutralize leukocytes including neutrophils, dead cells and cellular debris that form a potential pro-inflammatory threat. In contrast to live cells, dead cells generally expose extracellular phosphatidylserine, efficiently recognized as an “eat me” signal to phagocytize cellular remains (Schwertfeger, Rosen et al. 2006). Through this elegant strategy, dead immune cells decompose safely, a crucial process supporting homeostasis.

Lymphocytes are organized in Lymph Nodes (LN) and are part of an elaborate lymphatic system (Van den Broeck, Derore et al. 2006). T-lymphocytes become activated in these mammary-associated LNs by antigens captured either by mammary dendrocytes, by macrophages or by B-lymphocytes or other resident APCs. In contrast to other mammals, dairy cow udders do not have mammary LN but are drained by retro-mammary LNs and contain a Furstenberg’s Rosette where lymphocytes are numerous. Moreover, bovine lymphocytes are predominantly cytotoxic T-cells ($CD8^+$) compared to a lower number of T-helper cells ($CD4^+$). Both these subtypes are released in different ratios in milk depending on the lactational stage (Taylor, Dellinger et al. 1994; Asai, Kai et al. 1998). Overall, lymphocytes have important implications for all mammals in the mammary defense against exogenous agents (Denis, Lacy-Hulbert et al. 2011), protection of the milk-fed offspring (Sabbaj, Ibegbu et al. 2012) and also in breast cancer metastasis (Tan, Zhang et al. 2011). The number of lymphocytes increases following bacterial infection of the mammary gland (Cao, Jing et al. 2012). Interaction with antigens such as *S. aureus* enterotoxins induces T-lymphocyte activation, immunosuppression (i.e. decreased T-cell proliferation) or influences cell viability (Park, Fox et al. 1993; Webb and Gascoigne 1994; Slama, Sladek et al. 2009). Activated T-lymphocytes secrete a specific panel of pro-inflammatory cytokines aiding the efficient clearance of bacteria (Persson Waller, Colditz et al. 2003; Riollet, Mutuel et al. 2006). This potential makes them key players in chronic mammary gland infections and therefore suggests innovative prevention perspectives in the context of staphylococcal mastitis vaccination (Wallemacq, Bedoret et al. 2012).

The most prominent cell type in the mammary gland is the epithelial cell which is perceived as an imminent part of the mammary gland innate immune system (Monks, Rosner et al. 2005). As the mammary gland regresses very fast during its involution post-weaning, a scary accumulation of dead cells occurs in the lumen. These might expose their intracellular compounds and thus imply an inflammatory danger (Monks, Smith-Steinhart et al. 2008). Mammary epithelial cells can act as (non-professional) phagocytes and engulf either partly damaged or already dead cells to resolve or prevent such undesired inflammatory conditions. Although less efficient in this elimination function than the two professional leukocytes (macrophages and neutrophils), they again utilize specific receptors such as the phosphatidylserine receptor, CD36, vitronectin receptor, CD91 to scavenge the vast lumen for damaged cells. Upon the detection hereof, they respond by excreting anti-inflammatory mediators like Transforming Growth Factor (TGF)-β1, while reducing the amount of pro-inflammatory mediators like Tumor Necrosis Factor (TNF)-α and IL-8/CXCL8 thus bridging the critical time frame before massive influx of both the innate immune cells (Monks, Rosner et al. 2005). Mammary epithelial cells should undoubtedly be considered as non-professional immune cells as they express Pathogen Recognition

Receptors (PRRs) embedded in their membrane, which enable them not only to sense exogenous bacterial ligands but moreover to react against this threat as an essential part of the innate immunity (Gunther, Koczan et al. 2009).

In healthy functional mammary glands, other leukocytes besides the predominating macrophages and lymphocytes are only present in low amount (e.g. neutrophils, natural killer-cells, dendritic cells, eosinophils, basophils, B-lymphocytes). Nevertheless, they rapidly become the principal innate immune cell type found in the inflamed udder and in its secretions. The numerical values of immune cells in bovine milk have been reported by differentiating the SCC in normal cows and in mastitis cases (Schwarz, Diesterbeck et al. 2011). Indeed, the non-specific innate immune cells called neutrophils are initially recruited to the site of infection. This recruitment is a result of a cocktail of inflammatory mediators which are orchestrated by the activated PRR-expressing cells (described in detail in Section II.1). Furthermore, stimulated local endothelial cells become fenestrated so that blood neutrophils leak in the infected mammary tissue further guided by the latter chemoattractants. Once directly exposed to pathogens, neutrophils also use the process of phagocytosis now complemented by bactericidal means (both oxygen-dependent as mediated by the respiratory burst involving ROS and RNS and oxygen-independent via small antibacterial molecules like cathepsins and calpains) to eliminate the bacterial hazard as rapidly as possible (Paape, Mehrzad et al. 2002).

II.1. Toll-like Receptors, Pathogen Recognition Receptors for the Host Innate Immunity

The resident innate defense system has evolutionary roots and comprises the unique anatomy of the gland (e.g. the teat canal with its keratin plug) as well as an extensive humoral and cellular component to protect the host against mastitis pathogens such as *E. coli* or *S. aureus* (Craven 1985). To prevent their excessive activation, these defense systems have to be appropriately controlled both in time and space. For this purpose, antigens expressed by pathogens are distinguished from host factors by PRRs (Fig. 2). The main PRR expressed by mammary cells to recognize bacteria are Toll-Like Receptors (TLRs). These receptors are evolutionary conserved protecting even invertebrates from pathogenic assaults. The specificity of a certain TLR is determined by the MAMP it recognizes. For example, TLR2 recognizes LTA of Gram-positive bacteria, TLR4 recognizes LPS from Gram-negative bacteria and TLR5 interacts with bacterial flagellin (Rainard and Riollet 2006). Furthermore, PGN is expressed by both Gram-negative and -positive bacteria and is suggested to bind bovine TLR2 although this is a matter of debate (Girardin, Travassos et al. 2003; Farhat, Sauter et al. 2008). Remark that each of these MAMPs does not bind a fully designated TLR, but that rather binding is possible to several TLRs (Yang, Mark et al. 1998; Takeuchi, Hoshino et al. 2000). Furthermore, the formation of lipid rafts within clathrin-coated pits is required for the endocytosis of stimulators and their subsequent intracellular signal transduction. Consequently, blockage of these lipid rafts inhibits the TLR response by the mammary epithelium (Kim, Im et al. 2011).

The intensity with which a mammary resident cell type reacts to a MAMP is typically coordinated by its co-receptors. A well-known example is that of CD14, a receptor with a soluble (sCD14) and a membrane bound (mCD14) isoform (Zanoni and Granucci 2013). LPS is recognized by its LPS-binding protein (LBP) and these LPS/LBP complexes interact with (s or m)CD14 to trigger TLR4

(Oviedo-Boyso, Valdez-Alarcon et al. 2007). Cell types bearing mCD14 in the vicinity of their TLR4, such as macrophages or neutrophils, have an advantage over cell types lacking mCD14 and relying on less easy accessible sCD14 such as endothelial or epithelial cells (Frey, Miller et al. 1992; Sohn, Paape et al. 2004). Moreover, CD14 expression is not restricted to Gram-negative bacteria because it also recognizes PGN (Rainard and Riollet 2006).

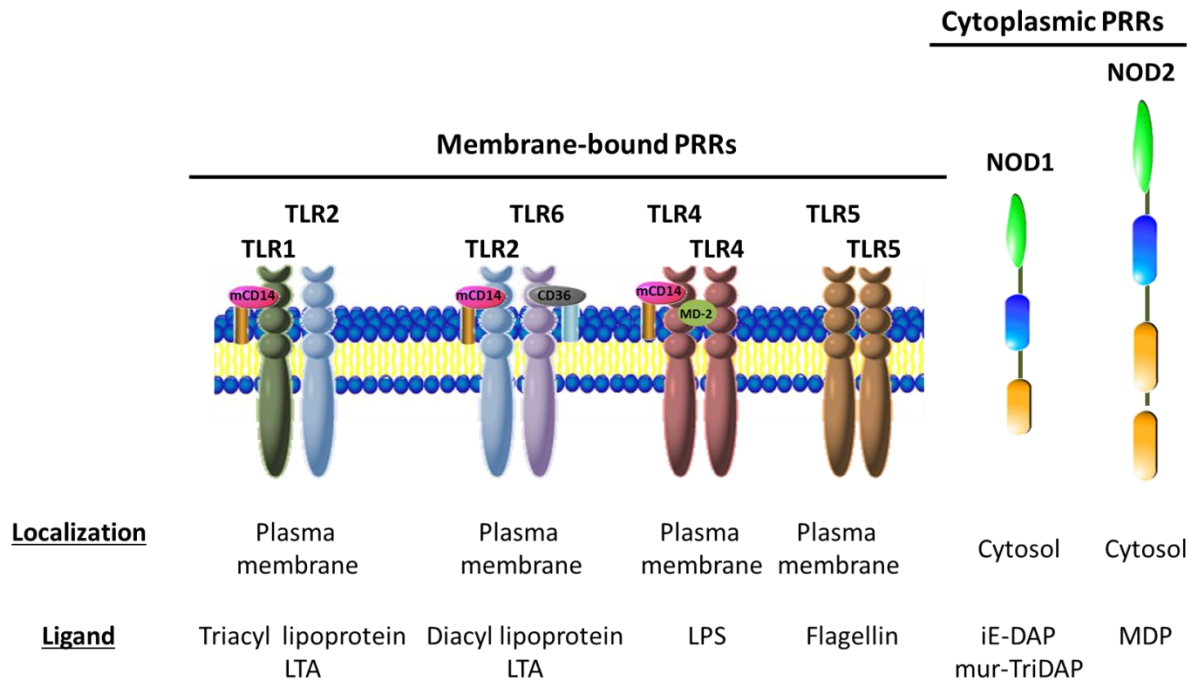

Figure 2: Pathogen Recognition Receptors (PRR) of interest for bacterial induced mastitis in dairy cows. Toll-like receptors (TLR) recognize extracellular MAMPs while Nod-Like Receptors (NLR) recognize intracellular MAMPs. Agonists of TLRs are LipoTeichoic Acid (LTA), Lipopolysaccharide (LPS) and Flagellin. Agonists of NLRs are PeptidoGlycaN constituents gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP), N-acetylmuramyl-L-alanyl-g-D-glutamyl-meso-diaminopimelic acid (mur-TriDAP) and MuramylDiPeptide (MDP). Made with software from www.proteinlounge.com.

In the context of this PhD thesis, the focus lies on the TLRs as main PRR during the host innate immune response. However this host defense process is so crucial that important “back-up” PRRs exist with overall signaling pathways that appear partially redundant. Furthermore, MAMPs are not only detected extracellularly. There are also intracellular PRRs such as Absent In Melanoma 2 (AIM2) and NOD-like receptors (NLR) (Takeuchi and Akira 2010). For instance, microbial DNA can be detected by the extracellular part of the TLR9 but the intracellular receptor HIN200 can also bind this MAMP. Reports on this other group of PRRs are scarce in the context of mastitis, but nevertheless have already been stated as important (Bercot, Kannengiesser et al. 2009). More specifically, Muramyl DiPeptide (MDP) and LTA are both essential constituents of the cell wall of *S. aureus.* When these MAMPs are injected in bovine udders, they synergistically and selectively increase several local

chemoattractants and recruit neutrophils. A comparable neutrophil influx ensues when LTA triggers TLR2 signaling or when MDP triggers NOD2, but the cooperation of both receptors raises a synergetic response (Bougarn, Cunha et al. 2010). Based on other infection models, these intracellular PRRs are not just characterized as back-up PRRs but seem important to sense intracellular bacteria and toxins. This property is suggested to discriminate between commensal and invasive pathogens (Franchi, Kamada et al. 2012). Next to their function to mediate the antimicrobial defense, the presence of these PRRs and downstream effectors have recently also been implicated in the homeostasis of gut microbiota. NLR-deficient mice may develop a dysregulation of the gut flora (dysbiosis) and make them more susceptible to persistent pathogenic infections (Gagliani, Palm et al. 2014).

II.2. NF-kappaB, a Transcription Factor for Pro- and Anti-Inflammatory Signaling

Interaction between MAMPs and PRRs of either professional or non-professional innate immune cells initiates signaling pathways. These eventually lead to the production of cytokines and other endogenous mediators, which are essential in the host protection against pathogens. The nuclear *de novo* production of cytokines occurs through Transcription Factors (TFs) activated downstream of PRRs (Fig. 3).

Most of the inflammatory proteins involved in neutrophil influx and activation have been shown to contain kappaB sites within their promoter (Pahl 1999). The latter are necessary for the Nuclear Factor-kappaB (NF-kappaB) TF to bind, activate and transcribe the accommodating gene. NF-kappaB is the best described TF in mastitis, mammary gland development, the lactation cycle and even mammary tumorigenesis (Safieh-Garabedian, Mouneimne et al. 2004; Notebaert, Duchateau et al. 2005; Lerebours, Vacher et al. 2008; Notebaert, Carlsen et al. 2008; Connelly, Barham et al. 2010; Demeyere, Remijsen et al. 2013; Matusik, Jin et al. 2014). As a plethora of genes can be induced by a handful of TFs such as NF-kappaB, strict regulatory systems are hence required.

Interference of the TF to bind its promoter can be effectuated on many levels. First, the TF can be prevented to translocate to the nucleus by influencing several upstream mediators in a post-translational way. Indeed, NF-kappaB is a dimer comprising 5 TFs that have to coordinate an adequate inflammatory response i.e. RelA/p65, RelB, c-Rel, p50, and p52. The variable composition of the NF-kappaB dimer induces a differential gene expression. Furthermore, two subunits (i.e. p50 and p52) are expressed as p105 and p100 protein precursors, respectively, that contain ankyrin repeats which need to be post-translationally cleaved and unlike the former 3 TFs (RelA/p65, RelB, c-Rel) have no transcriptional activation domain. All NF-kappaB subunits contain a highly conserved Rel Homology Domain (RHD) necessary to bind DNA, other subunits and the Inhibitor of NF-kappaB (IkappaB). Through the RHD binding of these IkappaB subunits, NF-kappaB can be kept inactive in the cytoplasm. Only when a stimulus such as a complexed TLR triggers the canonical pathway the IkappaB Kinase (IKK) complex can phosphorylate IkappaBalpha. This phosphorylation induces ubiqitination and subsequent degradation of the IkappaB in turn releasing NFkappaB for translocation to the nucleus (Bradford and Baldwin 2014).

Secondly, a TF can be hindered to bind its promoter. NF-kappaB activity can be regulated at the promotor-site either by the local presence of modulating TFs or by the chromatin density. NF-kappaB co-operates with other factors to fine-tune the expression of genes such as IL-8 and a beta-defensin upon TLR triggering of bovine mammary epithelial cells (Liu, Shi et al. 2011). IL-8 is very fastly and transiently expressed while beta-defensin is slowly upregulated. The kinetics of this enhancement occur simultaneously with a fast DNA binding activity of the CAAT Box Enhancer Binding Proteins (C/EBP) TF concomitant with a slower and more steady NF-kappaB activity. Remarkably, both IL-8 and the beta-defensin have a composite promotor (Liu, Shi et al. 2011). On the other hand, the beta-defensin /EBPbeta a.k.a. NF-IL-6, a member of the TF C/EBP, interacts with the NF-kappaB subunit RelA/p65 (Zwergal, Quirling et al. 2006) and is induced by RelA/p65 but inhibited by C/EBPbeta. IL-8 is inhibited with increasing NF-kappaB activity while increased with C/EBPbeta activity. Furthermore, chromatin accessibility is also important for NF-kappaB transcription (Liu, Shi et al. 2011).

Other inflammatory signaling of key importance to mastitis is the induction by PRRs of two additional TFs i.e.Activator Protein-1 (AP-1) and Interferon Regulatory Factor (IRF). These are also known to induce cytokine and chemokine production with both inflammatory and microbicidal properties. Although MyD88-dependent NF-kappaB signaling is generally accepted, MAPK signaling (AP-1), cAMP response element-binding protein (CREB) and IRF3 are only occasionally suggested for *E. coli* and *S. aureus* mastitis (Kim, Im et al. 2011; Wang, Song et al. 2014). Clearly, some signaling induced by these different TFs is redundant, but marked differences also exist. NF-kappaB is proven to be important for internalization of *S. aureus* bacteria in epithelial cells (Wei, Fu et al. 2014). In contrast, although activated, inhibition of MAPK kinases does not change the internalization of the latter (Oviedo-Boyso, Barriga-Rivera et al. 2008).
At last, different TFs and resulting cytokines can also be indirectly activated by PRR. For example, the InterLeukin (IL)-receptor can bind MyD88 and thus activate both NF-kappaB and MAPK pathways to induce antimicrobial defensins (Froy 2005). This latter strategy is suggested for cells that require activation by a secondary signal different from but related to either the initial infection, or redundant systems or additional stimuli next to the MAMPs. This can be demonstrated by supplementing IL-1beta or TNF-alpha to a *S. aureus* inoculum resulting in a synergetic internalization of the bacterium in bovine cells by additional NF-kappaB activation of the cytokines compared to that of the MAMPs alone (Oviedo-Boyso, Barriga-Rivera et al. 2008).

II.3. Caspases, Mediators of Inflammation and Cell Death

Inflammatory mediators, such as cytokines and chemokines sometimes need additional regulation to perform their activity. A well-known example is the pro-inflammatory cytokine IL-1beta (Fig. 3). Following bacterial infection, PRRs in bovine epithelial cells get triggered, internalized and express the proIL-1beta gene through activation of multiple TFs such as NF-kappa, AP-1 and CRE (Kim, Im et al. 2011). Despite its fast transcription, IL-1beta is not always detected in milk post-exposure to a mastitis pathogen (Bougarn, Cunha et al. 2010).

Transcription of the IL-1beta gene results in an inactive 31kDa proform that needs processing into an active form detectable by the IL-1Receptor (IL-1R). Following infection with mastitis-associated *E. coli* or *S. aureus* isolates, transcripts of IL-1beta are enhanced in mammary epithelial cells and protein levels are detected in milk of infected cows. Remarkably, the kinetics of this increase in milk differ between *E. coli* and *S. aureus* (Bannerman, Paape et al. 2004; Lahouassa, Moussay et al. 2007). Based on other models, IL-1beta is hypothesized to be a product of the inflammasome (Thacker, Balin et al. 2012). Inflammasomes are intracellular multiproteins that mediate activation of inflammatory cysteine-aspartic proteases (caspases) -1. This latter protein is a zymogen (procaspase) that contains a large prodomain to recruit caspase-1 into an activating complexes such as the inflammasome. Following this recruitment the inactivating prodomain is released through autocleavage. Different inflammasomes exist depending on the cytosolic Damage-Associated Molecular Pattern molecules (DAMPs) and MAMPs they detect and their name reflects the receptor bound. Furthermore, these receptors can either directly or indirectly bind procaspases-1 the latter through an adaptor protein such as Apoptosis-associated SpeCk-like protein (ASC) (Lamkanfi and Dixit 2012).This canonical pathway activates caspase-1 and IL-1beta as well as IL-18 but not IL-1 alpha, nevertheless this inflammasome activity is important for the secretion of all these proteins. A redundant system is caspase-11 which can also induce the release of IL-1beta/IL-18 through non-canonical inflammasomes (Vanden Berghe, Demon et al. 2014). Indeed, functional caspase-11 activates caspase-1 in the absence of NLR NLrp3 or ASC, thus also activating IL-18. Moreover, the latter pathway has unique features as IL-1alpha release *in vivo* is fully dependent on caspase-11 activity (Vigano and Mortellaro 2013).

Both caspase 1 and -11 do not only influence the production and secretion of cytokines, they also induce an unique cell death program called pyroptosis which is already suggested in infected bovine neutrophils (Demeyere, Remijsen et al. 2013). Clearly, PRR signaling is not merely related to inflammation. Indeed, it has been shown in mice that TLR4 triggering reduces the milk secreting epithelium and inhibits the nuclear localization of phosphorylated STAT5 which is important for the production of milk proteins (Glynn, Hutchinson et al. 2014). Furthermore, this shrinking of the mammary epithelial cell number is associated with shedding of the alveolar epithelial cells into the lumen. This process happens cleanly without disrupting the blood milk barrier or distorting the tight junctions (Kobayashi, Oyama et al. 2013). The increase in mammary epithelial cell death seen during *E. coli* and *S. aureus* mastitis is believed to be an enhanced involution that is accompanied by high levels of pro-apoptotic caspases such as caspase-3 (Wesson, Deringer et al. 2000; Long, Capuco et al. 2001). Similar to inflammatory caspases, these pro-apoptotic caspases are assembled in a multiprotein complex (apoptosome) with adaptor proteins (apaf together with cyt c and ATP) inducing (auto)cleavage of the involving procaspases (Bao and Shi 2007).

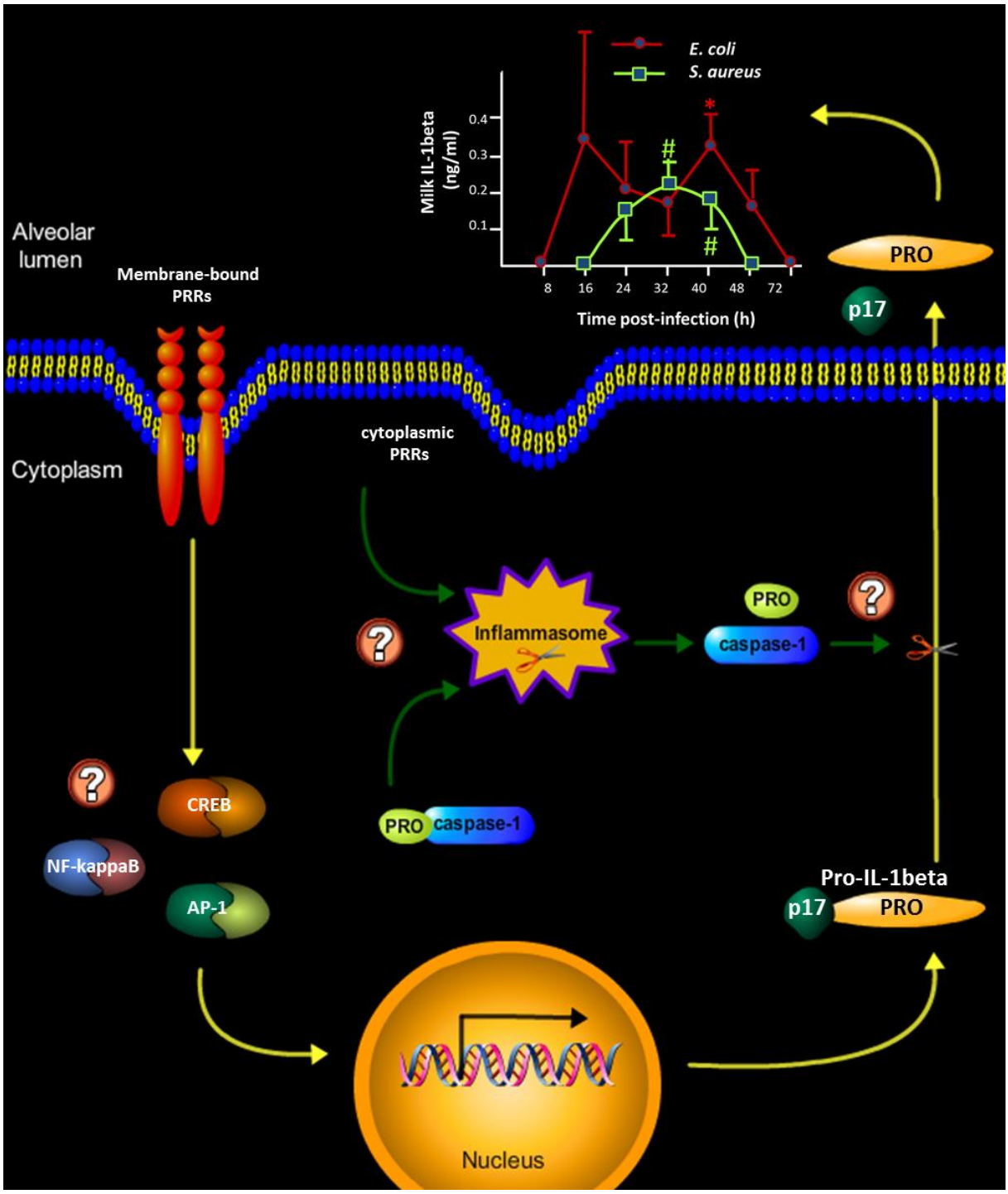

Figure 3: Hypothetic scheme of pathogen dependent processing of proIL-1beta in resident mammary cells (e.g. epithelia, macrophages, …) . Membrane bound Pathogen Recognition Receptors (PRRs) get triggered and induce pro-IL-1beta production through several transcription factors (NF-kappaB, CREB or AP-1). Intracellular PRRs also get triggered and cleave pro-caspase-1 in the inflammasome. As a resulting caspase-1 may cleave pro-IL-1beta into IL-1beta. *E. coli* and *S. aureus* are believed to trigger different PRRs and to induce different transcription factors that result in different IL-1beta levels in the milk. Made with software from www.proteinlounge.com.

III. MOUSE MODELS CONTRIBUTE TO NOVEL INSIGHT IN MASTITIS

To study the molecular mechanism of bovine mastitis and improve therapies, several experimental infection models have been developed. *In vitro* models typically characterize mastitis-associated bacterial isolates (i.e. their MIC, biofilm production, antibiotic resistance genes; Section I) and/or their interaction with host cells. Inflammatory signaling data obtained from such model studies using either mammary epithelial cell lines or isolated udder/blood cells exposed to mastitis-associated bacteria or MAMPs are of great value for elucidating the pathogenesis of bovine mastitis. Although these models are of high interest to the field, in the context of this PhD thesis the focus will be mainly on the *in vivo* mouse model while *in vitro* models are only briefly mentioned.

III.1. Mastitis Models

Dairy cows have been genetically selected for their unique linked or pleiotropic traits to achieve high production efficiency. However, the heritability of these features brings along a negative energy balance as body reserves are in a major part being used for milk anabolism. Moreover they affect other highly energy-dependent anabolism, more specifically also the animals' immunity (Rauw, Kanis et al. 1998). Generally, it is well recognized that a high milk yield and suboptimal management predisposes dairy cows to mastitis by impairing their defense system (Sordillo and Streicher 2002). The unique milk composition, the high milk production and the immune system of the cow (Werling, Jungi 2003) and the possible bacterial adaptation to the milk environment of the udder (see Section I.2) imply that confirmatory mastitis studies should always be performed within the target-animal species. Nevertheless, such experiments are severely limited with respect to research tools and infrastructure, and are also very expensive and labour-intensive. As a result, more specific culture models and *in vivo* mouse models have been developed to tackle these major drawbacks of *in vivo* bovine studies. These *in vitro* models culture isolated primary udder cells but are still hampered by both the limited number of cell types that can be studied to interact within a single set-up and their lack of dynamism. Indeed, the constant change of cells that influence one another during an inflammation process in the udder cannot be addressed by any *in vitro* approach. Therefore, preclinical *in vivo* models which mimic ruminant udder infections with mastitis-associated bacteria isolated from field cases are increasingly used. Unlike *in vitro* models the latter do include a dynamic multicellular system yet in another genetic background. As mice are homologous to other mammals, the murine mammary gland inflammation data can at least partly be extrapolated to cows as well as to breastfeeding women. Nevertheless, some aspects of both the overall physiology and the specific traits of udders or breasts differ from those of murine mammary glands. Therefore, care should be taken when attempting to extrapolate results from the mouse to other mammals. Several examples illustrate that the pathogenicity of some inflammatory agents and the acquired host response can be species-dependent (Andersen-Nissen, Smith et al. 2007; Werling, Jann et al. 2009). Although differences may be present, this will be less the case for the innate immunity aspects of the mammary gland biology, which are quite conserved between mammals. Another argument in favour of the mouse mastitis

model is that the use of laboratory Specific-Pathogen-Free (SPF) mice facilitates mastitis experiments and allows to downsizing of the number of animals required to achieve a statistically conclusive result. This is mainly due to the fact that these strains have the same genotype and are easily housed and/or managed. Last but not least, mouse models are extensively investigated for a whole array of predominantly human diseases. The use of mice opens up a wide panel of sampling, immunological reagents and research tools that are either very difficult or even impossible to perform in cows.

III.2. Experimental Bacterial Infection of the Murine Mammary Gland

To more conveniently address the molecular biology of mastitis focusing on key innate signaling pathways in the mammary gland, experimental mouse models were first developed and characterized already in the 1970s (Chandler 1969; Chandler 1970; Chandler 1970; Chandler 1971). Cow udders and mice mammary glands both manifest glands in the inguinal region. In mice, the fourth inguinal gland pair is the biggest and therefore preferred one for suckling by the pups. The litter is nursed by the mother for about 10 to 14 days. During this lactation period the mammary gland is fully functional and can expand to about seven-fold its original volume. Consequently, the teat canal ducts become easily accessible for experimental infection (Notebaert and Meyer 2006).

III.2.1. Inoculation Methods of Mastitis-Associated Bacteria in the Murine Mammary Gland

There are several methods to mimic bovine mastitis in lactating mice with bovine mastitis isolates. The introduction method - as well as the choice of mastitis-associated pathogen - greatly influences the elicited inflammation process in these models.

The first method consists of simply dropping a pathogen-dense inoculum on top of the nipple, enabling bacteria to descend through the duct orifice into the mammary gland. With this non-invasive procedure, that probably best reflects the natural infection route, the number of bacteria infecting the gland is however not controllable. Inter-animal differences are consequently very high. Therefore, other techniques were developed that focused on introducing the concentrated bacterial load directly into the teat orifice. For this purpose researchers tried to optimize the pipetting of the bacterial suspension into the ducts through the use of smaller tips, illumination fibers, microscopes and micro-manipulators.

This second method was successfully achieved when small volumes were reproducibly introduced with the help of pediatric blunted needles (> 30 Gauge). The point of entry can optionally be enlarged by an additional minimal cut in the nipple top. The needle is then positioned at an angle of about 180 degrees and can thus enter the primary duct fluently when performed by an experienced researcher (Fig. 4 A). The released volume spreads through the accessible lactating lobules as visualized by Chinese ink administration or even more elegantly with *in vivo* imaging of luminescent bacteria (Fig. 4 B-C) and subsequently interacts with the hosts' tissue. The latter intraductal (or intramammary) injection technique is superior to the first strategy as the infection rate is about 100% when the mammary glands are fully lactating and thus have become easily accessible. When the inoculum is administered correctly, the Colony-Forming Units (CFU) can be as low as 10 CFU/injection and still

induce mastitis. Nevertheless, higher inocula have the advantage of lower inter-animal variation. It should be remarked that the induction implicates a low-grade induction of inflammation. Therefore, sham-inoculated mammary glands should always be included in these experiments (Barham, Sherrill et al. 2012).

The intraductal injection model has been successfully used with different mastitis-associated bacteria including *E. coli, S. aureus* and *S. uberis*, but still needs to be validated for others such as bovine CNS (Sordillo and Nickerson 1986; Tuchscherr, Buzzola et al. 2005; Gonen, Vallon-Eberhard et al. 2007). Furthermore, mammary gland inflammation can alternatively be induced by intramammary injection of multiple exogenous factors in mice. These intramammary introduced compounds vary from antibiotics, host factors (such as cytokines) to MAMPs (e.g. toxins and wall fragments) which all aided in unraveling the molecular pathways involved in the complex disease mechanism of mastitis (Brouillette, Grondin et al. 2004; Cao, Fu et al. 2014; Elazar, Gonen et al. 2010). A major added value is that these findings can be confirmed or elaborated by the use of genetically modified mice.

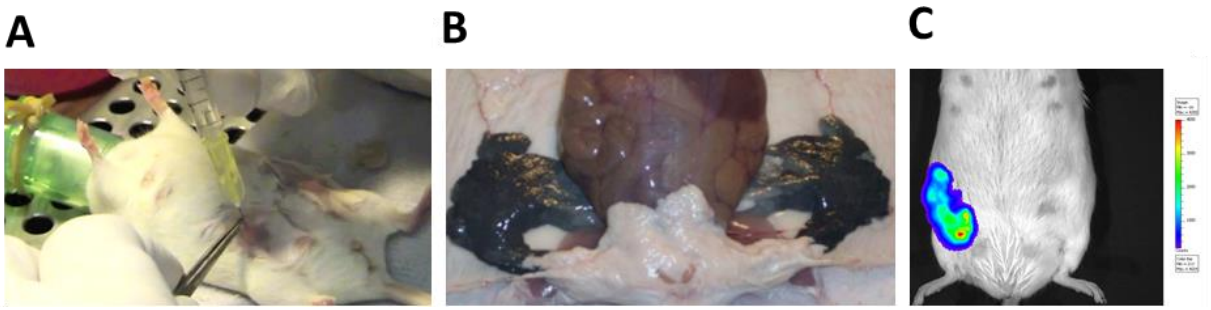

Figure 4: Experimental induction of mastitis pathogens in the murine mammary gland. Mastitis isolates or bacterial toxins can be intraductally introduced in anesthetized lactating mice by means of a blunted pediatric needle to induce an inflammatory reaction in the murine mammary gland simulating bovine mastitis (A). The inoculum injected in the mammary gland remains local, as visualized with Chinese ink in both glands of the fourth pair (B). Bioluminescent *E. coli* bacteria injected in the right mammary gland of the fourth pair can be tracked through *in vivo* imaging and remain local in a time-lapse of 24h (C).

III.2.2. Applications of Murine Models to Study the Molecular Biology of Mastitis

III.2.2.1. Mouse Mastitis Models Corroborate *in vitro* MIC Data

Non-transgenic or line bred wild Type (wT) mice are used to study the pathogenesis of bovine mastitis. This more simple approach has been most valuable either to improve current treatments or to test novel molecular targets for innovative treatment strategies. As described in section I., antimicrobial treatment is frequently used as therapeutic strategy for mastitis in dairy cows. Antimicrobial testing of putative active substances is primarily screened *in vitro* by Minimum Inhibitory Concentration (MIC) determination. This general microbiological technique has been applied in the mastitis field by exposing mastitis-associated isolates to a library of compounds. As such, the MIC defines the lowest concentration of an antimicrobial substance that kills or inhibits any visible growth of a selected bovine mastitis-associated isolate. These dilution-based (culture) methods reveal the susceptibility of mastitis-

associated pathogens to candidate antibiotic molecules, which is an important first step that will aid to select an optimal therapy.

Over the past decades this curative strategy along with other measures such as proper management has succeeded to significantly decrease the prevalence and incidence of udder infections and has also positively affected animal health and milk productivity (Piepers, De Meulemeester et al. 2007). Nevertheless, in recent years, both pathogen adaptation to the udder and the observation that the current antibiotics are selecting for minor pathogens (e.g. CNS) has resulted in an urgent need to improve the treatment of mastitis. Although MIC research certainly provides valuable data, the Effective Dose (ED) of an antibiotic substance can differ from the MIC value and influences the clinical outcome. The optimal drug concentration *in vitro* differs from that *in vivo* due to several factors including the dosing regimen, the host-pathogen inflammatory interaction and species-differences. Therefore, mouse mastitis models are valuable as an intermediate step because they can extend the MIC data of potential antibiotic treatments before going into clinical trials with experimentally-infected cows (Brouillette, Grondin et al. 2004; Brouillette, Grondin et al. 2005). These preclinical models provide a platform that mimics the pathogen properties such as the exponential growth of bacteria in a very dense mammary gland compartment and the expression of bacterial toxins, in addition to host-related factors such as multicellular interactions, infiltration of (innate) inflammatory cells and milk antibacterial factors. In these *in vivo* set-ups the selected live pathogens are inoculated through the teat canal in the mammary gland and another intramammary injection – now with an antibiotic agent - is subsequently performed. The use of mice has the advantage that different conditions can be compared on a statistically relevant scale in one experiment. The efficacy of one compound can be validated by a dose-response control. The lower the concentration of the candidate antibiotic agent, the better it performs compared to a reference test compound taken along in the set-up (positive control).

III.2.2.2. Mouse Mastitis Models Corroborate the Importance of MAMP Detection and Induced Inflammatory Signaling

The mouse mastitis model demonstrated that an increased MAMP detection can improve bacterial clearance (see Section II). Locally administered sCD14 sensitizes the mammary epithelium for an upcoming bacterial hazard and enhanced alertness for future infections (Wang, Zarlenga et al. 2002). Upon a intramammary *E. coli* infection, the host scavenger receptor pretreatment lowered local TNF-α levels and lowered mammary bacterial growth compared to the non-sCD14 pretreated controls (Lee, Paape et al. 2003). Of relevance, this marked preclinical effect was extrapolated to dairy cows where an udder injection of *E. coli* with sCD14 elicited lower TNF-α and IL-8 levels concomitant with a decline of the bacterial growth rate due to a faster neutrophil influx (Lee, Paape et al. 2003). In analogy with this innovative sCD14 treatment, other novel therapies focusing on the host immunity rather than on antibacterial substances are now rapidly emerging and first evaluated with the preclinical mastitis models.

An innovative application to boost the host immune system with MAMPs was achieved by engineering a fusion protein attaching the streptococcal endolysin endopeptidase domain to the staphylococcal cell wall binding domain from the staphylococcal phage K endolysin. This newly bioengineered molecule was injected in the mammary gland mice post-infection with *S. aureus* mastitis. It was able to significantly reduce the mammary CFU load and this effect was enhanced when co-injected with bacterial lysostaphin (Schmelcher, Powell et al. 2012). Cyclic diguanylate (c-di-GMP) is also a bacterial molecule, but which is used as a preventive therapy instead of a curative one. Intramammary injection of c-di-GMP 12h and 6h prior to *S. aureus* reduced the CFU post-infection (Brouillette, Hyodo et al. 2005). Furthermore, a preventive treatment to induce easier recognition of an intramammary infection with *S. aureus* by the host has been attempted with other *S. aureus* MAMPs such as fibrinogen and collagen binding proteins. This experiment resulted in a reduction of bacterial growth post-infection with fibrinogen binding proteins but not with the collagen binding proteins (Mamo, Boden et al. 1994; Mamo, Jonsson et al. 1994; Hu, Gong et al. 2010).

Instead of injecting just the MAMPs in mice, live bacteria can also be altered to identify MAMPs essential for host recognition by PRRs. Mastitis-associated *S. aureus* isolates have been pre-incubated with sera from previously vaccinated mice and subsequently injected in the mammary gland of mice. At first, mice were injected with the DNA plasmid expressing the virulence/Clumping factor A (ClfA) that led to a raise of specific antibody in blood. These antibodies opsonized the circulating pathogens released from blood and aided the mouse to clear the bacteria from the mammary gland post-infection (Brouillette, Lacasse et al. 2002) When bacterial ClfA Knock-Out (KO) mutants were tested in the mouse model, only a delayed infection was seen, implicating that ClfA is not such a crucial MAMP of *S. aureus* after all (Brouillette, Talbot et al. 2003). Moreover, Sortase A (SrtA), mutants of *S. aureus* were made and this affected the attachment of *S. aureus* to host cells, thus attenuating the activation of the NF-kappaB and MAPK signaling pathways (Chen, Liu et al. 2014).

Next to improved host detection of mastitis inducing bacteria, preventive therapies interfering with downstream signaling were also attempted with line bred mice by an increasing number of research groups (Chen, Mo et al. 2013; Li, Liang et al. 2013; Boulanger, Brouillette et al. 2007). One of these reported an anti-inflammatory compound such as helenalin, acting as an NF-kappaB inhibitor which upon intraperitoneal (i.p.) injection reduces mammary *S. aureus* proliferation and leukocyte infiltration in the mammary glands *S. aureus* inoculation (Boulanger, Brouillette et al. 2007). Other NF-kappaB inhibitors that are believed to signal through either Myd88-dependent (Liver X receptor -alpha agonists Leonurine, Kaempferol, Alpinetin, Curcumin, Astragalin) or -independent signaling (Glycyrrhizin) were shown to reduce the symptoms of LPS-mastitis (Chen, Mo et al. 2013; Li, Liang et al. 2013; Cao, Fu et al. 2014; Fu, Gao et al. 2014; Fu, Tian et al. 2014; Fu, Zhou et al. 2014; Song, Wang et al. 2014). Similarly, an i.p. injection of Salidroside, Emodin, Magnolol or oxymatrin were suggested to reduce next to NF-kappaB also MAPK signaling during LPS-induced mammary inflammation (Li, Fu et al. 2013; Li, Zhang et al. 2013; Wei, Dejie et al. 2014; Yang, Yin et al. 2014). Another molecule cyanidin-3-O-beta-glucoside is even believed to inhibit IRF3 activation post-inducing LPS stimulation (Fu, Wei

et al. 2014). Furthermore, Selenium deficiency in the food of the mice has been proven to exacerbate LPS-mastitis (Wei, Yao et al. 2014).

III.2.2.3. Transgenic Mouse Models Identify Key Host Factors in the Pathogenesis of Bovine Mastitis

The ultimate goal of mastitis research combines economical with animal health issues. In essence, a dairy cow that requires a low amount of dry matter intake to produce a high amount of good quality milk and is protected against pathogenic udder infections. Historical breeding programs that rely on population genetics have led to distinct beef and dairy cattle, where the latter became specialized in high milk yield which compromised their immunity. In contrast, current breeding programs are selecting for cows that are more resistant to mastitis. Unfortunately, these latter programs are limited by the genes or gene combinations that already reside in the cattle pool (Rainard 2005). Therefore, implementation of other genes into cows may provide protection against mastitis. One report described the production of lysostaphin in the milk of transgenic cow. Lysostaphin is a staphylolytic enzyme secreted by *Staphylococcus simulans.* Due to the expression of the antibacterial resistance gene in the udder, *S. aureus* was hindered to infect, colonize or grow in the milk of these cows (Wall, Powell et al. 2005). Notably, the development of the transgenic cow was based on previous findings in mice (Kerr, Plaut et al. 2001).

Despite the fact that this approach is very valuable, it still remains controversial with regard to public acceptance. Consequently, genetically modified cows have not yet been created to study specific inflammatory pathways during mastitis. Rather, these innovative approaches have lingered on the production of complex human biomolecules such as lactoferrin, α-lactalbumin and lysozyme (van Berkel, Welling et al. 2002; Wang, Yang et al. 2008; Yang, Wang et al. 2011; Wu, Ouyang et al. 2012). In marked contrast, transgenic mouse models have been employed for decades to study a vast number of pathologies. The contribution of a selected gene can be verified either through the lack, or the adaptable expression, or the overexpression, or the presence of an additional gene in that host. Mastitis research has greatly benefited from the use of different genetically mouse models as summarized in Table 2.

In section II, it was described how bacteria are sensed by host receptors enabling production and/or activation of cytokines to guide an elaborate local innate immune response. As such, TLR KO mice were tested following intramammary mastitis-associated pathogen exposure (De Schepper, De Ketelaere et al. 2008). The use of TLR4 KO mice demonstrated that *E. coli* triggers more than one PRR, while LPS inflicted a neutrophil influx by mere needs of TLR4-expression on the residing macrophages (Gonen, Vallon-Eberhard et al. 2007). The latter group elegantly demonstrated this by intraductal injection of either a bovine mastitis *E. coli* isolate or LPS in TLR4 C57BL/6 KO mice with or without macrophage transfer from wT C57BL/6 mice. Partly corroborating this pioneer study, a recent paper suggested that LPS signaling is not fully TLR4-dependent albeit using another mouse strain (Glynn, Hutchinson et al. 2014). Furthermore other PRR such as TLR2 that could be of essence for recognizing *E. coli* were also verified by the latter group. TLR2 KO similarly abrogated Pam_3CSK_4-

induced inflammation of the mammary gland. The latter molecule is a synthetic analogue of the triacylated lipoproteins present on the cell membrane of Gram-negative bacteria as MAMPs (Fig. 2). Interestingly, the KO of TLR2 could not prevent *E. coli*-induced mastitis. In marked contrast to the TLR4 signaling pathway, the TLR2 signaling pathway was found to be macrophage-independent but could nevertheless also restrict bacteria from invading the mammary tissue (Mintz, Mintz et al. 2013).

Overall, stimulation of several TLRs clearly results in a proper influx of neutrophils in the infected udder during bovine mastitis. The neutrophil diapedesis process has been suggested to be the result the induction of different cytokines and chemokines such as TNF-α (primordially resulting from a macrophage-dependent response), IL-1 (primordially resulting from a neutrophil- dependent response), CXCL8/IL-8 (with 3 chemokines i.e. CXCL1/KC, CXCL5-6/LIX and CXCL2/MIP-2 being the orthologues in mice (Hol, Wilhelmsen et al. 2010), and inducible nitric oxide synthase (iNOS). Noteworthy, little is known about their individual contribution to the overall inflammatory network. Therefore, each contribution has been investigated with proper TNF-α KO, IL-1R KO, IL-8R KO or iNOS KO mice, respectively (Elazar, Gonen et al. 2010). With these KO animals it was shown that cytokine signaling can influence the influx of neutrophils during LPS- or *E. coli*- induced mastitis and that different cell types (macrophages, mammary epithelia and neutrophils) are involved in the production or response of these cytokines. However, a complete map of the complex interplay between the different cytokines and their receptors is still lacking.

The corresponding genes of these key cytokines and chemokines are regulated by TFs such as NF-kappaB. Already one decade ago, NF-kappaB has been shown by other Belgian researchers as well as by our research group to be activated in milk cells derived from mastitic cows (Boulanger, Bureau et al. 2003), respectively to inhibit cell death in isolated bovine blood neutrophils (Notebaert, Duchateau et al. 2005). By means of transgenic mice as an elegant tool, NF-kappaB activity was also at first monitored *in vivo* by our group. When live *E. coli* were injected, a transient activation signal was observed in these transgenic mice (Notebaert, Carlsen et al. 2008). In a later study, LPS was shown to induce cell death pathways and milk loss after local inoculation. The inflamed mammary glands were again concomitant with – albeit after a faster onset – transient NF-kappaB activity (Connelly, Barham et al. 2010).

Next to transgenic mice that have an altered the innate immune response (PRR, TF and cytokines), mice have also been used to study antibacterial genes and their effect against bacterial mastitis. As previously mentioned in this Section, lysostaphin Knock-In (KI) mice had an improved clearance of a mammary *S. aureus* infection compared to non-transgenic mice and the concept could be extrapolated to cow. Another immuno-modulating host factor that holds promise is L-amino Acid Oxidase (LAO). LAO is an antibacterial enzyme present in mouse milk during the first 10 days of lactation. Its antibacterial function is considered to occur through the production of H_2O_2 by oxidative processing of milk L-amino acids. One research group generated LAO KO mice and introduced *S. aureus* mastitis pathogens in the mammary gland of these genetically modified animal strain. It resulted in a far higher susceptibility compared to the non-transgenic mice of the same animal strain (Nagaoka, Aoki et al. 2009).

Table 2: Overview of the types of transgenic mice that have been employed to study the molecular biology of bovine mastitis.

