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MASTITIS: ASPECTS OF THE HOST-PATHOGEN INTERACTION

Tese apresentada ao Programa de Pós-Graduação em Ciência Animal da Escola de Veterinária da Universidade Federal de Minas Gerais como requisito parcial para obtenção do título de Doutor em Ciência Animal

Área de concentração: Medicina Veterinária Preventiva

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Belo Horizonte
UFMG - Escola de Veterinária
2015

Souza, Fernando Nogueira de, 1982-
S729m Mastitis: aspects of the host-pathogen interaction / Fernando Nogueira de Souza. – 2015.
119 p. : il.

Orientadora: Mônica Maria Oliveira Pinho Cerqueira
Co-orientadores: Marcos Bryan Heinemann, Alice Maria Melville Paiva Della Libera
Tese (doutorado) – Universidade Federal de Minas Gerais, Escola de Veterinária.
Inclui bibliografia

1. Bovino de leite – Doenças – Teses. 2. Mastite – Teses. 3. Resposta imune – Teses. 4. Estafilococos áureos – Teses. I. Cerqueira, Mônica Maria Oliveira Pinho. II. Heinemann, Marcos Bryan. III. Della Libera, Alice Maria Melville Paiva. IV. Universidade Federal de Minas Gerais. Escola de Veterinária. V. Título.

CDD – 636.214 089 69

UFMG UNIVERSIDADE FEDERAL DE MINAS GERAIS

FOLHA DE APROVAÇÃO

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Tese submetida à banca examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIA ANIMAL, como requisito para obtenção do grau e DOUTOR em CIÊNCIA ANIMAL, área de concentração MEDICINA VETERINÁRIA PREVENTIVA.

Aprovada em 27 de fevereiro de 2015, pela banca constituida pelos membros:

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DEDICATORY

To my mother Helena Maria Nogueira Venâncio
I am sure that it would not be possible without her dedication, care, encouragement, support and trust. I love you!!!

ACKNOWLEDGMENTS

This thesis symbolizes the end of an extremely rich phase of exchange of knowledge, experiences and emotions, and many people have contributed to this achievement. I thank everyone that somehow, even with a simple sincere smile, took part in this story of personal and professional growth.

Initially, I thank all my family, my safe haven, which supports me unconditionally in every step of my walk, always believing in my growth as a human being and as a professional. Without you none of this would make sense. I record here my eternal gratitude. I love you !!!

To my promoter, Professor. Dr. Monica Maria Oliveira Pinho Cerqueira. The person who guided me during this period, not strictly in the professional life, but also as important as, in the personal aspect. At this point it is time to thank for trust on me, and for the teachings. "Being master is not only teaching, teaching is not only to convey the curriculum. Being master is to be a supervisor and a friend, to guide, is walking with the student step by step. You forward the secrets of this walk. Being master is to be an example of dedication, giving, personal dignity and love." And with this lesson, I truly hope to transmit it to others.

To my co-promoter, Prof. Dr. Marcos Bryan Heinemann, wonderful person who had the pleasure to have crossed in my life. ... Essential… a key person who made all efforts in directing, facilitating and conducting this work. Thanks to his teachings and diligence that turn this work possible. I come here to thank the extreme dedication and tremendous confidence placed in me.

To my co-promoter, Professor. Dr. Maria Alice Melville Paiva Della Libera, counselor, sincere, helpful, and always present in the most joyful and difficult moments of this journey. Thank you to trust on me, and for all efforts to improve my personal and professional growth. Striking icon in building this story. In addition to giving me the honor of sharing his teachings, gives me the greatest of all honors, to have her as a friend.

To Prof. Dr. Sarne De Vliegher and Dr. Sofie Piepers, the Faculty of Veterinary Medicine. - Ghent University - Belgium, for welcoming me in the other side of the ocean. The opportunity and honor to work with you, I am and will always be grateful.

To my dear brother Rodrigo Nogueira de Souza, and his small family - Denia Rocha and the young child Ana Clara, who have always been by my side with your shoulders friends.

To my friends Eduardo Milton Ramos Sanchez and Luiza Campos Reis, who were with me at all times, with words, advice and shoulders friends who helped me to walk this journey. Without your help none of this would be possible!!!

To the dear Maiara Garcia Blagitz, a great friend who became my sister. Always present in all joyful and difficult moments of this journey. Literally, "companion for all hours" ... Without her dedication, this work would not be possible...

To Kristine Picciart, Joren Verbeke, Dimitri Valckenier, Lars Hulpio, Anneleen de Visscher, Kristel Demeyere, Marina Stevens and Reshat Jashari from the Faculty of Veterinary Medicine -

Ghent University - Belgium. People who welcome me in the other side of the world. Thank you for pleasant living!!!

To Prof. Dr. Evelyne Meyer, Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine - Ghent University - Belgium, always smiling and in good spirits. Thank you for enable the accomplishment of this work.

To Prof. Dr. Magnus Ake Gidlund, from the Departamento de Imunologia do Instituto de Ciências Biomédicas of Universidade de São Paulo (USP), and Prof. Dr. Hiro Goto, from the Instituto de Medicina Tropical of USP. Reference, vanguard and excellence are their names. People who provided me valuable lessons and with whom I had the great honor of working. Here, I show my deep appreciation.

To PhD students Christiane Yumi Ozaki and Flaviane Alves de Paulo, from the Instituto de Medicina Tropical of USP, for the help and pleasant living.

To Prof. Fernando Ferreira, from the Faculdade de Medicina Veterinária e Zootecnia from USP, for his help with the statistical analysis.

To Prof. Flávio Guimarães da Fonseca and Edel Figueiredo Barbosa Stancioli for sharing knowledge and allow the improvement of this work.

To Prof. Andrey Pereira Lage, your help was of great value, and contributes to the progress of this work. My sincere and heartfelt thanks.

To my friends Prof. Marcos Silva Xavier, Prof. João Paulo Amaral Haddad, and Soraia Araujo Diniz, who marked their participation in this story far beyond collaboration in the statistical analysis, so thank you for the support, care, advice and companionship.

To the Researchers Maria Aparecida Vasconcelos Paiva and José Brito and Renaldi Feitosa and Brito, EMBRAPA - Dairy Cattle, the teachings, provision and care. People that I have always admired. I am grateful and I will always be grateful!!!

To the my friends and researcher Alessandro de Sá Guimarães, Guilherme Nunes Souza, and Letícia Caldas Mendonça EMBRAPA - Dairy Cattle, which undoubtedly contributed immensely to the implementation of this work !!!

To the PhD students Adriano França da Cunha and Dalila Lapinha Silva Oliveira Rosa, always willing to help even the most difficult times and with great skills, dedication and good mood. Make all efforts. I share with you the merits of this achievement.

To the PhD students Denise Freitas Ribeiro and Cristiane Viana Guimarães Ladeira, thank you for the friendship, care and support. Without their companies, everything would have been more difficult.

To the team "Della Libera", especially to Camila Freitas Batista, Bruna Parapinski dos Santos, Andrea Parra and Kamila Reis. My sincere thanks for the help and pleasant living!!!

To my friends Rafael Pontara and Wagner Ribeiro, who shared the good moments that made this a less arduous step.

To Claudia Regina Stricagnolo, from the Departmento de Clínica Médica at FMVZ-USP, who became a great friend. Get my sincere thanks for the debt I have with you!!!

To Clara Satsuki Mori, from the Departmento de Clínica Médica at FMVZ-USP. Always willing to help with her skill and good mood. My deepest and sincerest thanks.

The staff of the LabUFMG at EV- UFMG, without exception, always willing to help as best as they can.

To the staff of the Departmento de Tecnologia e Inspeção de Produtos de Origem Animal and the Departmento de Medicina Veterinária Preventiva at EV-UFMG, for all the help with good willing. Thank you a million !!!

To the veterinarian Guilherme Bartholomew, from GEPEC, and farm workers, especially Robson Araújo Rodrigues and Marcus Vinícius. Your help was essential to conclude this work.

To the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for granting scholarships.

To the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the grant of aid for research (Process n. 480102/2011-2 and 481950/2013-3) which enabled the implementation of this work.

To the Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP - granting of aid for research (Processes n. 2009/50672-0 and 2012/ 08982-4) that made possible the implementation of this work.

To the animals, unwittingly involved in this research, without which such an effort was not made in vain.

“Não sei se estou perto ou longe demais, se peguei o rumo certo ou errado. Sei apenas que sigo em frente, vivendo dias iguais de forma diferente. Já não caminho mais sozinho, levo comigo cada recordação, cada vivência, cada lição. E, mesmo que tudo não ande da forma que eu gostaria, saber que já não sou a mesma pessoa de ontem me faz perceber que valeu a pena.”

Albert Einstein

"Bom mesmo é ir à luta com determinação, abraçar a vida com paixão, perder com classe e vencer com ousadia, pois o mundo pertence a quem se atreve, e a vida é muito pra ser insignificante"

Charles Chaplin

ABSTRACT

Mastitis, generally defined as the inflammation of the mammary gland, is the most costly of the infectious, endemic diseases to affect dairy cows and other dairy species. The greatest obstacle to reduce mastitis is the implementation of effective mastitis control measures in dairy farmers, which mainly depends on a better understanding of the complex interaction between an infecting pathogen and the host immune system. Host-pathogen interaction is a broad, important area of research encompassing both basic and clinical sciences. Thus, here it was addressed some aspects of the host-pathogen interactions, as follows: 1) Review of the role of pattern-recognition receptors in the innate immunity of bovine mastitis with focuses on the major mastitis pathogens: *Escherichia coli* and *Staphylococcus aureus*; 2) Determine a set of rules for classifying the infection status of an udder at quarter (single and duplicate milk samples) and cow (composite milk sample) levels and compare with distincts somatic cell count (SCC) thresholds (at quarter and cow levels); 3) Evaluation of the interdependence of the quarters by evaluating milk neutrophil function and milk lymphocyte profiles in uninfected quarters from infected and uninfected udders by flow cytometry analysis; 4) Function of milk neutrophils and the milk lymphocyte profile in *Streptococcus dysgalactiae*-infected mammary glands; 5) Capacity of *S. aureus*, *S. fleurettii*, and two dissimilar strains of *S. chromogenes* species to adhere and internalize into bovine mammary epithelial cells; 6) Efficacy of a commercial vaccine under field condition in two dairy herds with high bulk milk somatic cell count (SCC) and a high prevalence of IMIs by *S. aureus*.

Key words: intramammary infection, *Staphylococcus aureus*, somatic cell count, immune response, vaccine, dairy cow.

RESUMO

Mastite, geralmente definida como a inflamação da glândula mamária, é a enfermidade de maior custo para a pecuária leiteira mundial. O maior obstáculo para reduzir da incidência da mastite, e consequentemente as implicações na produção e qualidade do leite, é a implementação de medidas efetivas no controle da mastite nas fazendas leiteiras, que depende principalmente da melhor compreensão da complexa interação patógeno-hospedeiro. A interação patógeno hospedeiro é uma importante e ampla área de pesquisa básica e clínica. Desta forma, o presente trabalho buscou elucidar alguns aspectos da interação patógeno-hospedeiro: 1) revisão sobre a importância dos receptores do tipo *Toll* na mastite bovina, especialmente considerando os patógenos: *Escherichia coli* e *Staphylococcus aureus*; 2) avaliação do diagnóstico da infecção intramamária pelo exame microbiológico do leite de amostras individuais por quarto, em duplicata por quarto e compostas e sua relação com a contagem de células somáticas; 3) avaliação por citometria de fluxo da interdependência dos quartos mamários pela função neutrofílica e perfil linfocítico; 4) estudo da função neutrofílica e perfil linfocítico em quartos mamários infectados por *Streptococcus dysgalactiae*; 5) determinação a capacidade de aderência e internalização em células epiteliais mamárias por *S. aureus*, *S. fleurettii*, e duas cepas de *S. chromogenes*; e 6) efeito da vacinação sobre a dinâmica da infecção intramamária por *S. aureus* e estafilococos coagulase-negativo.

Palavras-chave: infecção intramamária, *Staphylococcus aureus*, contagem de células somáticas, resposta immune, vacinação, vaca leiteira.

1 INTRODUCTION

Mastitis, generally defined as the inflammation of the mammary gland, is the most costly of the infectious, endemic diseases to affect dairy cows and other dairy species. The greatest obstacle to reduce mastitis is the implementation of effective mastitis control measures in dairy farmers, which mainly depends on a better understanding of the complex interactions between an infecting pathogen and the host immune system[1].

Host-pathogen interaction is a broad, important area of research encompassing both basic and clinical sciences. From the perspective of the pathogen, the cross-talking between microorganim and the immune cells may also be involved in the selection of microorganisms well-adapted to the host[2], which may be related to the establishment, persistence and severity of the infection, and the outcome to possible therapeutic and prevention interventions[3]. On the other hand, from the perspective of the host, immune response to pathogens is greatly determined by variable properties of the pathogens, which in turn can affect their lifecycle[4].

As milk production and milk quality are mainly affected by mastitis, the economical viability of dairy chain requires effective control measures of this disease which in turn include a broad comprehension of the defense mechanisms of the mammary gland[5], the evasion of the immune system by pathogens[6] and an appropriate use and interpretation of diagnosis measures and prevention strategies[7].

Regarding that, in last years, the Brazilian Normative Instruction and quality based payment program for milk quality established by many Brazilian dairy plants have been proposed as an attempt to increase Brazilian milk quality parameters. Nevertheless, we supposed that without an effective mastitis control program, the Brazilian legislation requirements for milk quality, especially for bulk tank SCC, proposed by Normative Instructions 51 and 62 (Brazil, 2002[8]; Brazil, 2011[9]) will not be easily met.