Genetically modified mouse model	Mouse strain	Inoculum	Dose		Duration	Phenotype in mammary gland compared to non-transgenic mice	Reference
TLR4 KO	C3H/HeJ	*E. coli*	300	CFU	24h / 48h	*similar neutrophil recruitment* *megalocytosis* *formation of intracelullar bacterial communities in mammary epithelia*	(Gonen, Vallon-Eberhard et al. 2007)
	C3H/HeJ	LPS	10	µg	24h	*less neutrophil recruitment*	
	C3H/HeJ	*E. coli*	100-1000	CFU	24h	*similar TNF-alpha, IL-1beta, CXCL1/KC, CXCL2/MIP-2 levels*	(Elazar, Gonen et al. 2010)
	C3H/HeJ	LPS	10	µg	24h	*lower TNF-alpha, IL-1beta, CXCL1/KC, CXCL2/MIP-2 levels*	(Elazar, Gonen et al. 2010)
	BALB/c	LPS	10	µg	7 days	*more neutrophil and macrophage recruitment* *lower TNF-alpha, IL-1beta, CXCL1/KC, IL-6, IL-10, IL-10, CCL2/MCP-1 levels in blood* *higher nuclear pSTAT5* *higher milkproduction*	(Glynn, Hutchinson et al. 2014)
TLR2 KO	C57BL/6	*E. coli*	1000	CFU	24h	*formation of intracelullar bacterial communities in mammary epithelia*	(Mintz, Mintz et al. 2013)
	C57BL/6	LPS	10	µg	24h	*no comparison with wT strain*	
	C57BL/6	Pam3CSK4	10	µg	24h	*less neutrophil recruitment*	
IL-1R KO	C57BL/6	*E. coli*	100-1000	CFU	24h/48h	*more neutrophil recruitment* *neutrophils are trapped in the alvolear space and alveolar lumen* *formation of intracelullar bacterial communities in mammary epithelia at 48h*	(Elazar, Gonen et al. 2010)
	C57BL/6	LPS	10	µg	24h	*more neutrophil recruitment* *neutrophils are trapped in the alveolar space and blood vessels*	(Elazar, Gonen et al. 2010)
CXCR2 KO	BALB/c	LPS	10	µg	24h	*less neutrophil recruitment* *recruited neutrophils trapped on the basolateral side of the alveolar epithelium*	(Elazar, Gonen et al. 2010)
iNOS KO	C57BL/6	*E. coli*	100-1000	CFU	24h/48h	*formation of intracelullar bacterial communities in mammary epithelia at 48h*	(Elazar, Gonen et al. 2010)
	C57BL/6	LPS	10	µg	24h	*more neutrophil recruitment*	(Elazar, Gonen et al. 2010)
TNF-α KO	C57BL/6	LPS	10	µg	24h	*lower neutrophil recruitment*	(Elazar, Gonen et al. 2010)
Caspase-1/-11 double KO	C57BL/6	*E. coli*	20000	CFU	24h	*caspase-1/-11 independent cleavage of IL-1beta*	(Notebaert, Demon et al. 2008)
NF-kappaB luciferase KI	C57BL/6	*E. coli*	10000	CFU	24h	*NF-kB activity in the mammary gland raised between 4h and 10h post-infection* *lowNF-kB activity in the liver*	(Notebaert, Carlsen et al. 2008)
	C57BL/6	LPS	5	µg	24h	*strong transient NF-kB activity in the liver between 4h and 10h post-infection*	
	C57BL/6	LPS	5	µg	6h	*NF-kB activity in the mammary gland was visualized*	(Connelly, Barham et al. 2010); (Barham, Sherrill et al. 2012)
CX3CR1 GFP/+	BALB/c	LPS	10	µg	24h / 48h	*detectable dendrocytes stay belowthe basal membrane* *no upregulation of markers CD80,CD86, CD103 and CD44*	(Gonen, Vallon-Eberhard et al. 2007)
Gln(125,232)-lysostaphin KI	not mentioned	*S. aureus*	10000	CFU	24h	*lowneutrophil recruitment* *reduction of mammary CFU* *Gln125,232-lysostaphin expression in milk*	(Kerr, Plaut et al. 2001)
LAO KO	C57BL/6	*S. aureus*	10-20	CFU	24h	*higher mammary CFU* *less survival* *higher IL-1beta*	(Nagaoka, Aoki et al. 2009)

S. aureus = *Staphylococcus aureus*, *E. coli* = *Escherichia coli*, LPS = lipopolysaccharide, Pam3CSK4 = synthetic bacterial lipopeptide, a Toll-like receptor (TLR) 2 agonist; IL = interleukin, LAO = L-amino acid oxidase, TNF-α = Tumor necrosis factor -α, iNOS = inducible nitric oxide synthase, NF-kappaB = nuclear factor-kappaB, CX3CR1 GFP/+= CX3C chemokine receptor 1 Green fluorescent protein, KO = Knock-out, KI = Knock-In, Colony Forming Units = CFU, h = hours

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Aims Of The Thesis

Virulence is one of a number of possible outcomes of the host-microbe interaction. Unfortunately, the specific host responses against different bovine mastitis isolates are not yet fully characterized. Previous research from our group focused on the host innate immune response induced by a bovine mastitis-associated *E. coli* isolate. Experimental infection studies were performed with a validated mouse mastitis model. The current PhD research aims to elaborate the immune response induced by bovine-associated bacterial isolates with this elegant *in vivo* model. Unravelling differential pathogenesis may aid to assess essential inflammatory signaling that needs to be triggered in the host to prevent new infections of the mammary gland. Complementary to preventive treatment, mastitis can also be treated curatively with antibiotics. However, the prediction of the efficacy of novel antibiotics for mastitis may also be influenced by host-related factors which are challenging to simulate under *in vitro* conditions. A laboratory animal mastitis model can implement these factors and thus aid to evaluate *in vivo* candidate antimicrobial compounds with high *in vitro* efficacy.

The specific objectives of this thesis were:

- To explore the **differential inflammatory signaling** induced by **bovine-associated bacterial isolates** in the mouse mastitis model (Chapter I). Chapter I.1 compares the local innate immune response induced by two major mastitis pathogens i.e. *E. coli* versus *S. aureus* focusing on the processing of IL-1beta. Chapter I.2 validates the mouse mastitis model for the first time with a CNS strain isolated from a chronically infected dairy cow and then compares the host response induced by three different CNS isolates with that of *S. aureus*.

- To examine whether pretreatment of the murine mammary gland with LPS, a MAMP of *E. coli*, can elicit a beneficial local inflammatory signaling as a basis for a novel **preventive strategy** against *S. aureus*-induced mastitis (Chapter II).

- To evaluate the efficacy of antimicrobial compounds as a **curative strategy** for bacterial-induced mastitis in the mouse mastitis model (Chapter III). Chapter III.1 wants to evaluate a series of existing antibiotics from the first generation of cephalosporins, while Chapter III.2 assessed a novel candidate from the biphenomycin class against three bovine mastitis pathogens.

Experimental Studies

CHAPTER I

MICROBE-DEPENDENT INFLAMMATORY SIGNALING

The entrance of a pathogen into the cows' udder results in an inflammatory response called mastitis. Key indicators for a mastitic cow are the presence of the microbe in combination with the increase in SCC in the milk. The latter are leucocytes, primarily neutrophils. As previously shown by our group, *E. coli*-infected bovine neutrophils activate NF-kappaB, cytokine production, caspases and cell death (Notebaert, Duchateau et al. 2005; Demeyere, Remijsen et al. 2013), which could also be demonstrated in the mouse model (Notebaert, Carlsen et al. 2008; Notebaert, Demon et al. 2008). For *S. aureus*, a major Gram-positive mastitis pathogen, the information covering all these inflammatory parameters is scarce. Therefore, the **first aim of CHAPTER I** was to assess the involvement of NF-kappaB activity, cytokine profiling, the presence of caspases and neutrophil influx for *S. aureus* mastitis. Furthermore, we compared these observations with *E. coli* mastitis and determined whether the inflammation is pathogen-dependent.

CNS have been isolated from the teat apex of lactating dairy cows without symptoms of mastitis (Braem, Stijlemans et al. 2014) as well as from milk samples inducing a mild inflammatory response (Pyorala and Taponen 2009). Nevertheless, little is known about the CNS and their interaction with the host. Therefore, the **second aim of CHAPTER I** was to assess whether the mouse mastitis model can be used as a complementary *in vivo* tool for future CNS mastitis research and whether a differential host innate immune response can be observed between epidemiologically different bovine CNS species/strains.

First aim of CHAPTER I is adapted from: Breyne K., Cool S.K., Demon D., Demeyere K., Vandenberghe T., Vandenabeele P., Carlsen H., Van Den Broeck W., Sanders N.N., Meyer E., Non-Classical proIL-1beta Activation during Mammary Gland Infection is Pathogen-Dependent but Caspase-1 Independent, PLoS One. (2014) 9:e105680.

Second aim of CHAPTER I is adapted from: Breyne, K., S. De Vliegher, A. De Visscher, S. Piepers, and E. Meyer. 2015. Technical note: A pilot study using a mouse mastitis model to study differences between bovine associated coagulase-negative staphylococci. J Dairy Sci 98(2):1090-1100.

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I.1. Non-Classical ProIL-1beta Processing during Mammary Gland Infection is Pathogen-Dependent but Caspase-1 Independent

I.1.1. Abstract

Infection of the mammary gland with live bacteria elicits a pathogen-specific host inflammatory response. To study these host-pathogen interactions mammary glands of wild type mice, NF-kappaB reporter C57BL/6 mice as well as caspase-1/11 and IL-1beta knockout C57BL/6 mice were challenged with *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). The murine mastitis model allowed to compare the kinetics of the induced cytokine protein profiles and their underlying pathways. *In vivo* and *ex vivo* imaging showed that *E. coli* rapidly induced NF-kappaB inflammatory signaling concomitant with high mammary levels of TNF-alpha, IL-1 alpha and MCP-1 as determined by multiplex analysis. In contrast, an equal number of *S. aureus* bacteria induced a low NF-kappaB activity concomitant with high mammary levels of the classical IL-1beta fragment. These quantitative and qualitative differences in local inflammatory mediators resulted in an earlier neutrophil influx and in a more extensive epithelial remodeling post-infection with *E. coli* compared to *S. aureus*. Western blot analysis revealed that the inactive proIL-1beta precursor was processed into pathogen-specific IL-1beta fragmentation patterns as confirmed with IL-1beta knockout C57BL/6 animals. Additionally, caspase-1 knockout animals allowed to investigate whether IL-1beta fragmentation depended on the conventional inflammasome pathway. The lack of caspase-1 did not prevent extensive proIL-1beta fragmentation by either of *S. aureus* or *E. coli*. These non-classical IL-1beta patterns were likely caused by different proteases and suggest a sentinel function of IL-1beta during mammary gland infection. Thus, a key signaling nodule can be defined in the differential host innate immune defense upon *E. coli* versus *S. aureus* mammary gland infection, which is independent of caspase-1.

I.1.2. Introduction

Infectious mastitis is defined as the inflammatory response initiated when microorganisms enter the mammary gland challenging the host defense (Kebaier, Chamberland et al. 2012). This common disease has either clinical or asymptomatic (subclinical) characteristics and is generally perceived as a significant burden for the well-being of mammals and especially dairy animals (Barkema, Green et al. 2009). Two main bacterial species that cause bovine mastitis are *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) (Schukken, Gunther et al. 2011). Although both these pathogens grow in the mammary gland evoking a host immune response, they activate specific inflammatory signaling pathways which result in discriminatory stress profiles (Riollet, Rainard et al. 2000; Bannerman, Paape et al. 2004; Farhat, Sauter et al. 2008). This distinctive pathobiology can be explained by the microbe-associated molecular patterns (MAMPs) dictating the expression and subsequent release of specific pro-inflammatory cytokines (Brand, Hartmann et al. 2011; Jensen, Gunther et al. 2013). In the initial phase, these mediators orchestrate the diapedesis of predominantly neutrophils into the mammary alveoli activating phagocytic innate immune cells to eliminate pathogens or at least prevent their spreading (Bougarn, Cunha et al. 2010; Elazar, Gonen et al. 2010; Stevens, De Spiegeleer et al. 2012). Most of the observations seen in cows have been further elaborated at the

molecular level through *in vivo* studies in mouse mastitis models (Gonen, Vallon-Eberhard et al. 2007; Elazar, Gonen et al. 2010).

Our group previously reported that *in vitro* exposure of bovine neutrophils to live *E. coli* rapidly activates a complex series of molecular pathways involving cell death, the cleaving of the protease procaspase-1 and the transcription factor nuclear factor-kappaB (NF-kappaB). This activity occurs concomitant with the secretion of the pro-inflammatory cytokine IL-1beta (Notebaert, Duchateau et al. 2005; Demeyere, Remijsen et al. 2013). Two studies from our group using intramammary infections (IMI) in mice confirmed the relevance of these key parameters *in vivo* (Notebaert, Carlsen et al. 2008; Notebaert, Demon et al. 2008). However, elucidation of the link between these innate mammary host defense factors and their relevance for other mastitis pathogens than coliforms is just starting to emerge. It was already demonstrated for the bovine species that both *E. coli* and *S. aureus* bind mammary epithelial Toll-like receptor (TLR)4 and TLR2, but that both these pathogens differently modulate NF-kappaB (Yang, Zerbe et al. 2008). Indeed, *E. coli* increases mammary epithelial NF-kappaB activity through myD88-dependent signaling followed by enhanced mRNA cytokine expressions (Yang, Zerbe et al. 2008). In contrast, mammary *S. aureus* infections are characterized by reduced local NF-kappaB levels. These observations are linked to the internalization of these bacteria in bovine epithelial cells (Lara-Zárate, López-Meza et al. 2011). To date, NF-kappaB transcriptional activity is accepted to induce proIL-1beta transcription and the subsequent release of pro-inflammatory cytokines either locally and/or systemically. Nevertheless, reported mastitis data are still partly contradictory as they on the one hand state that active IL-1beta predominates upon infection with *S. aureus* compared to *E. coli*, while adverse data have also been published (Persson-Waller, Colditz et al. 1997; Gunther, Esch et al. 2011). Additionally, it was recently shown that total IL-1 signaling is of critical importance for the proper influx of neutrophils into the alveolar lumen following an infection with *E. coli* (Elazar, Gonen et al. 2010). Overall, little is known about the source of proIL-1beta or about its maturation process during mastitis. In a variety of other infectious pathologies, the production of this inactive pro-form is mediated by NF-kappaB, while its biological activity is regulated by a cytoplasmic multi-protein complex named the inflammasome. The inflammasome binds procaspase-1 and enables its activation; in turn the latter protease activity mediates the maturation of proIL-1beta (Lamkanfi and Dixit 2012).

The current *in vivo* study aims to further elucidate the main mechanistic differences or similarities between mammary *S. aureus* or *E. coli* infections as a basis for novel intervention strategies. Key findings highlight the non-classical maturation of proIL-1beta independently of caspase-1 during mammary inflammation. The resulting cleavage patterns were pathogen-specific and occurred concomitant with important steps in the innate immune response of the mammary gland such as the NF-kappaB activation, the expression of specific cytokine profiles, the influx of neutrophils and changes in the integrity of the epithelial layer.

I.1.3. Materials and Methods

I.1.3.1. Mice

Female albino NF-kappaB *luc* C57BL/6 mice were kindly provided by Harald Carlsen (University of Oslo, Department of Nutrition, Institute of Basic Medical Sciences) (Carlsen, Moskaug et al. 2002). Genetically these mice are heterozygous for the NF-kappaB *luc* transgene in a Tyr Knock-out (KO) C57BL/6 background and thus easier to visualize for *in vivo* imaging purposes than C57BL/6 animals. Caspase-1/Caspase-11 double KO C57BL/6 and IL-1beta KO C57BL/6 mice were kindly provided by Tom Vandenberghe. (Ghent University, Molecular Signaling and Cell Death Unit, Department for Molecular Biomedical Research, VIB). The latter were compared with wild type (wT) C57BL/6 mice provided by Harlan. All mice were conventionally housed with water and food supplied ad libitum, and maintained with a 12 hours (h) light/dark cycle. Sentinels were routinely screened to verify the pathogen-free conditions. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Ghent University (Permit Number: EC2011/042 and EC2010/148). All efforts were made to minimize suffering. Surgery was performed under isoflurane anesthesia combined with a long-acting analgesic buprenorphine, while a ketamine and xylazine containing cocktail was administered prior to euthanasia.

I.1.3.2. Intramammary infection model

Eight-week-old mice (wT or transgenic C57BL/6 mice) were allowed to mate with a ten-week-old male. Following parturition, the pups were weaned only after ±10 days to enhance mammary gland development. One hour after weaning, mice were inoculated using a 32-gauge blunt needle (fourth gland pair) with $3.20 \times 10^3 \pm 374.6$ CFU *S. aureus* Newbould 305 or *E. coli* P4:032, or PBS under inhalational anesthesia. These bacteria are relevant bovine mastitis isolates inducing a specific inflammatory response in our established mouse mastitis model (Notebaert, Carlsen et al. 2008; Demon, Ludwig et al. 2012).

During the Intramammary Infection (IMI) experiment, core body temperature of the mice was measured with a rectal thermistor and compared to the body temperature pre-IMI. Blood was harvested through the tail vain or cardiac puncture, incubated for 1h at 37°C and centrifuged (12250 g) for another 1h. Following sacrifice (cervical dislocation) the inoculated glands (and untreated livers) were isolated, homogenized and plated in serial logarithmic dilutions on Tryptic soy agar plates to determine CFU/g gland. Supernatant/serum samples were stored individually at −80°C until use.

I.1.3.3. *In vivo* and *ex vivo* bioimaging

Imaging of luciferase activity in intramammary injected transgenic mice was performed with the IVIS lumina II (Caliper) (Cool, Breyne et al. 2013). To visualize the NF-kappaB signal a suspension of D-luciferin (2mg/100µl) dissolved in PBS was injected. NF-kappaB *luc* C57BL/6 mice were pre-anesthetized by isoflurane, injected right and left of the abdomen with luciferin, and imaged about 10

minutes following injection. Briefly, bioluminescence is visualized when NF- kappaB gets activated, translocates to the nucleus where it binds the kappaB sites and transcribes the luciferase gene. As a result the injected luciferin is oxidized by the newly produced luciferase together with ATP, which yields a bioluminescent light signal. The transgenic mice were measured before (at -2h) and kinetically after IMI (at 2h, 4h, 8h, 10h, 12h and 24h). At 12h (n = 11) and 24h (n = 32) mice were sacrificed, organs harvested and separately visualized through imaging. The intensity of the bioluminescent signal is linked with the level of NF-kappaB activity and normalized through the division of the total flux data by the selected area as provided by the living image software 3.2 (Caliper).

I.1.3.4. Cytokine analysis

Hundred microliters of mammary gland homogenate were mixed with 200 µl lysis buffer supplemented with protease inhibitors (200 mM NaCl, 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 1% Nonidet P-40, 10% glycerol, 1 mM oxidized L-glutathion, 100 µM PMSF, 2.1 µM leupeptin and 0.15 µM aprotinin) to extract cellular proteins. The suspensions rested overnight at -20°C, were centrifuged (12.250 g) for 1h and finally the supernatant was centrifuged for another 30 minutes (min) to precipitating the pellet. The protein concentration in the supernatant was spectrophotometrically (Genesys 10S) determined with Bio-Rad Protein Assay (Biorad). Cytokine quantification in the lysates and serum was performed with specific Cytometric Bead Array kits (CBA, Becton Dickinson) for mouse IL-1beta, IL-6, MCP-1, TNF-alpha, and IL-1alpha and specific Aimplex multiplex assay kits (YSL Bioprocess Development Co.) with minor modifications for mouse KC and MIP-2 on a FACSArray instrument (Becton Dickinson). Fifty µg lysate or ¼ diluted serum were applied to each well.

I.1.3.5. Immunohistochemistry

Two mammary glands per condition (isolated from the NF-kappaB *luc* mice) were fixed in buffered 3.5% formaldehyde for 24h at room temperature and embedded in paraffin wax. Sections were deparaffinized, hydrated, and antigens retrieved in a citrate buffer through microwaves and cooker pressure for p50 and p65, respectively. To mask endogenous peroxidase activity, specimens were pre-treated with 3% hydrogen peroxide in methanol for 5 min at room temperature. Non-specific binding sites were blocked by 30% goat serum and 1% BSA for 30 min at 25°C. The tissues were incubated overnight at 4°C with p65 (rabbit anti mouse, sc-372, Santa Cruz) and p50/p105 (rabbit anti-mouse, ab7971, Abcam). The next day, samples were incubated with a biotinylated secondary antibody (goat anti-rabbit) that binds streptavidin conjugated to horseradish peroxidase (HRP). A 3,3'-Diaminobenzidine chromogen generates a brownish peroxidase-based signal visualized through the HRP reaction together with a hematoxylin counterstain. All rinsing steps were performed with Tris-buffered saline (TBS) supplied with Triton-X 100.

I.1.3.6. Western analysis

Mammary gland lysates (prepared as described sub cytokine analysis) were loaded in equal volumes (18 µg protein/20 µl buffer), proteins were separated on a 12% polyacrylamide Amersham ECL Gel (GE Healthcare) and the proteins transferred to a 0.45 µm nitrocellulose transfer membrane (Biorad).

A donkey anti-goat IgG (Bioconnect) was used to detect IL-1beta (R&D) and donkey anti-rabbit IgG (GE Healthcare) was used to detect caspase-1 (kindly provided by Wim Declercq).

I.1.3.7. Statistical analysis

P-values of normal distributed data were calculated by ANOVA testing. Depending on the Levene's test, mean values were compared by Tukey post hoc comparisons. If necessary, normalization occurred through log10 transformation and if normalization was impossible, median values were compared with non-parametic statistics and Mann-withney U post hoc-testing.

I.1.4. Results

I.1.4.1. The intramammary bacterial growth post-intramammary infection with *S. aureus* and *E. coli*

Sham-inoculated (PBS) C57BL/6 mice (n = 9; data not shown) were compared to C57BL/6 mice inoculated with (3.203 ± 0.38) x 10^3 CFU of either *E. coli* (n = 12) or *S. aureus* (n = 12). Both at 12h and 24h post-IMI, mammary glands were isolated and their CFU count was determined (Fig. 1). There was no statistically significant median difference (MD) between both treatment groups at the two time points of sacrifice. Nevertheless, significant intramammary growth of each of both pathogens was detected between these time points (*E. coli*, P < 0.001; *S. aureus*, P < 0.01).

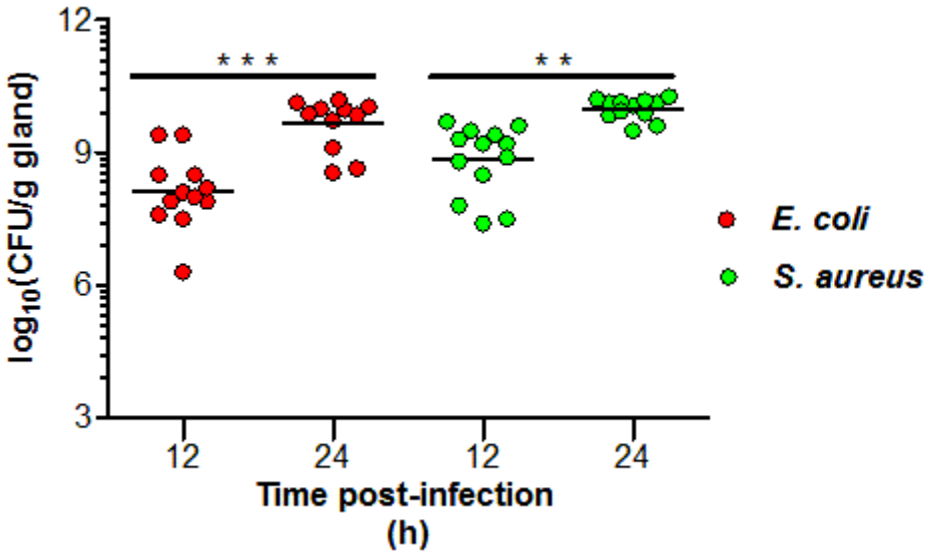


Figure 1. Changes in mammary bacterial counts post-IMI with *E. coli* or *S. aureus*. At 12h and 24h post-IMI, mammary tissue was harvested and evaluated for bacterial growth. Results are presented as mean ± standard deviation (P < 0.01 **, P < 0.001*).**

I.1.4.2. Mammary cytokine profiles post-intramammary infection with *E. coli* compared to *S. aureus*

Differential cytokine patterns were observed in the mammary gland post-IMI with *E. coli* versus *S. aureus* at 12h or 24h (n = 10 for all groups).

At the local level, *E. coli* dominated the innate immune response on both time points by a strong and continuing increase of TNF-alpha (at 12h, $P < 0.001$; at 24h, $P < 0.001$), IL-1alpha (at 12h, $P < 0.001$; at 24h, $P = 0.001$) and MCP-1 albeit only at the later time point (at 12h, $P = 0.051$; at 24h, $P = 0.001$) compared to *S. aureus* (Fig. 2A). Concentrations of these cytokines in all untreated lactating glands (at 0h, n = 6) and sham-inoculated glands (PBS, n = 8 at 12h and at 24h) at 12h and 24h remained below the detection limit (DL). Absolute MCP-1 levels were up to 8-fold higher than those of TNF-alpha and IL-1alpha. The increase of TNF-alpha and IL-1alpha was similar when comparing *E. coli* to *S. aureus* ($R^2 = 0.81$; $P < 0.001$) i.e. about 3-fold higher for *E. coli* compared to *S. aureus*, while this relative difference between both pathogens was about 2-fold for MCP- 1 (Fig. 2A). An IMI with both pathogens induced a strong and continuing local increase in IL-6 (Fig. 2B).

In marked contrast to TNF-alpha, IL-1alpha and MCP-1, this response did not significantly differ between the *E. coli* and *S. aureus* but did significantly differ for each pathogen compared to the sham-inoculated (PBS) glands (at 12h, $P < 0.001$; at 24h, $P < 0.001$) and lactating glands (at 0h, $P < 0.001$).

Post- IMI with *S. aureus*, a strong response albeit only at a later time point was observed for IL-1beta. At 12h post-IMI local levels were low and did not differ significantly between both pathogens, while at 24h post-IMI *S. aureus* strongly induced high local levels of this cytokine compared to *E. coli* ($P < 0.001$) (Fig. 2C). Concentrations of IL-1beta in the untreated lactating glands (at 0h) and sham-inoculated glands (at 12h and 24h) remained below the DL (detection limit). Pathogen-specific profiles in serum or liver samples were also detected and are discussed in the supporting information section (see Fig S1A and S1B).

I.1.4.3. Maturation of proIL-1beta and procaspase-1 post-intramammary infection with *E. coli* compared to *S. aureus*

The observed quantitative differences in cytokines do not necessarily imply biological activity of these proteins as for several cytokines this is the result of post-translational modification of their proforms. More specifically, activation of proIL-1beta is achieved by cytoplasmic multi-protein complexes named inflammasomes. The best known inflammasome enables procaspase-1 (p45) maturation upon vita-MAMP recognition. This active caspase-1 then mediates the maturation of active IL-1beta as characterized by its classical p17 fragment (Lamkanfi and Dixit 2012).

Based on multiplex results the local active IL-1beta concentration at 24h was higher post-IMI with *S. aureus* than with *E. coli* (Fig. 2C). This quantitative analysis measures the number of active p17 fragment. The absence of mammary IL-1beta in IL-1beta KO C57BL/6 animals was confirmed at both

time points through multiplex analysis, no significant differences in the local levels of other cytokines could be detected either (data not shown). However, using Western blot analysis as a complementary immunoassay, the complete fragmentation pattern of mammary proIL-1beta was evaluated post-IMI with *E. coli* compared to *S. aureus* (Fig. 3A and B).

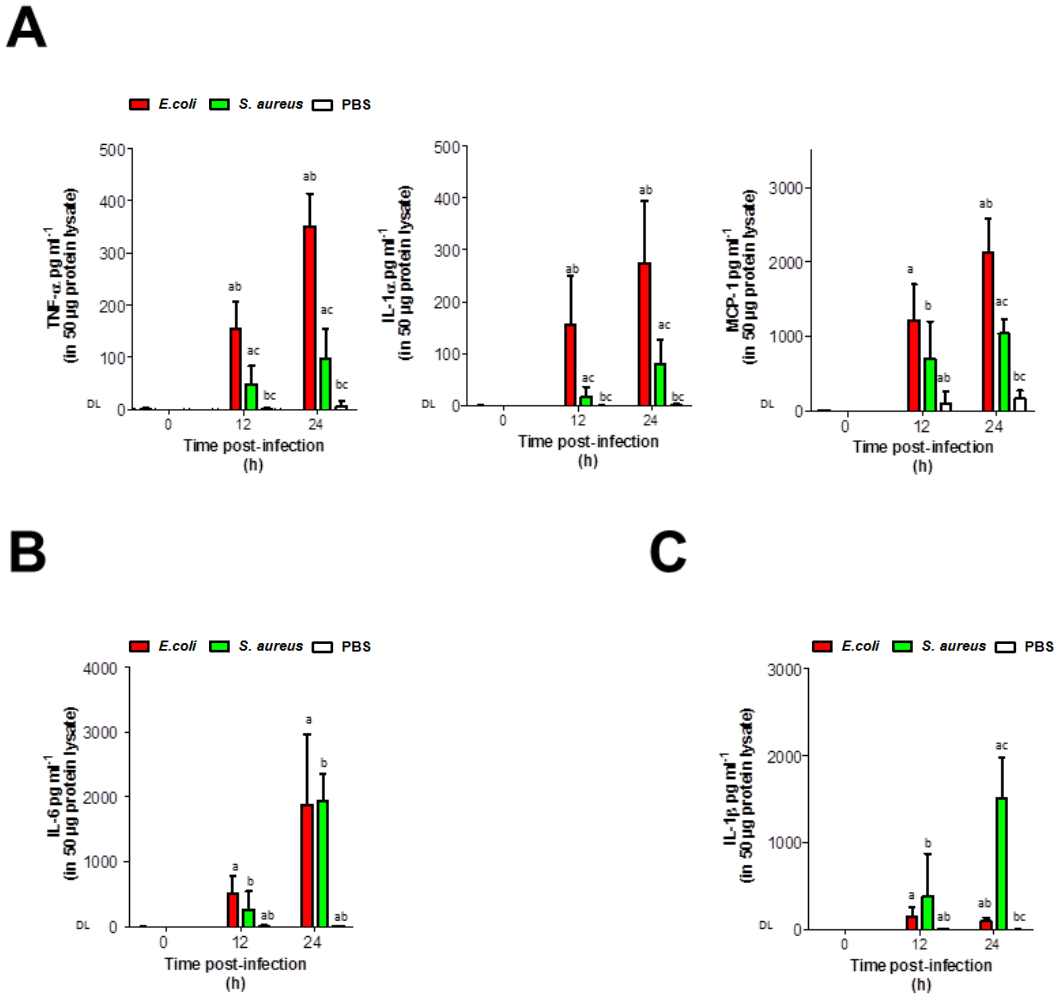


Figure 2. Differential mammary innate immune response post-IMI with *E. coli* versus *S. aureus*. (A) Local TNF-alpha, IL-1alpha, and MCP-1 concentrations in lactating mammary glands compared to mammary glands post-IMI with *E. coli, S. aureus* or sham-inoculated (PBS). (B) Local IL-6 concentrations in lactating mammary glands compared to mammary glands post-IMI with *E. coli* or *S. aureus* or sham-inoculated (PBS) mice. (C) Local concentrations of active IL-1beta in lactating mammary glands compared to mammary glands post-IMI with *E. coli, S. aureus* or sham-inoculated (PBS) mice. Results are presented as mean ± standard deviation. Letters of homogeneous subsets were marked if the difference between treatments were statistically significant (P < 0.001) (DL = detection limit).

Multiplex analysis showed that an IMI in C57BL/6 mice with *E. coli* induced the fast but transient increase of active IL-1beta (p17) compared to sham-inoculated (PBS) mammary glands (at 12h, $P < 0.001$; at 24h, $P < 0.001$) (Fig. 2C). Western blotting of these samples revealed that this increase of p17 the result of a fast proIL-1beta (p31 band, fragment 1) maturation into multiple fragments which

were absent post-inoculation with PBS. Indeed, at 12h post-IMI a complex pattern of at least six IL-1beta fragments were detected concomitant with procaspase fragmentation i.e. at ± 30 kDa, ± 25 kDa, ± 20 kDa, ± 17 kDa, ± 15 kDa and ± 10 kDa (fragments 1, 2, 3, p17, 4 and 5, respectively; Fig. 3A). In marked contrast, only one band (i.e. fragment 1, p31) of the proIL-1beta maturation remained at 24h post-IMI with *E. coli* although procaspase-1 cleavage was induced at that later time point (Fig. 3A).

As expected, IL-1beta fragments were largely absent in similarly infected IL-1beta KO C57BL/6 mammary glands while procaspase-1 fragmentation was still observed (Fig. 3A). Interestingly, extensive proIL-1beta (p31) maturation was also seen in caspase-1/-11 double KO C57BL/6 mammary glands at 12h albeit with a lower number of bands (i.e. no fragment 2; Fig 3A). Multiplex analysis further showed that IMI with *S. aureus* also induced active IL-1beta (p17) compared to sham-inoculated (PBS) mammary glands (at 12h, $P < 0.01$; at 24h, $P < 0.001$) (Fig. 2C). This raise of p17 occurred predominantly at 24h and was also higher than post-IMI with *E. coli* ($P < 0.001$). Western blotting of mammary gland lysates of C57BL/6 confirmed this late increase of p17 (Fig. 3B). While the fragmentation pattern at 12h post-IMI with *S. aureus* was similar to that of *E. coli* (except for the fragments 4 and 5) the intensity of the bands was lower. In marked contrast, a different cleavage pattern was seen at 24h post-IMI with *S. aureus* compared to that at 12h post-IMI with *E. coli* (there was an additional band, fragment 6) *,* this maturation was now totally caspase-1 independent as very limited fragmentation of procaspase-1 was observed. Compared to post-IMI with *E. coli*, the IL-1beta pattern contained a lower number of fragments post-IMI with *S. aureus* i.e. at ± 30 kDa, 20 kDa, ± 18 kDa and 17 kDa (corresponding to fragments 1, 3, 6 and p17, respectively; Fig. 3B). As for *E. coli*, these IL-1beta fragments were all absent in similarly affected IL-1beta KO mammary glands. Again in marked contrast to IMI with *E. coli*, no procaspase-1 was observed at 24h in IL-1beta KO mammary glands while an extensive IL-1beta maturation (p17 fragment) was seen in caspase-1/-11 double KO mammary glands post-IMI with *S. aureus.*

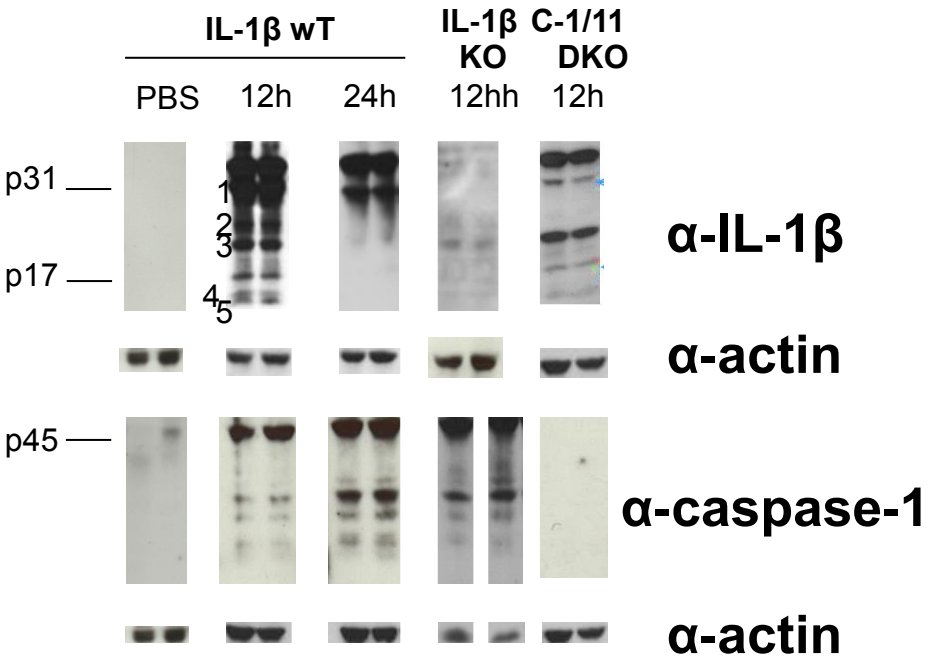
When comparing bacterial growth in IL-1beta KO C57BL/6 mammary glands to their C57BL/6 counterparts at the selected relevant time points for each of both pathogens, a significantly higher number of CFU was seen for *E. coli* and to a lower extent also for *S. aureus* ($P < 0.01$, $n = 4$ and $P < 0.05$, $n = 6$ Fig. 3C).

I.1.4.4. The mammary NF-kappaB activity post-intramammary infection with *E. coli* compared to *S. aureus*

Our group at first described the fast and transient induction of mammary NF-kappaB transcription post-IMI with *E. coli* (Notebaert, Carlsen et al. 2008). Monitoring the NF-kappaB activity with *in vivo* imaging in reporter mice*,* a gradual and significant increase was again observed at 4-11h post-IMI with *E. coli* and at first also with *S. aureus* albeit non-significant (Fig. 4A). The maximal NF-kappaB activity was about a 3-fold higher post-IMI with *E. coli* compared to *S. aureus.* This correlated with the local TNF-alpha and IL-1alpha levels that were stronger upregulated post-IMI with *E. coli*, compared to *S. aureus*. No significant increase was seen in the sham-inoculated (PBS, $n = 6$) glands compared to pre-IMI. The increase in mammary NF-kappaB activity at 4h, at 6h, at 8h, at 10h and at 11h post-IMI

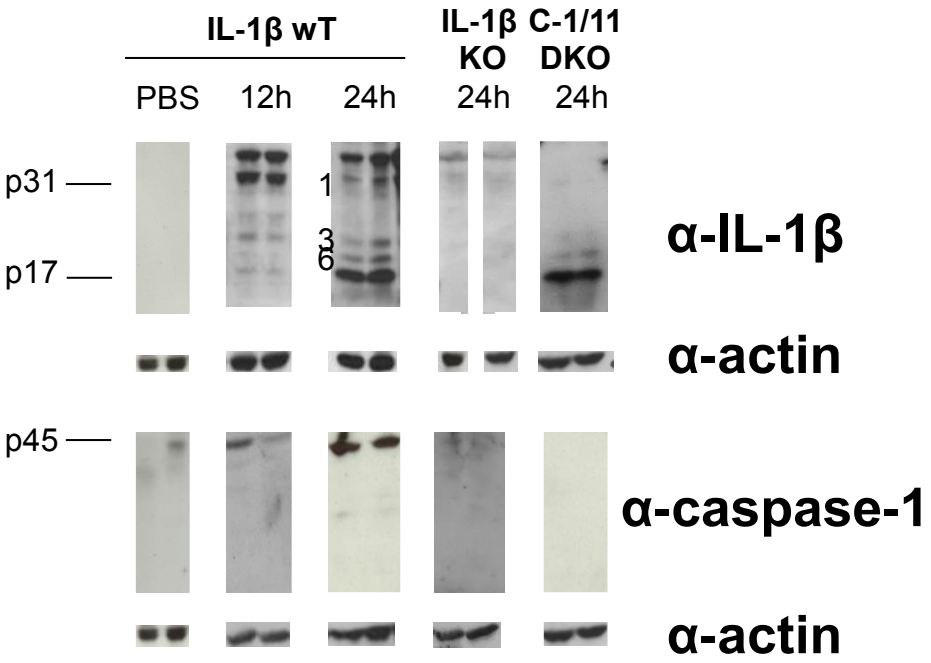
A

E. coli



B

S. aureus



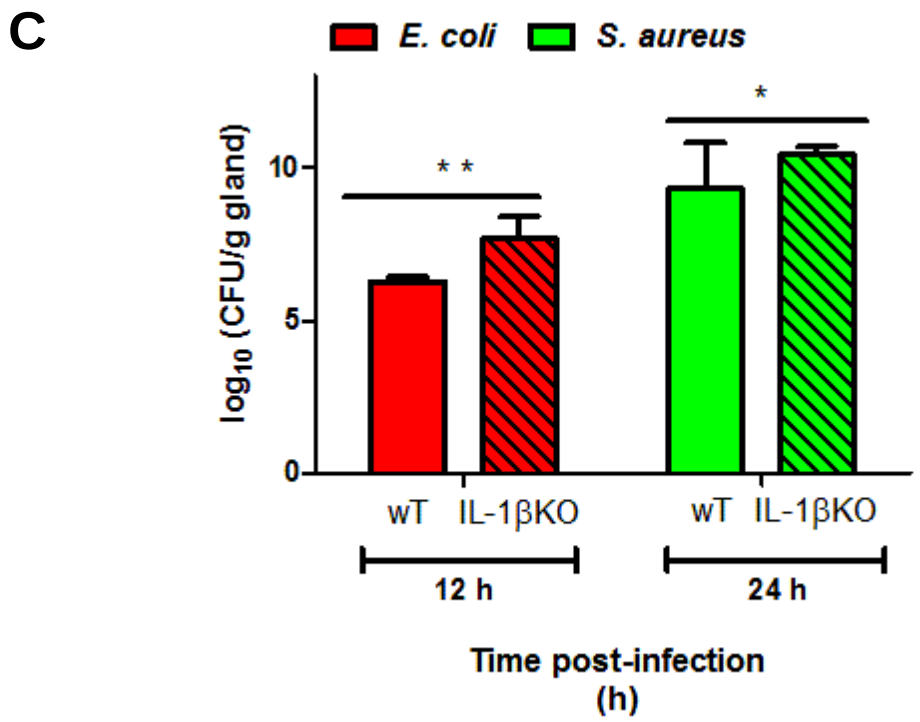


Figure 3. Differential mammary IL-1beta fragmentation post-IMI with *E. coli* versus *S. aureus* and effect on bacterial growth. (A) Cleavage of IL-1beta in the mammary gland post-IMI with *E. coli* shows a fast, transient IL-1beta maturation with six IL-1beta fragments at 12h i.e. ± 30 kDa, 25 kDa, ± 20 kDa, ± 17 kDa, ± 15 kDa and ± 10 kDa (fragments 1, 2, 3, p17, 4 and 5, respectively). At 24h (only fragment 1 and p31 proform) is seen. In IL-1beta KO C57BL/6 **and in sham-inoculated (PBS) mice no fragments or p31 proform are detected. Despite clear procaspase-1 maturation, the early complex IL-1beta pattern is not the result of caspase-1 cleavage as the latter only occurs extensively at 24 h and as cleavage of pro-IL-1beta still occurs in caspase-1/-11 double KO** C57BL/6 **glands (right Western blot images). No caspase-1 maturation was detected in caspase-1/-11 double KO** C57BL/6 **or in sham-inoculated mammary glands. (B) Cleavage of IL-1beta in the mammary gland post-IMI with *S. aureus* shows a slower IL-1beta maturation with four IL-1beta fragments at 24h i.e. ± 30 kDa, ± 20 kDa, ± 18 kDa and ± 17 kDa (fragments 1, 3, 6 and p17, respectively). At 12h (only fragment 1 i.e the preform p31, 2, 3,and p17). The late IL-1beta maturation is not the result of caspase-1 cleavage as IL-1beta cleavage still occurs in caspase-1 / -11 double KO** C57BL/6 **glands (strong p17 fragment albeit in the absence of the p31 proform). Both procaspase-1 and its cleavage were low, respectively absent in IL-1beta KO** C57BL/6 **glands (right Western blot images). No IL-1beta or caspase-1 was detected in sham-inoculated mammary glands of** C57BL/6 **mice. (C) ProIL-1beta fragmentation affects bacterial growth as in IL-1beta KO** C57BL/6 **mammary glands on time points of interest (12h for *E. coli*, 24h for *S. aureus*) CFU counts are significantly higher for both pathogens than in mammary glands of** C57BL/6**, especially for *E. coli.* Results are presented as mean ± standard error of the mean. (P < 0.01 *, P < 0.001**). DL = detection limit.**

was stronger for *E. coli* ($P < 0.01$, $n = 8$) compared to sham-inoculated glands. Upon *in vivo* imaging at 12h post-IMI this difference no longer remained, while *ex vivo* imaging still displayed a significant difference compared to sham-inoculated glands at 12h ($P < 0.01$) and 24h ($P < 0.01$; Fig. 4C and D). Although not significant *in vivo*; there was a difference between *E. coli* infected and *S. aureus* infected glands ($n = 8$) through *ex vivo* imaging at 12h post-IMI ($P < 0.05$) and at 24h post-IMI ($P < 0.001$).

I.1.4.5. Histology confirms the differential local NF-kappaB activation, demonstrates a delayed immune cell influx and less pathophysiological changes of the mammary epithelium post-IMI with *S. aureus* compared to *E. coli*

NF-kappaB-mediated transcription is only possible through translocation of its subunits to the nucleus. Immunohistochemistry was performed at 12h and 24h post-IMI with both pathogens on mammary gland sections.

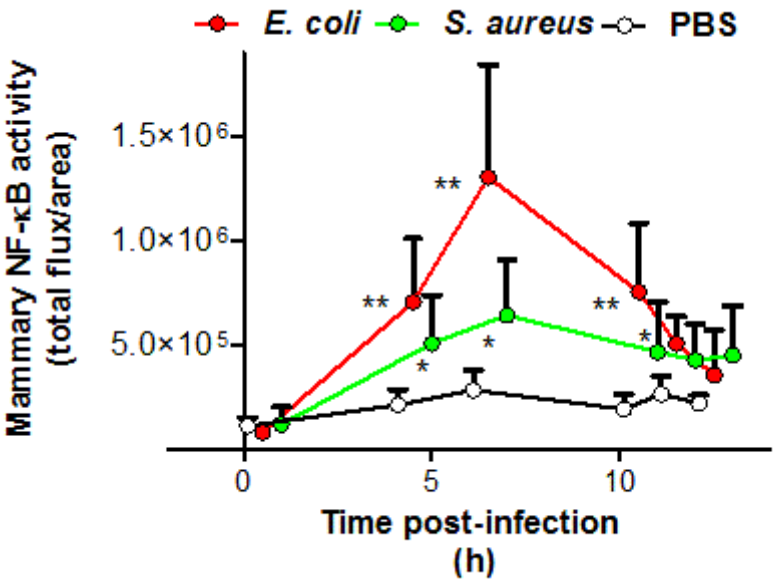
The NF-kappaB p65 subunit was detected in the nucleus of mammary epithelial cells and immune cells post-IMI with *E. coli* (Fig. 5A: -1a- and -3b-) as well as post-IMI with *S. aureus* (Fig. 5A: -4a- and -8a-) but was absent in both sham-inoculated (Fig. 5A -11-) and lactating mammary glands (Fig. 5A -12-). As suggested by the NF-kappaB activity imaging data, the nuclear NF-kappaB subunit p65 in the mammary epithelia was mainly visualized at 12h and less pronounced at 24h post-IMI with *E. coli* (Fig. 5A; -1a- compared to -6a-) and post-IMI with *S. aureus* (Fig. 5A; -4a- compared to -10a-). In marked contrast to post-IMI with *E. coli,* the p65 subunit couldn't be detected in all nuclei of the epithelial cells at 12h post-IMI with *S. aureus* (Fig. 5A; -4a- compared to -4b-) again confirming the higher NF-kappa activity post-IMI with *E. coli* compared to post-IMI with *S. aureus*.

Small cells with a characteristic multilobular polymorphonuclear morphology of neutrophils were detected post-IMI with *E. coli* (Fig. 5A: -3a- and -7a-) and *S. aureus* (Fig. 5A -8a-) but not in sham-inoculated (Fig. 5A -11-) or in lactating mammary glands (Fig. 5A -12-). At 12h post-IMI with *E. coli* the alveolar lumen contained a mixture of neutrophils either with (Fig. 5A -3a-) or without (Fig. 5A -3b-) nuclear p65 the NF-kappaB subunit. However, at 24h post-IMI, no p65 subunit was detected in these neutrophils (Fig. 5A -7a-). In contrast, for *S. aureus*, neutrophils could only be detected at 24h post-IMI and with (Fig. 5A -8a-) or without (Fig. 5A -8b-) nuclear p65 subunit. Remarkably, post-IMI with *S. aureus* also cells without nuclei (likely red blood cells) were observed outside the blood vessels, which was not the case post-IMI *E. coli* (Fig. 5A -9a-). This observed delay in neutrophil influx post-IMI with *S. aureus* was confirmed by quantitative IL-8-like (i.e. KC and MIP-2) multiplex analysis (Fig. 5B). At 12h post-IMI, there was already a significant difference for *E. coli* compared to sham-inoculated glands (PBS) for both these chemokines which persisted at 24h post-IMI ($P < 0.01$ and $P < 0.001$, respectively). In marked contrast, post-IMI with *S. aureus* this IL-8 like chemoattractant signaling was delayed until 24h for both chemokines compared to sham-inoculated glands ($P < 0.05$ and $P < 0.001$, respectively) .

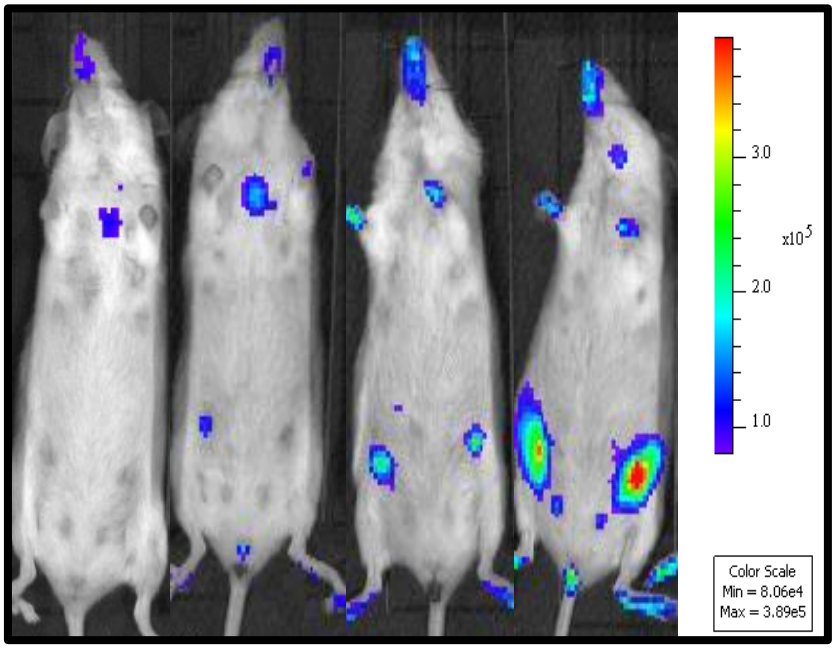
Additionally, as a result of the strong *E. coli*-inflicted immune response, the mammary epithelium was less structured compared to sham -inoculated and lactating mammary glands (Fig. 5A -2a- compared to -11- and -12-). At 12h post-IMI with *E. coli* mammary epithelial cells started to protrude from the

mammary epithelial layer (Fig. 5A -1b-) resulting in “empty spots” (Fig. 5A: -2b-), and many large cells drifting (Fig. 5A: -2b-) next to the polymorphonuclear cells in the alveolar lumen at 24h post-IMI (Fig. 5A: -2a- and -3c-). This major histological finding at 12h post-IMI with *E. coli* resulted in even more cell budding (Fig. 5A -5b-), empty spots (Fig. 5A -5c-), and discarded cells (Fig. 5A: -6b- and -5a-) at 24h post-IMI. Remarkably, this cell budding phenomenon was far less pronounced in the *S. aureus* infected glands and again clearly delayed as it could only be detected at 24h post-IMI (Fig. 5A: -10b-).