[1] Chapter I and Appendix I
[2] Chapter II
[3] Chapter VI
[4] Chapters I, III, IV and VI, and Appendix I
[5] Chapters I, III and IV, and Appendix I
[6] Chapter V
[7] Chapters II and VI
[8] Brasil 2002. Regulamento técnico de produção, identidade e qualidade do leite tipo A, do leite tipo B, do leite tipo C, do leite pasteurizado e do leite cru refrigerado e o regulamento técnico da coleta de leite cru refrigerado e seu transporte a granel. Instrução Normativa nº 51, de 18 de setembro de 2002. Diário Oficial da União, 18 de setembro de 2002, Ministério da Agricultura, Pecuária e Abastecimento, Brasília, DF.
[9] Brasil 2011. Regulamento técnico de produção, identidade e qualidade do leite tipo A, o regulamento técnico de identidade e qualidade de leite cru refrigerado, o regulamento técnico de identidade e qualidade de leite pasteurizado e o regulamento técnico da coleta de leite cru refrigerado e seu transporte a granel. Instrução Normativa nº 62, de 29 de dezembro de 2011. Diário Oficial da União, 29 de dezembro de 2011, Ministério da Agricultura, Pecuária e Abastecimento, Brasília, DF.

2 OBJECTIVES

The present study addressed some aspects of the host-pathogen interactions, as follows:

1. Review of the role of pattern-recognition receptors in the innate immunity of bovine mastitis with focuses on the major mastitis pathogens: *Escherichia coli* and *Staphylococcus aureus*;
2. Determine a set of rules for classifying the infection status of an udder at quarter (single and duplicate milk samples) and cow (composite milk sample) levels and compare with distincts somatic cell count (SCC) thresholds (at quarter and cow levels);
3. Evaluate the interdependence of the quarters by evaluating milk neutrophil function and milk lymphocyte profiles in uninfected quarters from infected and uninfected udders using flow cytometry analysis;
4. Study the function of milk neutrophils and the milk lymphocyte profile in *Streptococcus dysgalactiae*-infected mammary glands;
5. Determine the capacity of *S. aureus*, *S. fleurettii*, and two dissimilar strains of *S. chromogenes* species to adhere and internalize into bovine mammary epithelial cells;
6. Evaluate the effect of vaccination on the dynamic of intramammary infection by *S. aureus* and coagulase negative staphylococci.

3 CHAPTER I: The innate immunity in bovine mastitis: the role of pattern-recognition receptors

Mastitis is the most costly disease for dairy farmers and industry (Hujips et al., 2008; Hogeveen et al., 2011). More than 130 microorganisms can cause mastitis, although this disease is usually caused by some groups of bacteria (Wellenberg et al., 2002; Hillerton and Berry, 2005). Bovine mastitis is defined as an inflammatory condition of the mammary gland in response to injury, which serve to destroy and neutralize infectious agents and promote healing and the return to normal function. In the last few years, antimicrobial resistance has been growing concern worldwide. Thus, in an attempt to reduce the impact of mastitis and decrease the use of antimicrobials on dairy farms, there have been numerous efforts to try to exploit the immune capacity of the bovine mammary gland to stimulate the animal's natural defense mechanisms. These facts can reduce the use of antimicrobials and also minimize the development of resistance of bacterial strains (Wellnitz and Bruckmaier, 2012).

The role of toll-like receptors (TLRs) in innate and adaptive immunity has been subject of many good reviews (Medzhitov, 2001; Takeda et al., 2003; Akira and Takeda. 2004; Iwasaki and Medzhitov, 2004; Takeda and Akira, 2004; Hornung and Latz, 2010; Takeda et al., 2010; Kawai and Akira, 2011; Prince et al., 2011). Therefore, there is a need to summarize the role of these new concepts in bovine mastitis. In this review, we focus on the most recent knowledge about TLRs in bovine mastitis regarding their role in the major mastitis pathogens: *Escherichia coli* and *Staphylococcus aureus*.

Innate Immunity

Bovine mastitis is initiated by the entry of bacteria through the teat canal, and soon after is characterized by an important inflammatory response. Shortly after entry of the invading pathogen, the resident leukocytes together with epithelial cells initiate the inflammatory response necessary to eliminate the invading bacteria (Paape et al., 2003; Rainard and Riollet, 2006; Aitken et al., 2011). These cells release chemoattractants for the rapid recruitment of polymorphonuclear neutrophil leukocytes to the site of infection and consequently the somatic cell count (SCC) increases, which represents different cells types present in milk, including leukocytes and epithelial cells (Paape et al., 2003; Souza et al., 2012). The marked increase in milk SCC during infection is mainly due to influx of neutrophils from blood to the mammary gland, which neutrophils can represent over 90% of leukocyte population in milk from infected udder quarters in contrast to low numbers of this cell population in uninfected ones (Paape et al., 2003; Pyörälä et al., 2003; Souza et al., 2012).

Neutrophils are essential for innate host defense against invading microorganisms and eliminate pathogens by a process known as phagocytosis. During phagocytosis, neutrophils produce reactive oxygen species, including superoxide, hydrogen peroxide and hypochlorous acid, and release granule compounds into pathogen-containing vacuoles to kill the invading pathogen (Paape et al. 2003; Mehrzad et al., 2005; Rainard e Riollet, 2006; Prince et al., 2011). Thus, the rapid influx of neutrophils with high antimicrobial activity to the foci of infection is the main process that leads to the elimination of infection (Mehrzad et al., 2005). This importance was demonstrated by Mehrzad et al. (2005) who described that SCC in moderate cows increase faster than the colony forming units (CFU) of *E. coli* bacteria, whereas in severe cows the results were reversed.

Conversely, inflammation and tissue injury, as caused by the influx of neutrophils, can result in the release of endogenous TLRs ligands, known as damage-associated molecular patterns (DAMPs). DAMPs act in an autocrine manner, alerting the host of damage, but can also amplify inflammation leading to further tissue damage (Prince et al., 2011). In contrast, an

apoptosis differentiation program facilitates the resolution of neutrophil-mediated inflammation, and it has been suggested that phagocytosis initiates molecular cascade of events that accelerates apoptosis of this leukocyte population. Thus, as neutrophils can accumulate rapidly at sites of infection, and there is a concomitant potential to cause severe tissue destruction should they undergo necrosis lysis and release cytotoxic granule and reactive oxygen species onto host tissues. Thus, apoptosis can be viewed as the terminal stage of PMN-induced inflammation (Kobayashi et al., 2003).