A



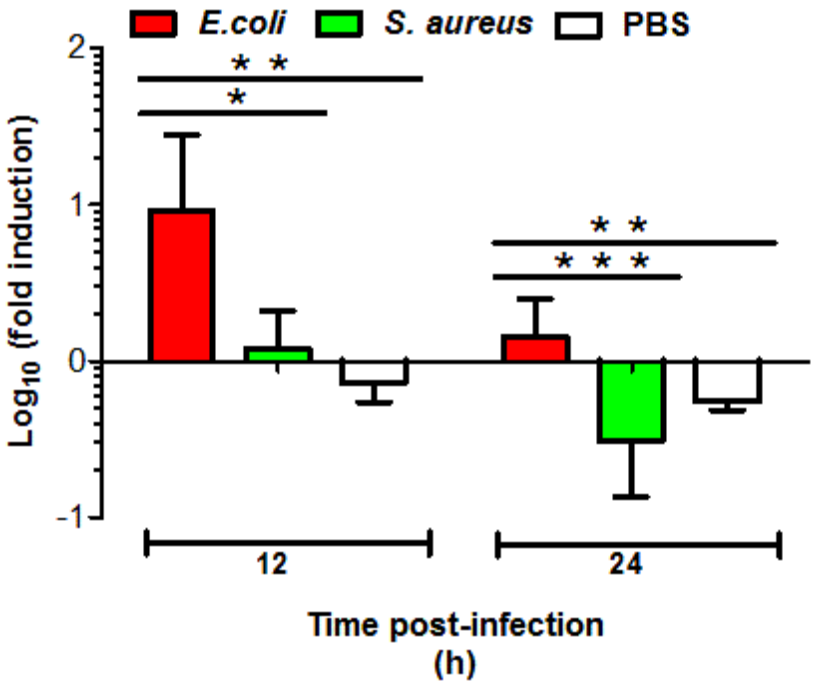
B



PBS ***E. coli***

LAC ***S. aureus***

C


E.coli
S. aureus
PBS
2
1
0
-1
* *
*
* *
* * *
Log10 (fold induction)
12
24
Time post-infection
(h)

D

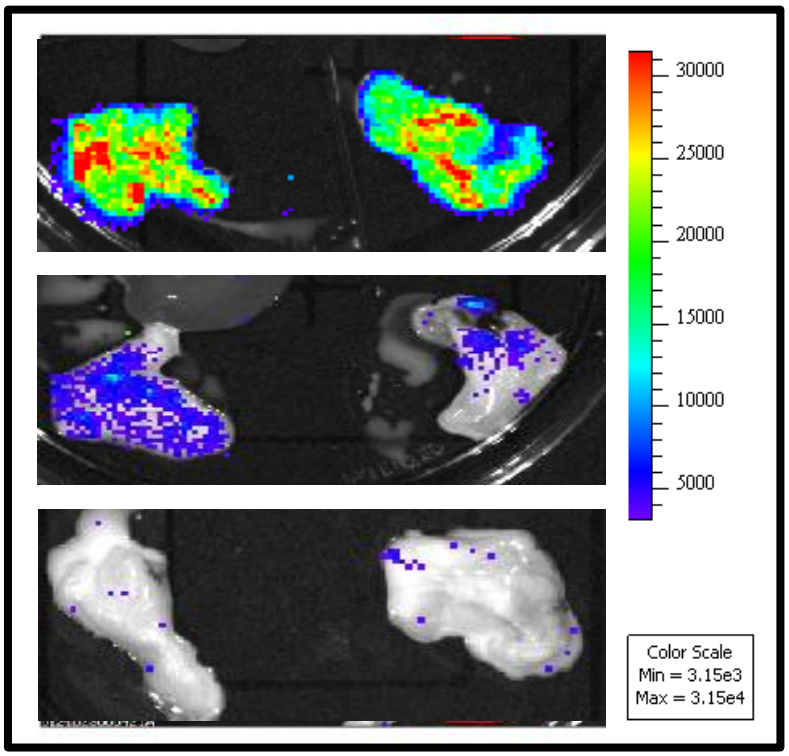
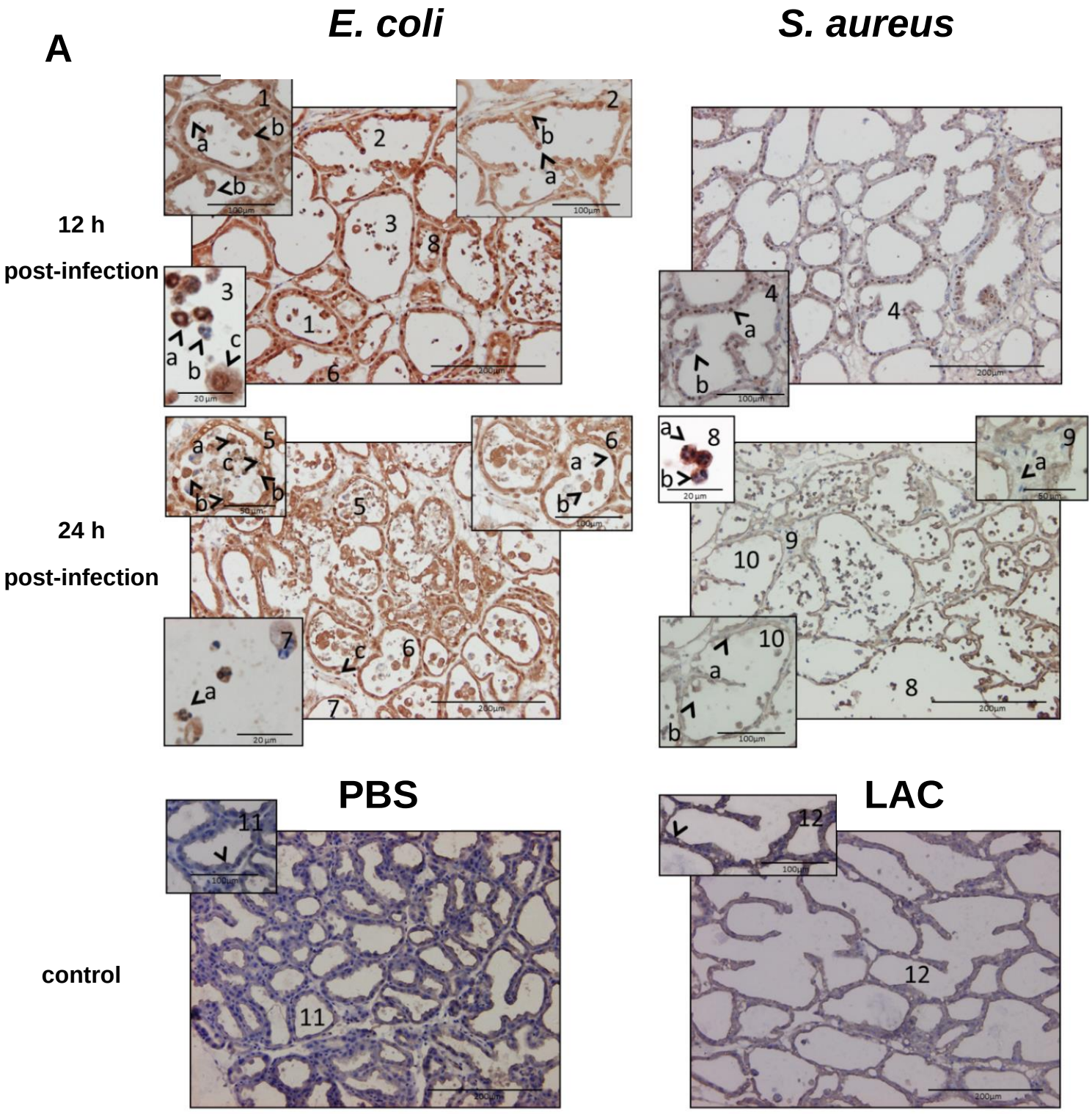

E. coli
S. aureus
PBS
30000
25000
20000
15000
10000
5000
Color Scale
Min = 3.15e3
Max = 3.15e4

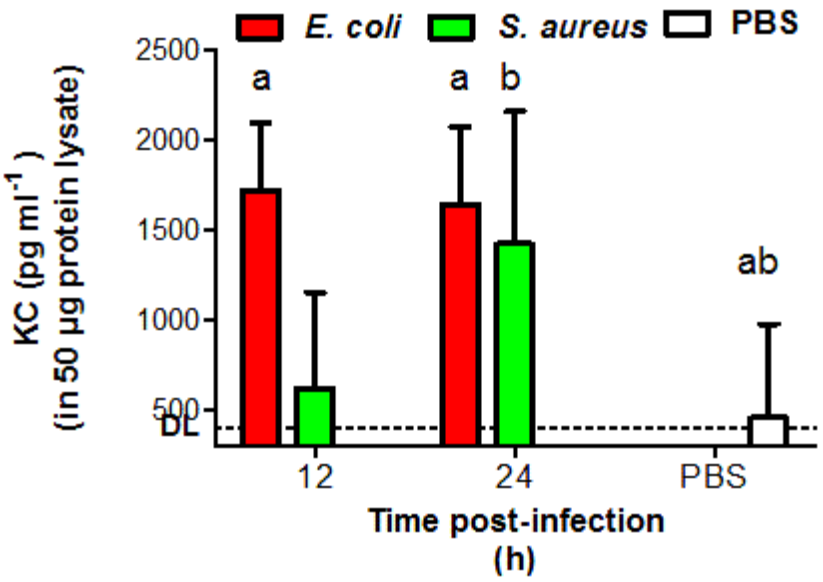
Figure 4. *In vivo* mammary NF-kappaB activity post-IMI with *E. coli* compared to *S. aureus*. (A) *In vivo* imaging of local NF-kappaB activity in mammary glands (ventral view) showed a transient and fast activation for both pathogens compared to sham-inoculated (PBS) glands and also about a 3-fold higher activation post-MI with *E. coli* compared to *S. aureus*. Data represent the flux density (total flux radiance per selected body area (in p*(s*m^2))) as a quantitative measure of the bioluminescent signal correlating with the NF-kappaB activity. (B) A representative photograph of lactating mice (at 0h, most left) compared to mice at 6h post-inoculation with sham (PBS, 2nd from left), at 6h post-IMI with *S. aureus* (3rd from left), or at 6h post-IMI with *E. coli* (most right) . The intensity of luminescence was scaled based on the radiance (in p*(s*m^2)). (C) Mammary glands of NF-kappaB reporter mice were excised at 12h and 24h post-inoculation and their *ex vivo* luminescence was measured. The graph represents the ratio between the total flux radiance per area (in p*(s*m^2)) of the inoculated gland and the value from the non-injected 3th gland in the same mouse. Although the maximal activation occurred earlier, the local NF-kappaB activity was still significantly higher in *E. coli* compared to *S. aureus* mammary glands at both these time points of relevance for cytokine transcription. (D) Representative photographical view of excised mammary glands post-IMI with *E. coli* (top), *S. aureus* (middle) or after sham-inoculation (PBS, bottom). Results are presented as mean ± standard deviation. Letters of homogeneous subsets were marked if the difference between treatments were statistically significant (P < 0.05) .

I.1.5. Discussion

Infectious mastitis is a complex bacteria-inflicted inflammatory disease that often affects dairy cows. As its traditional antibiotic treatment elicits public controversy and involves human health issues, an increased interest in novel superior therapeutic alternatives has emerged. Therapies that enhance the natural host defense systems by targeting specific pathogens and that limit antibiotic resistance would be very well received in this specific field. Therefore, the detailed molecular description of key signaling modules activated during different types of mammary gland infections is needed but lacking to date. In the current study, an acute murine mastitis model was used to compare the hosts' inflammatory response against the scientifically best documented Gram-negative and Gram-positive bovine mastitis pathogens i.e. *E. coli* strain P4:032 and *S. aureus* strain Newbould 305. Both pathogens multiplied rapidly in the murine mammary gland while triggering the release of pathogen-dependent as well as pathogen-independent immune responses. The different mammary multi-protein patterns each delineated a pathogen-specific infection and occurred concomitantly with discriminatory NF-kappaB activation. More specifically, our data emphasized the importance of evaluating proIL-1beta maturation by complementary immuno-assays as well as IL-1beta KO C57BL/6 and caspase-1/11 double KO C57BL/6 mice to gain insight in the IL-1beta activity in the mammary gland. While quantitative multiplex analysis showed only the high induction of the classical IL-1beta activity (p17) post-IMI with *S. aureus* compared to *E. coli,* Western blot analysis revealed a differential fragmentation pattern of mammary pro-IL-1 between both pathogens. This twice involved a non-classical pathway.


A
E. coli
S. aureus
12 h
post-infection
24 h
post-infection
PBS
LAC
control

B


E. coli
S. aureus
PBS
KC (pg ml-1)
(in 50 µg protein lysate)
2500
2000
1500
1000
500
DL
a
a
b
ab
12
24
PBS
Time post-infection
(h)

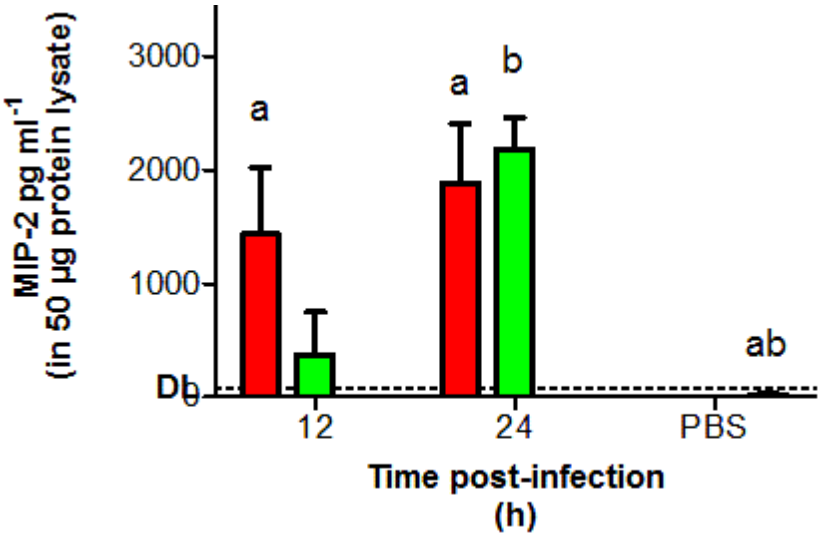

MIP-2 pg ml-1
(in 50 µg protein lysate)
3000
2000
1000
0
a
a
b
ab
12
24
PBS
Time post-infection
(h)

Figure 5. Differential nuclear translocation of mammary NF-kappaB p65, immune cell influx and the less structured mammary epithelium post-IMI with *S. aureus* compared to *E. coli*. (A) At 12h post-IMI, a nuclear translocation of NF-kappaB subunit p65 was observed in the epithelia of both *E. coli* (-1a-) and *S. aureus*-infected glands (-4a-). At this early time point, a strong cytoplasmatic NF-kappaB p65 signal was only seen post-IMI with *E. coli.* At 24h post-IMI, NF-kappaB p65 was mainly detected in the cytoplasm in the mammary epithelium of both *E. coli*-(-6-) and *S. aureus*-infected glands (-10-). while at this time both sham-inoculated (PBS, -11-) and lactating (at 0h,-12-) glands displayed very low basal levels of latent expressed cytoplasmic NF-kappaB p65. At 12h post-IMI with *E. coli* the polymorphonuclear cells in the alveolar lumen could morphologically be identified as neutrophils (-3a- and -3b-), while these immune cells were still absent at that time post-IMI with *S. aureus* (-4-) or in sham-inoculated (PBS, -11-) and lactating (at 0h,-12-) glands. In contrast, the influx of neutrophils could only be detected at 24h post-IMI with *S. aureus* (-8-). A clear nuclear translocation of NF-kappaB p65 could be detected at 12h post-IMI with *E. coli* and 24h post-IMI with *S. aureus* (-3a- and -8a-) in these immune cells. At 24h post-IMI with *E. coli,* epithelial structure at mammary alveoli was characterized by budding of mammary epithelia (-5b-) and resulted in cells in the lumen (-5a-) and empty spots in the luminal layer (-5c-), while at 24h post-IMI with *S. aureus* only some cells started to protrude from the epithelial layer (-10b-). (B) IL-8 like chemokines (i.e. KC and MIP-2) confirmed this difference in kinetics of the neutrophil influx between both pathogens. Both chemokines were significantly induced in *E. coli* infected glands compared to sham-inoculated glands (PBS) already at 12h post-IMI (a), while for *S. aureus* this strong induction was only seen at 24h post-IMI (b). Results are presented as mean ± standard deviation. Letters of homogeneous subsets were marked if the difference between treatments were statistically significant (P < 0.05)

Infection with *S. aureus* resulted in a later IL-1beta cleavage pattern and was associated with a markedly delayed influx of neutrophils compared to infection with *E. coli*. Furthermore, the mammary epithelium was already less structured at 12h post-IMI with *E. coli* as characterized by the presence of cells in the lumen and empty spots in the architecture, features which were observed later and less prominently post-IMI with *S. aureus*. These data are indicative for a protective cell death mechanism, especially in response to a Gram-negative infection, wherein mammary epithelial cells are massively shed to restrict bacterial growth as well as neutrophil cytotoxicity (Monks, Rosner et al. 2005).

Corroborating previous reports generated with either single protein detection analyses (i.e. ELISA) or multi-expression studies (i.e. microarray) of murine mammary glands (Li, Liang et al. 2013), udder tissue (Rinaldi, Li et al. 2010) or milk samples (Ibeagha-Awemu, Ibeagha et al. 2010), our initial quantitative mouse multiplex protein analysis approach demonstrated that an equal count of mammary CFU of *E. coli* and *S. aureus* each induced specific local cytokine profiles. The *E. coli* mediated immune response was characterized by a fast and substantial synthesis of active TNF-alpha, IL-1alpha and MCP-1. These three cytokines were also detected post-IMI with *S. aureus* although only to a relatively minor extent. Intramammary infection with *E. coli* or *S. aureus* was found to equally

enhance IL-6 secretion, which can thus be categorized as a Gram-independent immune response. This latter observation indicates that IL-6 induction is differently regulated compared to TNF-alpha, MCP-1 and IL-1alpha during an intramammary infection. In that respect, gene expression of IL-6 has been proposed to be induced by both an *E. coli* and a *S. aureus* infection of udder epithelia and occurs independently of MyD88 in contrast to TNF-alpha and IL-1alpha (Gunther, Esch et al. 2011).

In contrast to TNF-alpha, IL-1alpha, MCP-1 and IL-6, the active IL-1beta concentrations at 24h post-IMI were significantly higher in mammary glands exposed to *S. aureus* compared to *E. coli*. Apparently low levels of IL-1beta were induced at this later time point post-IMI with *E. coli*, nevertheless this pro-inflammatory cytokine was still significantly increased at both time points when compared to sham-inoculated glands and attained its highest level already at 12h post-IMI. Although this quantitative analysis allowed us to demonstrated different active IL-1beta levels in the mammary gland post-IMI with both pathogens, the proteolytic cleavage of proIL-1beta required for this key proinflammatory mediator to achieve its biological activity remained to be demonstrated as previously stated (Wittmann, Kingsbury et al. 2011). Therefore, the presence of IL-1beta fragments was at first verified upon Gram-negative versus the Gram-positive infection by Western blot analysis of the C57BL/6 mice-derived mammary glands. As suggested by our multiplex data, the classical p17 IL-1beta cleavage fragment was already detected at 12h post-IMI with *E. coli* albeit transiently as less p17 was detected at 24h post-IMI. In contrast, , the intensity of the p17 band was much higher at 24h post-IMI than at 12h post-IMI with *S. aureus*, indicating slower kinetics for this pathogen. Furthermore, Western blot analysis revealed several additional cleavage fragments besides the classical p17 fragment characterizing the markedly different IL-1beta fragmentation patterns upon infection with both pathogens. More specifically, *E. coli* promoted the formation of a fragment at 25 kDa and two fragments at 15 kDa, while *S. aureus* showed a unique band between 17 kDa and 20 kDa. Both bovine mastitis pathogens induced common bands at 17 kDa, 20 kDa and at 30 kDa. In a subsequent experiment, the comparative analysis of mammary glands from IL-1beta KO C57BL/6 mice-compared to C57BL/6 mice unequivocally confirmed that all these fragments did indeed derive from IL-1beta. Maturation of pro-IL-1beta is typically controlled by caspase-1 or -11 in large multi-molecular complexes called inflammasomes which sense (vita-)MAMPs from live bacteria (Lamkanfi and Dixit 2012). To determine whether mammary caspase-1 is also involved in proIL-1beta activation as recently questioned by our group (Breyne and Meyer 2013), the cleavage of procaspase-1 was investigated. Procaspase-1 fragmentation was clearly visible at 12h and at 24h post-IMI with *E. coli*, but unexpectedly far less present at both time points post-IMI with *S. aureus*. This important finding provided a first indication that IL-1beta maturation can also occur independently of caspase-1 in the murine mastitic mammary gland. This caspase-1 independency was confirmed in a subsequent experiment performed to further investigate this process through infection of caspase-1/-11 double KO C57BL/6 mice. These innovative data strongly supported the hypothesis of a non-conventional maturation of proIL-1beta into active p17 by other proteases than the conventional pro-inflammatory caspase-1, a hypothesis which also was recently postulated by our group (Notebaert, Demon et al. 2008). More specifically, the detected protein bands in the IL-1beta fragmentation patterns suggest the likely involvement of secreted neutrophil serine proteases (Wittmann, Kingsbury et al. 2011). Indeed, neutrophil elastase

and proteinase-3 are known to cleave proIL-1beta (i.e. 31kDa) into fragments of 20 kDa (Coeshott, Ohnemus et al. 1999) and of 17.5 kDa (Wittmann, Kingsbury et al. 2011) with different biological activities (Wittmann, Kingsbury et al. 2011). Cathepsin (Meyer-Hoffert and Wiedow 2011), another major neutrophil-associated protease, cleaves this immature protein proform directly into two identical fragments of 17.5 kDa (Meyer-Hoffert and Wiedow 2011). These reported fragments match with the low molecular weight bands retrieved around 20 kDa and p17 for both bovine mastitis pathogens in the current study. The alternative cleavage of pro-IL1beta was detected for *E. coli* at 12h post-IMI while only at 24h post-IMI with *S. aureus*. This nicely correlated both with the presence of neutrophils and with local NF-kappaB activation at each of these time points in the mammary gland. The concentrations of two mouse IL-8 like chemokines i.e. KC and MIP-2 were additionally measured in the mammary glands at the relevant time points for each pathogen. As expected, a massive influx of neutrophils was microscopically observed already at 12h for *E. coli*, while only at 24h for *S. aureus*. The local chemoattractant concentrations strongly supported this delayed influx as again a milder and slower innate immune response in the mammary gland was seen post-IMI with a Gram-positive compared to a Gram-negative bovine mastitis pathogen.

In contrast to the fragments seen post-IMI with *S. aureus*, *E. coli* infection induced less classical p17 as well as additional non-classical fragment formation upon proIL-1beta cleavage. We here suggest that these fragments can result from matrix metalloproteinases (MMPs) which are secreted by the either mammary epithelial cells (Kousidou, Roussidis et al. 2004) or neutrophils (Lin, Li et al. 2005). Interestingly, these extracellular enzymes are able to cleave proIL-1beta into either active, or less active, or even non-active IL-1beta fragments (Hazuda, Strickler et al. 1990). For example, MMP-9 can cleave proIL-1beta into an inactive 26 kDa fragment besides the classical active p17 fragment, while MMP-3 produces inactive 28 kDa as well as less active 14 kDa peptides. In addition, other papers - albeit not in a mammary gland context - describe that MMP-2 can cleave proIL-1beta into both a very low activity 16 kDa and an inactive 10 kDa fragment (Schonbeck, Mach et al. 1998; Mehrzad, Desrosiers et al. 2005). At least some of these reported fragments should correspond to fragments from the complex pattern of low-molecular weight bands found in the current study post-IMI with *E. coli* (ranging from 25 kDa to 10 kDa). Importantly however, they do not correspond with the molecular weight of those band found in the current study post-IMI with *S. aureus*. The suggestion that MMPs are induced during the hosts' innate immune response against *E. coli* to inactivate IL-1beta is strengthened by our histological findings (Ito, Mukaiyama et al. 1996). Indeed, the epithelial layer post-IMI with *E. coli* was clearly different as seen on mammary gland sections, a deterioration which again was only mildly present post-IMI with *S. aureus*. Of relevance, a high NF-kappaB activity during mammary gland infection increases caspase-3 mediated cell budding and shedding of epithelial cells (Connelly, Barham et al. 2010). Although not shown during this study, the loosening of epithelia suggests a form of accelerated involution which is suggested in literature to be associated with the production of MMPs. Finally, there was also one additional band with a MW between 17.5 kDa and 20 kDa that was selectively present post-IMI with *S. aureus* and not post-IMI with *E. coli*. It is here hypothesized that the latter cleavage fragment might be the product of pathogen-associated proteases (i.e. auto-cleavage) as previously described (Li, Zhao et al. 1999).

Above mentioned arguments implicate the involvement of epithelial (MMPs) and neutrophilic (serine proteases) proteases in the maturation of proIL-1beta. Surprisingly, in both these mammary cell types we could also detect NF-kappaB activity upon immunohistochemical evaluation. Furthermore, it should be highlighted that till date, the precise origin of the mammary proIL-1beta protein remains vague. Nevertheless, from the current study it is clear that the responsible transcription factor inducing the IL-1beta proform is certainly active prior to 12h post-IMI with both pathogens. However, its maturation occurs only shortly before 12h post-IMI with *E. coli*, while for *S. aureus* this process occurs about 12h later i.e. around 24h. Furthermore, our immunohistochemical data unequivocally demonstrated that for both bacteria the main subunit p65 of the transcription factor NF-kappaB is translocated to the nucleus of the murine mammary epithelial cells. The latter translocation is an essential step for activation of this key inflammatory transcription factor. To evaluate the level of NF-kappaB activation, both pathogens were compared with *in vivo* imaging in the reporter model previously established by our group for *E. coli* (Notebaert, Carlsen et al. 2008). As described for *E. coli* in this latter paper, a fast and strong NF-kappaB activation was again observed in the current study. However, in marked contrast, an on average 3 times lower NF-kappaB activity was detected in the mammary gland for *S. aureus*. Remarkably, the transient enhancement of this NF-kappaB activity already peaked for both pathogens at 6h post-IMI. This observation highlights that next to the production of TNF-alpha, IL-1alpha and MCP-1, the production of proIL-1beta can be NF-kappa-induced but its activation is likely not. Of relevance in this context, there is still the possibility of other transcription factors which have been reported in udder infections such as activator protein-1 (AP-1) (Kim, Im et al. 2011), or peroxisome proliferator-activated receptors (PPARs) (Mandard and Patsouris 2013), or regulatory factors such as CAAT box enhancer binding proteins (C/EBPs) (Liu, Shi et al. 2011).

Regardless the underlying transcription mechanism, our data overall imply that proIL-1beta is both highly and differentially processed within 12h to 24h post-IMI with both mastitis pathogens, which suggests its tight regulation. Based on the intramammary infections of IL-1beta KO C57BL/6 mice, IL-1beta affects local bacterial growth of Gram-negative to a low extent and the reduction of bacterial growth following a Gram-positive bacterial infection was even influenced to a lesser extent. Moreover, the revealed maturation process is certainly also non-classical as it occurs independently from the classical caspase-1 pathway. As a differential IL-1beta fragmentation pattern was observed after Gram-negative compared to Gram-positive mastitis, the cytokine protein profile data from the current study support the hypothesis that *E. coli* and *S. aureus* overall induce a markedly different IL-1beta activity. Our data also corroborate previous findings in KO C57BL/6 mice demonstrating that neutrophil IL-1R signaling in mammary gland inflammation mediates neutrophil influx from the interstitium and capillaries into the lumen of the alveoli (Elazar, Gonen et al. 2010). Additionally these innate immune cells restrict *E. coli* invasion into the mammary epithelial cells, which is only a characteristic of *S. aureus* infections in wild type mice (Elazar, Gonen et al. 2010). We hypothesize that proIL-1beta is locally secreted by the mammary epithelial cells and that multiple fragments likely result from the molding of bacterial proteases and/or NF-kappaB-induced non-caspase proteases secreted from neutrophils (i.e. serine proteases) and mammary epithelial cells (i.e. MMPs). Following infection with both pathogens, the IL-1beta proform is likely present in the interstitium where it is

cleaved by serine proteases produced by NF-kappaB activity in neutrophils, which are already sensitized by the preceding release of other pro-inflammatory cytokines following epithelial NF-kappaB signaling. Upon *E. coli* infection in mice, the host rapidly induces an efficient protective program that involves mammary NF-kappaB transcription to enhance TNF-alpha (*E. coli* mediated immune response). Interestingly, TNF-alpha is a well-known inducer of MMPs, which subsequently accelerates mammary gland involution (Black, Kronheim et al. 1988; Balasubramanian, Fan et al. 2011). Subsequently, proIL-1beta becomes inactivated by additional cleavage into fragments abolishing an additional neutrophil influx into the lumen but increasing the number of shedded epithelial cells in order to restrict bacterial growth as well as neutrophil cytotoxicity (Monks, Rosner et al. 2005). This explains the lower IL-1beta levels at 24h post-IMI versus 12h post-IMI with *E. coli*. In marked contrast, NF-kappaB is also rapidly but far less activated after *S. aureus* and thus a delayed influx of neutrophils occurs. Moreover, significantly less TNF-alpha is released and thus MMPs will not cause their typical IL-1beta cleavage patterns. In contrast to the early neutrophil-mediated *E. coli* mediated immune response, our data are indicative for the activity of *S. aureus*-derived proteases that alternatively cleave proIL-1beta enabling the typical evasion of these Gram-positive pathogens from the alveolar lumen.

These innovative insights obtained in mice revealed at first a sentinel function for IL-1beta during the early innate inflammatory response to mammary gland infections. Additionally, they highlight the limitations of single analysis methods. To acknowledge the proposed novel molecular mechanism of proIL-1beta in the early innate immune signaling during mastitis, additional studies are mandatory. Attractive options to extend our current findings are either the use of acute models with other relevant mastitis-associated germs (Demon, Breyne et al. 2013), or of more complex chronic mouse mastitis models (Brouillette and Malouin 2005) and last but not least of different mammalian target species, especially dairy animals.

I.1.6. Acknowledgements

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ADDENDUM

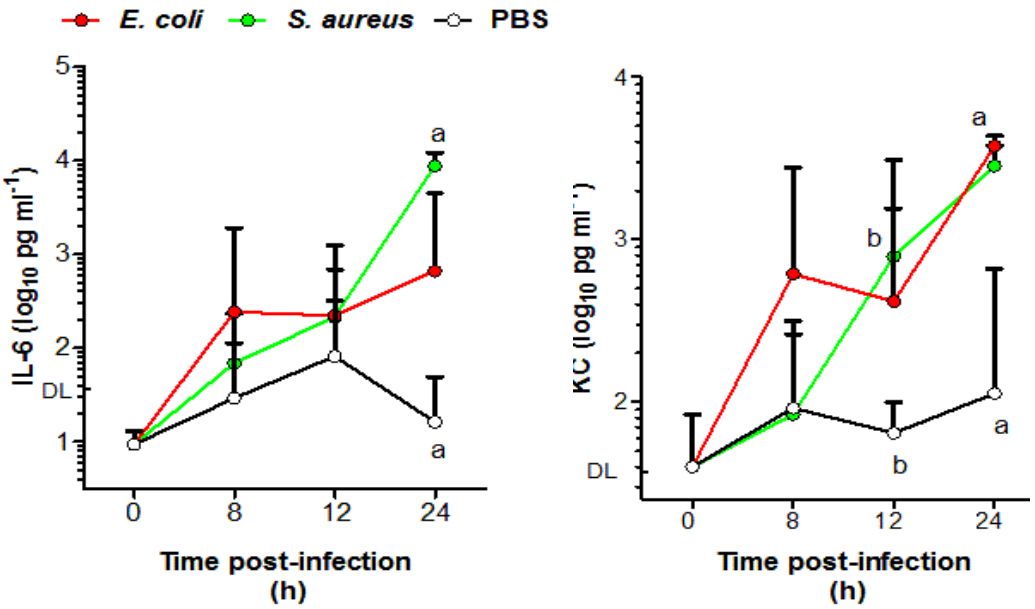
Differences secondary to the local immune response in mice post-IMI with *E. coli* versus post-IMI with *S. aureus* could be detected based on serum and hepatic cytokines, hepatic NFkappaB-activity and body temperature.

The levels of IL-6 and KC in blood were marked by a response post-IMI with *S. aureus* or post-IMI with *E. coli,* respectively (Fig. S1A). IL-6 levels in blood were higher post-IMI with *S. aureus* compared to sham-inoculated (PBS) glands and showed a trend to be higher than *E. coli*-inoculated animals. This observation could be linked to the susceptibility of mice to the virulence of *S. aureus* because temperature regulation is associated with systemic IL-6 levels (Shalaby, Waage et al. 1989; Chai, Gatti et al. 1996; Stewart, Landseadel et al. 2010) (see further). Therefore, the higher increase in systemic IL-6 levels at 24h post-IMI is associated with the hypothermia post-IMI with *S. aureus* (Fig. S1C). The KC blood levels were significantly higher post-IMI with *E. coli* compared to PBS. It is known that a chemotactic gradient of KC guides blood neutrophils into the mammary alveolar lumen (Bucerius, Naubereit et al. 2008). As such, this observation is in line with the earlier detected neutrophil influx post-IMI with *E. coli* compared to *S. aureus.* Furthermore, levels of TNF-alpha, IL-1alpha, MCP-1 and IL-1beta in the blood were not elevated compared to the sham-inoculated mice.

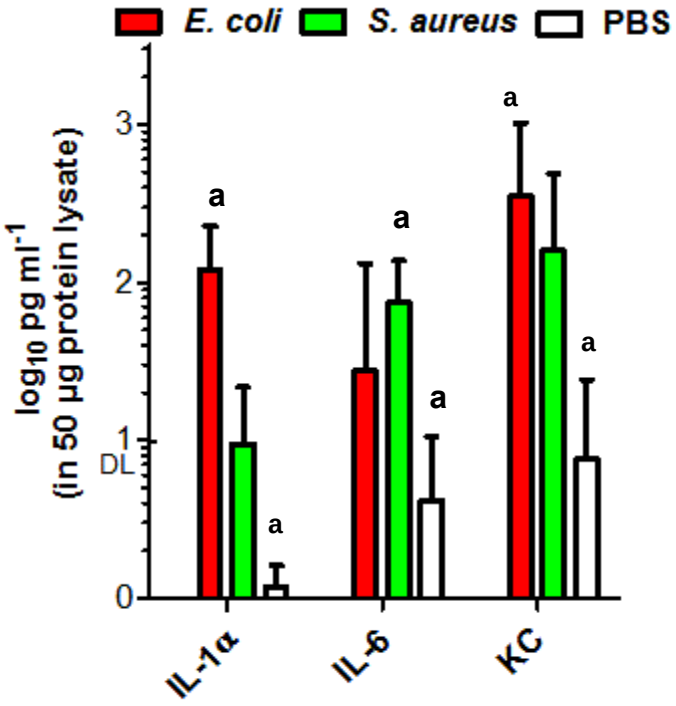
As shown in Fig. S1B and Fig. S2, elevated cytokines and NF-kappaB transcription were also detected in the liver at 24h post-IMI both pathogens compared to sham-inoculated (PBS) mice. Similarly as in the blood, hepatic IL-6 and KC were also elevated post-IMI with *S. aureus* and post-IMI with *E. coli* compared to the sham-inoculated mice. As the liver controls the homeostasis, hepatic production of cytokines (van Meijl, Popeijus et al. 2010) and acute phase proteins (Trautwein, Boker et al. 1994) are likely to control the systemic inflammatory responses (Tacke, Luedde et al. 2009). As already suggested by our group post-IMI with *E. coli* (Notebaert, Carlsen et al. 2008), both *E. coli* and *S. aureus* induced a high NF-kappaB activity in the liver.

The mice were significantly influenced by the *S. aureus* pathogenicity in contrast to *E. coli* or sham-inoculated (PBS) mice as reflected in the drop in body temperature (Fig. S1C). This observation is in line with previous reports. Corroborating characteristics of nearly all *S. aureus* strains, this subspecies is characterized by hemolysin and coagulase activity (Bhakdi and Tranum-Jensen 1991; Dinges, Orwin et al. 2000; Brouillette, Grondin et al. 2004; Bouchard, Peton et al. 2012). Both these exotoxins are associated with high virulence in the mouse mastitis model (Jonsson, Lindberg et al. 1985). Mice are in particular sensitive to the alpha-hemolysin/alpha-toxin (*hla*), especially when *S. aureus* bacteria are introduced in the mammary gland at a high inoculum dose. This was elegantly demonstrated by the fact that depletion of the toxin in the *S. aureus* strain -when inoculated at the same high dose- completely reversed the *S. aureus* lethality during mastitis in mice (Bramley, Patel et al. 1989).

A


E. coli
S. aureus
PBS
IL-6 (log10 pg ml-1)
KC (log10 pg ml-1)
DL
Time post-infection (h)
Time post-infection (h)
a
b

B


E. coli
S. aureus
PBS
log10 pg ml-1 (in 50 µg protein lysate)
DL
IL-1α
IL-6
KC
a

C

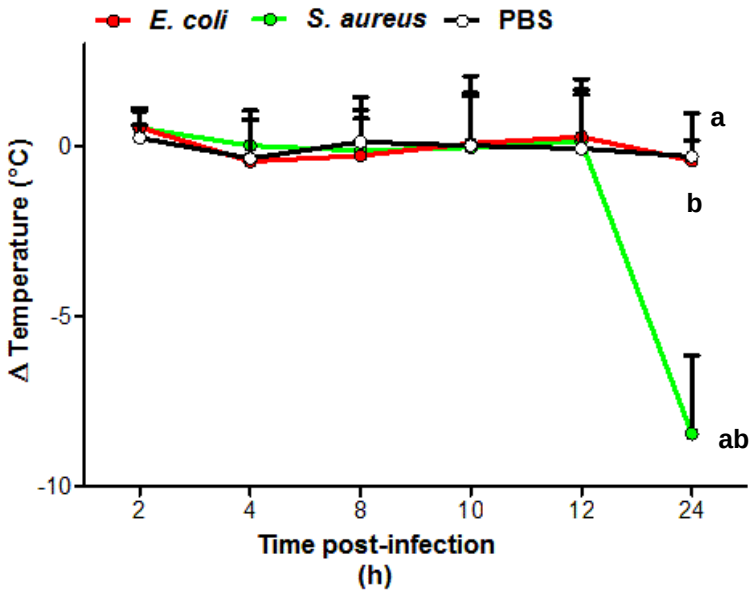
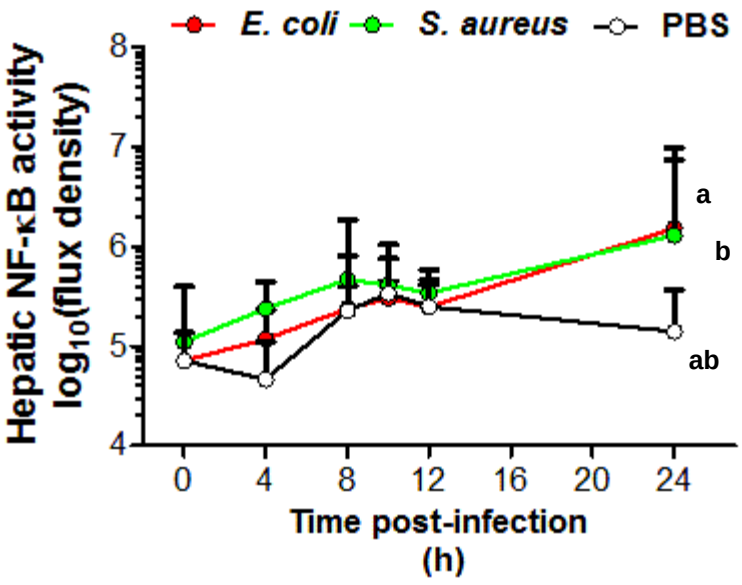
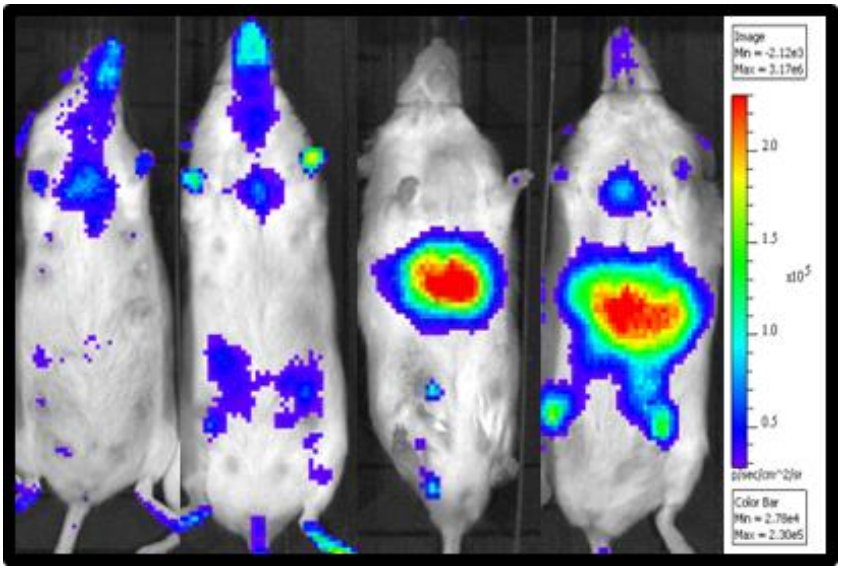


Figure S1. Secondary host response post-IMI with *E. coli* or *S. aureus*. (A) IL-6 and KC blood values post-IMI with *E. coli* and *S. aureus* compared to the sham-inoculated (PBS) and lactating (0h) negative controls. (B) Hepatic IL-1alpha, IL-6 and KC values 24h post-IMI with *E. coli* or with *S. aureus* compared to sham-inoculated (PBS) negative controls. (C) Changes in core body temperature post-IMI with *E. coli* or with *S. aureus*. Results are presented as mean ± standard deviation. Letters of homogeneous subsets were marked if the difference between treatments were statistically significant (P < 0.05). DL = detection limit.

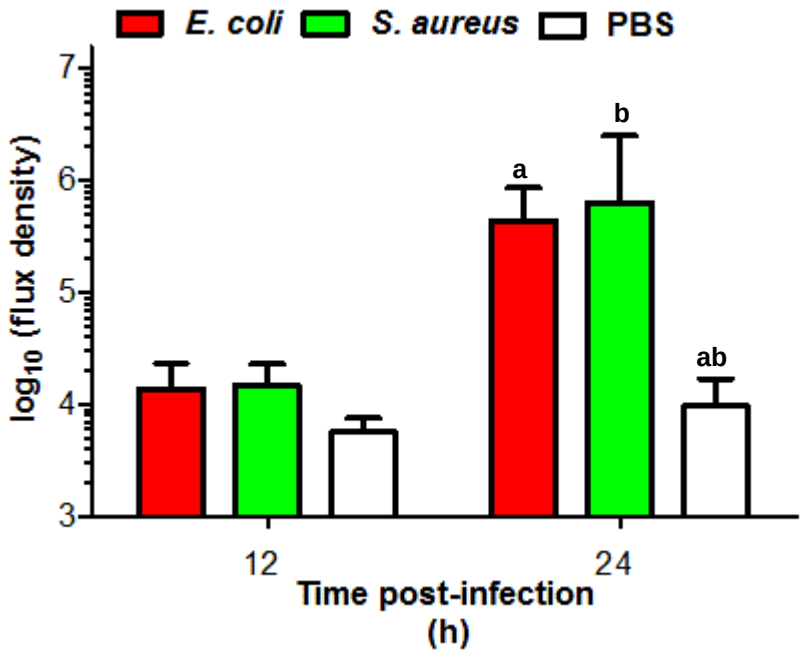
A



B


PBS
E. coli
LAC
S. aureus

C


E. coli
S. aureus
PBS
log10 (flux density)
7
6
5
4
3
a
b
ab
12
24
Time post-infection
(h)

D

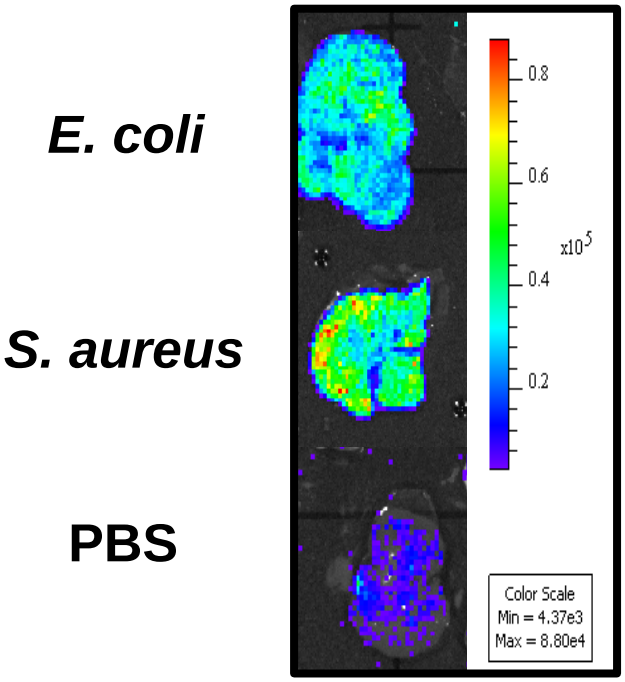


Figure S2. Hepatic NF-kappaB activity in transgenic reporter mice post-IMI with *E. coli* or *S. aureus*. (A) *In vivo* imaging follow-up of NF-kappaB activity in the liver (ventral view) after an IMI with *E. coli* or *S. aureus*. The data represents the flux density (total flux radiance per selected body area (in p*(s*m^2))). (B) Representative photograph of lactating mice (most left) compared to sham-inoculated (PBS, 2nd from left) mice at 24h post-IMI with *S. aureus* (3rd from left), or *E. coli* (most right). The intensity of luminescence was scaled based on the radiance (in p*(s*m^2)). (C) Livers of NF-kappaB reporter mice were excised at 12h and at 24h post-IMI, and their luminescence was measured *ex vivo.* The graph represents the total flux radiance per area (in p*(s*m^2)) as a measure for the NF-kappaB activity. (D) Representative photographical view of excised livers post-IMI with *E. coli* (top), *S. aureus* (middle) or PBS (bottom). Results are presented as mean ± standard deviation. Letters of homogeneous subsets were marked if the difference between treatments were statistically significant (P < 0.05).

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I.2. A Pilot Study using a Mouse Mastitis Model to study Differences between Bovine-Associated Coagulase-Negative Staphylococci

I.2.1. Abstract

Coagulase-negative staphylococci (CNS) are a group of bacteria classified as minor mastitis pathogens. Recent research suggests species- and even strain-related epidemiological and genetic differences within the large CNS group. The current pilot study investigated in two experiments whether a mouse mastitis model validated for bovine *Staphylococcus aureus* (*S. aureus*) can be used to explore further differences between CNS species and strains. In a first dose-titration experiment, a low inoculum dose of *S. aureus* Newbould 305 (positive control) was compared to increasing inoculum doses of a *S. chromogenes* strain originating from a chronic bovine intramammary infection (IMI) (*S. chromogenes* IM), and to sham-inoculated mammary glands (negative control). In contrast to the high bacterial growth following inoculation with *S. aureus*, *S. chromogenes* was retrieved in very low levels at 24h post-induction (p.i.). In a second experiment, the inflammation inflicted by three CNS strains was studied in mice. The host immune response induced by the *S. chromogenes* IM strain was compared with the one induced by a *S. fleurettii* strain originating from cow bedding sawdust and by a *S. chromogenes* strain originating from a teat apex of a heifer (*S. chromogenes* TA). As expected, at 28h and 48h p.i. low bacterial growth and local neutrophil influx in the mammary gland were induced by all CNS strains. As hypothesized, bacterial growth p.i. was the lowest for *S. fleurettii* compared to that induced by the two *S. chromogenes* strains and the overall immune response established by the three CNS strains was less pronounced compared with the one induced by *S. aureus*. Pro-inflammatory cytokine profiling revealed that *S. aureus* locally induced IL-6 and IL-1beta but not TNF-alpha, while overall CNS-inoculated glands lacked a strong cytokine host response but also induced IL-1beta locally. Compared to both other CNS strains, *S. chromogenes* TA inflicted a more variable IL-1beta response characterized by a more intense local reaction in several mice.

This pilot study suggests that an intraductal mouse model can mimic bovine CNS mastitis and has potential as a complementary *in vivo* tool for future CNS mastitis research. Furthermore, it indicates that epidemiologically different bovine CNS species/strains induce a differential host innate immune response in the murine mammary gland.

I.2.2. Introduction

Coagulase-negative staphylococci (CNS) are a group of bacteria that cause mastitis in both heifers and multiparous cows (Thorberg, Danielsson-Tham et al. 2009; De Vliegher, Fox et al. 2012; Ajitkumar, Barkema et al. 2013). Until recently, it was difficult to draw consistent conclusions on the relevance of CNS for bovine udder health. Some studies considered CNS as true mastitis pathogens although mostly retrieved from subclinical mastitis cases (Pyorala and Taponen 2009), while others considered CNS to be bacteria with limited or absent negative effects on SCC, milk quality and milk production (Schukken, Gonzalez et al. 2009; De Vliegher, Fox et al. 2012). The development and validation of molecular identification techniques specifically for bovine-associated CNS were the basis

to eliminate this confusion (Zadoks and Watts 2009). While older studies dealt with CNS as one homogenous and coherent group (Schukken, Gonzalez et al. 2009; Piepers, Opsomer et al. 2010; Piepers, Schukken et al. 2013), more recent studies addressed the CNS species separately revealing heterogeneous characteristics between them (Åvall-Jääskeläinen, Koort et al. 2013). In essence, 12 CNS species are frequently isolated from bovine milk. Evidence exists for diversity between and even within those species in their epidemiologic behavior and traits such as persistence, antibiotic resistance, susceptibility to phagocytosis by mouse macrophages and (putative) virulence (Piessens, Van Coillie et al. 2011; Supré, Haesebrouck et al. 2011; Åvall-Jääskeläinen, Koort et al. 2013; Vanderhaeghen, Piepers et al. 2014; Vanderhaeghen, Piepers et al. 2014).

To make further progress in our understanding of CNS mastitis, the interaction between CNS species and strains and the host should be studied in more detail. Ideally, such experimental infection studies are conducted using dairy cows. Unfortunately, these bovine studies are expensive and labour-intensive. For those reasons, a mouse mastitis model was developed and characterized (Chandler 1970) and has since been successfully validated for studying the specific host immune response to intramammary infection (IMI) with major mastitis pathogens such as *Escherichia coli* and *Staphylococcus aureus* (*S. aureus*) (Brouillette and Malouin 2005; Demon, Ludwig et al. 2012; Demon, Breyne et al. 2013), yet not for CNS.

The usefulness of a mouse mastitis model as a complementary tool to investigate differences between bovine CNS species and strains was explored. Specifically, two experiments were performed to answer clear-cut objectives: (1) do bovine-associated CNS grow in the murine mammary gland, (2) do they induce mastitis in mice, and (3) is it possible to detect differences in bacterial growth, clinical symptoms and host immune response between CNS species or strains with a different origin using this mouse mastitis model?

I.2.3. Material and Methods

The set-up of the two experiments is outlined in Figure 1. For both experiments, eight-week-old female Hsd:ICR (CD1) mice that mated with ten-week-old male Hsd:ICR (CD1) mice (Harlan labouratories, Horst, The Netherlands) were used. Pups were weaned ± 10 days after parturition to ease the intraductal accessibility. All inoculations were performed two hours post weaning under isoflurane anesthesia combined with a long-acting analgesic buprenorphine (10 µg/kg Vetergesic, Patheon UK Ltd, Swindon, UK) using a 32-gauge blunt needle. During both experiments, core body temperature of the mice was measured with a rectal thermistor. All experiments were approved by the committee on the ethics of animal experiments of Faculty of Veterinary Medicine, Ghent University (Permit Number: EC2013/166). In a first dose-titration experiment, 4 increasing inoculum doses (i.e. 2.5×10^2, 2.5×10^3, 2.5×10^4 and 2.5×10^5 colony forming units (CFU)/100µl) of a bovine-associated *S. chromogenes* strain originating from a chronic IMI (*S. chromogenes* IM (Supré, Haesebrouck et al. 2011) were injected in the 4th gland pair of each of the 12 mice ($n_{mice/dose}$ = 3; $n_{glands/dose}$ = 6) to evaluate both bacterial growth and immune cell influx in the mammary glands 24h post-induction (p.i.). An additional number of 6 mice were inoculated with either a 100 µl sham solution (PBS + 10% glycerol) (n_{mice} = 3)

or 1 x 10^2 CFU/100µl *S. aureus* Newbould 305 (n_{mice} = 3), and included as negative and positive controls, respectively (Fig. 1A). Twenty-four hours p.i., all mice were first sedated by administering a mixture of ketamine (100 mg/kg Anesketin, Eurovet Animal Health BV, Bladel, The Netherlands) with xylazine (10 mg/kg; Xylazini Hydrochloridum, Val d'Hony-Verdifarm, Beringen, Belgium) intraperitoneally and subsequently euthanized. After euthanasia, all *S. chromogenes* inoculated glands ($n_{glands/inoculum}$ = 6) were isolated to determine their bacterial load. Also, the *S. chromogenes*-inoculated glands with clinical signs of inflammation after dissection (i.e. red color, hard, swollen), as well as the 2 sham-inoculated glands and the 2 *S. aureus*-inoculated glands were further processed for histology. To quantify bacterial growth, the mammary glands were weighed, homogenized and spotted (20µl) in serial logarithmic dilutions on Tryptic soy agar plates (Oxoid, Drongen, Belgium) overnight at 37°C to determine CFU/100µl and divided by the exact weight of a mammary gland (g). For the histologic sections, mammary glands were divided in three transverse proportions to validate the homogeneity of the infection: near the nipple (i.e. near the inoculation site), near the lymph node and near the back. Two mammary glands for every inoculation (sham-, *S. aureus*- and 10^5 CFU *S. chromogenes* IM-inoculated glands) were fixed in buffered 3.5% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 24h at room temperature (24°C). Subsequently, the samples were dehydrated and embedded in paraffin wax. Sections were deparaffinised, hydrated and stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA). As the inoculated mammary glands displayed clinical symptoms of inflammation following a *S. chromogenes* IM inoculum dose as high as 10^5 CFU but not at lower doses, only these CNS-inoculated glands were collected for histological evaluation.

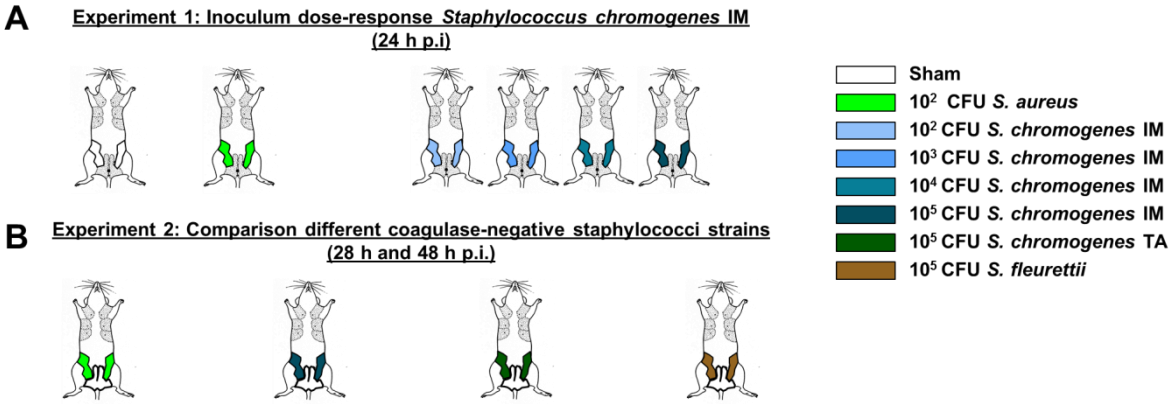

Figure 1. Schematic presentation of the set-up of the 2 experiments (A and B) performed in this study. The patterned glands represent the glands inoculated with *S. aureus*, or *S. chromogenes* intramammary (IM), or *S. chromogenes* teat apex (TA) or *S. fleurettii,* while the grey and dark grey mammary glands represent the unchallenged and the PBS/glycerol (sham)-inoculated mammary glands, respectively.

In the second experiment, two additional bovine CNS strains were included [a *S. fleurettii* strain originating from sawdust (Supré, Haesebrouck et al. 2011) and a *S. chromogenes* strain originating from a teat apex of a heifer (*S. chromogenes* TA) (De Vliegher, Opsomer et al. 2004)]. The 4th gland pair of each of the 18 mice ($n_{mice/CNS\ strain}$ = 6; $n_{glands/CNS\ strain}$ = 12) were injected with either *S. chromogenes* IM, *S. chromogenes* TA or *S. fleurettii* at an inoculum dose of approx. 10^5 CFU (9.6 x 10^4 CFU/100µl *S. chromogenes* IM, 9.3 x 10^4 CFU/100µl *S. chromogenes* TA and 8.5 x 10^4 CFU/100µl *S. fleurettii,* respectively). Three additional mice were inoculated with 2.5 x 10^2 CFU/100µl *S. aureus* Newbould 305 ($n_{mice/dose}$ = 3; $n_{glands/dose}$ = 6) and included as positive control (Fig. 1B). The 5th gland pair of each mouse was inoculated with 100µl sham solution and served as negative control. At 28h p.i., 12 mice (3 mice per CNS strain and 3 *S. aureus* Newbould 305-inoculated mice) were sedated and euthanized as earlier described. The other mice (n = 9) were sedated and euthanized at 48h p.i.. After euthanasia, the inoculated glands were isolated to determine bacterial growth, cytokine levels and histology. Bacterial growth was determined for every inoculum and time point as described. For cytokine quantification, mammary gland lysates were prepared from the mammary homogenates as previously reported (Breyne, Cool S. K. et al. 2014). Briefly, the IL-6, IL-1beta levels and TNF-alpha were determined in the protein suspensions (50µg) as described by the manufacturer with specific cytometric Bead Array kits (CBA, Becton Dickinson Biosciences, Erembodegem, Belgium) on a FACSArray instrument (Becton Dickinson Biosciences). For the histology, a sagittal section was used. Two mammary glands for every inoculum and time point were fixed and prepared as described. Statistical differences in bacterial growth at 28h and 48h p.i. and between the three CNS strains were determined using non-parametric or ANOVA tests (SPSS 20.0, IBM Corporation, Armonk, NY) with the log10-transformed CFU/ml, as outcome variables and the three CNS strains as categorical independent variable. A similar approach was followed to determine the association between the inoculum (*S. aureus*, *S. chromogenes* IM, *S. chromogenes* TA, or *S. fleurettii*) and the IL-6, IL-1beta and TNF-alpha concentration, respectively, either 28 h or 48h p.i.. For parametric testing, mean values of inoculation groups were compared through Tukey post-hoc testing. A *P*-value lower than 0.05 was considered statistically significant, values are represented as mean with standard deviation ($P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***).

Bacteria were extracted as described above from randomly chosen CNS-inoculated mammary glands ($n_{glands/CNS\ strain}$ = 4) to validate the results of both experiments and commensal microbiota was determined in unchallenged lactating glands and sham-inoculated mammary glands (n = 9 and n = 4). All colonies were counted on Tryptic soy agar plates and streaked onto Columbia agar (Oxoïd) to obtain pure cultures. After 24h aerobic incubation at 37°C, colonies were checked for purity and identified following National Mastitis Council (NMC) procedures (Hogan, Gonzalez et al. 1999). All CNS were identified to the species level using transfer RNA-intergenic spacer PCR (tDNA-PCR) (Supré, De Vliegher et al. 2009). If no identification could be obtained, isolates were subjected to sequencing of the 16S rRNA gene (Park, Fox et al. 2011).

I.2.4. Results

1.2.4.1. Experiment 1: Intramammary infection of mice with increasing inoculum doses of a *S. chromogenes* IM

Staphylococcus chromogenes IM-inoculated glands at 24h p.i. displayed higher bacterial levels compared to sham-inoculated glands. Still, bacterial growth in the *S. chromogenes* IM inoculated glands was limited compared to the *S. aureus* Newbould 305 inoculated glands, regardless of the inoculum dose (Fig. 2 and Table 1). The presence of *S. chromogenes* in the challenged glands was confirmed. A marked increase in mammary CFU p.i. was observed in *S. aureus* Newbould 305-inoculated glands compared to both the sham-inoculated glands as well as *S. chromogenes* IM inoculated glands (Fig. 2).

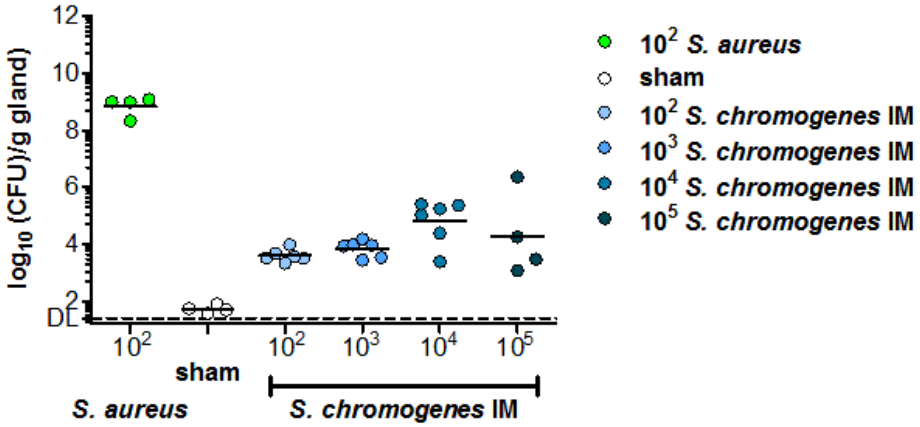

Figure 2. Bacterial growth (expressed as log_{10}CFU/g gland) in mammary glands at 24h post infection (p.i.) of sham- (PBS/glycerol), *S. aureus*- and *S. chromogenes* IM-inoculated mice of experiment 1. The line in each box represents the median of these individual datasets. The color and pattern code of each group correspond to these explained in Fig. 1. DL: detection limit.

Upon histological evaluation, both *S. aureus* and *S. chromogenes* IM induced an influx of immune cells in the alveoli (Fig. 3 – B and C) which was absent in sham-inoculated glands (Fig. 3 – A). The latter had an intact alveolar structure with a swollen luminal area at 24h p.i. with accumulated milk. In *S. aureus*-inoculated mammary glands, the general structural integrity of the alveoli was still present but the milk was replaced by a vast number of invading cells (Fig. 3 – B: white arrow heads) that were tentatively identified as neutrophils mainly based on their characteristic polymorphonuclear feature. In analogy, the alveoli from the *S. chromogenes* IM-inoculated glands were also packed with immune cells (Fig. 3 – C: white arrow heads). This influx of cells in the mouse mammary gland was however more invasive following *S. aureus* inoculation as red blood cells were clearly observed in the interstitium which was not the case after inoculation with *S. chromogenes* IM (Fig. 3 – B: black arrow heads).

Table 1. Overview of the amount of bacteria[1] in mammary glands at 24h post infection (p.i.) of sham- (PBS/glycerol), *S. aureus*- and *S. chromogenes* IM-inoculated mice of experiment 1.

	S. aureus	**sham**	***S. chromogenes* IM**			
CFU/inoculum	10^2	-	10^2	10^3	10^4	10^5
Minimum	8.32	1.57	3.34	3.44	3.38	3.08
25% Percentile	8.49	1.60	3.47	3.51	4.14	3.18
Median	9.00	1.72	3.54	3.95	5.13	3.87
75% Percentile	9.06	1.86	3.76	4.04	5.38	5.84
Maximum	9.08	1.89	3.99	4.19	5.41	6.37

[1]expressed as $\log_{10}$CFU/g gland

1.2.4.2. Experiment 2: Intramammary infection of mice with different CNS strains

For none of the 3 tested CNS strains, a significant overall change in the number of CFU between 28h and 48h p.i. was detected. At both time points p.i. no significant difference in bacterial growth was detected between *S. chromogenes* IM (6.94±1.56 $\log_{10}$CFU/g gland) and *S. chromogenes* TA (7.13±2.11 $\log_{10}$CFU/g gland) (Fig. 4). In contrast, *S. fleurettii* (4.41±1.01 $\log_{10}$CFU/g gland) was retrieved at significantly lower levels compared to both *S. chromogenes* strains ($P < 0.01$). Moreover, the first was not grown in the murine mammary gland but seems even decreased in $\log_{10}$CFU/g gland p.i. at 28h or 48h compared to the administered inoculum. In analogy with the first experiment, *S. aureus* displayed an exponential bacterial growth compared to each of the CNS p.i..

The presence of each inoculated strain p.i. was confirmed. No distinct differences in histological features were observed between both time-points p.i.. All tested strains induced an influx of immune cells of which the majority likely consisted of neutrophils. Still, in marked contrast to *S. aureus*, no vascular congestion was observed in the interstitium after challenge with each of the CNS strains (Fig. 5 – A, B, C and D). Corroborating the results of the first experiment, sham-inoculated control gland alveoli had an enlarged lumen filled with milk and lacked the presence of immune cells (Fig. 5 – E). Both *S. chromogenes* strains had a similar scattered distribution of immune cells in contrast with the densely packed neutrophils retrieved in the alveolar lumen of the mouse mammary glands upon inoculation with *S. fleurettii* (Fig. 5 – A , B and C).

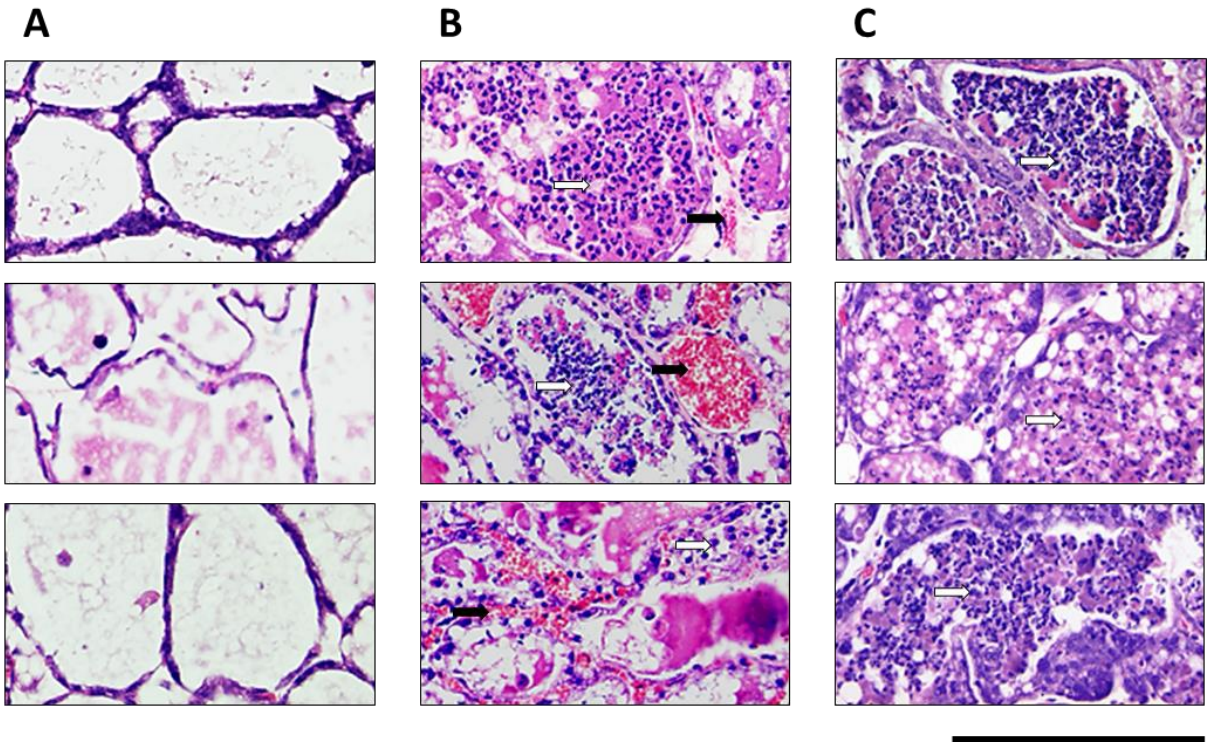

Figure 3. Representative hematoxylin and eosin-stained sections of (A) sham- (PBS/glycerol) (B) *S. aureus*- and (C) *S. chromogenes*-inoculated mammary glands isolated from mice of experiment 1. The histology images in the upper panel are taken from sections sampled near the nipple, the ones in the middle panel near the subiliac lymph node and the ones in the lowest panel near the dorsal gland tissue. The white arrows mark the immune cell content in the alveoli, while the black arrows point to the red blood cells in the interstitium. The scale bar represents a length of 200µm.

Although a lower inoculum dose was injected, cytokine quantification revealed significantly higher IL-6 levels in the *S. aureus*-inoculated glands (3.11±0.17 $\log_{10}$IL-6 pg/ml) than in the sham- (0.93±0.19 $\log_{10}$IL-6 pg/ml) or CNS-inoculated glands (1.15±0.56 $\log_{10}$IL-6 pg/ml, $P < 0.001$; Fig. 6 – A). Furthermore, significant differences in the local IL-6 concentrations were observed neither between the three CNS strains nor between the two time points p.i. within strains nor between the sham- and CNS-inoculated glands. A similar trend was observed for the local IL-1beta levels which were substantially higher in *S. aureus*-inoculated glands (2.97±0.14 $\log_{10}$IL-1beta pg/ml), compared to the sham- (1.06±0.42 $\log_{10}$IL-1beta pg/ml, $P < 0.001$) and CNS-inoculated glands (1.87±0.61 $\log_{10}$IL-1beta pg/ml, $P < 0.05$; Fig. 6 - B). Still, the differences in local IL-1beta concentrations induced by *S. aureus* compared to *S. chromogenes* IM, *S. chromogenes* TA and *S. fleurettii* was less pronounced between *S. aureus* and *S. chromogenes* TA (i.e. 0.9±0.24 $\log_{10}$IL-1beta pg/ml, n = 30; $P < 0.05$) than between *S. aureus* and the two other CNS-strains (i.e. 1.07±0.16 $\log_{10}$IL-1beta pg/ml for *S. chromogenes* IM, $P < 0.001$ and 1.34±0.15 $\log_{10}$IL-1beta pg/ml for *S. fleurettii*, $P < 0.001$) (Fig. 6 – B). There was again no significant difference in local IL-1beta concentrations between the two time points p.i. Notably, there was a small difference between the CNS- and sham-inoculated glands in their IL-1beta concentration

(i.e. 0.92±0.26 log_{10}IL-1beta pg/ml, n = 30; $P < 0.05$) for both *S. chromogenes* strains but not for *S. fleurettii* and sham-inoculated glands. TNF-alpha levels remained below the quantification limit for CNS-, *S. aureus*- and sham-inoculated glands. Two out of 6 mice inoculated with *S. chromogenes* TA had a severe local irritation (one mouse at 28h p.i. and one at 48h p.i.). In these glands (n = 4), a more pronounced bacterial growth as well as higher local IL-6 and IL-1beta concentrations were observed compared to other glands inoculated with the same strain, explaining the high variation for *S. chromogenes* TA in Fig. 6 A and B. Such distinct differences in response among mammary glands inoculated with the same strain were not observed for *S. chromogenes* IM or *S. fleurettii*. Upon infection with each of the three CNS strains the body temperature remained normothermic (36.6 ± 0.1 °C; n = 21). This was in marked contrast to *S. aureus*-inoculated mice, which initially also remained normothermic until at least 24h p.i. but became severely hypothermic (drop in body temperature of 7 ± 1.6 °C, $P < 0.001$; n = 3) within 48h p.i..

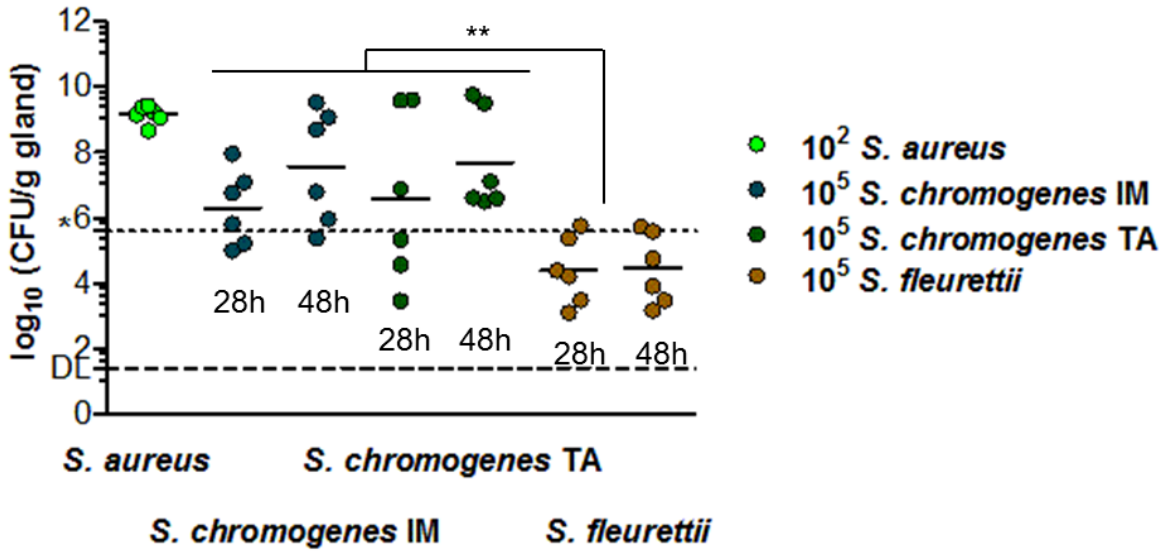

Figure 4. Bacterial growth (expressed as log_{10}CFU/g gland) in the *S. aureus*-, *S. chromogenes* IM-, *S. chromogenes* TA- and *S. fleurettii*-inoculated mammary glands isolated from mice of experiment 2 after 28h (left box) or 48h (right box). The line in each box represents the median of these individual datasets. ($P < 0.01$, **). The dotted line (*) represents the log_{10}CFU CNS/g gland injected in the mammary gland of CNS-inoculated mice. The color and pattern code of each group correspond to the one clarified in Fig. 1. DL: detection limit.

Because CNS can be retrieved in the milk of clinically healthy lactating dairy cows (Oikonomou, Bicalho et al. 2014), the residential microbiota in mammary glands of mice was verified in lactating and sham- inoculated glands of both experiments. Twenty-three percent of the unchallenged mammary glands were sterile. When non-inoculated glands contained bacteria (1.74 ± 0.77 log10CFU/g gland) a limited number of bacterial species were extracted and genotypically identified from the remaining glands: *Staphylococcus lentus* (23%), *Kocuria* spp. (7%), *Bacillus* spp. (7%), *Alcaligenaceae* spp. (15%), *Streptococcus* spp. (15%) and *Microbacterium* spp. (15%) (Table 2).

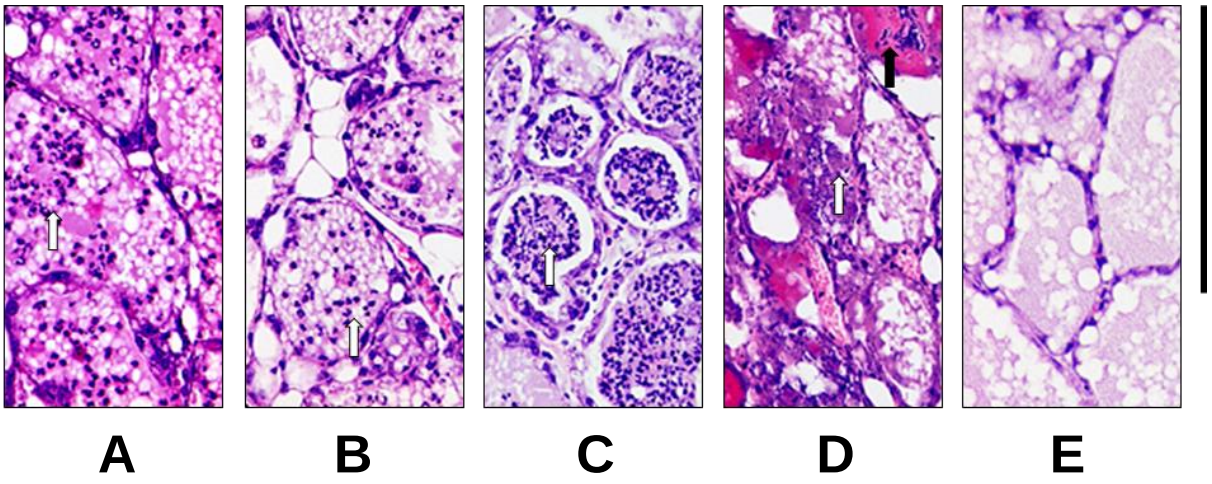

Figure 5. Representative hematoxylin and eosin-stained sections of (A) *S. chromogenes* IM-, (B) *S. chromogenes* TA-, (C) *S. fleurettii-,* (D) *S. aureus*- and (E) sham (PBS/glycerol)-inoculated mammary glands isolated from mice at 48h post inoculation of experiment 2. The white arrows mark the immune cell content in the alveoli, while the black arrows point to the red blood cells in the interstitium. The scale bar represents a length of 200µm.

I.2.5. Discussion

This pilot study explored the usefulness of a previously validated mouse mastitis model for bovine CNS research, which has been mentioned as relevant for human CNS research (Thomsen, Mogensen et al. 1985). A valid mouse model for CNS mastitis would circumvent the major drawbacks of cow challenge experiments. In the first experiment, our data unambiguously displayed limited growth in the murine mammary gland of the bovine CNS isolate that was administered. At 24h p.i., the glands inoculated with the highest inoculum dose of 10^5 CFU of CNS colored red and had a relatively mild alveolar influx of immune cells compared to the mammary glands that were inoculated with *S. aureus.* The latter results corroborate the observations in cows where high inocula of CNS are necessary to obtain colonization in the udder as described for *S. chromogenes*, *S. simulans* and *S. epidermis* (Simojoki, Orro et al. 2009; Simojoki, Salomaki et al. 2011). Moreover, -in contrast to mastitis caused by *S. aureus,* mastitis induced by CNS is less severe and accompanied by mild local signs such as slight swelling of the udder and color changes in the milk (Taponen and Pyorala 2009). Remarkably, sham-inoculated glands were only occasionally sterile. Molecular identification of the bacteria isolated from these glands suggests they originate from the pups as they are closely related to the commensal microbiota (Martin, Langa et al. 2004) from the murine gut and respiratory system (Rodrigue and Lavoie 1996; Shim, Seo et al. 2009; Dimitriu, Boyce et al. 2013). These bacteria were retrieved in limited number compared to the inoculum and are therefore not considered to have influenced our results to a large extent. Actually, the same species are also isolated from dairy products i.e. raw milk or cheese (Quigley, O'Sullivan et al. 2011). To our knowledge this is the first time that resident bacteria from murine mammary glands were considered in respect to experimentally induced mastitis in this laboratory animal. In order to have a global profile of the microbiota in the mammary gland of mice a meta-genomic analysis should be performed in future research.

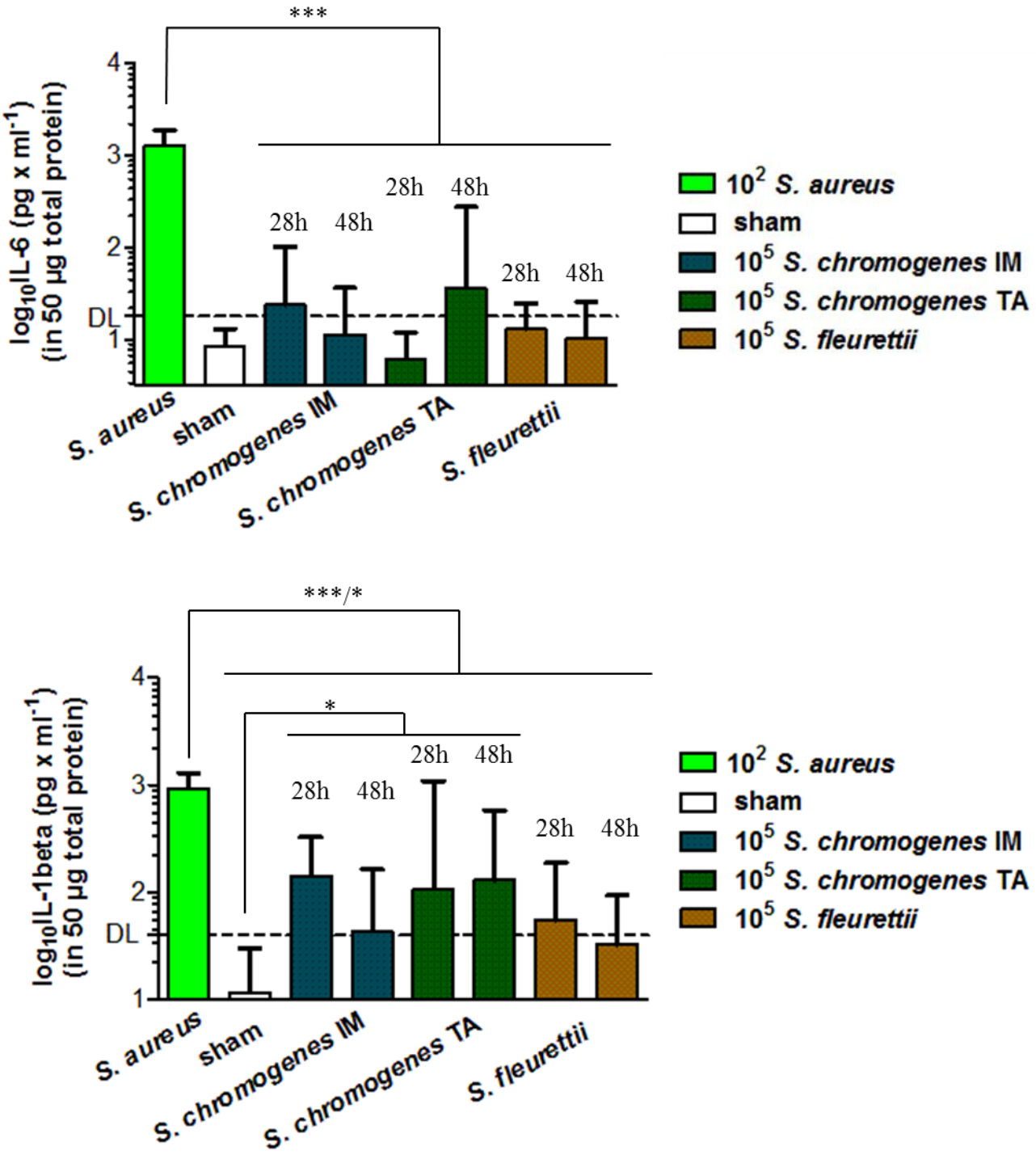

Figure 6. IL-6 (A) and IL-1beta (B) cytokine levels (expressed as pg/ml cytokine in 50µg total protein) of *S. aureus*-, sham (PBS/glycerol)- and CNS-inoculated mammary glands isolated from mice of experiment 2 after 28h (left box) or 48h (right box). The line in each box represents the median of these values. (*P* < 0.001, *; *P* < 0.05; *). The color and pattern code of each group correspond to the one clarified in Fig. 1. DL: detection limit.**

In the second experiment, the mouse mastitis model allowed the comparison between three CNS isolates differentiated by their anticipated main reservoirs. Again, limited growth of CNS p.i. was observed compared to a *S. aureus* challenge although each of the CNS strains as well as the *S. aureus* caused a neutrophil influx following challenge. Additional cytokine data implied in general only a moderate pro-inflammatory immune response to a challenge with a CNS strain in comparison with the one to *S. aureus*. This corroborated bovine studies as IL-6 is retrieved following *S. aureus* IMI in contrast to CNS IMI (Hagiwara, Yamanaka et al. 2001). TNF-alpha was not detectable post-inoculation with CNS and/or *S. aureus*. This corroborates TNF-alpha is highly induced by Gram-negative mastitis pathogens in cows (Bannerman 2009) and in mice (Notebaert, Carlsen et al. 2008) compared to Gram-positive infections in mice. The higher virulence of *S. aureus* versus each of the tested CNS isolates suggested through histology (experiment 1 and 2) and limited cytokine profiling (experiment 2) could be further endorsed with the overall clinical findings in the mice. It should be emphasized that the host innate immune response generated by *S. aureus* challenge was previously reported by several groups (Bramley, Patel et al. 1989; Bhakdi and Tranum-Jensen 1991; Dinges, Orwin et al. 2000; Brouillette, Grondin et al. 2004; Chinchali and Kaliwal 2014) and therefore not unexpected. Our results substantiate the high sensitivity of mice towards the toxins produced by *S. aureus* in this *in vivo* mastitis model (Jonsson, Lindberg et al. 1985). The absence of coagulase production in a transformed *S. aureus*, remarkably diminished its intramammary virulence in mice (Jonsson, Lindberg et al. 1985), and most likely also relates to the minimal virulence of CNS isolates in mice at first observed in the current study. This lower pathogenicity of the CNS isolates compared to *S. aureus* explains the high prevalence of the former pathogens in milk of clinically healthy cows and their less detrimental effects during bovine mastitis (Pate, Zdovc et al. 2012; Braem, De Vliegher et al. 2013; Oikonomou, Bicalho et al. 2014).

Importantly, our data also reflect - at least to some extent - the difference observed in cows between the so-called environmental *S. fleurettii* and the so-called host-adapted *S. chromogenes* IM and *S. chromogenes* TA. Corroborating our bacterial growth, cytokine level data, the discriminating grouping of presumed neutrophils in the murine mammary gland sections, and the low prevalence in milk samples of cows allowed to speculate that *S. fleurettii* is able to colonize the milk habitat but is less likely to multiply locally compared to both *S. chromogenes* isolates (Piessens, Van Coillie et al. 2011; De Visscher, Supré et al. 2014). Although the number of animals should be increased to allow for a precise interpretation of the statistical relevance of our results, these suggest an underlying difference in the hosts' innate immune response between *S. chromogenes* IM and *S. chromogenes* TA. More specifically, the *S. chromogenes* IM strain might be more adapted to the intramammary niche (milk environment) and as such induce less pathophysiological effects compared to the likely more teat-adapted *S. chromogenes* TA.

In conclusion, this study suggests that, as previously demonstrated for *S. aureus,* the mouse model can be an attractive complementary *in vivo* tool for bovine CNS mastitis research, however minding the limitations such as the presence of a commensal murine mammary gland microbiota. Nevertheless, it overall mimics the mild inflammation in cows induced by an IMI with CNS, through a

neutrophil influx accompanied either with or without a low induction of certain pro-inflammatory cytokines. Moreover, the limited data of our pilot study tentatively suggest that the CNS mouse model can distinguish the differences in host response inflicted by different isolates of the CNS group. Future research is however warranted and should include infection studies with longer duration besides the comparison of even more bovine CNS isolates. This would allow drawing reliable conclusions on the specific differences between CNS isolates as observed in cows. Moreover, analyzing the influence of the mouse strain would be valuable for the in-depth validation of our mouse mastitis CNS model. At last, this mouse model is potentially also an elegant tool to explore the inflammatory factors including cytokines that are enhanced by CNS when inducing a local neutrophil influx.

I.2.6. Acknowledgements

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ADDENDUM

Table 2. Residential microbiota in the mammary gland of mice.

treatment[1]	mammary gland[2]	replicates	dilution	number of colonies[3]	result
lactating	1	A	1	-	-
lactating	1	B	1	1	*Staphylococcus lentus*
lactating	2	A	1	1	*Staphylococcus lentus*
lactating	2	B	1	1	*Staphylococcus* spp.
lactating	3	A	1	1	*Kocuria* spp.
lactating	3	B	1	-	-
lactating	4	A	1	-	-
lactating	4	B	1	-	-
lactating	5	A	1	2	*Microbacterium* spp.
lactating	5	B	1	1	*Microbacterium* spp.
lactating	6	A	1	1	*Bacillaceae*
lactating	6	A	1	1	*Staphylococcus lentus*
lactating	6	B	1	1	*Staphylococcus lentus*
lactating	7	A	1	-	-
lactating	7	B	1	-	-
lactating	8	A	1	-	-
lactating	8	B	1	-	-
lactating	9	A	1	-	-
lactating	9	B	1	2	*Microbacterium* spp.
sham	1	A	1	-	-
sham	1	B	1	1	*Streptococcus* spp.
sham	1	A	2	55	*Streptococcus* spp.
sham	1	B	2	73	*Streptococcus acidominimus*
sham	2	A	1	6	*Streptococcus acidominimus*
sham	2	B	1	5	*Streptococcus* spp.
sham	2	A	2	31	*Streptococcus acidominimus*
sham	2	B	2	49	*Streptococcus acidominimus*
sham	3	A	1	31	*Alcaligenaceae*
sham	3	B	1	38	*Alcaligenaceae*
sham	3	A	2	5	*Alcaligenaceae*
sham	3	B	2	6	*Alcaligenaceae*
sham	3	A	1	22	*Alcaligenaceae*
sham	4	B	1	20	*Alcaligenaceae*
sham	4	A	2	2	*Alcaligenaceae*
sham	4	B	2	2	*Alcaligenaceae*

[1]lactating mammary glands were not injected and not weaned from the litter while sham-inoculated glands were weaned from the litter for at least 24h prior to inoculating with PBS. [2] Different number represents a different mammary gland. [3] The number of colonies with a distinct phenotype compared to other colonies.

CHAPTER

II

PREVENTIVE MASTITIS STRATEGY

Lipopolysaccharide (LPS) is the major cell wall component of the Gram-negative pathogen *Escherichia coli*. When host cells are exposed to this Microbe-Associated Molecular Pattern, LPS triggers host toll-like receptor 4 signalling that results in the production and secretion of a plethora of inflammatory mediators. In contrast, an initial LPS stimulus protectively dampens against the excessive inflammatory signalling due to endotoxin shock (Zingarelli, Hake et al. 2002; Morris and Li 2012). The phenomenon is confusingly referred to as endotoxin tolerance (ET) as it suggests a state of immune dysfunction or immunoparalysis (Varma, Durham et al. 2005). Although the molecular mechanisms are not yet fully understood, ET is now considered as a complex epigenetic reprogramming and not just a mere decrease in excessive sterile inflammation. Increasing reports using infectious mouse models with fungi, *Cryptococcus neoformans, Salmonella enteritica* or poly-microbial sepsis established that the altered inflammation during an ET phenotype is concomitant with either an increased bacterial clearance and/or decreased colonization of the infectious agent and an improved survival (Lehner, Ittner et al. 2001; Cavaillon and Adib-Conquy 2006; Wheeler, Lahni et al. 2008).

In the context of mastitis, repeated exposure to microbial pathogens or their MAMPs have high implications. The milkspace of the udder is rarely sterile and therefore superinfections are common. However, little is known about the phenomenon and somewhat contradictory reports have been published. Therefore, the **main aim of CHAPTER II** was to assess whether preconditioning of the mammary gland with LPS results in a beneficial effect during the later combat of bovine *S. aureus*-induced mastitis in mice.

The aim of CHAPTER II is adapted from: Breyne K., Vanden Berghe T., Demeyere K., and Meyer E., Immunomodulation by Lipopolysaccharide Protects while CCL5/RANTES Partially Protects Against an Intramammary Infection with Bovine *Staphylococcus aureus* in a Mouse Mastitis Model, *Manuscript in preparation*

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II.1. Immunomodulation by Lipopolysaccharide Protects While CCL5/RANTES Partially Protects Against an Intramammary Infection with Bovine Staphylococcus aureus in a Mouse Mastitis Model

II.1.1. Abstract

Studies with multiple mouse infection models demonstrated that pretreatment with lipopolysaccharide (LPS) - the major cell wall component of the Gram-negative pathogen *Escherichia coli* - can alter the hosts' immune state, increasing the resistance against infectious bacteria and reducing the severity of the symptoms. This phenomenon has been suggested to reduce the severity of *Escherichia coli*-mastitis in cows yet the underlying molecular mechanisms are not well understood. The present study aimed to demonstrate that LPS pretreatment can also be effective against *Staphylococcus aureus* (*S. aureus*) mastitis for preventive purposes. For this purpose, a mouse model was used and it was assessed how the host immune response is altered during this process. An intramammary LPS bolus prior to administration of the mastitis pathogen significantly decreased the local levels of inflammatory cytokines TNF-alpha, IL-1beta, IL-6, the IL-8 homologues (CXCL1/KC and CXCL2/MIP-2) and CCL3/MIP-1alpha. The observations were concomitant with a reduced bacterial load and severity of clinical symptoms upon exposure to *S. aureus*. In addition, a protein array analysis confirmed that the chemokines CCL5/RANTES and another IL-8 homologue (CXCL5/LIX) were selectively induced. Applying this novel insight, the increase of CCL5/RANTES prior to the intramammary *S. aureus* infection was shown to partially mimic the beneficial immunomodulation after pretreatment with LPS. These novel findings in a mouse mastitis model indicate that pretreatment LPS alters the host inflammatory response, resulting in a low local CFU of the infectious agent.

I.1.2. Introduction

Lipopolysaccharide (LPS) is the major cell wall component of the Gram-negative pathogen *Escherichia coli* (*E. coli*). When host cells are exposed to this Microbe-Associated Molecular Pattern (MAMP), it triggers host toll-like receptor 4 (TLR4) signalling that results in the production and secretion of a plethora of inflammatory mediators. In contrast, an initial LPS stimulus protectively dampens the excessive inflammatory signalling due to endotoxin shock (Zingarelli, Hake et al. 2002; Morris and Li 2012). This phenomenon is often and confusingly referred to as endotoxin tolerance (ET), a name which suggests a state of immune dysfunction or immunoparalysis (Varma, Durham et al. 2005). Although the underlying molecular mechanisms hereof are not yet fully understood, ET is now considered as a complex epigenetic reprogramming and its phenotype can be mediated by a multitude of agents such as TNF-alpha and IL-1beta (Ferlito, Romanenko et al. 2001), lipoteichoic acid (Lehner, Morath et al. 2001) and CpG DNA (Yeo, Yoon et al. 2003). In contrast to the initially assumed immunoparalysis state,– it is now clear that the suppression of pro-inflammatory factors (typically NF-kappaB induced signalling) during ET occurs concomitant with the activation of anti-microbial factors (Foster, Hargreaves et al. 2007; Biswas and Lopez-Collazo 2009; Liu, Yoza et al. 2011). It is now increasingly stated that this ET phenotype provides the host with an ideal strategy to reach a balance in its immune system. It allows to appropriately modify ongoing responses through cellular

reprogramming to focus on the phagocytosis of different infectious agents and dead cells and the healing of the affected site of infection (Pena, Pistolic et al. 2011). As such, increasing reports using infectious mouse models with fungi, Cryptococcus neoformans, Salmonella enteritica or poly-microbial sepsis established that the attenuated inflammation during an ET phenotype occurs concomitant with either an increased bacterial clearance and/or decreased colonization of the pathogen and improves survival (Lehner, Ittner et al. 2001; Cavaillon and Adib-Conquy 2006; Wheeler, Lahni et al. 2008). Remarkably, several aspects of the ET phenotype and its related anti-bacterial properties have widely been studied in macrophages, nevertheless, other innate immune cells such as neutrophils also seems to be prone to the ET concept (Landoni, Chiarella et al. 2012).

Extending the observations of LPS as the key MAMP for the ET concept, indications are now available showing that local pretreatment with LPS or bacteria can reduce the severity of a subsequent mammary gland infection (Petzl, Gunther et al. 2012). Such bacterial-induced inflammation is called mastitis and results from either chronically or acutely infected udders, being a major challenge in the dairy sector and affecting both milk quality and animal welfare (Waage, Mork et al. 1999; Piepers, De Meulemeester et al. 2007). Bacterial udder infections can be elicited by Gram-negative bacteria such as *E. coli* or by Gram-positive bacteria such as *Staphylococcus aureus* (*S. aureus*). Classical curative therapy to treat mastitis consists mainly of antibiotics (Vanderhaeghen, Cerpentier et al. 2010; Barlow 2011; Demon, Ludwig et al. 2012), while complementary anti-inflammatory treatment is given to ease clinical symptoms during acute mastitis (Shpigel, Chen et al. 1994). Despite all these efforts, mastitis is still difficult to eradicate from a herd. Therefore, the main objective of the current pilot study was to explore the potential of pretreatment with LPS in order to reduce the severity of a detrimental inflammatory host response, by decreasing the severity of symptoms while increasing bacterial clearance and/or colonization potential upon *S. aureus* mastitis.

II.1.3. Materials and Methods

II.1.3.1. Mice

Hsd:ICR (CD1) mice were purchased from a commercial supplier (Harlan labouratories, Horst, The Netherlands). The mice were fed *ad libitum* in a controlled environment that included light and dark cycles (12 hours (h) light:12 hours darkness). All the protocols that involved animals were approved by the Committee on the Ethics of Animal Experiments of the Faculty of Veterinary Medicine, Ghent University (Permit Number 2014/107). Eight-week-old female mice were mated with 10-week-old male mice. To ease the intraductal accessibility of the lactating mothers, the 10-day old pups were weaned 2 h before the first inoculation. All intraductal injections using a 32-gauge blunt needle were performed in the 4th gland pair of each of the mice under isoflurane anaesthesia combined with the long-acting analgesic buprenorphine (10 µg/kg Vetergesic, Patheon UK Ltd, Swindon, UK) and core body temperature was post-operative monitored with a rectal thermistor.