Recognition of microbial pathogens is an essential element for initiation of innate immune responses such as inflammation and is mediated by germline-encoded pattern-recognition receptors (PRRs) that recognize molecular structures that are broadly shared by pathogens, known as pathogen-associated molecular patterns (PAMPs). Upon PAMP recognition, PRRs initiate a serious of signaling programs that execute the first line of host defensive responses necessary for killing infectious microbes (Medzhitov, 2001; Takeda et al., 2003; Akira and Takeda. 2004; Brandley and Medzhitov, 2004; Iwasaki and Medzhitov, 2004; Takeda and Akira, 2004; Takeda et al., 2010; Kawai and Akira, 2011; Prince et al., 2011). TLRs were the first PRRs identified. They are also the best characterized PRRs and recognize a wide range of PAMPs. They are expressed either on the cell surface or associated with intracellular vesicles (Medzhitov, 2001; Takeda and Akira, 2004). To date, 10 functional TLRs have been identified in bovine (Menzies and Ingham, 2006). These 10 TLRs and nucleotide-binding oligomerization domain (NOD) 1 and 2 were detected in tissue from alveolar, ductal, gland cistern and teat canal from infected and healthy quarters, with TLR8 having the least expression in comparison to the other PRRs (Whelehan et al., 2011). Functional analysis of mammalian TLRs has revealed that they recognize specific patterns of microbial components that are conserved among pathogens (Takeda and Akira, 2004). Each TLR detect distinct PAMPs derived from bacteria, viruses, mycobacteria, fungi and parasites. For instance, these include lipoproteins (recognized by TLR1, TLR2, and TLR6), flagellin (TLR5) lipopolysaccharide (LPS) (TLR4) and a 6-base DNA motif consisting of an unmethylated CpG dinucleotide motifs (CpG DNA) that are rarely found in higher vertebrates (TLR9) (Table 1) (Medzhitov, 2001; Takeda et al., 2003; Akira and Takeda. 2004; Iwasaki and Medzhitov, 2004; Takeda and Akira, 2004; Takeda et al., 2010; Kawai and Akira, 2011).

Table 1. Toll-like receptors and their ligands*

Receptor	Ligand	Origin of Ligand
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria
TLR2	Lipoprotein/lipopeptides	Various pathogens
	Peptidoglycan	Gram-positive bacteria
	Lipoteichoic acid	Gram-positive bacteria
	Lipoarabinomannan	Mycobacteria
	Phenol-soluble modulin	*Staphylococcus epidermitis*
	Zymosan	Fungi
TLR3	Double-stranded RNA	Viruses
TLR4	Lypopolysaccharide	Gram-negative bacteria
	Fusion protein	Respiratoty syncytial virus
TLR5	Flagelin	Bacteria
TLR6	Diacil lipopeptides	Mycoplasma
	Lipoteichoic acid	Gram-positive bacteria

	Zymosan	Fungi
TLR7	Single stranded RNA	Viruses
TLR8	Single stranded RNA	Viruses
TLR9	CpG-containing DNA	Bacteria and viruses
TLR10	N.D.	N.D.

Adapted from Akira and Takeda (2004).
N.D.: not determined
*Only ligands that can be related to mastitis pathogens were included

For instance for the role of TLRs in bovine mammary gland, it was found that LPS induced the expression of the chemokines MCP-1, MCP-2, and MCP-3, and slightly increase in CXCL8. Conversely, peptidoglycan combined with lipotechoic acid (LTA) induced the expression of MCP-1 and a slight increase in MCP-3 expression. Indeed, no significant expression for any of the chemokines was observed when induced by CpG-DNA (Mount et al., 2009).

Furthermore, it should be noted that the TLRs can act together with other molecules or other TLRs. For instance, TLR4 requires other molecules in addition to TLR4 to recognize LPS. LPS binds to the LPS-binding protein (LBP) present in serum, and this LPS-LBP complex is subsequently recognized by CD14, which is expressed on monocytes/macrophages and neutrophils. Moreover, LPS stimulation is followed by the increased physical proximity between CD14 and TLR4 in the membrane, suggesting that CD14 and TLR4 may interact in LPS signaling. Indeed, the TLR2 act in cooperation with at least two other TLRs: TLR1 and TLR6. So, the formation of heterodimers between TRL2 and either TLR1 or TLR6 dictates the specificity of ligand recognition (Medzhitov, 2001; Takeda et al., 2003; Akira and Takeda. 2004; Iwasaki and Medzhitov, 2004; Takeda and Akira, 2004; Takeda et al., 2010; Kawai and Akira, 2011). Another factor that can influence the innate immune response in bovine is cell maturation, as demonstrated for the same monocytes subsets - monocytes, macrophages and dentritic cells, which have different responses to the same TLR agonist (Werling et al., 2004).

All TLR signal transduction pathways are known to activate NF-κB factors (Akira and Takeda, 2004). MyD88 (myeloid differentiation primary-response protein 88) dependent pathways are associated with early-phase NF-κB response whereas as MyD88 independent pathways are associated with late-phase NF-κB response. These NF-κB factors subsequently enter the nucleus and bind to target promoters. A wealth of pro-inflammatory regulated genes feature NF-κB attachment sites in their promoter region and transcription factor complex act as a main switch to orchestrate immune defense genes against bacterial infection, as production of several pro-inflammatory cytokines.

Thus, the innate immune system uses various PRRs that are expressed on the cell surface, in intracellular compartments, or secreted into the blood stream and tissue fluids. The principal functions of PRRs include: opsonization, activation of complement and coagulation cascade, phagocytosis, activation and induction of apoptosis (Medzhitov, 2001). For instance, TLR signaling pathway by bacteria regulated phagocytosis at multiple steps, including internalization and phagossome maturation (Blander and Medzhitoz, 2004).

The importance of the innate immunity (TLR2, tumor necrosis factor (TNF)-α, interleukin (IL-)1β, IL-6, IL-8 complement factor C3, lactoferrin and RANTES) was also demonstrated by the significantly elevated expression of these innate immune genes in less-susceptible cattle when compared to high susceptibility group by detection of quantitative trait loci (QTL) affecting mastitis (Griesbeck-Zilch et al., 2009).

Mastitis caused by *Escherichia coli*

E. coli is among the major mastitis pathogens responsible for clinical mastitis in dairy cows, but the infection is normally cleared by the immune system within a few days. Indeed, in the last few decades, with the improvement of mastitis control programs, which leads to herds with low SCC, the clinical mastitis has become a major problem in many well-managed dairy herds that successfully controlled contagious pathogens (Green et al., 2004). Gram-negative bacteria, such as *E. coli*, are generally regarded as environmental pathogens, however contagious behavior of these pathogens has been proposed (Burvenich et al., 2003; Dogan et al., 2006; Suojala et al., 2011).

E. coli express a variety of virulence factors, but no coherence between the severity of disease and specific virulence factors could be defined (Wenz et al., 2006; Suojala et al., 2011; Schukken et al., 2011). However, the ability to grow in mammary secretions and to liberate LPS is crucial in the pathogenesis of *E. coli* mastitis. The faster bacterial numbers increase in the mammary gland, more LPS is present in the mammary gland and faster inflammatory response and clinical disease may occur (Mehrzad et al., 2008). Sensing the pathogen and initiating an immune response depends on the initial number of bacteria present at the start of the IMI. Increasing the initial challenge dose of *E. coli* resulted in faster immune response in primiparous cows (Vangroenweghe et al., 2004; Schukken et al., 2011), which the cytokines synthesis, such as TNF-α and IL-8, in mammary epithelial cells positively correlated with the concentration of *E. coli* particles (Güntler et al., 2010). Congruently, expression of IL-8 and interferon (IFN)-γ by milk somatic cells was increased in *E. coli* challenged mammary glands (Lee et al., 2006).