II.1.3.2. Intramammary infection model

For the injection regimen described in Fig. 1A, mice were injected with LPS (10 µg, Sigma-Aldrich, St. Louis, MO, USA) diluted in 100 µl phosphate buffered saline (PBS) 6 h prior to inoculation with *S. aureus* Newbould 305 (2.5 x 10^2 colony forming units or CFU/100µl PBS; n_{mice} = 3; n_{glands} = 6). These mice were compared to control mice that received only the same *S. aureus* injection (n_{mice} = 3; n_{glands} = 6). For the injection regimen described in Fig. 2A, mice were injected with LPS (10 µg, Sigma-Aldrich, St. Louis, MO, USA) diluted in 100 µl PBS 6 h prior to inoculation with *S. aureus* (2.5 x 10^2 CFU/100µl PBS; n_{mice} = 6; n_{glands} = 12). These mice were compared to control mice that received twice a PBS injection (n_{mice} = 3; n_{glands} = 6) or a PBS injection prior to the *S. aureus* infection (n_{mice} = 6; n_{glands} = 12). For the injection regimen described in Fig. 3A, 2.5 x 10^2 CFU/100µl PBS of 2 bovine mastitis isolates i.e. *E. coli* P4 and *S. aureus* Newbould 305 were inoculated in mice (n_{mice} = 2; n_{glands} = 3). One additional mouse was included as sham control following injection of 100 µl PBS. For the injection regimen described in Fig. 4A, 3 groups of mice were injected with either PBS (n_{mice} = 3; n_{glands} = 6), or LPS (n_{mice} = 3; n_{glands} = 6) or recombinant mouse CCL5/RANTES (20 µg, Affymetrix, CA, USA) (n_{mice} = 4; n_{glands} = 8) at 6 h prior to *S. aureus* inoculation (2.5 x 10^2 CFU/100µl PBS).

II.1.3.3. Bacterial growth, Cytokine analysis and Histology

At relevant time-points post-infection (p.i.) mice were sedated by an intraperitoneal administered mixture of ketamine (100mg/kg Anesketin, Eurovet Animal Health BV, Bladel, The Netherlands) and xylazine (10mg/kg; Xylazini Hydrochloridum, Val d'Hony-Verdifarm, Beringen, Belgium) and subsequently euthanized through cervical dislocation. Mammary glands were harvested to determine local bacterial growth, analyse cytokine levels or perform histology.

Local bacterial growth (CFU/g gland) was determined as previously described (Breyne, Cool et al. 2014). For cytokine profiling, mammary gland lysates were prepared from homogenates as previously described (Breyne, Cool et al. 2014). The mouse cytokine panel A array and chemokine array (R&D systems, MN, USA) were performed according to the manual. Briefly, 400 µg total protein was incubated with a detection antibody cocktail and afterwards with a blocked nitrocellulose membrane containing different capture antibodies immobilized on spots. Chemiluminescent detection on a photographic film was performed following incubation of the membrane with streptavidin-horseradish peroxidase. The relative change in cyto-/chemokine levels of all inoculated glands was visualised with the ImageJ protein analyzer. Cyto-/chemokine quantification in the lysates was performed with specific Aimplex multiplex assay kits (YSL Bioprocess Development Co., CA, USA) for mouse CXCL5/LIX, CXCL1/KC, CCL5/RANTES, CCL3/MIP-1alpha and CXCL2/MIP-2 on a FACSArray instrument (Becton Dickinson, NJ, USA). For histology, mammary gland sections were stained with hematoxylin and eosin as previously described (Breyne, Cool et al. 2014).

II.1.3.4. Statistics

Statistical differences were determined for the injection regimen described in Fig.1A with an independent sample T-test (SPSS 20.0, IBM Corporation, Armonk, NY). The statistical differences for

the injection regimen described in Fig.2A and Fig.4A were determined using ANOVA tests for normalised data. Mean values between the different priming groups were determined through Tukey post-hoc testing. Values were expressed as mean with standard deviation (SD; levels of statistical significance $P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***).

II.1.4. Results

II.1.4.1. Pretreatment with LPS reduces the severity of clinical symptoms concomitant with a low local bacterial growth and low levels of TNF-alpha, IL-1beta, and IL-6 in the mouse mastitis model

Mammary glands of mice were pretreated with LPS prior to *S. aureus*-induced mastitis (Fig. 1A). The pretreatment with LPS insured that mice stayed normothermic until at least 48 h p.i. with *S. aureus* i.e. showed a mean change in body temperature of 0.4±0.6 °C at 28 h p.i. compared to 0 h., while non-pretreated mice had a mean change of 7.0±2.8 °C at 28 h p.i. compared to 0 h as a consequence of the infection (Fig. 1B). Furthermore, LPS resulted in a low local bacterial growth of *S. aureus* p.i. compared to non-pretreated mice (Fig. 1C).

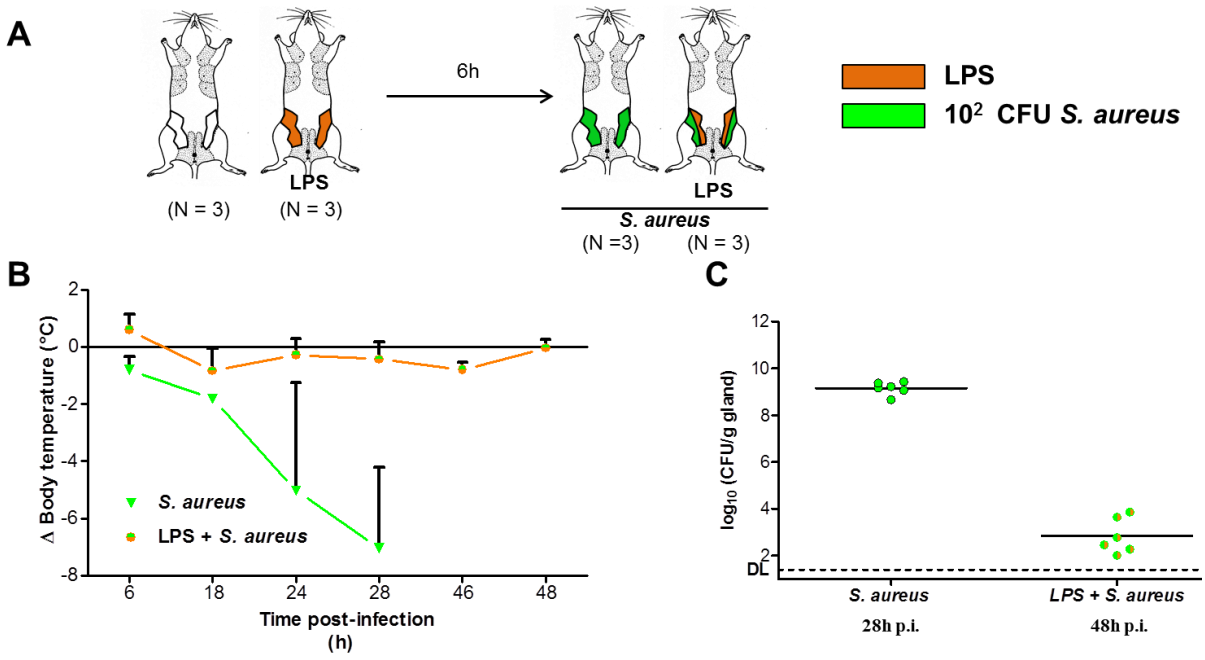

Figure 1. Effect of a local LPS pretreatment on the severity of *S. aureus* mastitis. (A) Schematic presentation of the experimental set-up. Highlighted in orange are the glands treated with LPS, in green are the glands inoculated with *S. aureus*. The effect on (B) the change in body temperature and in (C) local bacterial load (expressed as log10(CFU/g gland) was examined at 28 h p.i. without LPS injection and at 48 h p.i. with LPS pretreatment. Results are presented as mean ± standard deviation and DL = detection limit.

In the second experiment, the proinflammatory cytokines induced p.i. with *S. aureus* were additionally verified besides the change in body temperature and the bacterial growth, now using both once and

twice PBS-inoculated (sham) mice as control groups (Fig 2. A). Confirming the previous differences in change in body temperature and in bacterial growth, pretreatment with LPS again significantly reduced the change in body temperature triggered in both groups by *S. aureus* with a significant mean difference of 4.3 ± 1.3 °C at 48 h p.i. ($P < 0.05$) between these once with PBS- and the LPS-pretreated groups. The latter had a similar body temperature compared to the control mice that had been twice inoculated with PBS (sham). Indeed, these twice PBS-inoculated control mice (sham) differed significantly from the once PBS-inoculated and then *S. aureus*-infected mice with a mean difference of 4.2 ± 1.5 °C ($P < 0.05$). This observation was concomitant with a significant lower bacterial growth both in the infected glands pretreated with LPS (3.2 ± 0.2 $\log_{10}$CFU/g gland, $P < 0.001$) and the twice PBS-inoculated control mice (sham, 2.4 ± 0.2 $\log_{10}$CFU/g gland, $P < 0.001$), compared to the infected glands that were once PBS-inoculated (9.1 ± 0.2 $\log_{10}$CFU/g gland).

Furthermore, cytokines that are typically induced with a mastitic infection were determined in the glands of each group (Fig. 2 C). TNF-alpha levels in the infected glands once inoculated with PBS (145.9 ± 13.0 pg/ml) were significantly higher than in the infected glands pretreated with LPS (20.4 ± 11.8 pg/ml; $P < 0.001$) and even more so than in the twice PBS-inoculated control glands (5.6 ± 16.7 pg/ml; $P < 0.001$). Similarly, IL-1beta levels were significantly higher in the infected glands once inoculated with PBS (1479.3 ± 106.7 pg/ml) than in the infected glands pretreated with LPS (89.6 ± 97.4 pg/ml; $P < 0.001$) and even more so than in the twice with PBS-inoculated control glands (sham, 2.2 ± 137.8 pg/ml; $P < 0.001$).This was also true for the IL-6 levels which were significantly higher in the infected glands once inoculated with PBS (1407.2 ± 82.1 pg/ml; $P < 0.001$) than in the infected glands pretreated with LPS (3.6 ± 75.0 pg/ml; $P < 0.001$) and sham controls (0.3 ± 106.0 pg/ml; $P < 0.001$) (1407.1 ± 82.1 pg/ml; $P < 0.001$).

II.1.4.2.Candidate inflammatory mediators prone to immunomodulation are identified by differential protein array between *E. coli*- and *S. aureus*–infected glands

Single injection of mastitis pathogens induces high levels of cytokines. To identify the pathogen-dependent profiles, a comparative protein array on *E. coli-, S. aureus*- and PBS-inoculated mammary glands was performed (Fig. 3A and B). Mammary gland infection by *E. coli* and *S. aureus* both resulted in a neutrophil influx in the alveolar lumen at 18 h p.i., while these immune cells were absent in the PBS-inoculated control glands (Fig. 3C).

More specifically, the relative changes of 52 cyto- and chemokines were compared at the protein level for all 3 groups (Fig. 3B). Hereof, a selected panel of 14 cyto-/chemokines were analysed by both the mouse cytokine and the chemokine antibody array (Additional Figure 1). These protein arrays showed discrepancies in sensitivity. Nevertheless, except for C5a, qualitative differences in cytokine levels observed between *E. coli*- and *S. aureus*-infected glands were comparable. In the *E. coli*-infected glands 35 cytokines were relatively enhanced compared to PBS-inoculated (sham) control glands (Additional File 2). Of these, CCL5/RANTES, CCL3/MIP-1alpha and CXCL5/LIX had the highest values of fold induction. Moreover, when the relative levels of these 35 cytokines between both types of infection were compared, CCL5/RANTES and CXCL5/LIX again showed the highest fold induction

p.i. with *E. coli* compared to *S. aureus*. CXCL5/LIX is a murine homologue of IL-8 (Hol, Wilhelmsen et al. 2010). Interestingly, the two other murine IL-8 homologues CXCL2/MIP-2 and CXCL1/KC were differently induced in *E. coli* compared to *S. aureus*: while CXCL2/MIP-2 was equally induced by both pathogens compared to sham, CXCL1/KC had comparable levels in all three groups of inoculated glands.

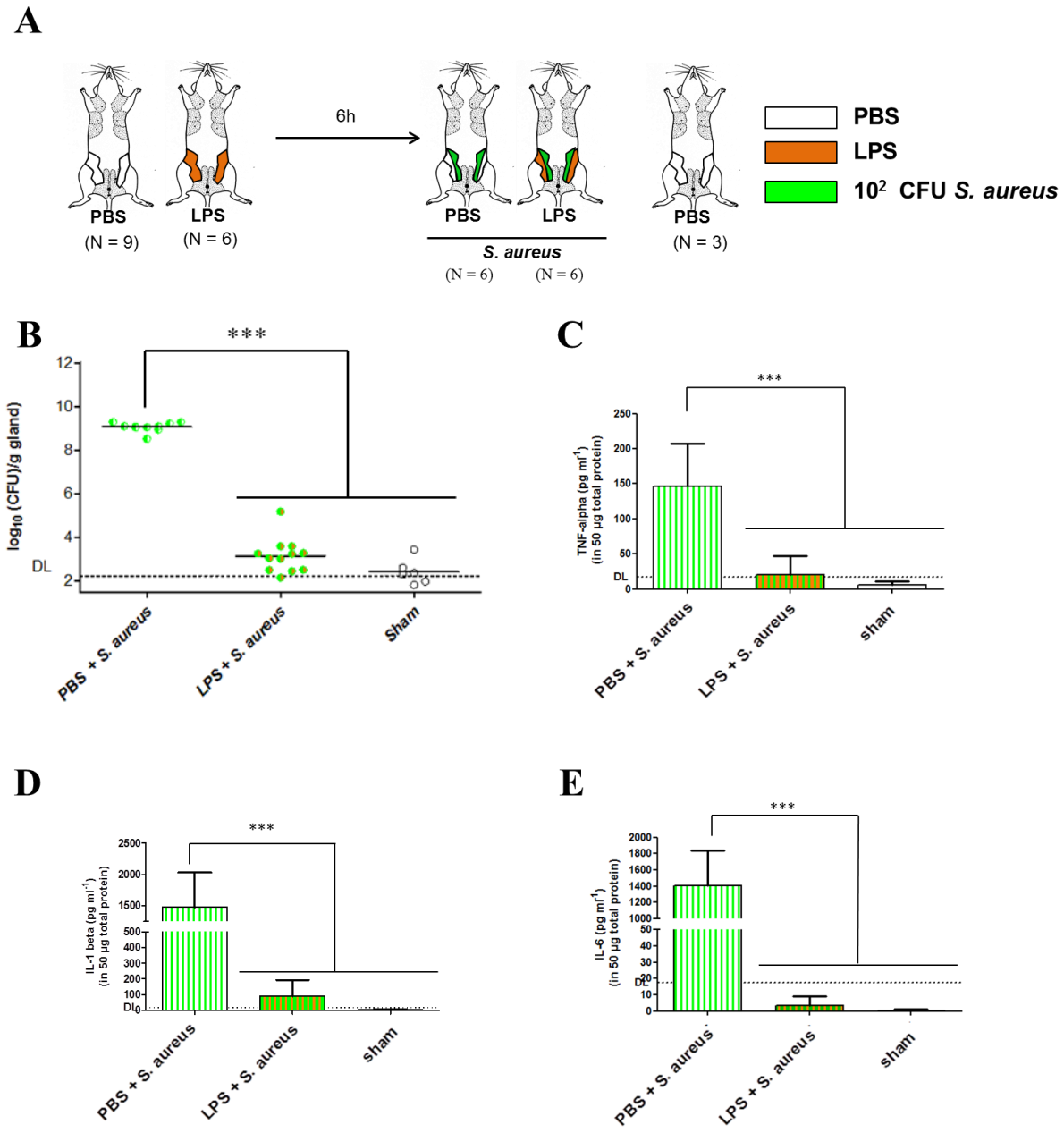

Figure 2. Effect of a local LPS pretreatment on the local bacterial load of *S. aureus* and the mammary inflammatory cytokine response. (A) Schematic presentation of the experimental set-up. Highlighted in white, orange and green are the glands inoculated with PBS (sham), LPS and *S. aureus*, respectively. (B) Local bacterial growth (expressed as log10(CFU/g gland) was examined next to the cytokines TNF-alpha (C), IL-1beta (D) and IL-6 (E). Results are presented as mean ± standard deviation, DL = detection limit.

II.1.4.3. LPS pretreatment modulates murine IL-8-like homologues differently and increases CCL5/RANTES, while pretreatment with the latter only partially mimics the immunomodulation and does not inhibit the bacterial growth

The previously with protein array screening identified chemokines of interest being the 3 IL-8 like homologues (i.e. CXCL5/LIX, CXCL2/MIP-2 and CXCL1/KC), CCL5/RANTES and CCL3/MIP-1alpha were now quantitatively analysed in a combined set-up, extending the LPS pretreatment with pretreatment CCL5/RANTES (Fig. 4A). Corroborating our previous results and again based on both body temperature and local CFU counts, pretreatment with LPS subsequently induced a milder inflammation phenotype compared to a PBS injection prior to *S. aureus* infection. At 28 h p.i., all LPS-pretreated mice had a normothermic body temperature differing 10.2±2.1 °C ($P < 0.01$, Fig. 4B) and 4.4±0.2 $\log_{10}$(CFU/g) ($P < 0.01$, Fig. 4C) with the values of the PBS-injected control mice. As expected from the previous protein array screening experiment, LPS pretreatment significantly altered the local levels of all the studied chemokines compared to PBS-injected control mice. LPS pretreatment decreased cytokines that - based on our previous experiment - were not considered to be very pathogen dependent i.e. CXCL1/KC CXCL2/MIP-2 were decreased with LPS treatment compared to the PBS-inoculated mammary glands prior to *S. aureus* infection (Table 1). Moreover, this LPS-pretreatment also decreased CCL3/MIP-1alpha levels compared to the non-pretreated glands. The LPS-induced immunomodulation was also characterized by an increase in chemokines that were in a previous experiment considered to be highly *E. coli*-dependent namely CCL5/RANTES and CXCL5/LIX.

Table 1. Concentrations (expressed as means ± standard deviation, pg/ml per 50 µg total protein) of the chemokines CCL5/RANTES, CXCL1/KC, CCL3/MIP-1alpha, CXCL2/MIP2 and CXCL5/LIX, in the mammary glands of the mice pretreated with either LPS, or CCL5/RANTES or PBS prior to a second PBS or *S. aureus* inoculation.

Item	PBS + *S. aureus*	LPS + *S. aureus*	CCL5/RANTES + PBS	CCL5/RANTES + *S. aureus*
CCL5/RANTES	47.6 ± 7.0^{a,b,c}	712.9 ± 104.2^{a,d,e}	1511.0 ± 243.5^{b,d}	1592.0 ± 213.8^{c,e}
CXCL1/KC	1265.0 ± 183.1^{a,b,c}	617.1 ± 137.6^{a,d}	204.3 ± 145.9^{b,d,e}	817.7 ± 300.5^{c,e}
CCL3/MIP-1alpha	4938.0 ± 1177.0^{a,b,c}	2111.0 ± 1015.0^{a,d}	26.5 ± 9.0^{b,d,e}	2379.0 ± 1244.0^{c,e}
CXCL2/MIP-2	7308.0 ± 371.4^{a,b}	4287.0 ± 1167.0^{a}	92.2 ± 25.6^{b}	5808.0 ± 774.4
CXCL5/LIX	157.0 ± 51.1^{a}	785.0 ± 66.2^{a,b,c}	128.9 ± 12.8^{b}	189.1 ± 17.8^{c}

$^{a\text{-}e}$ Letters of homogeneous subsets were marked if the difference between treatments were statistically significant ($P < 0.05$).

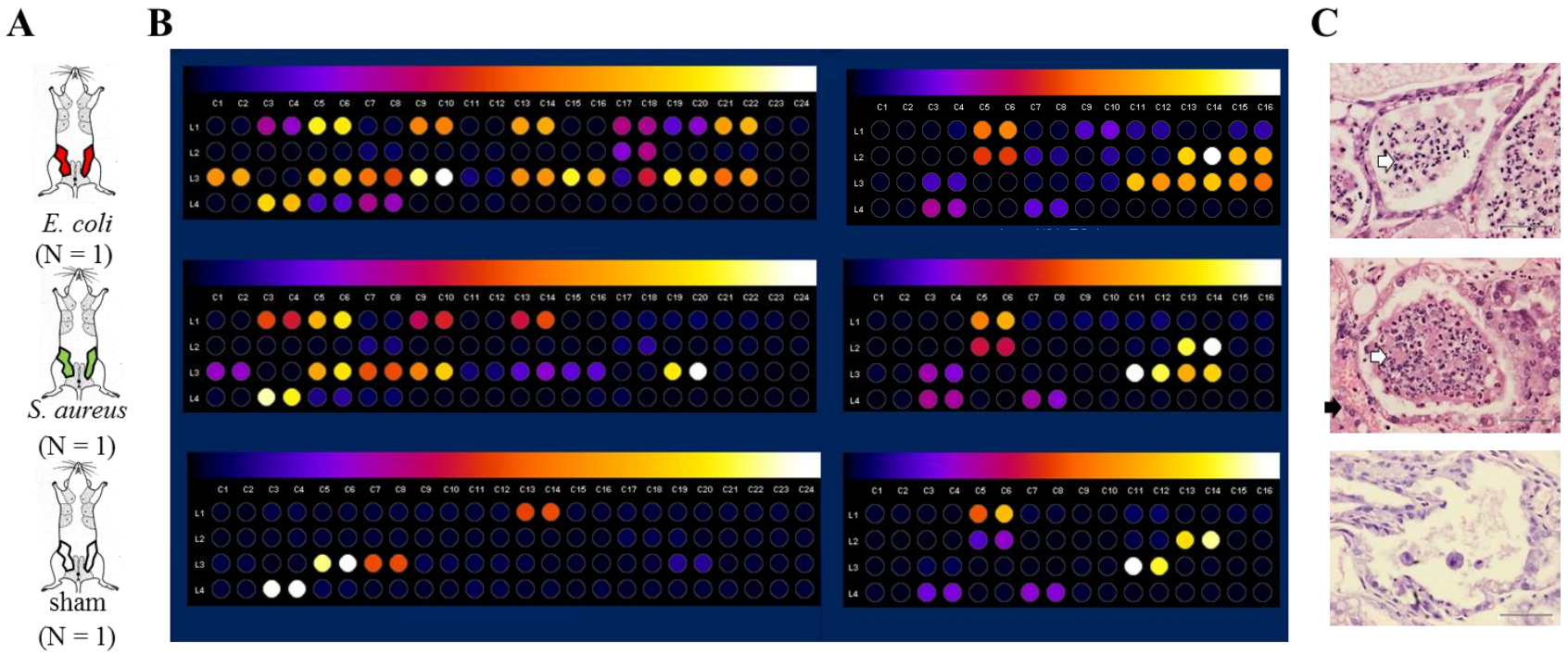

Fig. 3. Evaluation of differential cytokine profiles induced by *E. coli*-, *S. aureus*- and PBS (sham)-inoculation in mammary glands. (A) Schematic presentation of the experimental set-up. Highlighted in red or green are the glands inoculated with *E. coli* or *S. aureus* respectively and marked in white are the sham-inoculated mammary glands. The local inflammatory response of the mammary glands was examined at 18 h p.i. (B) Representative mouse cytokine protein array (left) and mouse chemokine protein array (right) images from *E. coli*-, *S. aureus*- and sham (PBS) -inoculated mammary glands are represented in the upper, middle and lower panels, respectively. The black/blue staining represents low protein levels, while the yellow/white staining represents highly upregulated protein levels. Samples were screened in duplo. (C) Representative hematoxylin- and eosin-stained sections of *E. coli*- (upper panel), *S. aureus*- (middle panel) and sham- (lower panel) inoculated glands at 18 h p.i. The white arrows mark the alveolar immune cell content, while the black arrow point to the interstitial red blood cells. The scale bar represents a length of 50 µm.

This experiment therefore additionally verified whether the previously identified CCL5/RANTES could induce a host-protecting effect during *S. aureus* mastitis (Fig. 4A). At 28 h p.i., the latter CCL5/RANTES induced moderate immunomodulation resulting in an intermediate change in bodytemperature of 5.5±2.0 °C compared to PBS-inoculation (P = 0.06; Fig. 4B). In marked contrast, PBS- and CCL5/RANTES-injected mammary glands did not significantly differ in their bacterial growth as both groups contained on average 4.3±0.2 $\log_{10}$(CFU/g gland) (P < 0.01) more bacteria than those pretreated with LPS (Fig. 4C). The CCL5/RANTES injection also induced generally more moderate changes in cytokine levels than observed after LPS pretreatment (Table 1).

Nevertheless, pretreatment with this chemokine still significantly decreased the local levels of two other chemokines CXCL1/KC (P < 0.01) and CCL3/MIP-1alpha (P = 0.01) compared to PBS-injected glands (sham), while only a trend was visible for CXCL2/MIP-2. Moreover, the local CXCL5/LIX levels were significantly lower (P < 0.01) with CCL5/RANTES compared to the LPS-pretreated mammary glands (Table 1). Of note, injection with CCL5/RANTES without subsequent infection (i.e. prior to sham) did not increase local CXCL1/KC, CCL3/MIP-1alpha, CXCL2/MIP-2 and CXCL5/LIX compared to LPS, CCL5/RANTES and PBS-injection with a subsequent *S. aureus* infection. In addition, the former injection regimen did not induce an influx of neutrophils in contrast to the the influx observed in the *S. aureus* infected glands pretreated either with LPS or CCL5/RANTES (Fig. 5). In essence, immunomodulation through CCL5/RANTES pretreatment has no detected disadvantage for the host.

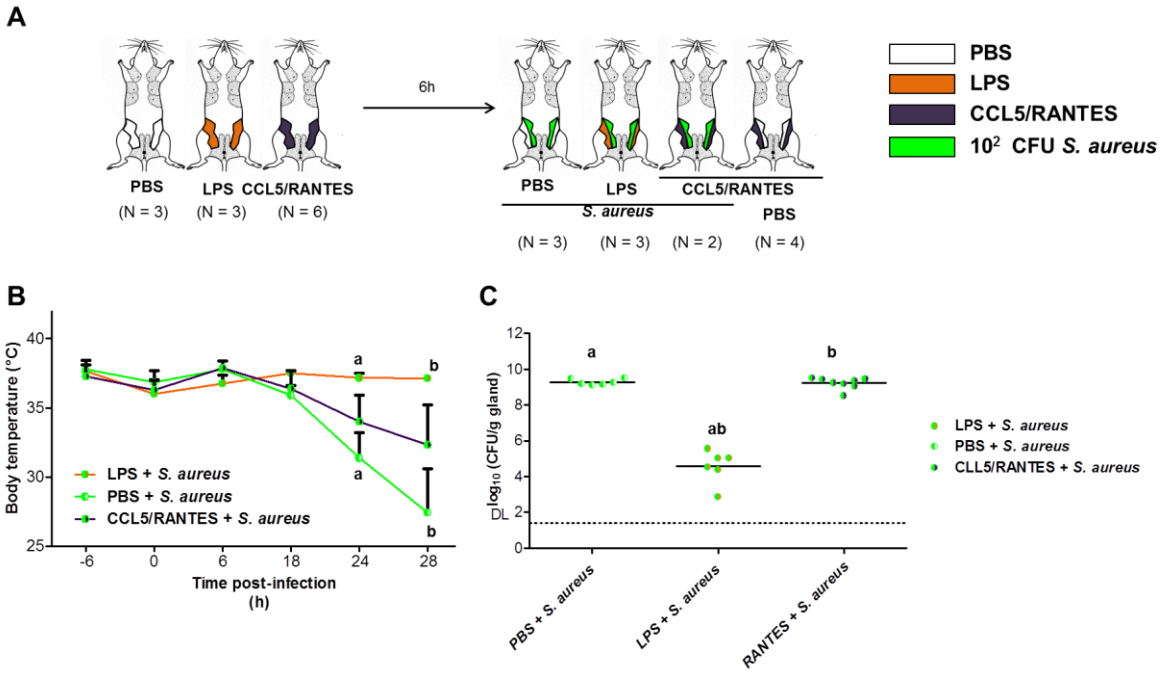

Fig. 4. Effect of a local CCL5/RANTES pretreatment on the severity of *S. aureus* mastitis . (A) Schematic presentation of the experimental set-up. Highlighted in orange, purple and green are the glands treated with LPS, CCL5/RANTES or inoculated with *S. aureus*, respectively while the ones marked in white are the PBS-inoculated mammary glands. Effect on the change in (B) body temperature and in (C) local bacterial load (expressed as log10(CFU/g gland) was

examined at 28 h p.i.. Letters of homogeneous subsets differ if the difference between treatments was statistically significant (P < 0.05). Results are presented as mean ± standard deviation, DL = detection limit.

II.1.5. Discussion

In current study, we first investigated whether a challenge with LPS before the establishment of a mammary infection may influence the detrimental immune response induced by *S. aureus.* Secondly, we searched for cytokines in the mammary gland that may be prone to altering during the LPS treatment based on differential cytokine array screening between *E. coli* - as a key source of LPS - and *S. aureus.* Thirdly, we examined the quantitative differences of these identified cytokines during LPS pretreatment. Finally, we checked whether cytokines upregulated as a result of LPS treatment such as CCL5/RANTES can mimic the immunomodulatory effect.

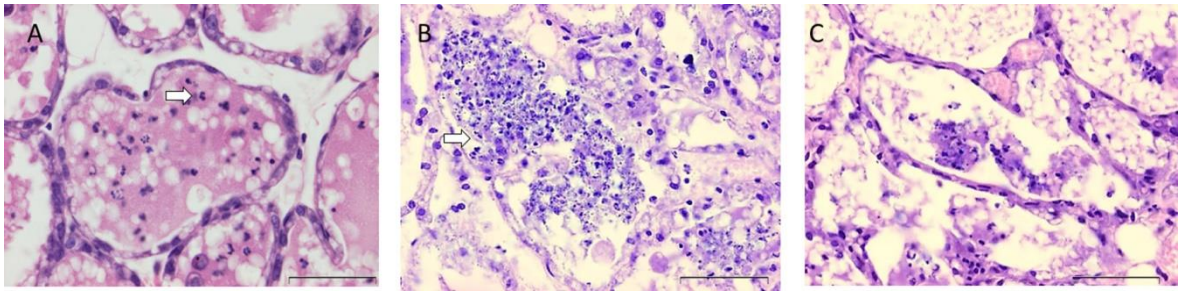

Fig. 5 Representative hematoxylin- and eosin-stained sections at 28 h p.i. of (A) LPS- or (B) CCL5/RANTES-pretreated mammary glands prior to *S. aureus* mastitis, (C) CCL5/RANTES-pretreated mammary glands prior to PBS-inoculation. The white arrows mark the alveolar immune cell content. The scale bar represents a length of 50 µm.

Our investigated concept is derived from ET, a complex immunomodulatory phenomenon predominantly described for macrophages (Biswas and Lopez-Collazo 2009). Nowadays, this concept has been shown to enhance bacterial clearance or decrease bacterial colonization and has been extrapolated to multiple innate immune cell types such as neutrophils through the formation of neutrophil extracellular traps (Landoni, Chiarella et al. 2012). In the context of bovine mastitis, the repeated exposure to either microbial pathogens or their MAMPs has important implications. Indeed, the milkspace of the udder is generally not sterile and therefore superinfections in milk are common in practical conditions. Nevertheless, little is known about the effect of these recurrent challenges of the udder and the few reports on this phenomenon are partly contradictory. One study infected cows twice with live *E. coli* which reduced the severity of the clinical symptoms associated with the second challenge (Suojala, Orro et al. 2008). Likewise, in an earlier study the first of both inflammation reactions was induced by LPS-infusion of the udder at a high dose (100 µg) which completely prevented the subsequent induction of *E. coli* mastitis in these cows (Lohuis, Van Leeuwen et al. 1988; Lohuis, Kremer et al. 1990). In a more recent study, a low LPS dose (1 µg) - that in contrast to the high one didn't induce visible clinical signs - still was able to reduce bacterial *E. coli* growth 10 days after this initial LPS bolus (Petzl, Gunther et al. 2012). When the latter second inoculation was

replaced by a sequence of multiple intermediate LPS doses (10 µg), the inflammation reaction induced by the first inoculation was gradually resolved and finally a complete recovery of the milk yield and other parameters was obtained despite a local increase in SCC (Shuster and Harmon 1991). In contrast, another report which also used an intermediate second LPS dose (33 µg) induced more inflammation as indicated by a subsequent higher TNF-alpha production (Rainard and Paape 1997). Furthermore, the effect of LPS-injection on the udder does not seem to be restricted to *E. coli* mastitis but seems to be altering the an *S. aureus* udder infection as well. *In vitro* inoculation of milk obtained from udder quarters infused with LPS demonstrated that the growth of *S. aureus* in milk from these udder quarters was significantly inhibited (Lohuis, Kremer et al. 1990; Kauf, Vinyard et al. 2007). Unfortunately, when LPS was injected post- instead of pre-infection with *S. aureus* the effect lasted only temporally.

Altogether it is important to know whether a preceding challenge with a MAMP or bacteria in the udder is beneficial to have. It is suggested that LPS pretreatment increases bovine epithelia to fight mastitis pathogens better and may enable them to protect against excessive reactions of the immune response (Gunther, Petzl et al. 2012). From a clinical standpoint, a preventive treatment that attenuates the host inflammatory response, yet improving bacterial clearance and/or decreasing of bacterial colonization, has obvious interest and a thorough mechanistic understanding is warranted. Such a beneficial effect can be the basis for novel innovative preventive treatments which are highly anticipated yet have remained relatively unsuccessful till date in this field (Barkema, Schukken et al. 2006). Preventive measures are preferred over curative ones against clinical and chronic *S. aureus* mastitis as the latter are hampered both by the production of biofilms as well as the complicating factor of the intracellular localization in the udder epithelium of these Gram-positive bacteria (Melchior, van Osch et al. 2011). Hence, this limits the action and penetrance of classical antibiotics to the region of interest, which is reflected by an effectiveness of only 30% depending on the cows' age and parity (Sol, Sampimon et al. 1997). Moreover, the overuse of antimicrobial agents in the dairy management is a fact and hence increases the risk of resistance to these latter drugs (Oliver, Murinda et al. 2011).

For reasons of costs and labour-intensity a mouse mastitis model was used that was previously reported by our group and others as a relevant tool in ruminant mastitis research (Brouillette and Malouin 2005). This preclinical *in vivo* model was validated, applied for the molecular study of bovine mastitis pathogenesis (Notebaert, Carlsen et al. 2008) and used for the screening of potential novel therapies (Demon, Breyne et al. 2013). To study LPS immunomodulation in our mastitis model, the host innate immunity has to be challenged either with a single non-lethal stimulus or by a combination of multiple stimuli prior to infection with the bovine pathogen. In other mouse models, a decrease of pro-inflammatory cytokines such as IL-6 and TNF-alpha can be achieved by one LPS bolus prior to an infection that can be dampened even further by additional LPS pretreating boli (Lehner, Ittner et al. 2001). The altered inflammatory response is believed to be due to a change in composition of the transcription factor NF-kappaB from a heterodimeric p65/p50 complex to a p50/p50 homodimer (Biswas and Lopez-Collazo 2009). In the mouse mammary endotoxin model, NF-kappaB activity occurs around 6-8 h p.i. which was therefore considered as the ideal time point to introduce a

subsequent bovine mastitis-associated *S. aureus* isolate (Notebaert, Carlsen et al. 2008). The transcription produces a large number of cytokines as seen with the cytokine and chemokine arrays in our study. These arrays indicated that single infections with bovine *S. aureus*- or *E. coli* isolates enhance a large number of - albeit sometimes to a different extent - cytokines in the murine mammary gland including IL-1beta, IL-6 and TNF-alpha. A pretreating bolus of LPS decreased the cytokines IL-1beta and TNF-alpha which is believed to be triggered through NF-kappaB and IL-6, the latter signalling being likely unrelated to NF-kappaB. Both these pathways are presumed to be essential for a major pathogen mastitis infection in cows (Gunther, Esch et al. 2011). Our results indicate that LPS pretreatment does not induce the excessive inflammation that could be expected of a single LPS bolus but decreases pathogen-induced inflammation. This anti-inflammatory strategy has been proven to be effective against *S. aureus* mouse mastitis (Boulanger, Brouillette et al. 2007).

Neutrophils are essential for bacterial clearance in the innate response of mastitis (Elazar, Gonen et al. 2010). Mice have a single receptor for CXC chemokines homologous to the bovine CXCR1 and CXCR2. This receptor (CXCR2) is activated by CXCL5/LIX, CXCL1/KC and CXCL2/MIP-2, the functional homologs of bovine CXCL8 (IL-8) (Lahouassa, Rainard et al. 2008). Although CXCL1/KC and CXCL2/MIP-2 are highly acknowledged, CXCL5/LIX is less well characterized but has proven its importance in guiding the critical mammary influx of circulating neutrophils in mice (McDonald and Kubes 2010). Furthermore, its homologue has occasionally been mentioned in the context of bovine mastitis (Pareek, Wellnitz et al. 2005; Le Marechal, Jardin et al. 2011; Petzl, Gunther et al. 2012). Moreover, other main neutrophil receptors - also present on macrophages - are CCR1 and CCR5 and they are believed to be stimulated by CCL5/RANTES and CCL3/MIP-1alpha. As such, these cell types are recruited to the site of infection (Diab, Abdalla et al. 1999; Pan, Parkyn et al. 2000; Hartl, Krauss-Etschmann et al. 2008). These chemokines and receptors are also expressed in cows and activated during mastitis (Buitenhuis, Rontved et al. 2011; Widdison and Coffey 2011). We verified whether these neutrophil-recruiting chemokines could be detected in our mouse model and whether they were modulated during LPS pretreatment. A broad protein array screening comparing early *E. coli-, S. aureus*- and sham-inoculated mammary glands of mice indicated whether they were of essence for mastitis. Unexpectedly, the host response patterns of these cytokines were differently influenced by the pathogens in the mammary gland. The chemokines CCL5/RANTES and CXCL5/LIX were selectively enhanced with an *E. coli* infection, while MIP-1alpha was present in both *S. aureus*- and *E. coli*-infected glands albeit much higher after *E. coli.* In contrast, CXCL2/MIP-2 was equally enhanced in both *S. aureus*- and *E. coli*-infected glands and CXCL1/KC was almost not enhanced by both these pathogens compared to sham. When LPS was injected prior to *S. aureus* we again detected a decrease in inflammatory signalling by the cytokines MIP-1alpha, CXCL2/MIP-2 and CXCL1/KC. Furthermore, we also showed that the LPS pretreatment is not a mere decrease of inflammation but can be better appreciated as immunomodulation because it also actively increased CCL5/RANTES and CXCL5/LIX.

Immunomodulation can be triggered by cytokines without LPS. It was shown that TNF-alpha treatment can decrease cytokines comparable to a LPS pretreatment (Ferlito, Romanenko et al. 2001). As such,

TNF-alpha primed bovine neutrophils have an enhanced oxidative burst upon their subsequent stimulation (Sample and Czuprynski 1991). While cytokines are decreased, other cytokines are preferably enhanced during LPS pretreatment and are suggested to be host-protecting. The latter authors detected also an increased gene expression of chemokines such as IL-8 and CCL5/RANTES in addition to antimicrobial and tissue protecting factors, concomitant with a decrease in key inflammatory cytokines (including IL-6) and other immune effectors (Gunther, Petzl et al. 2012). To evaluate its potential host-protecting effect, we injected the chemokine CCL5/RANTES. Although CCL5/RANTES did not elicit an immune response and immune cell influx *per se*, it could nonetheless decrease some immune-tolerant cytokines when injected prior to a *S. aureus* infection. More specifically, while the local levels of CXCL1/KC and CCL3/MIP-1alpha decreased comparably as upon LPS pretreatment and the decrease in local CXCL2/MIP-2 levels was far less pronounced. Furthermore, no increase of the other host-protecting chemokine CXCL5/LIX was observed following CCL5/RANTES pretreatment in contrast to LPS pretreatment.

In addition to the significant modulation of local cytokine levels upon LPS treatment, there an inhibited local bacterial growth and increased survival rate following a subsequent *S. aureus* infection. The phenotype is believed to be reasoned by an increased neutrophil influx and amplify the killing and antigen presentation state macrophages (Ariga, Abatepaulo et al. 2014; de Lima, Sampaio et al. 2014). This phenomenon is in contrast to what would be expected from just lowering immunity and suggests an active process through the enhancement of specific cytokines. As demonstrated here, the results cannot be solely attributed to the enhancement of CCL5/RANTES alone as the immunomodulation of this cytokine alone could not mimic the bacterial clearance and/or decrease in bacterial colonization and only partially - but not significantly – dampened the severity of the clinical symptoms. Nevertheless, a non-detrimental challenge seems to provoke an enhanced basal alertness of the mammary gland and our findings suggest that future mastitis research should further elaborate these functions of cytokines which are enhanced as a consequence of LPS pretreatment.

In conclusion, our results demonstrated that the mouse mastitis model can be used to study the complex innate immunity alterations by a LPS pretreatment as observed in experimentally infected cows. Furthermore, we at first showed that the concept of LPS pretreatment is able to modulate inflammatory response, bacterial growth and the reduction of *S. aureus* virulence *in vivo*. Moreover, although the beneficial effect elicited by the chemokine CCL5/RANTES were only moderate compared to that observed by LPS, we suggest that the former immunomodulation can be ameliorated with complementary cytokines.

II.1.6. Acknowledgements

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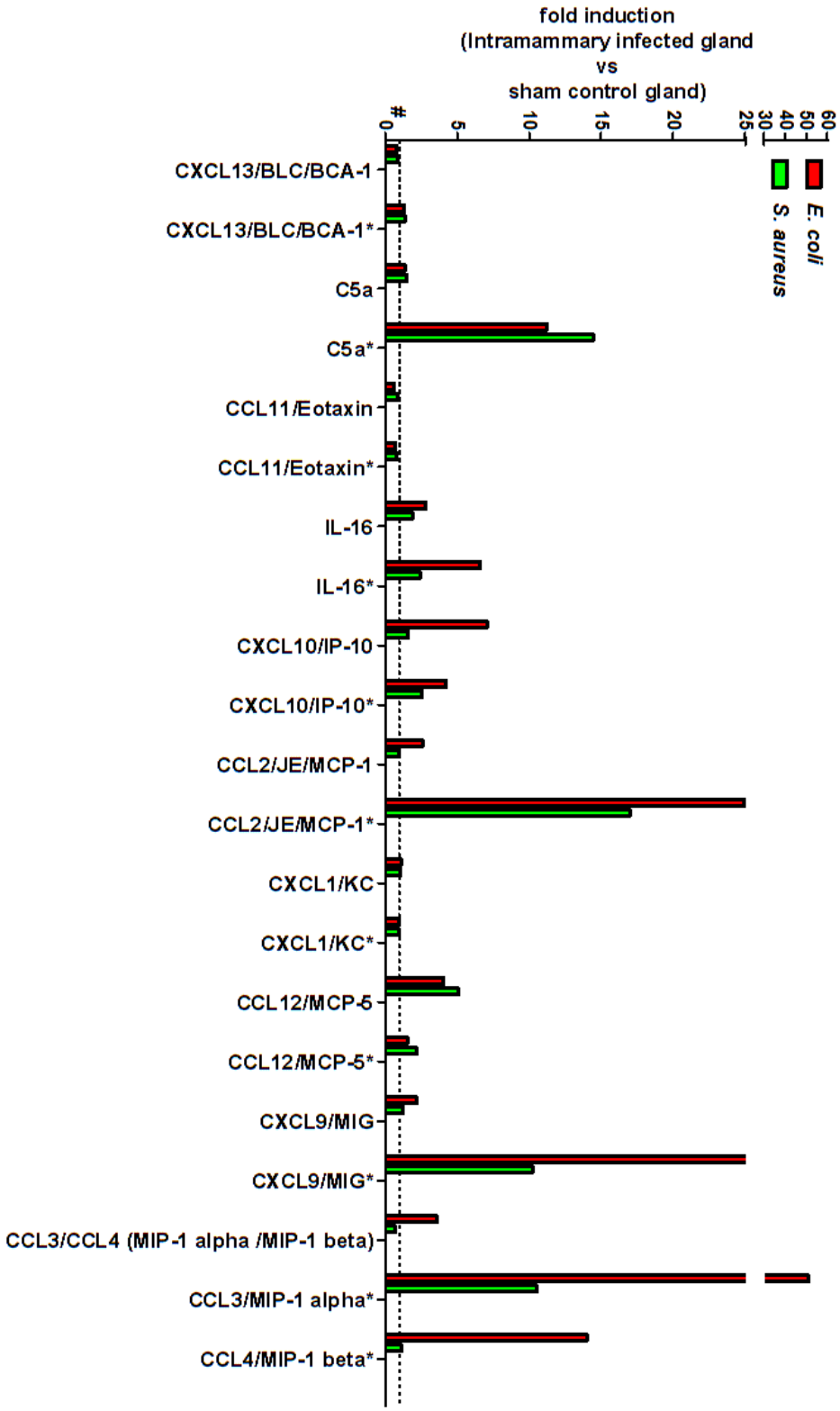
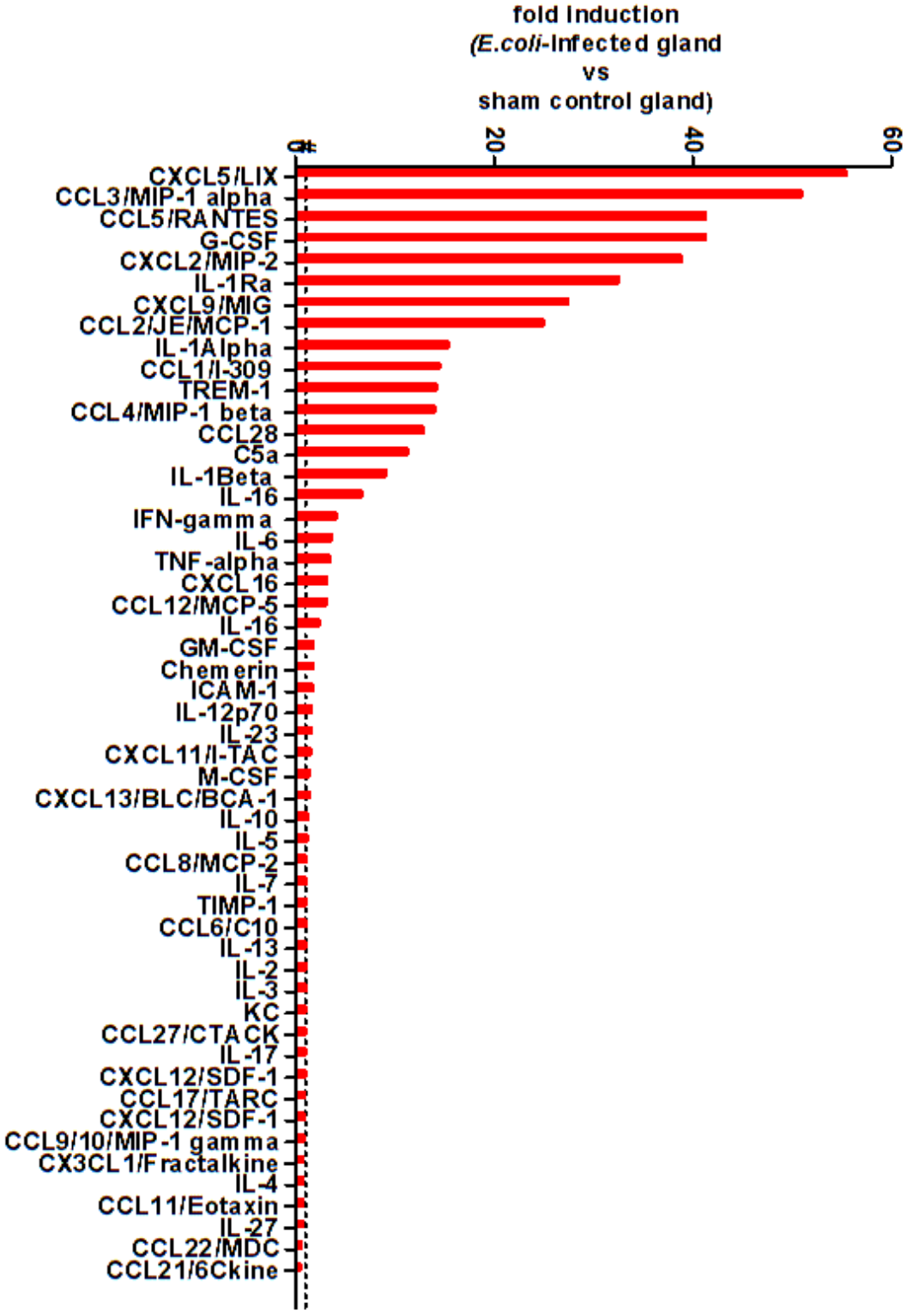
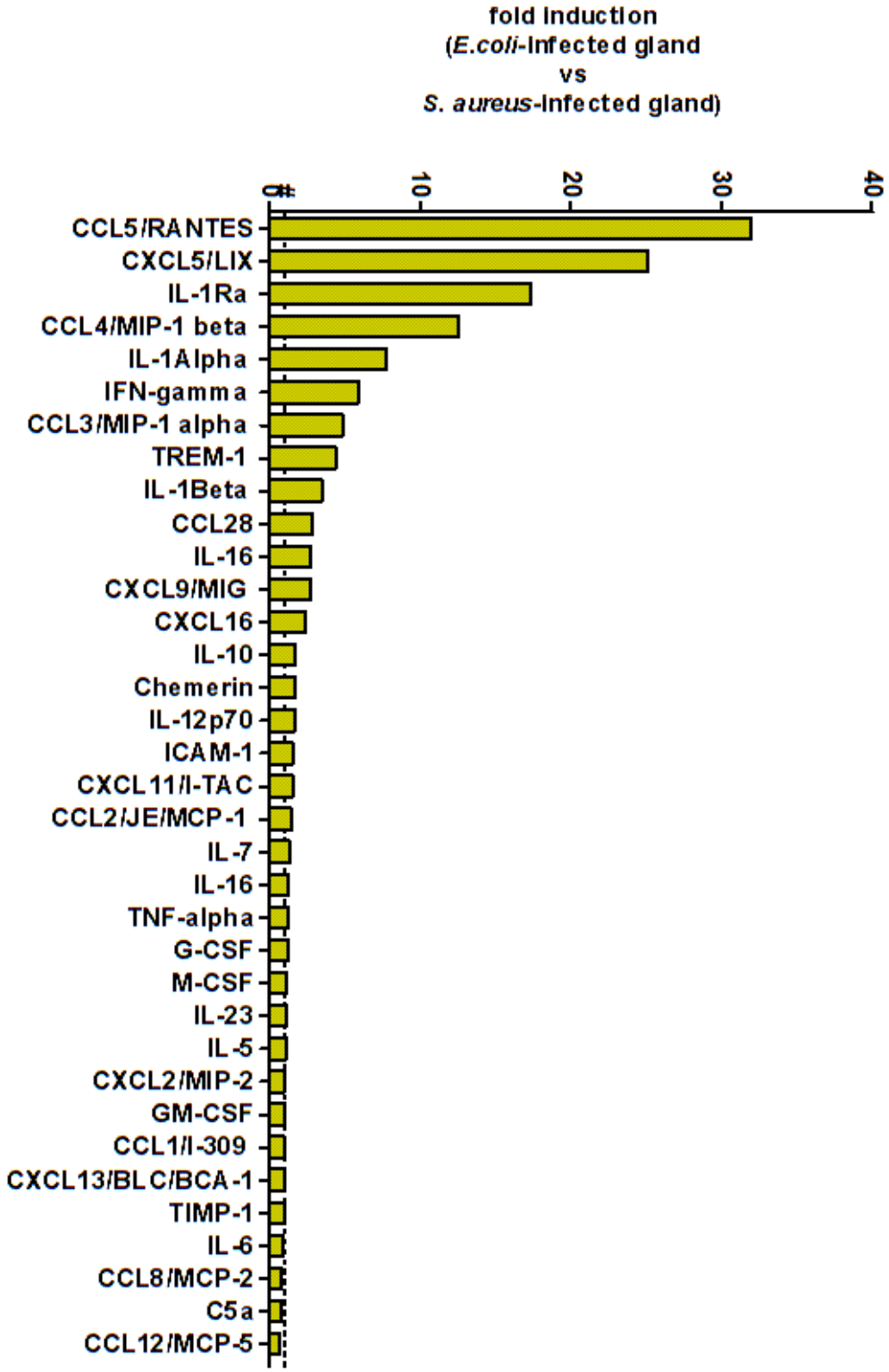
ADDENDUM



Additional Figure 1. Cytokines screened in both arrays 18 h post-inoculation. Bars represent the mean ratio of *E. coli*-or S. *aureus*-infected glands relatively to sham-inoculated glands. The * indicates the cytokine measured with the cytokine array, while the other was detected in the chemokine array. The line # represents the levels that equal the cytokine levels of the sham-inoculated glands.