Buitenhuis et al. (2011) also described that in the early stages of *E. coli* mastitis a large number of up-regulated transcripts were associated with immune response functions, mainly those involved in acute phase response, while the down-regulation transcripts were principally involved in fat metabolism, which is consistent with the milk fat content depression commonly observed during mastitis infection, and later the up-regulated transcripts were associated with tissue healing processes, and were independent of *E. coli* strain and dose and lactation stage and number. Another factor that should be considered is the linkage between lipid metabolism and inflammation, as the nuclear receptors known as peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs) that emerged as key regulators of lipid metabolism and inflammation (Lubick et al., 2006; Bensinger and Tontonoz, 2008; Rios et al., 2008; Aitken et al., 2011; Moyes et al., 2010a; Moyes et al., 2012b), as has been demonstrated in mammary glands infected with *Streptococcus uberis* (Moyes et al., 2010a).

The innate immune system represents the first line of defense in the host response to infection and is prepared to immediately recognize and respond to the earliest stages of infection. The inherent capability of the innate system to respond to a vast number of pathogens is mediated by its ability to recognize highly conserved motifs shared by diverse pathogens, commonly referred to as PAMPs. It has been shown that a prompt response of the mammary gland after *E. coli* entry into the lumen of the gland is required to control the infection, which means that early detection of bacteria are of prime of importance (Bannerman et al., 2004; Porcherie et al., 2012).

The incidence and severity of septic *E. coli* mastitis in dairy cattle is mainly dependent on cow factors. During the periparturient period, the non-specific or innate immunity of the cow is depressed, which makes cows more susceptible to intramammary infection (IMI) by environmental pathogens like *E. coli*, while cows in mid lactation cure spontaneously from such infections (Burvenich et al., 2003; Mehrzad et al., 2005). There is substancial evidence indicating that at these periods, the expected influx of neutrophils, which form the first cellular defense against infection (Paape et al., 2003), into the mammary gland is delayed during inflammation after IMI with *E. coli* (Mehrzad et al., 2005; De Schepper et al., 2008). In fact, the

disturbance in neutrophil functions during early lactation stages are accompanied by modulation of TLR4 pathway genes, as diapedesis and migration process (Stevens et al., 2011).

Furthermore, Mehrzad et al. (2005) when classified cows as moderate and severe responders according to clinical symptoms and milk production output, observed an inverse relationship between pre-infection milk neutrophils microbicidal activity and amount of *E. coli* bacteria in milk, while for moderate cows the pre-infection milk and blood neutrophils microbicidal activity was about two fold higher than for severe cows. Mammary epithelial cells challenged by *E. coli* bacteria must have the capacity to mountain a strong innate immune response in their own right and attract circulating immune effector cells such as neutrophils. The importance of these cells is demonstrated by their role in the production of cytokines (Riollet et al., 2000; Strandberg et al., 2005; Lahoussa et al., 2007; Griesbeck-Zilch et al., 2008; Güntler et al., 2011; Porcherie et al., 2012). The upregulation of cytokine production is a key component of the host innate immune response to infection (Bannerman et al., 2004; Schukken et al., 2011).

Regarding bovine mammary epithelial cells (bMEC), Porcherie et al. (2012) showed that these cells are key players in initiating neutrophil inflammation during *E. coli* mastitis, as for instance, by producing he chemotractic factor CXCL8 (IL-8). So, recognition of several PAMPs at a time could contribute to the onset of an early response of the cow after infection by *E. coli*. These authors showed that a repertoire of potential bacterial agonists can be sensed by bMEC and udder during *E. coli* mastitis, as which both bMEC and udder can express domain receptors for NOD1, NOD2, TLR1, TLR2, TLR4 and TLR6, but not hardly TLR5, and can act synergistically. Therefore, LPS upon activation of TLR4 present a central role in the pathogenesis of clinical mastitis caused by this pathogen (Gonen et al., 2007) in a dose-dependent manner (Baumert et al., 2009). The inflammation caused by LPS leads to dose alteration alteration in milk parameters, such as lactose and chloride levels, which are likely caused by tighter junction damage by higher LPS doses (Werner-Misof, 2007). These parameters are also used to evaluate the indicators of inflammation in bovine mastitis, and consequently in their diagnosis (Pyörälä et al., 2003).

Elazar et al. (2010a) demonstrated that neutrophil recruitment to the milk is mediated through TNF-α, which is produced by alveolar macrophages in response to LPS/TLR4 signaling and is dependent on IL-8 and IL-1β signaling and regulated by iNOS-derived NO in a murine mastitis model. The ability to recruit cells into the mammary gland during the bacterial growth phase represent a crucial role since a 1 h delay in recruiting neutrophils can result in an 8-fold increase of *E. coli* (Hill, 1981). Both the MtD99 dependent and independent pathways in TLR4 signaling were activated in bMEC model (Ibeagha-Awemu et al., 2007). Despite the importance of LPS/TLR4 signaling pathway, Gonen et al. (2007) described that IMI of mice with *E. coli* P4 resulted in inflammation even in absence of LPS/TLR signaling. This inflammation response points out to additional factors beyond LPS and additional cells beyond alveolar macrophages play a role in the inflammatory response to *E. coli*. It has been suggested that in the absence of functional TLR4 the infecting *E. coli* P4 invaded epithelial cells with high efficiency, forming intracellular micro-colonies, since invasion of epithelial cells by *E. coli* is limited by alveolar macrophages using a process dependent on TLR4 signaling (Gonen et al., 2007; Elazar et al., 2010b; Schukken et al., 2011).

Infections caused by *E. coli* are more typically, but not exclusively, associated with fast and more dramatic immune response (Lee et al., 2006; Schukken et al., 2011). IMI with *E. coli* elicited systemic changes, including a febrile response, and induction of acute-phase synthesis of LBP. In milk, this infection resulted in increased levels of insulin-like growth factor-1, IL-1β, IFN-γ, IL-12, IL-8, TNF-α, sCD14, LBP, the complement cleavage factor C5a, lactoferrin, lysozyme and lipid mediators, such as cyclooxygenase-2 and 5-lipoxygenase (Riollet et al., 2000; Bannerman et al., 2004; Schmitzh et al., 2004).

Petzl et al. (2008) reported that *E. coli* inoculation in the mammary gland strongly upregulated the expression of β-defensins, TLR2 and TLR4 in the pathogen inoculated udder quarters as well as in mammary lymph nodes. In constrast, *S. aureus* did not significantly regulate the expression of these genes during the first 24 h after pathogen inoculation. Only 84 h after inoculation, the expression of β-defensins, but not of TLRs was significantly upregulated (< 20 fold) in *S. aureus* inoculated mammary glands.

E. coli IMIs induce distinct local and systemic transcriptome responses in the mammary gland. The local response, only in infected quarters, mainly involved in immune response and inflammation, while the systemic reactions, in both infected and neighboring quarters, comprises antigen processing and presentation, cytokines, protein degradation and apoptosis. Enhanced expression of antimicrobial genes, acute phase genes and indicators of oxidative stress point out to an active defense reaction in infected and neighboring healthy quarters (Mitterhuemer et al., 2010).

In this concern, data support an important sentinel function for teats, as these tissues respond rapidly and intensively, with production of cytokines and antimicrobial peptides. For example, genomic analysis showed that at 12 h post-infection with *E. coli* the inflammatory response was greatest in teat cistern and gland cistern. Only 24 h post-infection, the lobulo-alveolar region responds, at the time the inflammatory response was greatest of all regions (Rinaldi et al., 2010).

Mastitis caused by *Staphylococcus aureus*

S. aureus mastitis remains a worldwide problem for the dairy industry and producers, and can cause both subclinical and clinical mastitis (Barkema et al., 2006), whose severity and outcome of infection depend, in part, on strain-factors (Le Maréchal et al., 2011) and cow factors (Barkema et al., 2006). The cure rate of antimicrobial treatments for this agent is low and, therefore, the disease has not been effectively eliminated and/or controlled in many herds (Barkema et al., 2006). Staphylococcal infections are characterized by an ability to colonize the mammary tissue and bacterial survival inside epithelial cells, macrophages and even neutrophils (Gresham et al., 2000; Hébert et al., 2000; Lowy, 2000).

It is commonly assumed that most IMI are result of cow-to-cow transmission, however other sources of *S. aureus* bacteria in the environment of dairy cows have been described. Presumably, contagious strains of *S. aureus* co-exist with a large collection of non-contagious strains (Zadoks et al., 2002). Haveri et al. (2007, 2008) compared bacterial genomics of strains from persistent and transient infections, and found that genetic elements such as clonal type and penicillin resistance were over-represented in *S. aureus* isolated from persistent IMI. This microorganism is characterized by dynamic fluctuations and cyclic bacterial shedding in milk, which leads to fluctuations in milk SCC that normally fluctuate depending on organism's number and viability (Schukken et al., 2011; Souza et al., 2012).

In contrast to *E. coli* mastitis, *S. aureus* mastitis is characterized by a more moderate and delayed SCC increase, due in part, to limited cytokine response (Bannerman et al., 2004). Riollet et al. (2000) also described no detection of IL-1β, TNF-α, IL-8, bovine serum albumin, in milk whey from *S. aureus* infected animals. Indeed, the ability of milk to generate the complement cleavage product C5a in whey samples after addition of zymosan through complement activation was evaluated, and *E. coli* lead to a huge augment of C5a production (up to 100-fold), in contrast to a lower production in whey milk from *S. aureus* infected animals. Rainard et al. (2008) also showed that LTA from *S. aureus* induced an increase in chemokine and IL-1β, but little TNF-α in the milk.

Although, *S. aureus* is regarded as a gram-positive bacteria, the expression of TLR2 were correlated with TLR4, in a coordinated regulation of these two PRRs (Goldammer et al., 2004; Ibeagha-Awemu et al., 2008), although the expression of TLR9 was not increased during mastitis (Goldammer et al., 2004).

Cytokine gene expression in mammary epithelial cells induced by *S. aureus* infection was delayed and less than 5% of the cytokine expression was observed with *E. coli* infection (Lee et al., 2006; Yang et al., 2008; Güntler et al., 2010). This impaired proinflammatory activation is simultaneous with a complete lack of NF-κB activation in primary bovine mammary epithelial cells infected by *S. aureus* or LTA. In contrast, *E. coli* and LPS that activate strongly NF-κB in these cells. A large proportion of this activation is attributed to TLR-mediated signaling, since dual transdominant negative DN-MyD88-DN-TRIF factor blocks more than 80% of the pathogen-related NF-κB activation in primary bovine mammary epithelial cells. These facts may contribute to the well-known ability of this bacterium to establish chronic intramammary infections.

For example, the interleukin (IL)-8 and TNF-α were not detected in milk from quarters experimentally infected with *S. aureus* (Riollet et al., 2000; Bannerman et al, 2004), although, the mRNA expression of TNF-α in mammary cells increases during infection (Alluwaimi et al., 2003). Expression of IL-8 by milk somatic cells was also increased in *S. aureus* challenged mammary glands, but in lower magnitude than *E. coli* challenged mammary glands. However, the expression of IFN-γ was not increased in milk somatic cells from *S. aureus* challenged quarters (Lee et al., 2006).

Mammary epithelial cells demonstrated *in vitro* greater mRNA expression of IL-1β, IL-8 and TNF-α 24 h after infection with *E. coli* than with *S. aureus* (Lahouassa et al., 2007). Wellnitz et al. (2011) also found that infusion of *E. coli* LPS induced an increased TNF-α in milk from glands given LPS, but not by *S. aureus* LTA. The levels of lactate dehydrogenase, an enzyme released by degenerating cells, was greater in milk from glands instilled with LPS than with LTA. LPS was also a stronger inducer of IL-8 and IL-1β.

Conversely, the ability to induce clinical or subclinical mastitis was dependent on the used dose. LTA strongly induced the secretion of the chemokines CXCL1, CXCL2, CXCL3 and CXCL8, which induced neutrophils recruitment. The complement-derived chemoattractant C5a was generated in milk only with the highest doses of LTA. Furthermore, the pro-inflammatory cytokine IL-1β was induced in milk, but small amounts of TNF-α, and no IFN-γ (Rainard et al., 2008).

The muramyl peptide (MDP), an elementary constituent of the bacterial peptidoglycan, induce a prompt influx of neutrophils mediated by chemoattractants for these leukocytes (CXCL1, CXCL2, CXCL3, CXCL8 and C5a), and the highest concentrations of these chemoattractants were followed after challenge in combination with LTA, whose signal transduction is mediated by TLR2, although they do not significantly contribute to pro-inflammatory cytokines. Thus, TLR2 and NOD2, a major sensor for MDP, pathways could cooperate to trigger an innate immune response to *S. aureus* mastitis (Bougarn et al., 2010).

Induction of immune functions in mammary epithelial cells is accomplished via the activation of the relevant TLR and their downstream signaling pathways. Induction of these genes by *S. aureus* is reduced, due to at least in part, the impairment of MyD88 signaling, and the downstream of the trans-membrane TLRs (e.g. TLR2, TLR4). *S. aureus* apparently prevents the formation of the so-called Myddossome around TIR domain of the TLR forming the structural platform for the attachment of further downstream acting factors (Motshwene et al., 2009; Lin et al., 2010; Schukken et al., 2011). As a consequence, *S. aureus* elicits an immune response in these cells mainly by IL-6, while *E. coli* also activates IL-1β and TNF-α (Güntler et al., 2011; Schukken et al., 2011). The upregulation of IL-6 by both bacteria may be due to a

MyD88 independent mechanism (Güntler et al., 2010; Güntler et al., 2011), which, as cited above, is associated with late-phase NF-κB response.