Additional Figure 2. Cytokines enhanced by *E. coli* 18h post-inoculation compared to sham in murine mammary glands. Bars represent the mean ratio of cytokine levels between *E. coli*- and sham-inoculated glands. The horizontal line (#) indicates that the cytokine levels in the *E. coli*-infected gland are equal to the levels of the sham-inoculated glands.



Additional Figure 3. Cytokines enhanced by *E. coli* compared to *S. aureus* in murine infected mammary glands 18h post-inoculation. Bars represent the mean ratio of cytokine levels between *E. coli*- and *S. aureus*-infected glands. The horizontal line (#) indicates that the cytokine levels in *E. coli*-infected gland are equal to the levels of the *S. aureus*-infected gland.

CHAPTER III

CURATIVE MASTITIS

Antibiotics have been and still are the main curative measure against bovine mastitis. The most effective therapy - as dictated by the National Mastitis Council (NMC_Guidelines 2014). - is to administer a long-acting antibiotic during the dry-off period, preferably after thorough characterization of the causal pathogen. Generally, the efficacy of antibiotics against these pathogens is at first demonstrated *in vitro*. The well-known dilution methods (Minimum Antibiotic Growth or MIC) (Tenhagen, Köster et al. 2006), disk diffusion methods (Pillar, Stoneburner et al. 2014), specific bioassays (Owens and Watts 1988), E-tests (Gentilini, Denamiel et al. 2002) or genotypic methods (Moon, Lee et al. 2007) all suggest which antibiotic should be used against a certain mastitis-associated pathogen and in which concentration. However, mastitis-associated bacteria are pathogens that thrive in the high-caloric milk matrix with changing composition, as well as in an anaerobic or facultative aerobic atmosphere, form biofilms, nestle intracellularly, and induce local inflammation. These complex features are very difficult to mimic *in vitro* and may hence lead to wrong conclusions drawn from the above-mentioned methods (Owens, Ray et al. 1997; Olson, Ceri et al. 2002). Moreover, different formulations of the antibiotics' excipients influence their efficacy in the udder, an aspect which is again not feasible to evaluate *in vitro*. Therefore, veterinary researchers as well as R&D departments from Animal Health Companies increasingly utilize an intermediate *in vivo* step to check which antibiotic is best fitted to treat a selected mastitic infection without the cost and other disadvantages of using cows (Brouillette, Grondin et al. 2004).

The **first aim of CHAPTER III** was to evaluate whether the mouse mastitis model is suited to test the efficacy of different first generation Cephalosporins and excipients against an intramammary bovine *S. aureus* infection. The **second aim of CHAPTER III** was to evaluate whether this mouse model is also suited to check a novel broad-spectrum antibiotic against different bovine mastitis-associated isolates.

It should be highlighted that although K. Breyne is not the first author of this work, he has made significant contributions to both papers. Indeed, under the direct leadership of Dr. D. Demon, K. Breyne helped in conceiving and designing the experimental set-up, assisted during the experiments, supported in analyzing the data and reviewed the manuscripts. Moreover, the latter have not and will not be used in other PhD theses.

First aim of CHAPTER III is adapted from: Demon D., Ludwig C., Breyne K., Guede D., Dorner J.C., Froyman R., Meyer E., The intramammary efficacy of first generation cephalosporins against *Staphylococcus aureus* mastitis in mice, Vet Microbiol. (2012) 160:141-150.

Second aim of CHAPTER III is adapted from: Demon D., Breyne K., Schiffer G., Meyer E., Short communication: Antimicrobial efficacy of intramammary treatment with a novel biphenomycin compound against *Staphylococcus aureus*, Streptococcus uberis, and *Escherichia coli*-induced mouse mastitis, Journal of Dairy Science. (2013) 96:7082-7087.

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III.1. Intramammary Efficacy of First Generation Cephalosporins against Staphylococcus aureus Mastitis in Mice.

III.1.1. Summary

Staphylococcus aureus-induced mastitis in cattle causes important financial losses in the dairy industry due to lower yield and bad milk quality. Although *S. aureus* is susceptible to many antimicrobials *in vitro*, treatment often fails to cure the infected udder. Hence, comprehensive evaluation of antimicrobials against *S. aureus* mastitis is desirable to direct treatment strategies. The mouse mastitis model is an elegant tool to evaluate antimicrobials *in vivo* while circumventing the high costs associated with bovine experiments. An evaluation of the antimicrobial efficacy of the intramammary applied first generation cephalosporins cefalexin, cefalonium, cefapirin and cefazolin, was performed using the *S. aureus* mouse mastitis model. *In vivo* determination of the effective dose $2\log_{10}$ ($ED_{2\log10}$), $ED_{4\log10}$, protective dose 50 (PD_{50}) and PD_{100} in mouse mastitis studies, support that *in vitro* MIC data of the cephalosporins did not fully concur with the *in vivo* clinical outcome. Cefazolin was shown to be the most efficacious first generation cephalosporin to treat *S. aureus* mastitis whereas the MIC data indicate that cefalonium and cefapirin were more active *in vitro*. Changing the excipient for intramammary application from mineral oil to miglyol 812 further improved the antimicrobial efficacy of cefazolin, confirming that the excipient can influence the *in vivo* efficacy. Additionally, statistical analysis of the variation of *S. aureus*-infected, excipient-treated mice from fourteen studies emphasizes the strength of the mouse mastitis model as a fast, cost-effective and highly reproducible screening tool to assess the efficacy of antimicrobial compounds against intramammary *S. aureus* infection.

III.1.2. Introduction

Mastitis provokes detrimental damage to epithelial cells. Hence, it jeopardizes milk production and entails prohibitive costs in the dairy industry (Piepers et al., 2007). *Staphylococcus aureus* is historically one of the most important causes of subclinical mastitis and clinical mastitis that becomes chronic (Piepers et al., 2007 and Tenhagen et al., 2009). Despite intensive research and multiple preventive measures (NMC, 2006), the dairy industry has not managed to eradicate *S. aureus* mastitis (Zadoks and Fitzpatrick, 2009). So, antimicrobial therapy remains an essential component of staphylococcal mastitis control programs. The major concern with *S. aureus* infections is that once established they are extremely difficult to eradicate from the mammary gland. Although *S. aureus* is susceptible to numerous antibiotics *in vitro* (Erskine et al., 2002 and EUCAST, 2011), this pathogen has developed several features to influence the host immune system and to escape from antimicrobial treatment (Barkema et al., 2006).

Over 50% of the single component preparations on the dairy market in Belgium are cephalosporins (BCFI, 2011). Together with penicillins they belong to the group of β-lactam antibiotics, but are less susceptible to inactivation by *S. aureus* β-lactamases. The first generation cephalosporins show good activity against bovine *S. aureus* species *in vitro* (Table 1). Notably, these species show very low resistance rates against first generation cephalosporins (Erskine et al., 2002, Kaspar, 2006 and

Maran, 2010), an encouraging advantage with regard to public safety and antimicrobial efficacy. The aim of the current study was to assess *in vivo* side-by-side the intramammary efficacy of the 4 first generation cephalosporins cefalexin, cefalonium, cefapirin and cefazolin in the *S. aureus* mouse mastitis model. In addition, the efficacy of cefazolin was evaluated using two different excipients. Finally, the reproducibility of the mouse mastitis model was statistically analyzed to evaluate its robustness.

Table 1. Distribution of the Minimal Inhibitory Concentration 50 (MIC_{50}), MIC_{90}, MIC range and number of samples for cefalexin, cefalonium, cefapirin and cefazolin against *Staphylococcus aureus* species isolated from bovine mastitis cases worldwide. MIC_{50} and MIC_{90} are the epidemiological MICs that indicate that 50% and 90%, respectively, of the *S. aureus* strains have a MIC below this value.

Cephalosporin	MIC50 (µg/ml)	MIC90 (µg/ml)	Range (µg/ml)	Number of samples	References
Cefalexin	4.0	4.0	1.0–8.0	119	Guerin-Faublee *et al.*
	4.0	128	0.50–256	102	EUCAST
Cefalonium	0.08	NR	0.04–0.08	330	Groothuis and Frik
Cefapirin	0.50	0.50	≤0.06–1.0	135	Watts et al.
	0.25	0.50	≤0.06–64	79	Salmon et al.
	0.125	0.25	≤0.06–0.25	20	Owens et al.
	0.12	0.25	0.03–0.50	119	Guerin-Faublee et al.
	≤1	≤1	≤1–4	331	Sato et al.
Cefazolin	0.39	0.39	0.20–1.56	51	Yoshimura et al.
	0.25	0.50	0.063–32	105	Brix
	0.50	0.50	0.12–1.0	119	Guerin-Faublee et al.
	0.25	1.0	0.125–2.0	30	Nunes *et al.*
	0.50	4.0	0.064–32	19,051	EUCAST

NR = Not Reported

III.1.3. Materials and methods

III.1.3.1. Antibiotics and excipients

Cefalexin (ABCR GmbH & Co. KG, Germany), cefalonium (Sigma–Aldrich, Germany), cefapirin (Fluka Analytical, Switzerland), cefazolin (ABCR GmbH & Co. KG, Germany) and excipients (mineral oil and miglyol 812) were provided as ready to use suspensions by Bayer Animal Health GmbH.

III.1.3.2. *S. aureus* inoculum preparation

S. aureus Newbould 305 (ATCC 29740) was used for infection. This bacterial strain was isolated from a clinical mastitis case in 1958 (Prasad and Newbould, 1968) and is since widely used for experimental intramammary infection of cows (Gudding et al., 1984 and Schukken et al., 1999) and mice (Brouillette et al., 2004). Overnight brain heart infusion (BHI; Oxoid Limited, Belgium) cultures of *S. aureus* were diluted in sterile phosphate-buffered saline (PBS; Gibco, Belgium) and quantified by

flow cytometry (BD Biosciences, Belgium). Briefly, 1 ml of a 1000-fold PBS-diluted bacterial suspension was added to a TRU count tube (BD Biosciences), which contained a known number of fluorescent beads. The number of bacteria was then calculated using the following equation:

$$Bacteria\ per\ ml = \frac{bacterial\ counts\ x\ total\ beads\ in\ tube\ x\ dilution}{bead\ counts}$$

The actual colony forming units (CFU) of the inoculum was confirmed by overnight culture of a serial logarithmic dilution on tryptic soy agar (TSA; Oxoid Limited) plates.

III.1.3.3. Minimal inhibitory concentration (MIC) determination

Determination of the MIC of the 4 first generation cephalosporins for the bovine mastitis isolate *S. aureus* Newbould 305 was performed using the Mueller–Hinton agar dilution assay according to CLSI guidelines (CLSI, 2008). Plates were incubated at 35 °C (±2 °C) for 16–20 h in an aerobic atmosphere.

III.1.3.4. Staphylococcal mouse mastitis model

The general procedure for mouse mammary gland infection was adapted from the method used by Brouillette et al. (2004). Briefly, CD-1 lactating mice (Harlan Labouratories Inc., Netherlands) were utilized 12–14 days after birth of the offspring. The mice typically weigh 50 g at that time. The pups were weaned 1–2 h before bacterial inoculation of the mammary glands. A mixture of oxygen and isoflurane (2–3%) was used for inhalational anesthesia of the lactating mice. A syringe with 32-gauge blunt needle (Thiebaud Biomedical Devices, France) was applied to inoculate both L4 (on the left) and R4 (on the right) glands of the fourth abdominal mammary gland pair with approximately 150 CFU of *S. aureus*. Each orifice was exposed by a small cut at the near end of the teat and 100 µl of the inoculum was injected slowly through the teat canal. The cephalosporin formulations were instilled into the mammary gland of anesthetized mice using the desired dose (µg/gland) and excipient, usually mineral oil, at 4 h after bacterial inoculation. This 4 h time point was chosen, because a relevant bacterial load of about $5\log_{10}$ CFU/g gland together with phagocytosed bacteria are typically observed at that time (Brouillette et al., 2004). Immediately thereafter the postoperative analgesic Buprecare (Codifar NV, Belgium) was administered intraperitoneally, i.e. at 4 h after bacterial inoculation. The animal experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2009/010). In all studies, a group with *S. aureus*-infected, excipient-treated mice ("excipient control group") was compared with several test groups, in which mice were infected with *S. aureus* and treated with a particular dose of a cephalosporin. The control groups in the different studies were generally composed of 2 mice (4 mammary glands). The test groups were generally composed of 4 mice (8 mammary glands). Detailed information on the used dosages and number of glands per group is provided in Table 2 and Table 3.

III.1.3.5. Mammary gland homogenization and CFU quantification

After sacrifice of the mice by cervical dislocation at 14 h post-treatment, mammary glands (two per mouse) were harvested, weighed and homogenized on ice in sterile PBS using a tissue ruptor (QIAGEN Benelux BV, Netherlands). The mammary glands, which are structurally separate, were considered as individual samples. Bacterial CFU counts were obtained after quantification of serial logarithmic dilutions of mammary gland homogenates on TSA plates and transformation of the raw CFU counts into base 10 logarithm values. The detection limit (DL) was $3\log_{10}$ CFU/g gland weight for the cefalonium and cefapirin dose-titration studies. Further optimization of the serial dilution technique for plating resulted in a decrease of the DL to $1.7\log_{10}$ CFU/g gland weight. This lower DL was handled in the cefalexin and cefazolin dose titration, the intra-experimental comparative and the excipient studies. Importantly, the data collected in the studies with the 20-fold higher DL had no impact on the final conclusions, as was verified in the intra-experimental comparative study.

III.1.3.6. Calculation of the effective (ED) and protective dose (PD) for each cephalosporin

The mean intramammary CFU/g gland of *S. aureus*-infected glands from mice treated with different doses of each of the 4 cephalosporins ("test groups") are compared with each other and with the mean CFU/g gland of infected glands from mice injected with excipient only ("excipient control group"). To evaluate the dose–response relationship for each cephalosporin the ED_{2log10}, ED_{4log10}, PD_{50} and PD_{100} were calculated. The ED_{2log10} and ED_{4log10} values are the concentrations of the cephalosporin needed to reduce the mean CFU/g gland from the control group by $2\log_{10}$ and $4\log_{10}$, respectively. The PD_{50} and PD_{100} are the doses of cephalosporin needed to observe respectively 50% and 100% of cleared mammary glands (with CFU/g below DL). To calculate the ED values, the CFU/g gland were analyzed separately with a mixed-effect E_{max} model as a function of the dose. The model was parameterized as follows:

$$logCFU = E_0 + \frac{E_{max}\, x\, dose}{ED_{50} + dose} + animal + eps$$

eps (epsilon) is the residual variability, assuming an independent normal distribution: eps $\sim N(0, \sigma^2)$. *Animal* is the inter-animal variability, assuming an independent normal distribution: *Animal* $\sim N(0, \sigma^2_{sub})$. CFU/g gland below the DL were censored at $\log_{10}$(DL) and the cumulative normal distribution was specified in the likelihood function. E_0 estimates the overall arithmetic mean CFU/g gland in the untreated group and E_{max} estimates the maximum change to the CFU/g gland. Because the CFU/g gland for mice of the control group were all comparable (see Section III.1.3.7), they were pooled to increase the precision to estimate E_0. The ED_{50} is the dose required to produce 50% of the maximal effect.

The ED_{4log10} dose was determined as:

$$ED_{4log10} = -\frac{4\ x\ ED_{50}}{E_{max} + 4}$$

The ED_{2log10} dose was determined as:

$$ED_{2log10} = -\frac{2\ x\ ED_{50}}{E_{max} + 2}$$

To calculate the PD_{50}, the proportion of cleared glands *versus* the compound dose was analyzed using a logistic regression model, *i.e.* cleared/not cleared. The model was defined as:

$$\text{Logit(P.(gland is cleared))=intercept+slope×dose}$$

The PD_{50} was determined from the model as:

$$PD_{50} = -\frac{intercept}{slope}$$

The PD_{100} cannot be calculated from the model and was therefore deduced from the experimental data. To compare the efficacy of the cephalosporins in the separate dose-titration studies with their efficacy in the comparative study, the E_{max} model described above was estimated again for each compound separately and by pooling all data from the separate dose-titration and the comparative studies. For each cephalosporin, the mean CFU/g gland were compared between the separate and the comparative study with a mixed-effect analysis of variance (ANOVA) model. The model was parameterized as follows:

$$\text{Mu = mean(logCFU)|study + animal + eps}$$

$\text{mean(logCFU)}|_{study}$ is the mean value observed from the separate or the comparative study. The difference between the study means was calculated as:

$$\text{diff = mean (logCFU)|comparative study − mean(logCFU)|separate study}$$

All statistical analyses were carried out using SAS v 9.2 under PC windows. The estimations and tests were performed with the NLMixed and GenMod procedures in SAS.

II.1.3.7. Analysis of the reproducibility of the *S. aureus* mouse mastitis model

The CFU/g gland of 14 excipient control groups, *i.e.* mice inoculated with *S. aureus* and treated with mineral oil, were included in the statistical analysis. The data of 6 of the 14 groups is presented in the current manuscript. The data of the additional 8 excipient control groups were from other studies, in which mice were inoculated with *S. aureus* and treated with mineral oil, performed in our research unit (data not shown). The evaluation was performed according to the analysis of reproducibility based on linear mixed-effect models (Stanish and Taylor, 1983). Three items were addressed to evaluate the reproducibility of the model. At first, to assess change over time, the CFU/g gland were analyzed with a mixed-effect linear regression as a function of time. The model included the mean-centered study date as covariate. The significance of the change with time was assessed using an *F*-test at the 2-sided 5% level. If not significant, a reduced model without time effect was estimated. Then, to evaluate the main sources of variability within and across studies, a linear mixed-effect model was applied to the CFU/g gland. The total variability was broken down into 3 distinct sources, *i.e.* (a) the variability across studies (inter-study variability), (b) the variability across animals (intra-study variability) and (c) the variability between the left and right gland of each animal (intra-animal variability). The intra-class correlation coefficients (ICC) were calculated to quantify the degree of consistency:

- The intra-study level ICC:

$$ICC_{study} = \frac{\sigma^2_{study}}{\sigma^2_{study} + \sigma^2_{animal} + \sigma^2_{residual}}$$

- The intra-animal level ICC:

$$ICC_{animal} = -\frac{\sigma^2_{study} + \sigma^2_{animal}}{\sigma^2_{study} + \sigma^2_{animal} + \sigma^2_{residual}}$$

The ICC values range between 0 and 100%. A large (small) value indicates strong (weak) agreement in the response within the component. It is typical to consider that a value below 40% indicates poor correlation, whereas a value greater than 75% indicates excellent correlation. Prior to these calculations, the upper and lower outlier studies were removed by using the Cook's distance for each study to determine the influence of the study on the results. Finally, it was determined what could be considered as a normal CFU/g gland in any future study. The range of plausible individual and mean CFU/g gland was predicted using Bayesian posterior predictive methods. A flat prior was specified for all parameters, making the likelihood function the posterior. The predicted distribution of CFU/g gland was evaluated for several glands and mice per study. 95% prediction intervals were calculated to obtain acceptance ranges for subsequent studies.

Table 2. Parameters (at left side of the dashed vertical line) were calculated from the individual study data (at right side of the dashed vertical line) in the dose titration studies to evaluate the antimicrobial efficacy of cefalexin, cefalonium, cefapirin and cefazolin in the *S. aureus* mouse mastitis model. CFU: colony forming unit; ED: effective dose; Exp. DL: experimental detection limit, which is 3log10 CFU/g gland weight for the

Cephalosporin	MIC[(a)] (µg/ml)	$ED2log_{10}$ (µg/gland)	$ED4log_{10}$ (µg/ml)	PD_{50} (µg/gland)	PD_{100} (µg/gland)			CFU/g gland (log_{10})		Glands cleared (%)
						Dose (µg/gland)	# glands	Mean	SD	Exp. DL
Cefalexin	4	58	226	>2000(b)	N.O.	0	4	7.99	0.55	0
						100	8	5.03	0.42	0
						200	7	4.26	0.49	0
						400	8	3.26	1.06	25
Cefalonium	0.06	27	105	362	800	0	4	7.80	1.00	0
						200	4	3.09	0.16	0
						400	4	3.01	0.01	75
						800	4	3.00	0.00	100
Cefapirin	0.125	38	248	>2000(b)	N.O.	0	4	8.16	0.31	0
						100	6	4.82	0.51	0
						200	7	4.11	0.36	0
						400	8	3.64	0.52	0
Cefazolin	0.25	28	65	117	200	0	4	7.75	0.29	0
						100	8	2.75	0.92	38
						200	8	1.70	0.00	100
						400	6	1.70	0.00	100

(a) Experimentally determined MIC values for each of the cephalosporins against *S. aureus* Newbould 305 (ATCC 29740). (b) The predicted PD50 values are 2499 and 297415 µg/gland for cefapirin and cefalexin, respectively, but are indicated by >2000 because the former doses are of minimal biological relevance.

III.1.4.Results

III.1.4.1. Minimal inhibitory concentration of cephalosporins

The MIC of the 4 compared first generation cephalosporins against the bovine mastitis isolate *S. aureus* Newbould 305 ranged between 0.06 and 4 µg/ml, approximating previously determined MIC50 values (Table 2). The MIC value of the 4 cephalosporins overall indicates adequate antimicrobial activity against *S. aureus* Newbould 305.

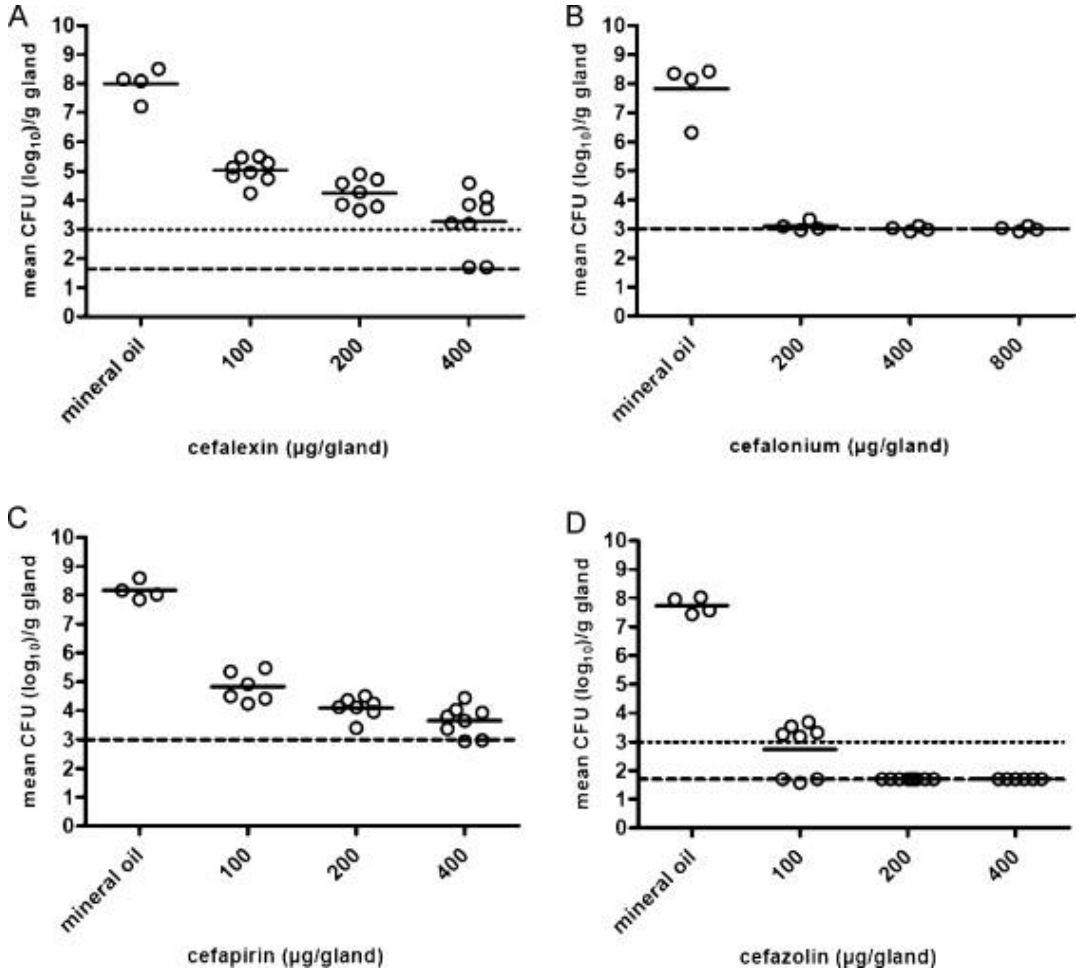

Fig.1. *S. aureus* count reduction (CFU/g gland) at 14 post-treatment in mouse mammary glands treated with multiple doses of cefalexin (A), cefalonium (B), cefapirin (C) and cefazolin (D). An artificial detection limit (DL) was drawn at 3log10 for cefalexin and cefazolin to allow straightforward comparison with cefalonium and cefapirin, for which 3log10 was the experimental DL. Data details are summarized in Table 2. Dashed line: experimental DL. Dotted line: artificial DL.

III.1.4.2. Assessment of cephalosporin efficacy in *S. aureus*-induced mouse mastitis

a. Dose titration studies

The *in vivo* dose–response relationship of each cephalosporin was analyzed in 4 separate dose titration studies, from which the ED_{2log10}, ED_{4log10}, PD_{50} and PD_{100} were calculated (Fig. 1 and Table 2). The ED_{2log10} value of the 4 cephalosporins was found to be similar, ranging between 27 and 58 µg/gland. This indicates that all 4 compounds can clear 99% of the bacteria at a low dose. In contrast, the ED_{4log10} value, i.e. 99.99% bacterial clearance, of the 4 cephalosporins varied substantially, ranging between 65 µg/gland for cefazolin and 248 µg/gland for cefapirin. The PD_{50} values also varied substantially between 117 µg/gland for cefazolin and >2000 µg/gland for cefapirin, indicating that the antibacterial efficacy of the 4 cephalosporins can be better discriminated when tested at higher doses (Table 2). This variation is also reflected in the dose needed to eradicate all bacteria from the glands (PD_{100}). For example, 200 µg/gland cefazolin eradicates all bacteria in all mammary glands to at least the DL, while not one of the analyzed cefalexin or cefapirin doses was able to achieve this objective (Table 2). Notably, the DL was $3log_{10}$ CFU/g gland weight for the cefalonium and cefapirin dose-titration studies and 1.7log10 CFU/g gland weight for the cefalexin and cefazolin studies. Importantly, the conclusion was identical when the DL was artificially drawn at 3log10 CFU/g gland weight for the cefalexin and cefazolin studies (Fig. 1 and Table 2), indicating the negligible influence of the alternative DLs on the data.

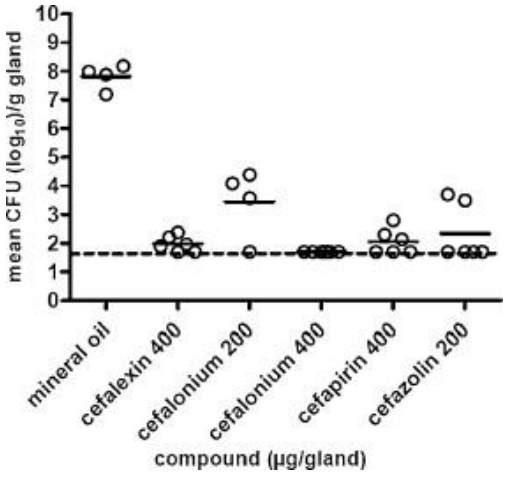

Figure 2. *S. aureus* count reduction (CFU/g gland) at 14 post-treatment in mouse mammary glands treated with a selected dose of cefalexin, cefalonium, cefapirin and cefazolin. Of note, cefalonium was analyzed in 2 concentrations, because the detection limit in the cefalonium study (Fig. 1) did not allow unambiguous identification of the optimal dose to be compared in this comparative study. Data details are summarized in Table 3. Dashed line: experimental detection limit.

b. Confirmatory study

To rule out inter-experimental variations, a selected dose of each of the 4 cephalosporins was compared in the same study (Fig. 2 and Table 3). The selected dose for each cephalosporin was the dose in the separate dose-titration studies that reduced the bacterial load virtually to the DL in the

majority of the mammary glands. This dose was 200 µg/gland for cefazolin and cefalonium. Nonetheless, the less sensitive DL in the cefalonium dose-titration study may have masked bacterial growth which would have been detected with the lower DL of 1.7log10. Therefore, a dose of 400 µg/gland cefalonium was also included. Since a dose that cleared all bacteria in nearly all mammary glands was not observed for cefalexin nor cefapirin, the highest dose of 400 µg/gland was chosen for both compounds. The mean CFU/g gland of cefalexin, cefalonium and cefazolin in the dose-titration studies did not differ significantly with its respective mean in the intra-study ($p = 0.15, 0.87, 0.14$ respectively), indicating adequate reproducibility. Although there was significantly more reduction in the mean CFU/g gland for cefapirin in the confirmatory study than in the dedicated dose-titration study ($p = 0.003$), the ranking of efficacy for the four cephalosporins in the former study corroborated well the efficacy results obtained in the dose titration studies.

Table 3. Intra-study comparison of (1) a selected dose of cefalexin, cefalonium, cefapirin and cefazolin, and (2) the influence of the excipient on the efficacy of cefazolin in the mouse mastitis model of *S. aureus* infection. CFU: colony forming unit; SD: standard deviation.

Treatment	Dose (µg/gland)	Number of Glands	CFU/g gland (log_{10})		Glands cleared (%)
			Mean	SD	
(1) Mineral oil	0	4	7.81	0.42	0
Cefalexin	400	6	1.98	0.28	33
Cefalonium	200	4	3.44	1.21	25
Cefalonium	400	6	1.70	0.00	100
Cefapirin	400	6	2.06	0.45	50
Cefazolin	200	6	2.33	0.98	66
(2) Mineral oil	0	10	7.64	0.18	0
Cefazolin in mineral	100	8	3.88	0.62	0
Cefazolin in Miglyol	100	8	1.89	0.53	88

c. Antibacterial efficacy of cefazolin suspended in two excipients

The influence of the excipient on the *in vivo* antibacterial activity was evaluated by comparing the therapeutic efficacy of 100 µg/gland cefazolin suspended in either mineral oil or miglyol 812 (Fig. 3 and Table 3). The mean CFU/g gland for mineral oil-suspended cefazolin was reduced with 3.7log10, when compared with the mean CFU/g gland from the negative control group. Miglyol 812-suspended cefazolin decreased the mean CFU/g gland nearly to the DL.

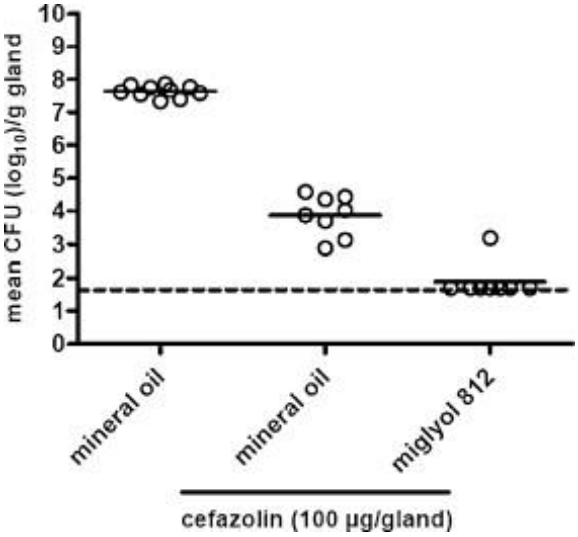

Figure 3. *S. aureus* count reduction (CFU/g gland) at 14 post-treatment in mouse mammary glands treated with 100 µg/gland cefazolin suspended in either mineral oil or miglyol 812. Data details are summarized in Table 3. Dashed line: experimental detection limit.

d. Assessment of the reproducibility of the mouse mastitis model

The mean CFU/g gland of the excipient control groups, i.e. *S. aureus*-inoculated mice treated with mineral oil, from the 6 studies included in the current manuscript and 8 other studies performed in our research unit (n = 14) were statistically analyzed on three levels to evaluate the robustness of the *S. aureus* mouse mastitis model. At first, the variation over time was assessed (Fig. 4A). The mean CFU/g gland across all studies is 8.06log10 ± 0.11. There is a negligible, non-significant (p = 0.133) trend toward an increase in CFU/g gland with time (0.02log10 ± 0.01 per month). Secondly, the inter-study, intra-study and intra-animal variability together with the intra-study and intra-animal level ICC estimates were evaluated. The inter-study variability is 0log10 CFU/g gland. The intra-study variability is 0.65log10 CFU/g gland and is identical to the total variability. The intra-study level ICC is 0.0%, indicating no reciprocal dependency among the animals within a study group. As a consequence of this, the results are completely reproducible and exchangeable across the studies. The intra-animal variability is 0.40log10 CFU/g gland, also showing a reasonable degree of consistency (ICC = 61.66%). Finally, to predict normal ranges for future studies, the distribution of plausible CFU/g gland in a future study counting 4 animals was calculated. In addition, the 98% prediction ranges for CFU/g gland in a single gland and in a single animal (2 glands) were predicted (Fig. 4B). The estimated CFU/g gland are close to 8.06log10, which is identical with the mean CFU/g gland across the studies. Logically, the more glands included, the more precise the estimation of the CFU/g gland becomes.

III.1.5. Discussion

Antimicrobial drug research both into novel antibiotics and improved formulations in cows associated with several limitations such as stringent ethical constraints and excessive costs. A valid strategy to circumvent the high costs associated with screening studies in cattle but still determine the *in vivo* potential of candidate antimicrobials, is their analysis in the experimental mouse mastitis model. The latter model was characterized for *S. aureus* in 2004 and suggested as a valuable and highly reproducible intermediate *in vivo* alternative for antimicrobial efficacy studies (Brouillette et al., 2004). This aspect is supported in the current study by the high reproducibility over time for the *S. aureus*

load (CFU/g gland) of the excipient control group from 14 independent studies performed in our research unit. Analysis of the inter-study, intra-study and intra-animal variability also indicated that the results are highly reproducible across the studies.

The first generation cephalosporins cefalexin, cefalonium, cefapirin and cefazolin make up one third of the single component antibiotics on the Belgian market currently used to treat mastitis (BCFI, 2011). Both *in vitro* and bovine field studies evaluated the efficacy of these 4 cephalosporins individually (Apparao et al., 2009, Bradley et al., 2010, Maneke et al., 2011, Moroni et al., 2006, Nunes et al., 2007 and Roy et al., 2009). However, a side-by-side comparison of their *in vivo* antimicrobial efficacy has never been performed. The present study addressed this issue by evaluating the dose–response relationship in a dose-titration experiment for each of the 4 cephalosporins and by a subsequent confirmatory comparison of a selected dose. In addition, the influence of the excipient on the efficacy of cefazolin was evaluated.

From the 7 cephalosporins, i.e. 4 first, 2 third and 1 fourth generation, that have been described for mastitis treatment, only cefapirin (1st), ceftiofur (3rd) and cefoperazone (3rd) have been analyzed in the *S. aureus*-induced mouse mastitis model (Anderson and Craven, 1984, Brouillette et al., 2004 and Yancey et al., 1987). Comparison of our study results with those of the aforementioned authors is informative. However, making strong statements based on such comparison would be inappropriate as the experiments were performed with different mouse and/or *S. aureus* strains, with variable bacterial inoculation doses, and with different antimicrobial doses, administration routes and incubation times. Converting the intramammary PD_{50} doses obtained in our studies into PD_{50} doses expressed as mg/kg bodyweight (BW), assuming a mean mouse BW of 50 g, results in PD_{50} doses of >40, 7.2, >40 and 2.3 mg/kg for cefalexin, cefalonium, cefapirin and cefazolin, respectively. The PD_{50} (confusingly named ED_{50} by Yancey) of ceftiofur and cefoperazone is respectively 0.8 and 1.8 mg/kg with a higher bacterial inoculation dose and a longer incubation time (Yancey et al., 1987). It is therefore likely that both these third generation cephalosporins have similar antimicrobial potential as cefazolin but are more efficacious than the other analyzed first generation cephalosporins. Likewise, intramammary injection of 8 mg/kg cefapirin in our study reduced the median bacterial growth with 4.5log10, which approximates the 3.6log10 reduction after intravenous injection of 10 mg/kg cefapirin but is lower than the 7.4log10 reduction upon intramuscular injection with the same dose (Brouillette et al., 2004). Intra-study comparison of the cephalosporins in the mouse mastitis model would be an excellent strategy to evaluate their relative efficacies whether or not in function of the administration route. Initial assessment of the efficacy of antimicrobials is based on *in vitro* analysis according to the guidelines of the CLSI (CLSI, 2008). However, it is generally accepted that *in vitro* results do not necessarily fully correlate with *in vivo* outcome of antimicrobials (Apparao et al., 2009). Interaction of *S. aureus* with host mammary and immune cells during the infection is impossible to mimic *in vitro* (Brouillette and Malouin, 2005).

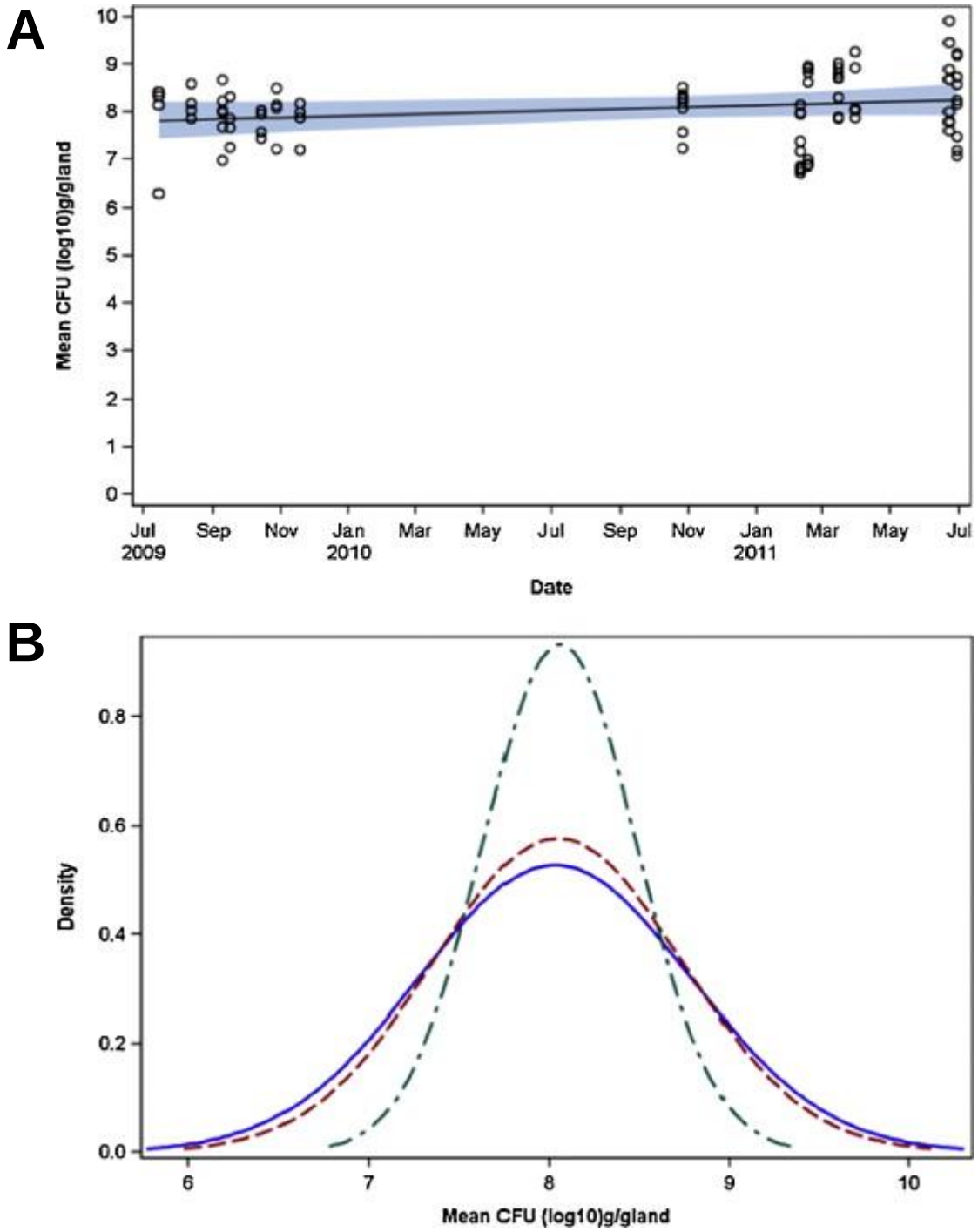

Figure 4. (A) Graphical presentation of the reproducibility over time of the predicted mean CFU/g gland (black line) with 95% confidence interval (blue band) for *S. aureus*-infected, paraffin oil-treated mice of 14 independent studies performed in our research group. Each circle corresponds to the CFU/g gland for an individual gland. The mean CFU/g gland was comparable between studies and the variability did not significantly change over time. (B) Graphical presentation of the 98% prediction range in CFU/g gland for individual glands (blue line), a single animal (2 glands; red line) or 4 animals (green line). The expected mean CFU/g gland are close to 8.04log10. The estimation of the CFU/g gland became more precise when more glands were used.

Moreover, the milk environment in the mammary gland influences bacterial growth, the expression of genes that control *S. aureus* pathogenesis, and the absorption, distribution, half-life and metabolism of the antimicrobial (Brouillette and Malouin, 2005 and Gehring and Smith, 2006). When the 4 first generation cephalosporins in the current study are compared according to their *in vitro* and *in vivo* efficacy, a different ranking is observed. More specifically, our *in vitro* MIC data indicate that cefalonium has the best antimicrobial efficacy against *S. aureus*, followed by cefapirin, cefazolin and cefalexin. In contrast, the objective efficacy parameters derived from the *in vivo* mouse mastitis data indicate that cefazolin is the most effective cephalosporin for intramammary treatment, followed by cefalonium, cefalexin and cefapirin. Although side-by-side comparison of these cephalosporins in cows is required to formulate definitive conclusions for the target species, their doses currently applied in the mastitis field support the classification of the *in vivo* mouse mastitis study (BCFI, 2011). This underscores the crucial importance of *in vivo* studies for antimicrobial drug evaluation.

Of interest for future treatment directions, the efficacy of an antibiotic can be analyzed in experimental mouse mastitis induced by other bovine mastitis isolates than *S. aureus* Newbould 305. In the current study, the *S. aureus* Newbould 305 was selected because of its historical use as mastitis-inducing pathogen in cow studies, but numerous bovine *S. aureus* strains with different persistence and therapeutic response have been identified (Zadoks et al., 2011). Several *S. aureus* strains have already been applied in the mouse mastitis model [reviewed in Brouillette and Malouin, 2005].

Optimizing the excipient is possibly a cost-effective strategy to improve the efficacy of existing compounds. In comparison to mineral oil, miglyol 812 has higher stability against oxidation, better spreadability and absorption, and improved compound stability and solubility characteristics (Sasol, 2004). These characteristics most likely explain why cefazolin suspended in miglyol 812 showed improved antimicrobial activity against *S. aureus*-induced mouse mastitis compared to the classical mineral oil-suspended cefazolin. This observation indicates that an inert substance is not necessarily an optimal excipient. The latter substance should support the active compound *in vivo* by providing improved adsorption, distribution, metabolism and excretion characteristics (Gehring and Smith, 2006, Gruet et al., 2001 and Van Bambeke et al., 2006).

In summary, our study supports that *in vivo* analysis of antimicrobials is of utmost importance to assess their therapeutic potential. It also indicates that cefazolin has the most efficient *in vivo* antimicrobial activity against *S. aureus* of the 4 tested first generation cephalosporins, as could be deduced from the ED_{2log10}, ED_{4log10}, PD_{50} and PD_{100} parameters calculated from the *in vivo* mouse mastitis experiments. Additionally, our data also highlight the influence of the excipient on the antimicrobial efficacy, an observation which is in line with previous findings. Overall, the current study emphasizes the strength of the mouse mastitis model as a fast, cheap and reproducible intermediate *in vivo* alternative for antimicrobial compound efficacy studies in cattle.

III.1.6. Acknowledgements

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III.2 Antimicrobial Efficacy of Intramammary Treatment with a Novel Biphenomycin Compound against Staphylococcus aureus, Streptococcus uberis, and Escherichia coli-induced Mouse Mastitis

III.2.1. Summary

Bovine mastitis undermines udder health, jeopardizes milk production, and entails prohibitive costs, estimated at $2 billion per year in the dairy industry of the United States. Despite intensive research, the dairy industry has not managed to eradicate the 3 major bovine mastitis-inducing pathogens: *Staphylococcus aureus*, Streptococcus uberis, and *Escherichia coli*. In this study, the antimicrobial efficacy of a newly formulated biphenomycin compound (AIC102827) was assessed against intramammary *Staph. aureus*, *Strep. uberis*, and *E. coli* infections, using an experimental mouse mastitis model. Based on its effective and protective doses, AIC102827 applied into the mammary gland was most efficient to treat *Staph. aureus*, but also adequately reduced growth of *Strep. uberis* or *E. coli*, indicating its potential as a broad-spectrum candidate to treat staphylococcal, streptococcal, and coliform mastitis in dairy cattle.

III.2.2. Introduction

Mammary gland inflammation, or mastitis, provokes detrimental damage to mammary epithelial cells and results in highly impaired milk production. As the most common disease in dairy cows, mastitis is the principal cause of economic losses in the dairy industry (Piepers et al., 2007; Pinzón-Sánchez et al., 2011). Clinical mastitis caused by environmental pathogens such as Streptococcus uberis and *Escherichia coli* can mostly be cured by antibiotic therapy (Pyörälä, 2009). Nevertheless, the incidence of coliform mastitis has been increasing relentlessly, and multidrug resistance is a typical feature for *E. coli* (Bradley, 2002; Saini et al., 2012). Although use of the preventive measures in the well-known 10-point checklist of the National Mastitis Council (NMC, 2006) has significantly improved the incidence of mastitis induced by contagious pathogens such as *Staphylococcus aureus*, staphylococcal mastitis is still frequently observed (Hogeveen et al., 2010; Zadoks et al., 2011). Therefore, novel antimicrobial therapies remain highly desired for treatment of not only *Strep. uberis* and *E. coli* mastitis but also *Staph. aureus* mastitis.

The antimicrobial AIC102827 belongs to the cyclopeptide biphenomycin class (Ezaki et al., 1985), acting at the level of bacterial protein synthesis; that is, inhibition of the thermo-unstable elongation factor (EF-Tu). Specifically, the thermo-unstable elongation factor mediates the entry of the aminoacyl-transfer (t)RNA into the free A-site of the ribosome (Agirrezabala and Frank, 2009). This mode of action makes no distinction between gram-positive and gram-negative pathogens, suggesting the potential for efficient antibiotic activity against gram-positive *Staph. aureus* and *Strep. uberis* and gram-negative *E. coli*. The objective of the current study was to screen, both *in vitro* and *in vivo*, the intramammary efficacy of the novel candidate antimicrobial AIC102827 against the bovine mastitis pathogens *Staph. aureus*, *Strep. uberis*, and *E. coli*. Bovine mastitis studies are time consuming and extremely costly. Therefore, the mouse model for bovine mastitis, which has been recognized as a fast, cheap, and reproducible intermediate *in vivo* alternative for antimicrobial compound efficacy

studies in cattle (Brouillette et al., 2004; Notebaert et al., 2008; Demon et al., 2012), was applied as an elegant screening tool in the current study.

III.2.3. Materials and methods

In the present study, the excipient (hydrogel) and different concentrations of AIC102827 (2, 4, 8, and 12 mg/mL) were provided as ready-to-use suspensions by AiCuris GmbH & Co. (Wuppertal, Germany). *Staphylococcus aureus* Newbould 305 (ATCC 29740), *Strep. uberis* NADC C-1 (ATCC 27958), and *E. coli* P4:O32 were used for infection of mice (challenge isolates). These bacterial strains were originally isolated from clinical mastitis cases (Prasad and Newbould, 1968; Bramley, 1976; McDonald and McDonald, 1976). Overnight cultures of the bacteria were diluted in sterile PBS and quantified by flow cytometry (BD Biosciences, Erembodegem, Belgium), as described in detail by Demon et al. (2012). *In vitro* determination of the MIC of AIC102827 against the challenge isolates *Staph. aureus* Newbould 305, *Strep. uberis* NADC C-1, and *E. coli* P4:O32, and a random selection of recent bovine mastitis field isolates (n = 10 for each species) was performed using the Mueller-Hinton agar dilution assay according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). The procedures for mouse mammary gland infection, postmortem mammary gland homogenization, and bacterial quantification have been described recently by our group (Demon et al., 2012). Briefly, CD-1 lactating mice (Harlan Labouratories Inc., Horst, the Netherlands) were anesthetized and intraductally inoculated with approximately 150 cfu of challenge isolate in 100 µL of PBS in both glands of the fourth abdominal mammary gland pair. Similarly, 100 µL of the excipient and test doses of AIC102827 were instilled at 4 h after bacterial inoculation. After killing of the mice at 14 h posttreatment, mammary glands were harvested, weighed, and homogenized on ice in sterile PBS. Bacterial counts (cfu) were obtained after quantification of serial log10 dilutions of mammary gland homogenates on tryptic soy agar plates and transformation of the raw counts into base 10 logarithmic (log10) values. The detection limit was 1.7log10 cfu/g of gland. In the dose-titration studies, a group with bacteria-infected, excipient-treated mice (excipient control group) was compared with 3 test groups, in which mice were infected with bacteria and treated with a particular dose of AIC102827. In the intra-experimental comparison study, the excipient control group was compared with one test group, in which mice were infected with bacteria and treated with 800 µg/gland of AIC102827. The groups in the different studies were composed of 4 mice (8 mammary glands), except the 3 mice (6 glands) in the 800 µg/gland test group in the *E. coli* dose-titration study. The animal experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium (EC2009/133). To evaluate the dose-response relationship for the antimicrobial, the effective dose $(ED)_{2log10}$, ED_{4log10}, protective dose $(PD)_{50}$ and PD_{100} values were calculated from the dose-titration studies. The ED_{2log10} and ED_{4log10} values are the concentrations of AIC102827 needed to reduce the mean bacterial count from the excipient control group by 2log10 and 4log10 cfu/g of gland, respectively. The PD_{50} and PD_{100} are the doses of AIC102827 needed to observe, respectively, 50 and 100% of cleared mammary glands (with mean bacterial count below the detection limit). The model parameters were calculated by nonlinear regression analysis using Microsoft Excel software (Microsoft Corp., Redmond, WA). The models that fitted best; that is, that yielded the highest R2 value, with the

dose-response relationship of the antibiotic were polynomial for *Staph. aureus* Newbould 305 (y = 7.10−5x2 − 0.0471x + 8.5966) and *Strep. uberis* NADC C-1 (y = 5.10−6x2 − 0.0101x + 6.1556) and exponential for *E. coli* P4:O32 (y = 8.6977e−0.002x). The ED_{2log10} and ED_{4log10} values were then computed by calculating x from these models, in which y is replaced by z − 2 and z − 4, respectively, with z being the mean bacterial count (log10 cfu/g of gland) from the excipient control group. To determine the volume of the secretory fluid in the bacterially infected mammary gland, mice (n = 8) were intraductally infected with *Staph. aureus* Newbould 305 as described. Mammary glands (n = 15; 1 gland was omitted due to improper inoculation) were harvested at 4 h postinoculation. The glands were weighed, placed on a cell strainer of 40 µm, and centrifuged at 2,000 × g for 1 h to expel their milk content. After centrifugation, the glands were weighed again and subtracted from the gland weight before centrifugation. The resulting weight was divided by the density of mouse milk (1.06 mg/mL) to calculate the corresponding volume.

III.2.4. Results

Table 1 reports MIC values of AIC102827, the newly formulated antimicrobial from the biphenomycin class, against the 3 major bovine mastitis pathogens. The MIC values of AIC102827 against 10 bovine mastitis field isolates of *Staph. aureus*, *Strep. uberis*, and *E. coli* were 4 to 8, ≥16, and 2 to 4 µg/mL, respectively.

Table 1. Antimicrobial efficacy of AIC102827 against *Staphylococcus aureus*, Streptococcus uberis, and *Escherichia coli* evaluated *in vitro* by determination of MIC values and *in vivo* by analyzing effective and protective doses using the mouse mastitis model.

	MIC[1] (µg/mL)		Effective dose[2] (µg/gland)		Protective dose[3] (µg/gland)	
Species	**Field**	**Mouse**	ED_{2log10}	ED_{4log10}	PD_{50}	PD_{100}
Staph. aureus	4–8	4	45	100	200	80
Strep. uberis	≥16	>16	215	530	400–800	800
E. coli	2–4	4	130	310	400–800	>800

1: Minimal concentrations of the antimicrobial required to inhibit visible growth of *Staph. aureus* (n = 10), *Strep. uberis* (n = 10), or *E. coli* (n = 10) field isolates and *Staph. aureus* Newbould 305, *Strep. uberis* NADC C1, or *E. coli* P4:O32 mouse mastitis challenge isolates .2: ED_{2log10} and ED_{4log10} values are the concentrations of AIC102827 needed to reduce the mean bacterial count (log10 cfu/g of gland) from the excipient control group by 2log10 and 4log10, respectively.3: The PD_{50} and PD_{100} values are the doses of AIC102827 needed to observe, respectively, 50% and 100% of cleared mammary glands; that is, with a count (log10 cfu/g of gland) below the detection limit.

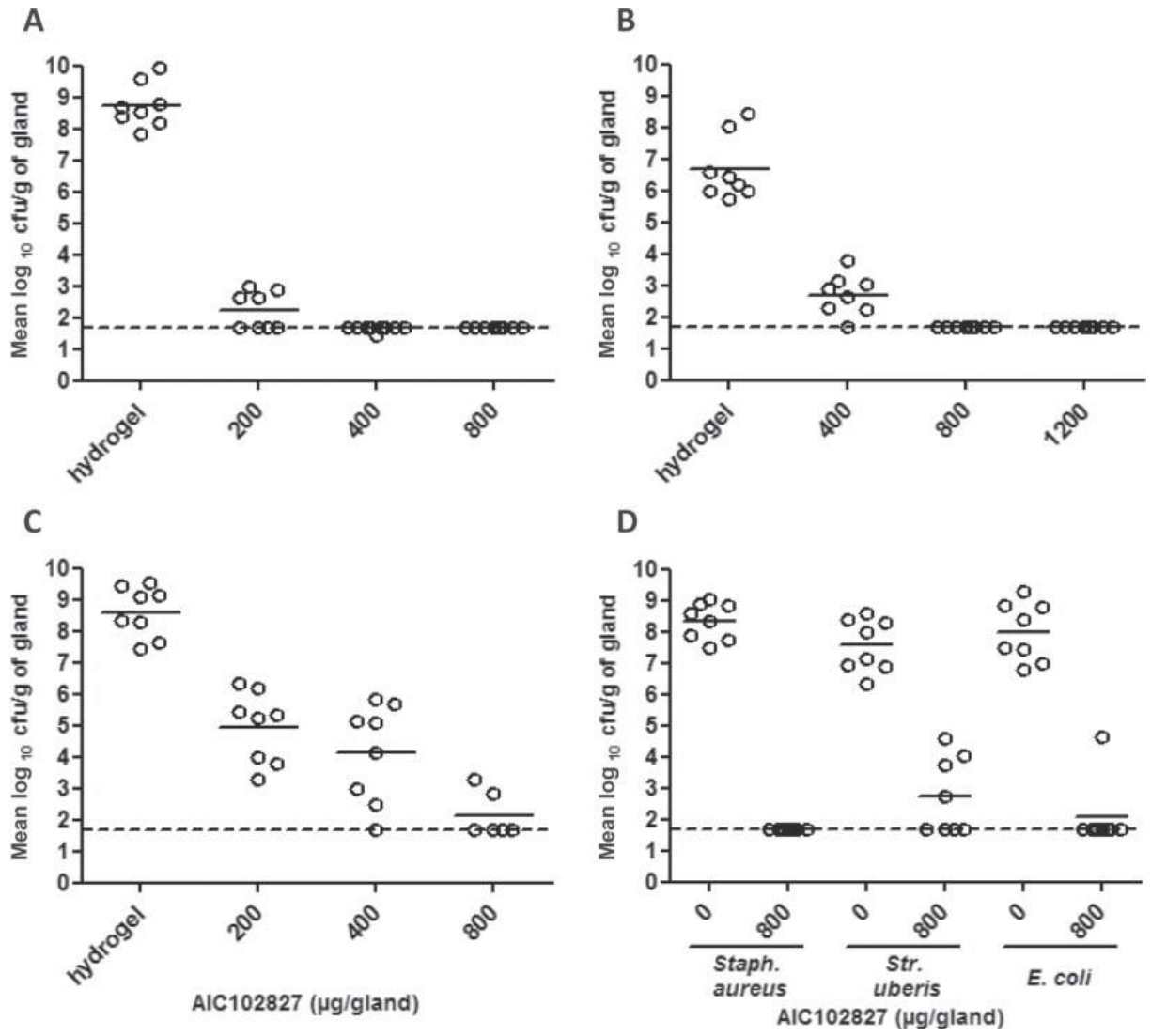

Figure 1. *Staphylococcus aureus* Newbould 305 (A), Streptococcus uberis NADC C-1 (B), and *Escherichia coli* P4:O32 (C) count reduction (log10 cfu/g of gland) at 14 h posttreatment in infected mouse mammary glands treated with increasing doses of the novel antimicrobial agent AIC102827; (D) intra-experimental comparison study indicating *Staph. aureus* Newbould 305, *Strep. uberis* NADC C-1, and *E. coli* P4:O32 count reduction (log10 cfu/g of gland) at 14 h posttreatment in mouse mammary glands treated with 800 µg/gland AIC102827. Dashed line indicates detection limit. Hydrogel = excipient only (no AIC102827).

These MIC values illustrate efficient *in vitro* antimicrobial activity of AIC102827 against *Staph. aureus* and *E. coli*, but less adequate activity against *Strep. uberis*. The adequate activity of AIC102827 against *Staph. aureus* and *E. coli* indicates its ability to act against both gram-positive and gram-negative bacteria. However, the inadequate activity against *Strep. uberis* shows that, although AIC102827 inhibits a universal bacterial growth mechanism, species-specific characteristics are

involved in the susceptibility to the antimicrobial. The MIC values of AIC102827 against the mouse mastitis challenge isolates of *Staph. aureus* Newbould 305, *Strep. uberis* NADC C-1, and *E. coli* P4:O32 were 4, >16, and 4 µg/mL, respectively, and strongly correlated with the sensitivity of the bovine mastitis field isolates, indicating the suitability of the latter as reference strains in the current *in vivo* mouse mastitis study. The *in vivo* dose-responses of mouse mastitis induced by *Staph. aureus* Newbould 305, *Strep. uberis* NADC C-1, and *E. coli* P4:O32 to hydrogel-suspended AIC102827 were analyzed in 3 separate dose titration studies (Figure 1A to C). These data were used to calculate the ED_{2log10} , ED_{4log10}, PD_{50}, and PD_{100} values (Table 1). The ED_{2log10} value (corresponding to 99% bacterial clearance) of AIC102827 was 45, 215, and 130 µg/gland for *Staph. aureus*, *Strep. uberis*, and *E. coli*, respectively. These values indicate that AIC102827 can clear 99% of the *Staph. aureus* bacteria from the lactating mammary gland, even at a low dose, whereas a higher dose is needed to clear *Strep. uberis* and *E. coli*. In line with this finding, the ED_{4log10} value (corresponding to 99.99% bacterial clearance) of AIC102827 was 100, 530, and 310 µg/gland for *Staph. aureus*, *Strep. uberis*, and *E. coli*, respectively. The PD_{50} and PD_{100} values of AIC102827 for the 3 mouse mastitis challenge isolates additionally indicated that its intramammary treatment is more efficient in curing glands infected by *Staph. aureus* than by *Strep. uberis* or *E. coli*. An intra-experimental study with one selected dose of AIC102827 was then performed to evaluate reproducibility and compare the antimicrobial efficacy of AIC102827 between the 3 pathogens in a study with minimal variability of experimental parameters such as the health status of the mice and environmental conditions such as humidity and temperature (Figure 1D). An AIC102827 dose of 800 µg/gland was selected for the intra-experimental comparison study, because it showed reduction of the bacterial load virtually to the detection limit in most mammary glands in the separate dose-titration studies. For all 3 species, the mean bacterial counts (log10 cfu/g of gland) in the intra-experimental study (Figure 1D) closely approximated their respective means in the dose-titration study (Figure 1A to C), illustrating the reproducibility of our data. Together, our *in vitro* MIC and *in vivo* mouse data indicate adequate efficacy of AIC102827 against the 3 mouse mastitis challenge isolates.

III.2.5. Discussion

The antimicrobial AIC102827 indicated similar *in vitro* MIC for *Staph. aureus* Newbould 305 and *E. coli* P4:O32. Consequently, analogous *in vivo* antimicrobial activity was expected against both pathogens. However, AIC102827 was less effective against *E. coli* than against *Staph. aureus* in the *in vivo* study. In contrast, efficacy against *Strep. uberis in vivo* was higher than expected based on the *in vitro* data. Together, these observations indicate that the mammary gland environment positively influences antimicrobial activity against the gram-positive bacteria and negatively affects activity against the gram-negative *E. coli*. A possible explanation for the increased *in vivo* activity against *Staph. aureus* and *Strep. uberis* could be that the concentration of AIC102827 in milk greatly exceeds the *in vitro* MIC required to inhibit bacterial growth. To assess this hypothesis, we evaluated the intramammary antimicrobial concentration in the bacterially infected mammary gland at 4 h postinfection; that is, at the time of compound treatment. On average, 52 µL (±19 µL) of secretory fluid was observed in the infected mammary gland, which had a mean weight of 635 mg (±163 mg) at the time of treatment

(Figure 2). Although substantial variability in gland volume was seen, these data indicate that the concentration of the compound suspension, which is injected in a volume of 100 µL, is reduced by approximately 30% after injection in the mammary gland. Treatment with the 8 mg/mL suspension led to a concentration of approximately 5 mg/mL in the mammary gland and is several-hundred-fold higher than the concentration that is used in *in vitro* experiments (in the µg/mL range) to determine the MIC values, corroborating this hypothesis. However, this does not explain the potentially lower *in vivo* antimicrobial efficacy against *E. coli*. The milk environment of the mammary gland is known to influence the *in vivo* efficacy of an antimicrobial agent. Critical aspects influenced by the milk matrix are local immune cell interactions, bacterial growth, and pharmacokinetic parameters (adsorption, distribution, metabolism, and excretion) of the antimicrobial (Brouillette and Malouin, 2005; Gehring and Smith, 2006; Van Bambeke et al., 2006). In this context, the extracellular residing *E. coli* has become better adapted to the milk environment than the typically intracellularly residing gram-positive pathogens *Staph. aureus* and *Strep. uberis*, at least partially clarifying the potentially lower than expected antimicrobial efficacy against *E. coli* observed in the current study. The observation that the *in vitro* antimicrobial activity of AIC102827 for *Staph. aureus*, *Strep. uberis*, or *E. coli* did not directly correlate with its *in vivo* efficacy corroborates previous reports by our group and others (Apparao et al., 2009; Pyörälä, 2009; Demon et al., 2012). Hence, our current data again highlight the importance of *in vivo* studies in addition to *in vitro* studies to screen antimicrobial candidates.

Several studies in cows describe strain-specific associations for *Staph. aureus*, *Strep. uberis*, and *E. coli* with respect to clinical signs, persistence, and response to therapy (Saini et al., 2012; and reviewed by Zadoks, 2007; Zadoks et al., 2011). Therefore, systematic comparison of the efficacy of antimicrobials against mastitis induced by different clinically relevant bovine strains would greatly help to guide veterinary practitioners in combining the most efficacious therapeutics as a strategy to target, for example, particular cases involving either a single resistant pathogen strain or multiple strains. Strains of *Staph. aureus* and *E. coli* other than Newbould 305 and P4:O32, respectively, have been applied in the mouse mastitis model (reviewed by Brouillette and Malouin, 2005; and Notebaert and Meyer, 2006), highlighting the diversity of its possible applications. In contrast to labour-intensive and costly experimental mastitis studies in cows, an intra-experimental comparison of a panel of selected clinically relevant bovine pathogen isolates is easily achievable in mice and therefore of major added value.

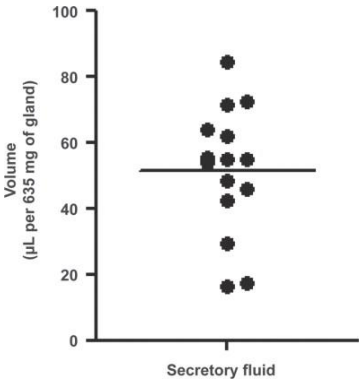

Figure 2. Volume of milky secretory fluid in the *Staphylococcus aureus* Newbould 305-infected mammary gland (n = 15) at 4 h postinfection. Volume is presented as microliters per 635 mg of mammary gland, because 635 mg is the average weight of an infected mammary gland at the moment of antimicrobial treatment.

The analysis in this *in vivo* mouse mastitis study showed the adequate antimicrobial efficacy of the novel biphenomycin compound AIC102827 against a reference strain of the 3 major bovine mastitis pathogens *Staph. aureus*, *Strep. uberis*, and *E. coli*. The field efficacy assessment of this promising compound in an appropriate formulation in dairy cattle is now required to validate these innovative intermediate data in the target species.

III.2.6. Acknowledgements

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General Discussion

Mastitis in dairy cattle has deleterious effects on both animal welfare and the dairy industry. The ensuing inflammatory response, mastitis, leads to annual costs estimated to be upwards $35 billion US dollars and is a major concern worldwide (Modi, Patel et al. 2012). These overall economic losses consist of the decrease in milk production, discarded milk post-treatment, cost of animal replacement, labour and veterinarian costs (Hamadani, Khan et al. 2013). Extensive research indicates that mastitis is a very complex disease triggering multiple molecular mechanisms that have to be taken into account when developing preventive or curative strategies. Here, these are discussed in the context of the results obtained in this thesis. The following paragraphs focus on the inflammatory signaling induced by different bovine-associated isolates in a mouse mastitis model which could provide some clues as basis for innovative treatment and prevention.

I. INFLAMMATORY CHANGES IN THE MAMMARY GLAND DEPEND ON THE BACTERIAL SPECIES: ROLE OF THE INFLAMMASOME AND LOCAL IL-1BETA LEVELS

IL-1 beta is detectable in milk proteins during an udder infection caused by either *E. coli* or *S. aureus.* The release of IL-1beta is concomitant with an increase in the mammary glands' vascular permeability which is necessary for the influx of somatic cells (Bannerman, Paape et al. 2004). This observation suggests that (vita)MAMPs of both pathogens trigger resident PRR-containing udder cells to produce, activate and secrete IL-1beta (Sander, Davis et al. 2011). Biologically active IL-1beta subsequently may trigger the IL-1R-containing endothelial cells so that neutrophils can be recruited at the site of infection. Based on other inflammatory models, a cellular apparatus responsible for proIL-1beta cleavage and secretion is the canonical inflammasome which engages and activates caspase-1 (Lamkanfi and Dixit 2012). This canonical pathway was provisionally accepted as a basis for cleaved IL-1beta in milk, considering that an *E. coli* infection induces gene expression of procaspase-1 in udder tissue (Long, Capuco et al. 2001). However, in contrast to *E. coli* mastitis, canonical inflammasome activation upon *S. aureus* infection is less obvious: although *S. aureus* also induces IL-1beta, caspase-1 activity is only limited or not at all present in bovine udder cells *in vitro* (Wesson, Deringer et al. 2000).

To appropriately test the hypothesis for caspase-1 dependency of IL-1beta triggered by *E. coli* and analyze this seemingly different host-response pattern between Gram-negative versus -positive pathogen infections, mammary glands of mice were exposed to a bovine *E. coli* or *S. aureus* isolate (Chapter I.1.). Bannerman, Paape et al. (2004) demonstrated that IL-1beta protein levels increased in milk following experimental infection of dairy cows with *E. coli* as well as *S. aureus*. Furthermore, this increase was suggested to occur faster with *E. coli* compared to *S. aureus* corroborating the delay in mammary IL-1beta processing post-infection with *S. aureus* compared to *E. coli* in mice. Bannerman, Paape et al. (2004) established experimental mastitis in cows with the same isolates and also detected a slower influx of neutrophils into the *S. aureus*- compared to the *E. coli*-infected gland comparable as seen during our experiments. However, these authors did not check IL-1beta processing necessary for its biological activity. Quantitative methods that detect active IL-1beta predominantly focus on antibodies targeting p17. Our results based on the Cytometric Bead Array,

similar to the Enzyme-Linked ImmunoSorbent Assay (ELISA) method used in Bannerman, Paape et al. (2004) may be specific for IL-1beta, but can produce different results when studied in detail with a qualitative method such as Western Blot focusing on the IL-1beta cleavage patterns. Unfortunately, antibodies of ELISA methods are rarely usable for the Western blot technique probably due to the different kinds of epitopes that they recognize (structural epitope for ELISA vs linear epitope for Western Blot). Nevertheless, for both techniques the specificity of the antibodies was confirmed with samples of IL-1beta KO mice. Therefore, we aimed to emphasize the importance of validating IL-1beta data by complementary techniques in order to get a clear understanding of its activity in the mammary gland. In this respect, the differences between Fig. 2C and Figure 3A/B of Chapter I.1 can be tentatively attributed to method-based restrictions. Our hypothesis described below indicates that during a *S. aureus* infection, IL-1beta cleavage is more focused on processing pro-IL-1beta into p17, while *E. coli*-inflammation generates another fragmentation pattern of IL-1beta.

In contrast to what was previously assumed (Figure 3 of the General Introduction), our results indicate that pro-IL-1beta cleavage does not require pro-inflammatory procaspase-1 activity - which reflects canonical inflammasome processing - for either of both pathogens. Notably, essential inflammatory pathways have backup mechanisms and this feature may also be true for proIL-1beta cleavage by caspases. Indeed, it is known that, besides caspase-1, also caspase-11 can induce IL-1beta secretion (Meunier, Dick et al. 2014; Shi, Zhao et al. 2014; Vigano and Mortellaro 2013). Although we did not selectively study caspase-11 during our experiments, it has been established that due to the way the transgenic mice were originally engineered, the caspase-1/-11 double KO mice are also deficient in caspase-11 (Kayagaki, Warming et al. 2011; Uchiyama and Tsutsui 2014). Furthermore, evidence has been published to suggest caspase-8 as an IL-1beta converting enzyme (Antonopoulos, El Sanadi et al. 2013; Man, Tourlomousis et al. 2013). Caspase-8 is activated during a *S. aureus* and an *E. coli* infection of bovine epithelia and believed to induce cell death in these cells (Aitken, Corl et al. 2011; Gilbert, Cunha et al. 2013; Hu, Cui et al. 2014). However, recombinant caspase-8 is able to cleave pro-IL-1beta *in vitro* at exactly the same site as caspase-1, an observation which does not correspond with our cleavage pattern data (Maelfait, Vercammen et al. 2008). Overall, caspases are very redundant and this is reflected by the many other caspases known to be enhanced during bovine mastitis (Wesson, Deringer et al. 2000; Long, Capuco et al. 2001; Hu, Cui et al. 2014). Of note, we detected that a known substrate of caspase-1, caspase-7 is also activated during an intramammary *E. coli* infection in mice (Lamkanfi, Kanneganti et al. 2008; Vanden Berghe, Demon et al. 2014). In a pilot experiment, IL-1beta levels in the mammary gland were higher in absence of caspase-7 than in the glands with capase-7 and this observation was concomitant with higher IL-6 and MCP-1. Nonetheless, single caspase-7 KO mice provided no significant difference in bacterial CFU post-infection, again indicating that IL-1beta is part of a redundant inflammatory process (Fig. 1, Breyne et al. *unpublished data*).

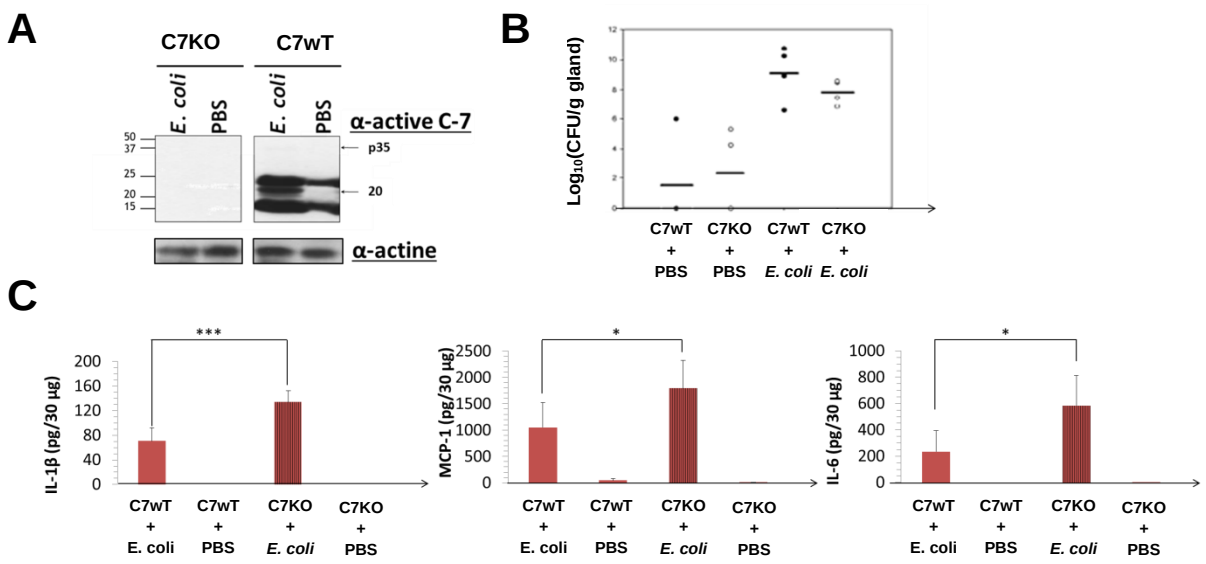

Figure 1. Minor differences in bacterial clearance could be observed during *E. coli* mastitis in caspase-7 (C7) knock-out (KO) mammary glands (n=6) compared to non-deficient mammary glands (C7wT, n=6) of C57BL/6 mice. (A) Cleavage of C7 was observed in infected C7wT C57BL/6 mammary glands and absent in C7KO mice. (B) No significant differences in bacterial loads seen between C7KO glands and C7wT glands. (C) Next to significant increases in IL-1beta, les significant differences in IL-6 or MCP-1 levels could be detected in C7KO mammary glands compared to C7wT mammary glands.

Despite the activation of several caspases during pathogen-induced mastitis, the different cleavage fragments of pro-IL1beta may indicate that processing occured rather following its secretion and through a multicellular mechanism (Fig. 2). Indeed, neutrophil proteases are the primary candidates for the suggested alternative IL-1 beta processing: these were already shown to contribute to proIL-1beta processing in a caspase-1-independent way during experimental mouse models of arthritis and neutrophil-dominated inflammation (Guma, Ronacher et al. 2009; Joosten, Netea et al. 2009). Elastase and cathepsins are the predominant proteases identified in somatic cells and can be retrieved from mastitic cow milk where they presumably hydrolyze casein during an *E. coli* infection (Moussaoui, Laurent et al. 2003; Haddadi, Prin-Mathieu et al. 2006; Watanabe, Hirota et al. 2012). Moreover, *S. aureus* is also known to induces elastase activity in the udder. In secretions of a *S. aureus* mastitic cow, this enzyme cleaves the bovine lactoferrin in low activity fragments which increase NF-kappaB activity and cytokine production when incubated with bovine epithelia *in vitro* (Komine, Kuroishi et al. 2006). Whether the elastase activity is correlated to neutrophil elastase or bacterial elastase is a matter of debate. Still, *S. aureus* proteases are known to cleave pro-IL-1beta (Black, Kronheim et al. 1988). In contrast to *S. aureus,* elastase activity is rarely associated with the pathogenicity of bovine *S. chromogenes* isolates and may establish lower processing of inflammatory proteins (Watts, Naidu et al. 1990). Nevertheless, little is known about the production and the activation of IL-1beta induced during a CNS exposure. Our data indicates that intramammary *S. chromogenes* isolates induce active IL-1beta at a far lower level compared to *S. aureus*. Furthermore,

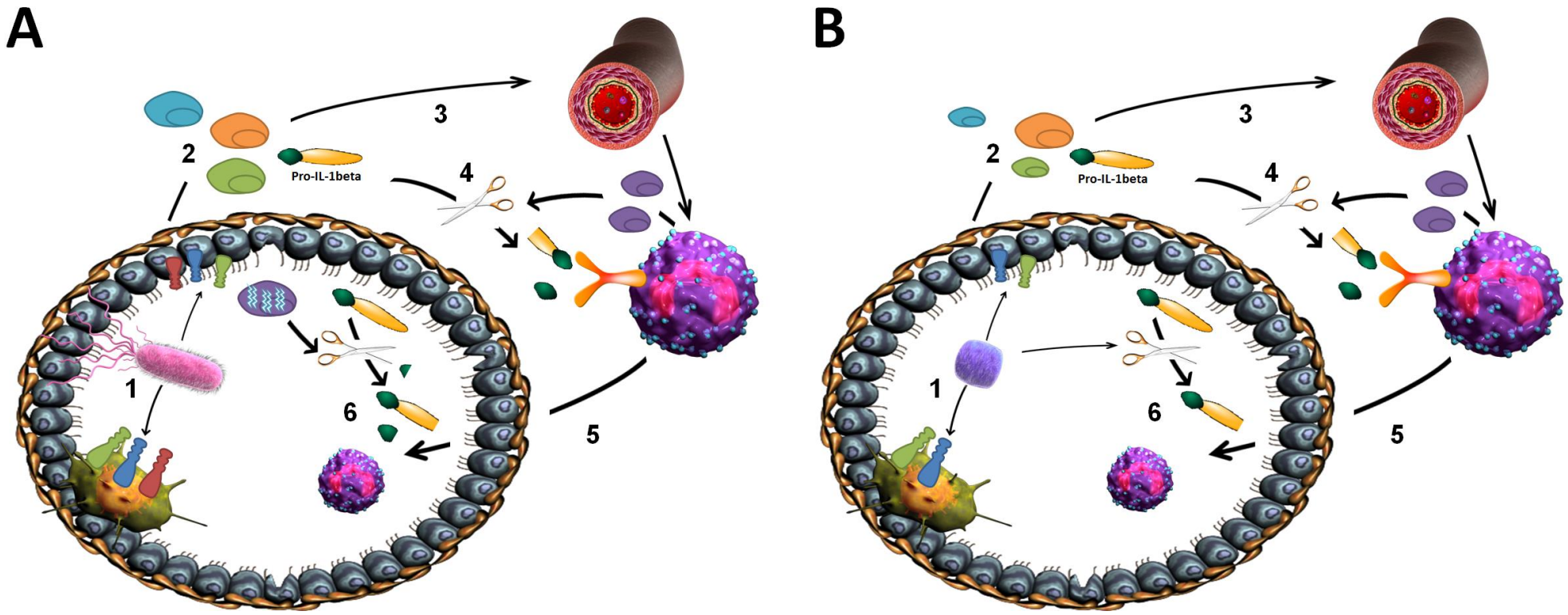

Figure 2. Hypothetical inflammatory host-response pattern of IL-1beta following exposure to pathogenic bovine-associated *E. coli* or *S. aureus* isolates. A. (1) Professional (macrophages and dendritic cells) and non-professional (mammary epithelia) sentinel PRR-containing cells recognize *E. coli* MAMPs and produce hereby a plethora of cytokines and chemokines through transcription of NF-kappaB and other TFs. (2) Cytokines (f.ex. IL-6, TNF-alpha, IL-1alpha and proIL-1beta) are released in the interstitium and trigger diapedesis whereby neutrophils enter the interstitial region. (3) Activated neutrophils release neutrophil serine proteases in the interstitium that are able to cleave proIL-1beta into fragments such as p17 and p20. (4,5) These IL-1beta fragments can stimulate the IL-1R on the neutrophils that enables them to cross the mammary epithelial barrier. (6) To control the excessive IL-1beta activity, proIL-1beta gets attenuated by the additional processing of MMPs of pro-IL-1beta into p25, p15 and p10 fragments. B. (1) MAMPs of *S. aureus* trigger some cytokines to a similar extend as *E. coli* in pathogen independent pathways (e.g. IL-6). (2,3) Nevertheless, a lower inflammatory response is in part resulting from a decreased NF-kappaB activation that elicits a delayed neutrophil infiltration into the interstitium and alveolar space compared to *E. coli* the lower cytokine. (4,5) Similarly to *E. coli*, activated neutrophils does release neutrophil serine proteases that enable them to cleave proIL-1beta into fragments such as p17 and p20 and subsequently enter the alveolar lumen. (6) *S. aureus* do not induce MMPs but contains proteases that can process pro-IL-1beta into active fragments.

S. fleurettii, another CNS that is not associated with the udder, did not significantly enhance IL-1beta compared to the sham controls. Despite the lower levels of active IL-1beta, CNS still manifested neutrophil recruitment in the mammary alveoli.

Moreover, a second candidate for IL-1beta cleavage are metalloproteinases (MMPs). MMPs are proIL-1beta processing enzymes that produce small fragments which correspond to our fragmentation results (Fig. 2). After the processing of pro-IL-1 beta into biological active fragments, MMP members can further degrade mature IL-1beta into inactive or less active fragments (Schonbeck, Mach et al. 1998). Unlike elastases, these proteolytic enzymes are not only expressed by neutrophils but also in the mammary epithelium where they are believed to degrade the extracellular matrix (Raulo, Sorsa et al. 2002; Gilbert, Cunha et al. 2013). This breakdown is accompanied by extensive remodelling of the mammary gland architecture while triggering detachment-induced cell death features (Green and Lund 2005; Watson 2006). Our histopathological observation of marked cell detachment already early in the course of an *E. coli* infection, compared to the delayed process upon *S. aureus* in the mouse mammary gland infection, suggests that MMPs have a higher significance during Gram-negative than - positive pro-IL-1beta processing. Supporting our findings in mice, MMPs are not expressed in bovine epithelial cells infected with *S. aureus* but only during an *E. coli* exposure (Gunther, Esch et al. 2011; Gilbert, Cunha et al. 2013; Im, Lee et al. 2014). Therefore, these *in vitro* data can at least partly explain our concept of a pathogen-dependent pro-IL-1beta cleavage pattern by MMPs. Notably, proMMPs can still be increased in the milk during a long lasting chronic *S. aureus* infection. Probably this phenomenon is at that time rather due to the continuous influx of neutrophils which are known to secrete MMPs (Higuchi, Ishizaka et al. 2007; Nagahata, Kawai et al. 2011).

What is the biological relevance of a local IL-1beta activation? During the innate immune response, bacterial growth is halted by recruited neutrophils that protect against lethal septic effects (Elazar, Gonen et al. 2010). The small and temporarily difference in pathogen loads between IL-1beta KO and wT animals observed in this PhD research indicates that the local production and processing of IL-1beta is not so essential for bacterial clearance and therefore may also not be so critical for neutrophil recruitment (see below). In line with this hypothesis, another experiment from this thesis can be referred to where different bovine-associated CNS isolates were inoculated at a high inoculum dose of 10^5 CFU in wT mice: these all had low bacterial loads post-infection and induced a recruitment of neutrophils without needing to highly increase local IL-1beta levels. Furthermore, similar conclusions could be made when a LPS pretreatment was applied before *S. aureus* exposure in the mammary gland. The attenuation of IL-1beta was concomitant with high bacterial clearance next to neutrophil recruitment in the mammary glands. At last, local IL-1beta levels could also be decreased through the injection of bovine-associated CNS isolates prior to an infection with *S. aureus* (Fig. 3, *unpublished data*). Whether this also implies an effective drop in *S. aureus* CFU concomitant with a neutrophil influx is subject of ongoing research.

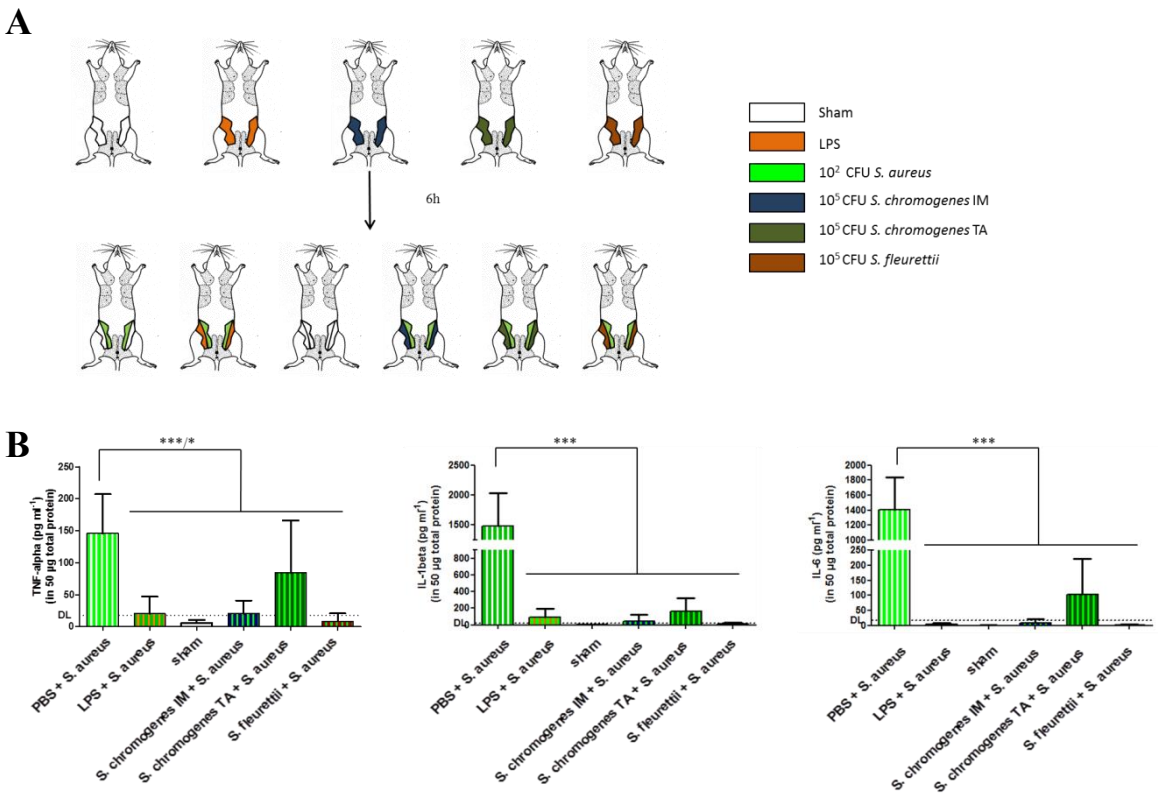

Figure 3. Local CNS pretreatment attenuates mammary levels of IL-1beta, IL-6 and TNF-alpha. (A) Schematic presentation of the experimental set-up. In short, 6h prior *S. aureus* infection, sham- , LPS- , *S. chromogenes* IM/TA and *S. fleurettii* were inoculated in mammary glands ($n_{treatment\ group}$ = 12) of mice, two times sham inoculation in the mammary glands (n=6) was also implemented as control. (B) A pretreatment with CNS reduces the levels of TNF-alpha, IL-1beta and IL-6. Results are presented as mean ± standard deviation and DL; detection limit.

Overall, these latter observations implicate that other cytokines are able to counter the function of IL-1beta signaling when this cytokine is lacking. Indeed, an attractive alternative for neutrophil recruitment instead of IL-1beta would be IL-1alpha. Similarly as IL-1beta, IL-1alpha stimulates a common IL-1R (IL-1 receptor) and shares similar biological activities (Dinarello 1996). Despite their related structure and biological activity, both subtypes are prone to a markedly different production and processing. IL-1alpha only needs NF-kappaB transcription to become expressed as a ~31 kDa polypeptide and hence has a direct biological activity (Mosley, Urdal et al. 1987; Hazuda, Strickler et al. 1990; Fettelschoss, Kistowska et al. 2011). Indeed, this uncleaved pro-IL-1alpha form is already capable of binding to the IL-1 receptor, although it exhibits less biological activity than the cleaved form. A second major difference is that pro-IL-1alpha is not processed either by caspase-1 or by MMPs in contrast to IL-1beta (Ito, Mukaiyama et al. 1996). Nevertheless, it can be processed by elastase, calpains, chymase or granzymes, the former being a common protease with the hypothetical

cleavage of IL-1beta (Carruth, Demczuk et al. 1991; Schonbeck, Mach et al. 1998; Afonina, Tynan et al. 2011).

As we observed in mastitic mice that the local NF-kappaB activity was lower after an *S. aureus* infection compared to an *E. coli* infection, the subsequent slower production of local IL-1alpha levels could explain the delay in neutrophils influx between both bovine pathogens. In line with this assumption, the high levels of IL-1alpha upon an *E. coli* infection may well compensate through IL-1R triggering even in the absence of IL-1beta. However, this is less plausible based on our of initial data of local IL-1Ra concentrations. This molecule is a naturally occurring inhibitor that prevents the interaction between IL-1alpha and IL-1beta and its cell-surface receptors (Burger, Dayer et al. 2006). We detected with a cytokine array that IL-1Ra was highly upregulated upon infection with *E. coli* compared to *S. aureus* which corroborate with reports in cows (Alluwaimi 2004). As IL-1Ra therapy failed in the latter study, It has been postulated that IL-1 has a marginal role in endotoxin-induced mastitis (Shuster and Kehrli Jr 1995). This assumption may be exaggerated. Neutrophils that are insensitive for IL-1 stimuli by the lack of the IL-1R, are unable to cross the mammary epithelium and get trapped in congested capillaries or interstitium upon LPS exposure (Elazar, Gonen et al. 2010; Elazar, Gonen et al. 2010). The phenotype is less pronounced with an *E. coli* infection, indicating that other MAMPs - PRR signaling may counter for the lack in IL-1R signaling and explain why IL-1beta KO or IL-1R KO mice in our model do not establish such an extreme phenotype (Porcherie, Cunha et al. 2012; Mintz, Mintz et al. 2013).

Next to the function of IL-1beta, the pathogen-dependent source of IL-1beta remains vague and is complicated *in vivo* by the fact that mastitis is a complex multi-factorial process with a continuous change in cell type mixture and cyto-/chemokine concentrations. MAMPs of bacteria can interact with different residential PRR-containing cells of the mammary stroma such as epithelial cells, macrophages, and neutrophils (Wellnitz, Reith et al. 2006). In addition to the translocation of NF-kappaB subunits in the mammary epithelia, we also detected NF-kappaB activity in the neutrophils following pathogen infection. When stimulated with mastitic bacteria, bovine blood neutrophils, milk cells, or mammary epithelia confirmed this activation of NF-kappaB and/or the cleavage of caspases in *in vitro* studies performed by our group and by other authors both in mice and cows (Notebaert, Duchateau et al. 2005; Demeyere, Remijsen et al. 2013). Furthermore, it is suggested for the bovine species that professional immune cells such as macrophages are more easily stimulated than non-professional bacterial sensing cells such as epithelia because they lack for example mCD14 (Sohn, Paape et al. 2004). The TLR4-NF-kappaB-IL-1beta signaling axis for *E. coli* is generally accepted, while TLR4-NF-kappaB activation by *S. aureus* does not seems to occur in the bovine cells *in vitro* (Yang, Zerbe et al. 2008; Fu, Zhou et al. 2013). However, TLR4 is not the only PRR expressed on bovine epithelial cells. Other PRRs such as TLR1/2/6 and NOD1/2 (Fig. 2 General Introduction) can be activated by both *E. coli* and *S. aureus* and have been shown to increase IL-8, a well-known chemokine responsible for neutrophil influx into the udder (Porcherie, Cunha et al. 2012). When bovine epithelial cells are exposed to MAMPs of *S. aureus,* IL-1beta is likely produced by other TFs such as AP-1 and CREB through the activation of MAP kinases (Kim, Im et al. 2011; Fig. 3 General

Introduction). Furthermore, interfering with MAPK signaling next to NF-kappaB pathways has also been effective attenuating the IL-1beta levels in LPS-induced mastitis (Gao, Guo et al. 2014). The pathogen-independent production of cytokines has been demonstrated in udder cells without MyD88 or TRIF (Gunther, Esch et al. 2011). Although these cells can no longer induce NF-kappaB, they still express IL-6. In that respect we also detected a different response in mice for TNF-alpha and IL-1alpha compared to IL-6 based on the comparison of *E. coli*- and *S. aureus*-infected mammary glands. Our experiments indicated that IL-6 was comparably enhanced in both *E. coli*- and *S. aureus*-infected glands, while generally IL-6 is less induced following *S. aureus* compared to *E. coli* (Gunther, Esch et al 2011). This discrepancy may be due to the fact that these literature data are typically obtained from *in vitro* experiments using a single cell type (epithelial) based system without macrophages and/or to a strain-dependent phenomenon of the different *S. aureus* isolates used in these papers and/or due to the use of heat-killed versus viable bacteria. Nevertheless, even in mammary epithelial cells, LPS and LTA may enhance IL-6 to a similar extend (Fu, Zhou et al. 2013). Complementary to IL-1beta, IL-6 bind both with neutrophil as well as macrophage receptors and regulates neutrophil trafficking during the inflammatory response by orchestrating chemokine production and leukocyte apoptosis (Fielding, McLoughlin et al. 2008). Of note in this context, our proof-of-concept study with CNS suggests that IL-6 is unlikely sufficient to counter the IL-1R signaling (Chapter I.2.). Indeed, CNS contain teichoic and lipoteichoic acids, but do not induce IL-1beta or IL-6. Still, CNS strains clearly induced a mild neutrophil influx. This indicates that upon infection of the mouse mammary gland other - currently unknown - cytokine signaling could be important for the influx of neutrophils into the lumen following bacterial infection.

At last we should mention that some of our observations concerning IL-1beta processing may be explained by the difference in the mouse strain used. In chapter I.1 we used C57BL/6 mice in accordance with the genetic background of the *luc* reporter and the IL-1beta KO and caspase-1/-11 KO mice, while in the other chapters we experimented on CD-1 outbred mice. LPS exposure of peritoneal macrophages was suggested to induce a slightly faster production of TNF-alpha in C57BL/6 background compared to the CD-1 mice (Wu, Vij et al. 2007). Next to the fact that different inocula were used (3000 CFU/ gland in chapter I.1 compared to 100 CFU/ gland in Chapter I.2); the genetic background of the used mice may also be a possible explanation why we could not detect TNF-alpha in Chapter I.2 at 28h post-infection with *S. aureus* with CD-1 mice while this was the case in Chapter I.1 (Fig 2 A 24h post-infection) with C57BL/6 mice. Furthermore, a similar concentration of TNF-alpha at 24h post-infection with C57BL/6 mice could only be reached at 48h post-infection with *S. aureus* in sham-pretreated CD-1 mice (Chapter II Fig. 2C). This seemingly different TNF-alpha response between CD-1 and C57BL/6 mice may also have consequences for an intramammary *E. coli* infection, as illustrated in our hypothesis (Fig. 2 General Discussion). Lower levels of TNF-alpha would imply less MMPs following an intramammary *E. coli* infection, less IL-1beta processing into less active fragments for *E. coli* compared to *S. aureus* and less histolopathological changes of the epithelial layer in the mammary gland following *E. coli*. Indeed, IL-1beta levels were higher in the *E. col*-infected mammary glands of CD-1 mice 18h post-infection compared to *S. aureus* (Add. Fig.3 Chapter II) and had an intact epithelial layer similar comparable to a *S. aureus* infection. It would therefore be very

relevant to compare the IL-1beta cleavage pattern in CD-1 mice with the IL-1beta processing in C57BL/6 mice next to the MMP and TNF-alpha levels.