It has also been suggested that *S. aureus* impaired NF-κB activation in mammary epithelial cells resulting in very low cytokine expression (Lara-Zárate et al., 2011). These authors reported that bovine prolactin stimulates *S. aureus* internalization in bovine mammary gland by regulating several innate immune elements, which is often modulated by NF-κB. On the other hand, prolactin induced NF-κB activation in bovine mammary epithelial cells; however, it was inhibited by *S. aureus* in presence of this hormone. When, these authors blocked NF-κB activation with acetylsalicylic acid, an inhibition of *S. aureus* internalization was found (48%) in prolactin stimulated cells. The infection of bovine mammary epithelial cells with *S. aureus* induced inhibition of NF-κB activation in the presence of prolactin which correlates with down regulation in prolactin-mediated TNF-α (27%) and nitric oxide production in mammary epithelial cells.

Curiously, Griesbeck-Zilch et al. (2008) encountered differences in expression of TLR2 and TLR4 by mammary epithelial cells in *S. aureus* and *E. coli* infections only after 24 h, when *S. aureus*-induced expression was significant lower. After 1 h *S. aureus* induced a significantly higher expression level of TNF-α and IL-1β, but after 6 and 24 h the transcription activity in *E. coli* treated cells was higher. *E. coli* induced a significant increase expression of IL-8 after 1h, but *S. aureus* caused no alteration in this chemokine. The RANTES (regulated upon activation, normal T-cell expressed and secreted) increased in *S. aureus* and *E. coli* treated bovine mammary epithelial cells after 1 h, whereas after 6 and 24 h the expression was significantly higher in *E. coli* treated cells. Lactoferrin showed a deviating expression pattern to pathogen stimulation, in which at 1 h *E. coli* induced a higher mRNA expression, whereas the highest level was reached after 24 h of *S. aureus* stimulation. The complement factor 3 was the only factor that responded equally to both microorganisms.

Genini et al. (2011) described that mastitis induced a prominence of metabolic and stress signals in the early stage and of the immune response and lipid metabolism in the late stage, both mechanisms apparently modulated by few genes. Comparison of *E. coli* and *S. aureus* infections in cattle revealed that affected genes with opposite regulation had the same altered biological functions and provided evidence that *E. coli* caused a stronger host response. The majority of genes with opposed regulation were associated with immune response belongs to antigen presentation, inflammatory response, cell-to-cell signaling and interaction network. Both cell death and lipid metabolism were among the most significant molecular functions altered in proteins of cows infected with both *E. coli* and *S. aureus*.

TLR1 was significantly expressed in ductal, gland cistern and teat canal after 48 h post-challenge with *S. aureus*, TLR3 showed a moderate increase in teat canal tissue, TLR6 and TLR7 presented a moderate increased in gland cistern tissue, TLR5 and TLR7 were also significantly increased in alveolar in alveolar tissue. Conversely, the genes encoding TLR4, NOD1 and NOD2 were significantly decreased in teat canal tissue, TLR6 in ductal tissue and TLR8 in gland cistern tissue. TLR2, TLR9 and TLR10 showed no differential expression across tissues of these regions. Chemokine and effector molecule expression was most significantly stimulated in alveolar tissue, in particular the expression of serum amyloid A and haptoglobulin, two acute phase proteins, and defensins-β 4 and 5 (Whelehan et al, 2011).

Thus, *S. aureus* appears to mostly circumvent the host immune response and IMI typically result in a very moderate host response with minimal observable innate immune response (Bannerman et al., 2004; Petzl et al., 2008; Bannerman et al., 2009; Schukken et al., 2011).

Therapeutic Opportunities

CD14 either in membrane or in soluble form (sCD14) is a high-affinity protein for the complex of bacterial LPS and LPS-LBP protein, and thus interact with TLR4 in LPS signaling (Medzhitov, 2001, Takeda et al., 2003; Nemchinov et al., 2006). Cells lacking mCD14, such as endothelial and epithelial cells, utilize sCD14 present in serum and milk to aid in LPS recognition by TLR4 (Aitken et al., 2011). Binding of soluble form of CD14 to LPS, found in the outer of *E. coli*, enhances the innate immune responses, reduces the severity of mastitis, and facilitates clearance and neutralization of LPS, thus preventing the development of endotoxic mastitis. Thus, Lee et al. (2003) found that the infusion of recombinant bovine sCD14 lead to an increase in SCC, due to more rapid recruitment of neutrophils that was accompanied by a faster clearance of bacteria, lower concentration of TNF-α and IL-8 in milk, and milder clinical symptoms. Congruently, Nemchinov et al. (2006) demonstrated that the recombinant bovine CD14 receptor produced in plants reduced the severity of *E. coli* mastitis, leading to enhancement of LPS-induced neutrophil recruitment, lowing the numbers of viable bacteria in milk and resulting in absence of clinical symptoms.

Kauf et al. (2007) in an attempt to increase the inflammatory response during *S. aureus* intramammary infection infused LPS in quarters experimentally infected with *S. aureus*. They found an increase in SCC in quarters between 24 and 72 h post LPS-infusion, as well as, an increase in bovine serum albumin in milk, which reflect alterations in the vascular permeability, between 4-48 and 480 h post LPS-infusion. There was no detection of TNF-α in *S. aureus*-infected quarters administrated PBS at any time during the study, but infected quarters infused with LPS showed an increased TNF-α concentrations in milk between 4-8 h post LPS-infusion. Moreover, a trend toward a lower recovery of viable bacteria from LPS- versus PBS-infused quarters between 4-13 h post LPS-infusion was observed. This trend occurred in the inflammatory responses elicited by LPS. *In vitro* inoculation of milk obtained from udder quarters infused with LPS or PBS demonstrated that the growth of *S. aureus* in milk from LPS-infused udder was significantly inhibited, and an overall negative correlation existed between milk SCC and *in vitro S. aureus* growth in milk inoculated with *S. aureus* and incubated for 6 or 12 h.

In a mastitis using rat model, the infusion of CpG-DNA in mammary glands stimulated the secretion of IL-6 and TNF-α at different points, reduced viable *S. aureus* (Zhu et al., 2007a) and *E. coli* (Zhu et al., 2008) in mammary tissues, decreased the activity of NAGase, promoted the expression of TLR9 and induced more rapid infiltration of neutrophils to mammary tissue at initial stages of experimentally induced mastitis induced in rat model (Zhu et al., 2007a; Zhu et al., 2008). In goats, Zhu et al. (2007b) demonstrated that the infusion of CpG-DNA in the mammary glands induced a decrease in viable *E. coli*, reduced bacteria counts in milk, promoted the expression of TLR9, stimulated the production of IL-6, attenuated the impact of inflammation mediators on cells, and significantly shortened the inflammation course.