In conclusion, further research is mandatory to decipher the input of different cell types in the suggested multicellular proIL-1beta processing and neutrophil recruitment into the alveolar lumen.

II. THE MOUSE MASTITIS MODEL, A BASIS FOR TESTING NOVEL CURATIVE AND PREVENTIVE STRATEGIES AGAINST MASTITIS

Despite the *in vitro* susceptibility of bacterial isolates to antibacterial agents, mastitis remains difficult to fully eradicate with the current curative strategies (Erskine, Walker et al. 2002; Apparao, Oliveira et al. 2009). Two studies of this PhD thesis compared the *in vitro* and *in vivo* antimicrobial efficacy against a bovine mastitis-associated *S. aureus* isolate in mice of four first generation cephalosporins used as curative therapy in cows, respectively the susceptibility of three major bovine mastitis pathogens to a candidate novel antimicrobial compound from the biphenomycin class.

In the first study (Chapter III.1.), the MIC data confirmed the previously reported efficacy of cefalonium (Groothuis and Frik 1982) over cefapirin (Owens, Ray et al. 1997), cefazolin (Brix 2007) and cefalexin (Guerin-Faublee, Carret et al. 2003), while the mouse mastitis data indicated that cefazolin had more antimicrobial efficacy *in vivo* than cefalonium, cephalexin or cefapirin. In favour of the latter model, the *in vivo* doses applied against bovine staphylococcal mastitis of the four cephalosporins are very similar as that in mice (BCFI 2011). In the second study (Chapter III.2.), the MIC data established a higher antimicrobial efficacy against *E. coli* and *S. aureus* compared to *S. uberis,* while *in vivo* the novel biphenomycin compound seemed less effective against *E. coli* than *S. aureus* and had a better efficacy against *S. uberis* than would be expected from the *in vitro* data. However, a pilot study with this compound on cows with mastitis would be of interest to demonstrate its antimicrobial efficacy in the target species (BAYER 2012). Overall, these discrepancies between *in vitro* and *in vivo* data applied on three bovine isolates indicate that the *in vivo* activity of a compound is influenced by factors that are not assessed by a simple MIC determination (Apparao, Ruegg et al. 2009). As reported, the used bacteria elicit different host response patterns and establish different absorption, distribution, half-life and metabolism of antimicrobials when they invade mammary cells compared to a laboratory growth medium (Craven and Anderson 1984; Sanchez, Ford et al. 1988; Gehring and Smith 2006; Schukken, Gunther et al. 2011). An additional intermediate step between MIC data with conventional growth media and *in vivo* data could be testing the efficacy of antimicrobials in milk. Indeed, different antibiotic activities have been observed between tryptone soy broth and delipidated milk *in vitro* (Amorena, Gracia et al. 1999). Nevertheless, a complete simulation of the *in vivo* situation that also includes a complex host-microbe interactions will not be attained. These arguments therefore argue against the extrapolation from these *in vitro* data to the *in vivo* situation in the cow (Apparao, Ruegg et al. 2009). Moreover, the mouse model has an additional advantage over the *in vitro* approach as it can improve existing curative treatment in the target species by modifying the delivery method (Brouillette, Grondin et al. 2004) or by optimizing the excipient formulation. As such, our data from the first curative

therapy study (Chapter III.1.) showed that the *in vivo* performance of an antibacterial compound such as cefazolin improved when formulated in an excipient with better solubility and absorption capacities (Sellers, Antman et al. 2005). As stated in the General Introduction, the studies of this PhD thesis confirm the conclusion drawn from previous studies on the advantage of the mouse model as a valuable intermediate step prior to evaluation of curative therapies in cows.

In the past decade, an extensive effort has been made with regard to alternative preventive strategies than the classical antimicrobial curative therapies. Indeed, the standard use of antibiotics may introduce resistance, beneficial commensal bacteria may have unwanted susceptibility to these compounds and per definition it does not intercept a future infection (Owens, Donskey et al. 2008). Nevertheless, as stated in the General Introduction, these attempts have been relatively unsuccessful so far in the mastitis field, more specifically for *S. aureus.*

Our studies with repeated challenge of the mammary glands in mice (Chapter II and Fig. 3) indicate a putative basis for an innovative preventive therapy. A proof-of-concept mouse study evaluated whether the immunomodulation of the innate immunity by LPS prior to a pathogenic mastitis infection is beneficial for the host or not. This strategy has been described by a handful of cow studies with at least partly contradictory results and fundamental mechanisms occurring at the molecular level remain to be fully elucidated (Rainard and Paape 1997; Kauf, Vinyard et al. 2007; Petzl, Gunther et al. 2012). While one report suggests that bacteria or MAMP exposure prior to another challenge in the udder attenuates excessive inflammation, enhances bacterial clearance and/or decreases bacterial colonization and reduces severity of clinical symptoms, another claims that such pretreatment on the contrary sensitizes the mammary gland (Rainard and Paape 1997; Petzl, Pfister et al. 2010; Gunther, Petzl et al. 2012; Hertl, Schukken et al. 2014). Elaborating on our previous findings, i.e. that a lack of IL-1beta as seen with KO mice did not induce a thorough change in the pathogenesis of mastitis, our alternative approach consisted of attenuating multiple cytokines through the injection of LPS prior to an intramammary infection with *S. aureus*. Indeed, when pretreating with the potent immunomodulator LPS - in addition to IL-1beta - other cytokines such as TNF-alpha, IL-6, or chemokines such as IL-8 (i.e. IL-8 homologues in mice CXCL1/KC and CXCL2/MIP-2) and CLL3/MIP-1alpha also decreased upon subsequent experimental mastitis. Surprisingly, this decrease in cytokine levels was concomitant with significantly lower bacterial loads in the mammary gland and a decrease in severity of symptoms. Although a mere immunoparalysis can decrease the severity of the inflammatory symptoms, it does not guarantee bacterial resolution without appropriate curative antibiotic treatment (Aitken, Corl et al. 2011; Guo, Zhang et al. 2013). Whether the observed lower number of CFU is the result of a reduced bacterial colonization and/or an increased bacterial clearance, is a matter of debate and should be studied in detail. Indeed the high LPS dose during the pretreatment given might induce a massive neutrophil influx in the mammary glands and thereby circumvent the bacterial colonization rather than influence the clearance of bacteria. Therefore, 6h after the LPS-injection we should verify whether neutrophils are present compared to sham-pretreated glands. Alternatively this could be checked with pretreatment of mammary glands with a low concentration of LPS that does not recruit neutrophils. Nevertheless, it should be emphasized that the observed number of CFU following LPS pretreatment

and *S. aureus* infection is rarely not detectable. Therefore, it can be suggested that if the pretreatment would only reduce the colonization and not their growth, the remaining bacteria would only reduce the colonization and not their growth, the remaining bacteria might still present grow in time. Although a time-growth experiment did not been conducted, we didn't observe an increase in bacterial loads between bacterial loads determined at experiments 48h post-infection (Fig. 1A & Fig. 2B of Chapter II) and at 28h post-infection (Fig. 4B) but rather a small decrease. As such, the effect of the LPS pretreatment is possibly a combination of both a reduction of colonization as maybe also of bacterial clearance. Next to the bacterial and anti-inflammatory features, our results revealed that LPS pretreatment that the immunomodulation is a rather active process through the enhancement of specific cytokines such as CCL5/RANTES and IL-8 (i.e. the IL-8 homologue in mice CXCL5/LIX) as confirmed *in vitro* with udder epithelia (Petzl, Pfister et al. 2010; Gunther, Petzl et al. 2012). These authors suggested that LPS pretreatment induces a higher expression of the latter genes to promote the sentinel function of the mammary epithelia. However, we showed that CCL5/RANTES only has a mild immunomodulating function without a significant effect on the clearance and/or decrease in colonization of bacteria in the mammary gland. Based on findings concerning plant defense mechanisms and also on a variety sepsis models, the host defense against pathogens may be subdivided into two conceptually different components: targeting the ability to reduce bacterial loads (resistance) and the ability to limit the damage caused by a given pathogen burden (tolerance) (Raberg, Graham et al. 2009). In line with the second concept, the host may thus be strongly protected when subjected to severe infections without a negative effect on the performance of the pathogen. We can thus tentatively suggest that CCL5/RANTES may be implicated in the host tolerance rather than the resistance component of the mammary defense against mastitis pathogens, as we observed attenuation of inflammation and a suggestive possible amelioration of the lethality due to the *S. aureus* infection however without a concomitant reduction in bacterial loads. Of relevance, a recent study has demonstrated that a low non-lethal dose of anthracyclines reduces TNF-alpha, IL-1beta and IL-6 and confer strong protection against severe sepsis (Figueiredo, Chora et al. 2013). This treatment was not able to lower the bacterial loads in absence of a supplementing an antibiotic treatment. The underlying mechanism of this effect is suggested to be a result of controlled Ataxia Telangiectasia Mutated-dependent autophagy induction. Therefore, it would be interesting to evaluate whether such tolerance-inducing pretreatments would also help to ameliorate the efficacy of classical therapeutic antibiotics in a mastitis set-up.

Nevertheless, the concept of LPS pretreatment is very valuable as it seems not to be restricted to one infectious pathogen. Corroborating this literature, this strategy also reduced *E. coli* mastitis in cows while our *in vivo* study demonstrated protection against *S. aureus* mastitis (Lohuis, Van Leeuwen et al. 1988; Lohuis, Kremer et al. 1990; Petzl, Pfister et al. 2010; Petzl, Gunther et al. 2012). Unfortunately, it should be stated that LPS pretreatment has also some major drawbacks as it has decreased yields and quality of the milk to some extend (Shuster and Harmon 1991) and the protective effect in cow has - until now - only been demonstrated for 10 days (Petzl, Pfister et al. 2010; Gunther, Petzl et al. 2012).

As previously indicated, next to LPS, bacteria can also alter the immune system. Reports on dairy cows that have been infected twice with an *E. coli* isolate in the udder were accompanied by a drop in severity of the clinical signs for the second infection (Suojala, Orro et al. 2008). Although colonizing the udder with pathogenic bacteria to fight a possible next challenge is not really a desired option for treatment, probiotic bacteria create an attractive alternative. Probiotic bacteria can modulate the immune system in a host as they downregulate the inflammatory response in challenged subjects, while they obtain an immunostimulatory effect in healthy ones (Pelto, Isolauri et al. 1998). Ideally, for mastitis this would imply that cows with probiotic microorganisms are protected against pathogenic udder infections while they have beneficial effects for the milk production or quality. As far as known, the application of probiotic bacteria as a potential treatment shown to combat bacterial-induced mastitis is limited. It has been shown that *Lactococcus lactis* was able to treat natural occurring chronical or clinical mastitis cows in the field as efficacious as common antibiotic treatments (Klostermann, Crispie et al. 2008). Notably, this concept does not seems not to be host-related and was demonstrated in women with infectious mastitis. In the latter study, two orally given lactobacillus strains isolated from breast milk were shown to have more efficacy than conventional antibiotic therapy (Arroyo, Martin et al. 2010). Although, these studies did not colonize the gland with probiotic bacteria to induce immunomodulation, they clearly indicate an antimicrobial effect by the production of bacteriocins (Klostermann, Crispie et al. 2010; Ryan, Flynn et al. 1999; Šušković, Kos et al. 2010). In collaboration with the group of De Vliegher S. *et al*., we evaluated in another pilot study whether CNS are suitable to induce an altered immunity response prior to a challenge with a mastitis-associated *S. aureus* (Fig. 3). CNS are natural occurring udder bacteria that can inhibit major mastitis pathogens and also produce bacteriocins (De Vliegher, Opsomer et al. 2004; Braem, Stijlemans et al. 2014). Moreover, they are believed to induce higher milk production in the first lactation of heifers and do not show milk loss associated with occurrence of CNS (Piepers, Opsomer et al. 2010; Hertl, Schukken et al. 2014). These bovine isolates induced only a limited inflammatory response as suggested by the low local levels of IL-1beta and IL-6 in our mouse mastitis model (Chapter I.2). A colonization with *S. chromogenes* IM*, S. chromogenes* TA and *S. fleurettii* decreased local TNF-alpha, IL-1beta and IL-6 levels upon bovine *S. aureus* (Fig. 3). Whether the pretreatment with CNS bacteria also lowers the bacterial load of *S. aureus* in the mammary gland, is a matter of ongoing research.

The previously mentioned injection of cow with *Lactococcus lactis* cleared the chronic pathogenic infections but the SCC remained high unchanged regardless of treatment (Klostermann, Crispie et al. 2008). These data raises the question whether a very low SCC and sterile milk are necessary features for a healthy cow? On one hand, too many active neutrophils may harm the udder tissue, but on the other hand our preconditioning experiments (Chapter II and Fig. 3) suggest that there is *in se* no detrimental effect associated with the mere enhanced alertness of the mammary gland. Moreover, an increased number of leukocytes in the mammary gland supports bacterial clearance and/or reduced colonization (Mehrzad, Duchateau et al. 2005; Elazar, Gonen et al. 2010). Unfortunately, the preventive use of antibiotics in the field of bovine mastitis makes it difficult to draw conclusions concerning this subject. However, there are some indications demonstrating a comparatively better health performance in organic husbandry (Weller and Bowling 2000). In contrast to conventional

herds, these cows are subjected to a lower degree of antibiotic pressure, which makes them prone to Gram-negative infections (KouřimsKá, LegaroVá et al. 2014). Although comprehensive information is scarce, these cows seem to have similar features as cows subjected to multiple challenges of endotoxin. These reports indicate that organic herds are associated with lower milk yield, higher SCC with increasing lactations, and lower incidence of mastitis compared to conventional farming (Hardeng and Edge 2001).

In summary, a non-detrimental challenge seems to provoke an altered inflammation to a subsequent exposure of the mammary gland and the contribution of the induced cytokines during this process remains largely unknown and should be focused on in future research.

III. COMMENSAL AND PATHOGENIC BACTERIA OF THE UDDER

Commensal bacteria have a mutually beneficial relationship with the host. On the one hand they prevent colonization by invasive pathogens that would induce inflammation in the host, on the other hand they thrive in a relative stable nutrient-rich environment provided by the host. This kind of benefit for the host is well-known for the gut, where non-pathogenic commensal bacteria exclude pathogens from the gut surface, enhance nutrient uptake and produce anti-pathogenic metabolites (Abt and Pamer 2014). Nevertheless, the answer to the question why commensals are beneficial and pathogens are detrimental while they both have similar MAMPs remains partly unknown till date. It is believed that a specific MAMP-TLR interaction can induce a dual response: (1) under normal steady-state conditions, MAMPs are recognized by TLRs and may induce protective factors which are essential for epithelial homeostasis (Rakoff-Nahoum, Paglino et al. 2004), in contrast (2) excessive TLR-MAMP interaction may ignite a cytokine response as a consequence to uncontrolled inflammation and results in a variety of pathological conditions, including, at the extreme, septic shock (Michelsen and Arditi 2007).

As milk is an important source to establish and maintain the probiotic gut microbiota of mammalian offspring, it is likely that a variety of commensal milk bacteria thrive and function in the mammary gland (Martín, Langa et al. 2003; Newburg and Walker 2007; Adlerberth and Wold 2009). Next to milk flora, the teat apex also contains a wide diversity of skin microbiota (Braem, De Vliegher et al. 2012) which are known to inhibit Gram-positive and/or -negative mastitis pathogens (Woodward, Besser et al. 1987). More specifically, CNS are natural colonizers of the bovine skin and other mucous membranes but they can also grow in the environment (Linhardt, Ziebuhr et al. 1992). Indeed, some CNS isolates harbor the teat apex of non-inflamed udders (Braem, De Vliegher et al. 2013), some the milk and others the stable (Piessens, De Vliegher et al. 2012; De Visscher, Supré et al. 2014). It is still a matter of debate whether CNS - generally considered as a group of minor mastitis pathogens - really contain harmless commensal isolates of importance for mastitis (Pyorala and Taponen 2009). The milk microbiota of healthy dairy cows is difficult to determine with standard culture techniques and culture conditions. A recent study revealed through pyrosequencing of 16S rRNA that even the culture-negative milk from cows with a SSC lower than 20,000 contained *Staphylococcus* spp. among *S. epidermidis* and *S. equorum* (Oikonomou, Bicalho et al. 2014). Although they are present in the

udder of dairy cows without inflammation symptoms, it is not demonstrated yet whether these isolates are commensals and not just pathobionts. A possible approach to identify commensal CNS isolates can be attempted based on IgA discrimination. In analogy with gut microbiota, potentially pathogenic bacteria are believed to have a higher affinity and specificity to IgA compared to commensals (Pabst 2012). Highly IgA-coated bacteria from the gut were proven to induce detrimental effects when sterile colitis is induced compared to low IgA-coated bacteria (Palm, de Zoete et al. 2014). As such, low IgA-binding CNS species from the milk of healthy cows could be injected in germ-free mice with our intraductal model (chapter I.2.). This experiment would enable us to profoundly evaluate the contribution of commensals to innate immunity of the mammary gland (Cash and Hooper 2005).

Nevertheless, some bovine-associated CNS isolates may contain beneficial traits for the host. Based on a prevalence study, CNS colonization at the teat apex prepartum appears to be associated with lower SSC in the first few days of lactation compared to their non-colonized counterparts (De Vliegher, Laevens et al. 2003) and moreover are able to inhibit major mastitis pathogens *in vitro* (De Vliegher, Opsomer et al. 2004; Braem, Stijlemans et al. 2014). Interestingly, this likely is no species-specific feature as it is not restricted to cows, because as an *S. epidermis* isolated from breast milk of healthy women inhibited pathogenic *S. aureus* too (Heikkila and Saris 2003). Another remarkable observation is that heifers with CNS in their milk during early lactation have a significantly lower amount of clinical infections with mastitis-associated major pathogens, and seem to have a higher milk yield compared to CNS-negative heifers (Piepers, Opsomer et al. 2010; Piepers, Schukken et al. 2013).

The fact that CNS species interact differently with their host implicates that conclusions based on epidemiological studies comprising several residential CNS is very challenging. Further complicating the interpretations of these studies, is that a marked intra-species heterogeneity among bovine *S. chromogenes* isolates was revealed in cows (Piessens, De Vliegher et al. 2012). In experimentally infected mice an intramammary isolated *S. chromogenes* IM showed a similar growth as one isolated from the teat apex (*S. chromogenes* TA), while inflammation with the latter strain was more severe in 30% of the in our pilot study (Chapter I.2.). An explanation for these species-independent differences should likely be looked for in the ongoing process that started from the evolutionary precursors of *S. chromogenes*. Indeed, as CNS originate from skin bacteria and the mammary gland is derived from skin folds (see General Introduction, Section II), it is plausible that some CNS evolved along with the formation of this gland structure. This suggests that selection pressure and adaptation shaped the group of CNS and especially some *S. chromogenes* strains to acquire available nutrients and resources from the skin or teat apex, while other CNS members specialized into obtaining their advantage from nutrients typically available in the milk environment. Although this hypothesis has not been demonstrated for CNS, it has already been put forward for *S. aureus*. It is believed that this pathogen acquired its colonization potential of mammary epithelial cells by gene decay. By means of this latter evolutionary process, mastitis-associated *S. aureus* isolates probably evolved from a free-living to an intracellular lifestyle, whereby biosynthetic components are lost in favour of parasitizing on nutrients obtained from the udder cells they reside in (Herron-Olson, Fitzgerald et al. 2007).

In this PhD thesis, CNS isolates obtained from different niches were compared for their capacity to induce a host immune response (Chapter I.2.). Furthermore, we compared the bacterial growth and cytokine profiles to these of a major *S. aureus* mastitis pathogen characterized by a high virulence in mice (Le Marechal, Jardin et al. 2011). It should be remarked that the mouse mastitis model has not yet been used to evaluate CNS mastitis but primarily to assess the pathophysiology of *S. aureus* and the role of its virulence factors (Bhakdi and Tranum-Jensen 1991; Dinges, Orwin et al. 2000; Brouillette, Grondin et al. 2004; Bouchard, Peton et al. 2012; Demon, Ludwig et al. 2012; Demon, Breyne et al. 2013). Mice have the physical disadvantage of a high surface area in relation to their body volume. Due to their difference in body volume, the used bovine *S. aureus* Newbould 305 - isolated from a clinical mastitis case (Bouchard, Peton et al. 2012) - typically causes already a more severe pathophysiological change in small ruminants than in cows (Noujaim, Lucca et al. 2004). For mice, this effect is even more pronounced with a severe systemic response to the *S. aureus* exotoxins characterized by a major drop in body temperature (Chapter I and II) (Bramley, Patel et al. 1989; Ragle and Bubeck Wardenburg 2009; Kebaier, Chamberland et al. 2012; Chinchali and Kaliwal 2014). This is probably due to alterations in the cardiovascular parameters i.e. a lower pulse distention and peripheral blood flow, which are inherent to topor in small animals (Cauwels, Janssen et al. 2006). Of relevance for the CNS findings described in this PhD thesis, it has been shown that lacking coagulase following mutagenesis decreased the virulence of *S. aureus* in the mouse mastitis model (Jonsson, Lindberg et al. 1985). However, other authors attributed this decrease as being due to the interference of multiple clotting factors (Phonimdaeng, O'reilly et al. 1990; Cheng, McAdow et al. 2010) present in CNS. In accordance with the former rather than the latter interpretation, it has been shown in a pioneer study that CNS virulence can be enhanced through the addition of coagulase activity (Ekstedt and Yotis 1960). Nontheless, before general conclusions concerning the virulence of CNS versus *S. aureus* can be drawn, more CNS species should be taken into account with the mouse mastitis model.

IV. MAIN CONCLUSIONS

- ✓ Differential inflammatory signaling of two major bovine-associated mastitis pathogens can be comparatively explored with the mouse mastitis model.

- ✓ A mastitis-associated *E. coli* isolate induces an early influx of neutrophils in the mammary glands of mice compared to mastitis caused by a *S. aureus* isolate.

- ✓ *E. coli*- and *S. aureus*-infections establish quantitative differences in NF-kappaB activity, inflammatory cytokine profiles, and IL-1beta processing in the mammary gland. The latter cytokine has negligible effect on the bacterial loads post-infection with *E. coli* or *S. aureus* and occurs independently of caspase-1/-11.

- ✓ Bovine-associated CNS isolates can be compared in parallel with the mouse mastitis model.

- ✓ A CNS-induced neutrophil influx in the mammary gland is characterized by low bacterial growth, as well as cytokine induction.

- ✓ CNS-inflicted mastitis is mild compared to *S. aureus* mastitis. Small differences between CNS isolated from niches such as the saw bedding, the teat apex and milk can be observed based on cytokine response and bacterial growth.

- ✓ Preventive LPS pretreatment modulates the mammary innate immune signaling beneficially during *S. aureus*-induced mastitis.

- ✓ LPS pretreatment followed by a *S. aureus* infection is characterized with a reduction of bacterial loads, IL-1beta, TNF-alpha and IL-6 as well as an increase of CCL5/RANTES and CXCL5/LIX.

- ✓ CCL5/RANTES in the mammary gland likely has only a limited tolerant and no resistance function during *S. aureus*-induced mastitis.

- ✓ Curative antimicrobial compounds for bacterial-induced mastitis can be evaluated in the mouse mastitis model.

- ✓ The intramammary efficacy of first generation cephalosporins against *S. aureus*-induced mastitis partly corroborates the MIC data obtained with this major bovine-associated isolate *in vitro*.

- ✓ The efficacy of cephazolin against *S. aureus*-induced mastitis can be increased through a change of excipient.

- ✓ A novel biphenomycin compound can be assessed as candidate curative treatment against bovine-associated pathogens with the mouse mastitis model and holds promise as a broad-spectrum antimicrobial drug

V. FUTURE PERSPECTIVES

- ✓ The underlying working mechanism of pro-IL-1beta cleavage is hypothesized to be associated with neutrophil-associated proteases and MMP activity instead of caspase-1/-11 during *S. aureus*- and/or *E. coli*-induced mastitis (Fig. 2 of the General Discussion). It would be worthwhile to verify whether IL-1beta processing occurs in absence of MMPs and/or neutrophil-associated proteases during *S. aureus*- and/or *E. coli*-induced mastitis and whether other major mastitis pathogens may also elicit IL-1beta processing. Other cytokines such as IL-18, may be similarly processed as IL-1beta and are also worthwhile to investigate (Vanden Berghe, Demon et al. 2014). Nevertheless, caspase-1/-11 is activated during *E. coli*-induced mastitis and its function is not yet known in this pathology. It would be interesting to examine whether caspase-1/-11 restricts bacterial invasion.

- ✓ Our hypothetic model indicates that mammary gland inflammation results from a multicellular process. Future research should focus on integrating effects of multiple sentinel cells (i.e. PRR-containing cells of the mammary such as mammary epithelia, macrophages and neutrophils) gland responding to different MAMPs of mastitis-causing pathogens. Notably, enzymes released by infiltrating neutrophils are known to be responsible for temporally elevated levels of inflammatory cytokines unrelated to the inflammasome (Bank and Ansorge 2001). Identifying and altering the activity of neutrophil-associated proteases would unravel the essential signaling to control the local cytokine bioactivity and availability.

- ✓ We observed that IL-6 production is activated by both *E. coli* and *S. aureus* and cannot be explained straightforwardly by NF-kappaB activity. This observation indicates that alternative signaling pathways triggered by MAMPs of both pathogens activate essential TFs other than NF-kappaB such as AP-1 or CREB (Wang, Song et al. 2014). Identifying genes that are controlled by these TFs during mastitis would help us to better understand the pathologenesis.

- ✓ Our observations in the mouse mastitis model suggest that bovine-associated CNS isolates have low bacterial fitness compared to major mastitis pathogens. Correlation between bacterial loads, highly induced cytokines and neutrophil influx would help us to determine whether the fitness of the studied CNS isolates is lower than the *S. aureus* isolate. Cytokine profiling of CNS-infected mammary glands would help to determine which elevated cytokines are necessary for the influx of immune cells. Furthermore, CNS isolates from healthy dairy cows may be evaluated in germ-free mice to determine their pathogenicity (e.g. minor pathogenic vs commensal).

- ✓ Colonization of the mammary gland with beneficial CNS isolates can potentially be used as a novel long-term probiotic treatment. Preliminary data confirmed the advantages of CNS (Figure 3 of the General Discussion) but findings should be more exploited in the future. Moreover, different isolates should be validated *in vivo* to establish which CNS isolate is responsible for the beneficial traits suggested in dairy cows such as production of anti-major pathogenic bacteriocins (De Vliegher, Opsomer et al. 2004; Braem, Stijlemans et al. 2014).

- ✓ LPS pretreatment suggests an increase in resistance and tolerance in the host against *S. aureus*-induced mastitis. Determining the anti-bacterial and protective parameters induced during this process would help to identify essential host signaling that could be exploited for preventive treatment. Alternatively, an increase in tolerance through low doses of chemotherapeutics prior to *S. aureus*-induced mastitis can be investigated to attenuate the severe damage induced by this major pathogen.

- ✓ The mouse mastitis model opens perspectives for testing the *in vivo* efficacy of novel candidate antibiotics compared to those generally used. Nevertheless, validation in cows always is required. Parameters influencing the efficacy of curative antibiotic treatment should be determined *in vivo* as they may explain why antibiotics sometimes fail to do their assignment based on MIC data with conventional culture media. Furthermore, our data indicate that an elaborate comparison of the excipient formulation may offer more possibilities in increasing the efficacy of already available antibiotics.

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Summary

Bacterial-induced mastitis is considered a major issue worldwide for both animal welfare and the dairy cow industry. The **General Introduction** explains how different pathogens cause mastitis in dairy cattle to a varying degree. For example, a limited number of colony forming units (CFU) of either a Gram-negative pathogen, such as *Escherichia coli* (*E. coli*), or a Gram-positive pathogen, such as *Staphylococcus aureus* (*S. aureus*), already cause mastitis, while a much larger number of CFU from a coagulase-negative staphylococcus (CNS), such as *Staphylococcus chromogenes* (*S. chromogenes*), induces only mild mastitis. The innate immune response in the host upon infection with bovine-associated isolates that display differences in their virulence is not well characterized. However, such mechanistical knowledge concerning host–microbe interactions is necessary to develop more efficient curative or preventive treatments for mastitis. Ideally, experimental mastitis is studied in dairy cows. However, those infection studies are very expensive and labour intensive. Therefore, an easily accessible mouse mastitis model was introduced in the 1970s. This validated *in vivo* model has since been used by several research groups to study the immune response induced by several bovine mastitis isolates, but not yet for CNS. Although results obtained in mice are undoubtedly valuable, they should always be confirmed in the target animal species, i.e. dairy cows.

The **Aims of the Thesis** are threefold and always assessed using the mouse mastitis model with bovine-associated bacterial isolates to simulate the disease in dairy cows. The first objective is to compare the microbe-driven innate immune signaling triggered by *E. coli, S. aureus* or CNS isolated from different niches. It is then evaluated whether pretreatment with a membrane component of *E. coli*, lipopolysaccharide (LPS), may dampen a subsequent intramammary *S. aureus* infection as a basis for a potential preventative mastitis strategy. Finally, the efficacy of existing and novel candidate antibiotics against bovine mastitis isolates is compared *in vitro* and *in vivo*, as applications of a curative mastitis strategy.

In **Chapter I**, the pathogen-dependent innate immune response was studied during mastitis. Until now it was accepted that microbe-associated molecular patterns (MAMPs), exposed by various bacteria, bind to pathogen-recognition receptors on udder cells to induce an influx of immune cells during mastitis. It was suggested that this influx results from cytokines secreted by these udder cells following activation of specific transcription factors, in particular Nuclear Factor (NF)-kappaB. Some pro-inflammatory cytokines produced during this process, such as the inactive pro-form of interleukin (IL)-1 beta, have to be processed post-translationally to obtain their biological activity. For proIL-1beta, this processing generally involves the inflammasome, a multiprotein complex that requires caspase-1 and/or -11 proteolytic activity. In Chapter I.1, the inflammatory signaling in the mammary gland induced in parallel by an *E. coli* and a *S. aureus* infection was assessed. In comparison to the *S. aureus*-induced inflammation, *E. coli* induced a rapid influx of immune cells in the mammary gland, which was associated with histopathological changes. In addition, quantitative differences in NF-kappaB activation and in local inflammatory cytokine profiles between infections with the Gram-negative and -positive pathogens were also detected. More specifically, *E. coli* induced, both at 12 and 24 hours after inoculation, a high NF-kappaB activity and substantial concentrations of IL-1alpha, tumor necrosis factor-alpha and monocyte chemotactic protein-1 in the mammary gland in comparison

to *S. aureus*. In contrast, IL-6 was upregulated equally for both types of infections, while IL-1beta concentrations were equally upregulated for both pathogens at 12 hours and significantly higher at 24 hours for *S. aureus*. These intriguing differences in kinetics of IL-1beta between both infectious pathogens were studied in detail. Interestingly, proIL-1beta processing occurred in both *E. coli*- and *S. aureus*-infected glands. However, the cleavage pattern was pathogen dependent yet surprisingly independent of the inflammasome. This conclusion could be made based on similar infections in IL-1beta knock-out and caspase-1/11 double knock-out mice, respectively. Pro-IL-1beta cleavage occurred for *E. coli* already at the early time-point, while for *S. aureus* it was observed only at the late time-point. To verify inflammasome dependency of this process, samples were taken at both time points in caspase-1/11 knock-out mice. Interestingly, the IL-1beta fragments were still present in the absence of caspase-1/11 activity. In Chapter I.2, the mouse model was validated for the first time to study CNS induced mastitis. The different CNS used in this study were isolated from various niches such as the udder of a dairy cow with chronic mastitis (IM), the teat tip of a heifer (TA) and the sawdust bedding. In contrast to the exponential growth of *S. aureus* after inoculation, *S. chromogenes* IM showed only very limited bacterial growth and this at several tested inoculum doses. This was also the case for *S. chromogenes* TA and *S. fleurettii* from the sawdust bedding. Although infection with each of the three bovine CNS isolates was accompanied with a local neutrophil influx, the corresponding innate immune response was characterized by much lower pro-inflammatory cytokine profiles (including IL-1beta) than that caused by *S. aureus*.

It has previously been demonstrated that immunomodulation following pretreatment with LPS, a MAMP of *E. coli*, improves bacterial clearance and/or decreases bacterial colonization in various mouse infection models. There are indications that such an LPS injection in the udder can also be useful in the context of a preventive mastitis strategy in cows. Therefore, in **Chapter II** LPS was injected shortly before the induction of an intramammary *S. aureus* infection. This local pretreatment markedly reduced the severity of the symptoms, the local CFU and the pro-inflammatory cytokine response, compared with the non-pretreated animals. A comparative protein-array analysis between *E. coli*- and *S. aureus*-infected mammary glands identified that the chemokine CCL5/RANTES was induced by *E. coli* and not by *S. aureus*. However, in contrast to the LPS pretreatment, pretreatment with CCL5/RANTES was only associated with a minor immunomodulation in the mammary gland and could not reduce the local growth or decrease the colonization potential of bacteria.

Antibiotics are frequently used as a curative therapy of bovine mastitis. Therefore, **Chapter III** compared the efficacy of antimicrobial agents against different mastitis pathogens *in vitro*, based on minimum inhibitory concentration (MIC) data, and *in vivo,* using the mouse mastitis model. In Chapter III.1, the antimicrobial activity of the first generation cephalosporins; cephalexin, cefalonium, cephapirin and cefazolin was analyzed. From the *in vivo* determination of the effective doses and the protective doses, cefazolin was shown to be the most efficacious antibiotic for the treatment of *S. aureus* mastitis while cefalonium and cephapirin were more active *in vitro*. Moreover, it was demonstrated in the mouse mastitis model that the antimicrobial efficacy of cefazolin could be enhanced by modifying the excipient. In Chapter III.2, the antimicrobial efficacy of a novel candidate

antibiotic was compared against different mastitis pathogens. As with the cephalosporins, the *in vitro* results with this biphenomycin class compound were not fully consistent with the *in vivo* data. Based on the effective and protective doses established in the mouse mastitis model, the screened compound shows potential as a broad-spectrum antibiotic against staphylococcal, streptococcal and coliform bovine mastitis pathogens.

In the **General Discussion**, a hypothetical model is proposed based on the differences observed in proIL-1beta processing during intramammary infections with the major mastitis pathogens *E. coli* and *S. aureus* (Chapter I.1). This multicellular model demonstrates a prominent role for local cells such as macrophages or epithelial cells together with the infiltrating neutrophils. Local cells are believed to be activated by various MAMPs of both *E. coli* and *S. aureus* which secrete proIL-1beta into the interstitial space in an inflammasome-independent manner. Depending on the combination of MAMPs present on the bovine mastitis-associated bacterial isolate, the intensity and the kinetics of the innate immune response will differ in the mammary tissue. Indeed, MAMPs of *E. coli* will strongly activate NF-kappaB and induce a pathogen-dependent cytokine profile that allows rapid recruitment of circulating neutrophils in contrast to the MAMPs of *S. aureus*. The activated neutrophils may produce neutrophilic proteases that are supposed to cleave pro-IL-1beta into multiple fragments. Furthermore, the *E. coli* infection is associated with the loosening of mammary epithelial cells presumably as a result of matrix metalloproteinases. These host-derived cellular proteases may provide the additional cleavage of pro-IL-1beta observed in an *E. coli* infection, whereas *S. aureus*-associated bacterial proteases may be responsible for the observed alternative pro-IL-1beta cleavage pattern. In conclusion, this molecular model shows that the inflammatory signaling during mastitis is strongly pathogen-dependent. In addition, alternative pathways can induce an immune cell influx, since bacterial isolates associated with mild mastitis, such as CNS, may lack the IL-1beta response characteristic for major mastitis pathogens, such as *E. coli* and *S. aureus* (Chapter I.2). Moreover, the local immune response following *S. aureus* mastitis can be dampened by pretreatment with LPS and thereby reduce the symptoms associated with high virulence of this pathogen. This observation provides prospects for alternative preventive strategies to favourably change the hosts‘ immune response. The comparison between data generated with MIC with conventional growth medium and the mouse mastitis model indicates that host-related factors may also influence the potential of antibiotics. As such, the efficacy of antimicrobial compounds can be screened with the mouse mastitis model in a fast and cost-effective manner prior to *in vivo* confirmation in dairy cows.

SAMENVATTING

Mastitis of uierontsteking bij melkvee vormt een wereldwijd probleem voor zowel het dierenwelzijn als voor de melkproductie. De **Algemene Inleiding** beschrijft hoe kiemen in verschillende mate mastitis kunnen veroorzaken bij melkvee. Zo kan een beperkt aantal kolonievormende eenheden (KVE) van hetzij een Gram-negatieve pathogeen zoals *Escherichia coli* (*E. coli*), hetzij een Gram-positieve pathogeen zoals *Staphylococcus aureus* (*S. aureus*) reeds mastitis veroorzaken, terwijl een veel groter aantal KVE vereist is om met mastitis ziekteverwekkers zoals de coagulase-negatieve stafylokok (CNS) *Staphylococcus chromogenes* (*S. chromogenes*) een uierontsteking te induceren. De reden waarom de immuunrespons van de gastheer tussen bacteriële isolaten uit koeien met mastitis in dergelijke mate verschilt, is onvoldoende gekend. Deze kennis is echter noodzakelijk om een efficiëntere curatieve of preventieve behandeling voor mastitis te ontwikkelen. Idealiter worden experimentele mastitis studies uitgevoerd bij melkkoeien. Echter, die infectiestudies zijn zeer duur, arbeidsintensief en kampen met een tekort aan onderzoeksinstrumenten. Om de moleculaire biologie van de aangeboren immuunrespons tijdens mastitis beter in kaart te brengen werd naar aanvullende onderzoeksmethoden gezocht. Zo introduceerde en karakteriseerde men in de jaren ‘70 het muis mastitis model dat sindsdien door verschillende onderzoeksgroepen werd gebruikt om de interactie van boviene bacteriële isolaten met de melkklier te bestuderen. Resultaten verkregen bij muizen zijn zeer waardevol, niettemin moeten ze steeds bevestigd worden in de melkkoe.

De **Doelstellingen** van het proefschrift zijn drieledig en worden steeds benaderd met behulp het muis mastitis model dat gebruikt maakt van boviene-geassocieerde bacteriële isolaten om de situatie bij de melkkoe na te bootsen. De eerste doelstelling is om de immuunrespons te onderzoeken veroorzaakt door verschillende koe-geassocieerde kiemen namelijk *E. coli*, *S. aureus* en CNS geïsoleerd uit verschillende niches. Vervolgens wordt nagegaan of de voorbehandeling met een membraanfragment van *E. coli*, lipopolysaccharide (LPS), als een preventieve strategie kan fungeren om de daaropvolgende *S. aureus* mastitis te temperen. Ten slotte wordt de werking van zowel bestaande als kandidaat nieuwe antibiotica als curatieve behandeling tegen boviene mastitis vergeleken *in vitro* en in het muis mastitis model.

In **Hoofdstuk I** werd de kiemafhankelijke immuunrespons in de melkklier bestudeerd. Tot op heden werd aanvaard dat microob-geassocieerde moleculaire patronen (MAMP), blootgesteld door verschillende mastitis kiemen, interageren met pathogeen herkennende receptoren op uiercellen wat een instroom van immuuncellen met zich meebrengt tijdens mastitis. Er werd gesuggereerd dat deze influx het gevolg is van de cytokines uitscheiden door lokale uiercellen na activatie van specifieke transcriptiefactoren met name Nuclear Factor (NF)-kappa B. Sommige pro-inflammatoire cytokines, zoals de inactieve pro-vorm van interleukine (IL)-1 beta, moeten post-translationeel worden verwerkt om hun activiteit te verkrijgen. Voor IL-1beta gebeurt dit in het inflammasoom, een complex dat caspase-1 en -11 omvat. In Hoofdstuk I.1 onderzochten we deze inflammatoire signalisatie in parallel bij een *E. coli* en een *S. aureus* infectie in de melkklier. In vergelijking met de *S. aureus*-geïnduceerde inflammatie, induceerde *E. coli* een snelle instroom van immuuncellen in de melkklier die gepaard ging met histopathologische veranderingen. Daarenboven waren tussen de Gram-negatieve en Gram-positieve pathogenen ook kwantitatieve verschillen in NF-kappaB activatie en in lokale inflammatoire

cytokine- profielen. Meer specifiek induceerde *E. coli* zowel op 12 uur als op 24 uur na inoculatie een hoge NF-kappaB activiteit en hoge concentraties aan IL-1alpha, tumor necrosis factor-alfa en monocyte chemotactic protein-1 in de melkklier in vergelijking met *S. aureus*. De lokale IL-6 concentraties stegen vergelijkbaar na beide infecties, terwijl dit voor IL-1beta enkel het geval was bij het vroege tijdspunt maar niet bij het latere tijdspunt. Na 24 uur inoculatie waren de IL-1beta concentraties significant hoger in de melkklier na een infectie met *S. aureus*. Deze intrigerende kiemafhankelijke kinetiek van IL-1beta werd in detail bestudeerd via Western blot analyse. Door middel van deze techniek observeerden we een pathogeen-afhankelijk verknippingspatroon, dat onafhankelijk tot stand kwam van het inflammasoom. Dit laatste werd duidelijk door het gebruik van IL-1beta knock-out muizen en caspase-1/-11 dubbel knock-out muizen. In Hoofdstuk I.2 werd het muismodel voor de eerste maal gebruikt om CNS infecties met elkaar te vergelijken. Deze CNS werden geïsoleerd uit verschillende niches zoals de uier van een melkkoe met chronische mastitis (IM), de speentop van een vaars (TA) en de stalbedding. In contrast met de exponentiële groei van *S. aureus* na inoculatie vertoonde *S. chromogenes* IM beperkte bacteriële groei en dit bij meerdere getestte inoculum dosissen. Dit was tevens het geval voor *S. chromogenes* TA en een *S. fleurettii* afkomstig uit de stalbedding. Alhoewel de drie boviene CNS isolaten een lokale neutrofiel-influx induceerden, was de corresponderende immuunrespons gekarakteriseerd door de pro-inflammatoire cytokine profielen (incl. IL-1beta) veel zwakker dan die veroorzaakt door *S. aureus*.

Studies in diverse muis infectiemodellen toonden aan dat immunomodulatie door voorbehandeling met LPS, een MAMP van *E. coli*, geassocieerd is met een betere bacteriële opruiming en/of kolonisatie. Er zijn indicaties dat een dergelijke preventieve LPS injectie in de uier ook nuttig kan zijn in het kader van mastitis bij koeien. Daarom werd in **Hoofdstuk II** LPS geïnjecteerd kort voor een intramammaire *S. aureus* infectie. Deze lokale voorbehandeling verlaagde de pro-inflammatoire cytokine respons, de ernst van de symptomen en de lokale KVE in de melkklieren vergeleken met de niet voorbehandelde dieren. Een vergelijkende proteine-array tussen *E. coli*- en *S. aureus*-geïnfecteerde melkklieren identificeerde o.a. het chemokines CCL5/RANTES dat werd geïnduceerd door *E. coli* en niet door *S. aureus*. In tegenstelling tot de LPS voorbehandeling ging een voorbehandeling met CCL5/RANTES slechts gepaard met een geringe immunomodulatie en kon deze de lokale groei en/of kolonisatie van bacteriën niet verminderen.

Als curatieve therapie van boviene mastitis worden antibiotica nog steeds frequent gebruikt. Daarom vergeleek **Hoofdstuk III** de werkzaamheid van antimicrobiële middelen tegen verschillende mastitis ziekteverwekkers *in vitro*, gebaseerd op minimale inhibitorische concentratie (MIC) gegevens, ten opzichte van *in vivo* met het muis mastitis screening model. In Hoofdstuk III.1 werd de antimicrobiële werking van de eerste generatie cefalosporinen cefalexine, cefalonium, cefapirine en cefazoline geanalyseerd. Uit de *in vivo* bepaling van de effectieve en de beschermende dosissen blijkt dat cefazoline het meest werkzame antibioticum is voor de behandeling van *S. aureus* mastitis terwijl cefalonium en cefapirine actiever zijn *in vitro*. Bovendien kon in het muis mastitis model aangetoond worden dat de antimicrobiële werkzaamheid van cefazoline verhoogt door de hulpstof te wijzigen. In Hoofdstuk III.2 werd de antimicrobiële werkzaamheid van een nieuw kandidaat antibioticum uit de

biphenomycine klasse bestudeerd ten opzichte van verschillende mastitis ziekteverwekkers. Net als bij de cefalosporines, stemden de *in vitro* resultaten niet volledig overeen met de *in vivo* data. Onze gegevens op basis van het muis mastitis model, wijzen op het potentieel van het getestte biphenomycine als breedspectrum antibioticum.

In de **Algemene Discussie** wordt een hypothetisch model voorgesteld dat de waargenomen verschillen in proIL-1beta verknippingspatronen tijdens een intramammaire infectie met mastitis ziekteverwekkers *E. coli* en *S. aureus* kan verklaren (Hoofdstuk I.1). In dit meercellige model is een prominente rol weggelegd voor lokale cellen zoals macrofagen of epitheliale cellen naast de infiltrerende neutrofielen. Deze cellen worden geactiveerd door verschillende MAMPs van zowel *E. coli* als *S. aureus* waardoor proIL-1beta wordt uitgescheiden in de interstitiële ruimte op een inflammasoom-onafhankelijke wijze. Afhankelijk van het MAMP zal de intensiteit en de kinetiek van de aangeboren afweer in het melkklierweefsel verschillen. Zo zullen de MAMPs van *E. coli* een sterke NF-kappaB activatie induceren en met een pathogeen-afhankelijk cytokineprofiel dat voor een snelle rekrutering van circulerende neutrofielen zorgt in tegenstelling tot de MAMP van *S. aureus*. Bij hun activatie, kunnen neutrofielen proteasen produceren die verondersteld worden proIL-1beta te klieven in meerdere fragmenten. Verder wordt een *E. coli* infectie geassocieerd met het loskomen van mammaire epitheelcellen vermoedelijk als gevolg van matrix metalloproteinasen. Deze proteasen zorgen voor de additionele verknipping van pro-IL-1beta tijdens een *E. coli* infectie, terwijl *S. aureus* geassocieerde bacteriële proteasen zouden instaan voor een alternatief pro-IL-1beta verknippingspatroon. In conclusie toont dit model aan dat de inflammatoire signalisatie tijdens mastitis op een kiemafhankelijke manier verloopt. Bovendien zijn er alternatieven voor het induceren van een immuuncel influx aangezien bacteriële isolaten geassocieerd met lage virulentie, zoals CNS, deze IL-1beta respons karakteristiek voor hoog-virulente kiemen zoals *E. coli* en *S. aureus* ontbreken (Hoofdstuk I.2). Het preventief temperen van de lokale immuunrespons door een voorbehandeling met LPS kan bovendien de symptomen geassocieerd met hoge virulentie verminderen. Deze observatie biedt toekomstperspectieven waarbij alternatieve preventieve strategieën de gastheer-immuniteit gunstig wijzigen. De interactie tussen de immuunrespons van de gastheer en bacteriële mastitis isolaten blijkt ook de antimicrobiële activiteit van antibiotica te kunnen beïnvloeden. Om dit na te gaan kan men steeds op een snelle en kosteneffectieve manier de werkzaamheid van antibiotica screenen met het muis mastitis model voorafgaand aan *in vivo* bevestiging bij de melkkoe.

Curriculum Vitae

Koen Breyne werd geboren op 10 oktober 1985 te Ieper. Na het beëindigen van het hoger secundair onderwijs aan het Sint-Jans College te Poperinge (richting Moderne talen Wetenschappen), startte hij in 2004 met de studie Biochemie en Biotechnologie aan de Universiteit Gent. Hij behaalde in 2009 met onderscheiding het diploma Master of Science in de Biochemie en Biotechnologie, met als opties biomedische biotechnologie; en biochemie en structurele biologie.
Onmiddellijk na zijn afstuderen trad hij in dienst bij de onderzoeksgroep Biochemie die deel uitmaakt van de vakgroep Farmacologie, Toxicologie en Biochemie aan de faculteit Diergeneeskunde. Sinds oktober 2009 verrichtte hij onder begeleiding van Prof. dr. Evelyne Meyer doctoraatsonderzoek met als doel uierontsteking bij de koe te bestuderen aan de hand van een gevalideerd muismodel. Verder begeleidde hij als assistent de practica biochemie en was hij promotor van meerdere studenten in het kader van hun masterproef. In 2015 vervolledigde hij het trainingsprogramma van de Doctoral School of Life Sciences and Medicine van de Universiteit Gent.

Koen Breyne is auteur en mede-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Tevens nam hij actief deel aan nationale en internationale bijeenkomsten, symposia en congressen over mastitis, inflammatie, celdood en borstkanker.

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