The retinoid, a group of derivates of vitamin A, exert various immunomodulatory actions. It has been demonstrated that the administration of retinoid acids protects rats against neutrophil-induced oxidative stress in acute experimental mastitis (Gu et al., 2009a; Gu et al., 2009b). A mechanism by which this protection is conferred is through TLR4. Gu et al. (2010) found that TLR4 gene expression reached its peak earlier in retinoid acid-treated rats, and that retinoid acid decreased NF-κB DNA binding activity and the level of IL-1β protein expression in mammary gland. So, retinoid acid leads to attenuation of LPS-induced inflammation response by repression of TLR4/NF-κB signaling system. Another mechanism that can also be involved was demonstrated by Uematsu et al. (2008) who found that retinoid acids, in a dose-dependent manner, regulated the differentiation of interleukin 17-producing T helper cells, which in turn mediated neutrophil response (Schukken et al., 2011).

Pheromonicin-SA (Ph-SA) is an enginnered multidomain bactericidal peptide (Qiu et al., 2003) that has effect against *S. aureus*. Zhu et al. (2012) when exposed primary mammary epithelial cells to Ph-SA found that this compound increases the expression of TLR2, TNF-α, IL-1β, IL-8 and lactoferrin, and later the expression of TLR4. Thus, Ph-SA may be value as an antimicrobial in promoting innate immune response by *S. aureus aureus*-infected bovine mammary epithelial cells, especially regarding the inhibition of innate immune response induced by *S. aureus* which leads to the chronic inflammatory response (Bannerman et al., 2004; Lahouassa et al., 2007; Yang et al., 2008; Motshwene et al., 2009; Güntler et al., 2010; Lin et al., 2010; Lara-Zárate et al., 2011; Schukken et al., 2011; Wellnitz et al., 2012).

TLR signaling induces 25-hydroxyvitamin D3 1α-hydrolase expression in macrophages. The 25-hydroxyvitamin D_3 1α-hydrolase is the primary enzyme that converts 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3, the active vitamin D_3 metabolite. It was shown that the expression 25-hydroxyvitamin D3 1α-hydrolase was significantly increased in tissue and cells from infected mammary glands, and was predominantly expressed in $CD14^+$ cells (Nelson et al., 2010), which is expressed in both neutrophils and macrophages in milk (Paape et al., 1996; Blagitz et al., 2012). Thus, regarding the importance of innate immunity for mammary gland health (Paape et al., 2003, Rainard and Riollet, 2006; Elazar et al. 2010a, Elazar et al. 2010b), efforts to find optimal range of 1,25-dihydroxyvitamin D_3 concentrations for proper immune function in cattle has implications for bovine health.

Conclusion

The innate immunity is crucial to maintain mammary gland healthy, which is mediate through recognition of pattern recognition receptors (PRRs). The PRRs recognized specific patterns of microbial components that are conserved among pathogens known as pathogen-associated molecular patterns (PAMPs). The interaction of PRRs and PAMPs mediate the inflammatory response characterized by each mastitis-causing pathogen that can contribute to the development of severe acute or chronic mastitis.

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4 Chapter II. Somatic cell count and microbiological detection of mastitis pathogens by composite milk samples, single or duplicate quarter milk samples

ABSTRACT

The most acceptable criteria for diagnosis of bovine intramammary infections (IMIs) includes both culture results and measures of inflammation. Therefore, information on operating characteristics, sensitivity (SE) and specificity (SP) of procedures used to identify infected quarters is required. Thus, this study was designed to evaluate a set of criteria for classifying the infectious status of an udder at quarter (single and duplicate milk samples) and cow (composite milk sample) levels and compare with somatic cell count (SCC). Here, the SCC thresholds determined by ROC curve analysis showed highest Youden index using mammary quarter duplicate milk samples, and can be regarded as a gold standard test, especially considering a sample bacteriologically positive with at least one pair of the duplicate with positive bacteriological result regardless the mastitis pathogen isolated, when compared to milk quarter single samples and composite milk samples. The kappa coefficient between the milk quarter single samples (single S1 and S2) was 0.85 $\pm$ 0.019 indicating that single quarter milk sampling can be useful in mastitis control programs. Therefore, although composite milk sampling are usually collect by veterinarians on a routine basis to detect mastitis pathogens, their use may be limited to detect major pathogens taken into account their predictive values (SE and SP using $\leq$ 200,000 cells/mL as a SCC threshold for infected cows, and its Youden index determined by ROC curve analysis). Thus, our findings suggests that the milk SCC and bacteriological examinations, although regarded as the most reliable indicators of ongoing mastitis, should be used in an integrated manner in mastitis control programs. Furthermore, the accuracy of single, duplicate and composite bacteriological analysis to diagnosis mastitis should be considered in mastitis control strategies.

Key words: mastitis, sensitivity, specificity, udder health status, dairy cow.

INTRODUCTION

Worldwide, mastitis is the most common infectious disease affecting dairy cows and the most economically important disease of the dairy industry. Mastitis implies inflammation within the udder, but not necessarily infection; however mastitis is usually caused by bacterial infection (Pyörällä, 2003, Dohoo et al., 2011a, Dohoo et al., 2011b, Reyher and Dohoo, 2011, Oliveira et al., 2013). Given the central role of intramammary infections (IMIs) in bovine mastitis, high-quality information on the operating characteristics, such as sensitivity (Se) and specificity (Sp) of procedures used to identify infected quarters, are required (Dohoo et al., 2011a, Dohoo et al., 2011b, Reyher and Dohoo, 2011).

Guidelines include both culture results and measures of inflammation, and discussion about the number of samples that should be taken to maximize sensitivity and specificity parameters (Dohoo et al., 2011a). In face of, the use of composite, single, duplicate or triplicate milk samples can affect the predictive values of the bacteriological culture (Dohoo et al., 2011a, Dohoo et al., 2011b, Reyher and Dohoo, 2011), the most expensive component in the diagnosis of IMIs. Then, in attempt to minimize costs, a relevant question would be whether composite milk sampling, or at least individual quarter milk sampling, would be useful for detecting culture-positive milk samples, regardless of cow or quarter-level characteristics.

Furthermore, milk somatic cell count (SCC) has been extensively used as an indicator of IMI and as a component of the definition of mastitis since milk SCC increases during infection mainly due to the influx of neutrophils from the blood to the mammary gland (Pyörälä, 2003, Schukken et al., 2003, Dufour and Dohoo, 2013, Mira et al., 2013). Altogether, we hypothesized that the culture results affects the SCC thresholds used to detect infected quarters or cows, as bacteriological culture has long been considered the gold standard and compared with other mastitis diagnostic tests (Pyörälä, 2003).

Thus, this study was designed to evaluate a set of criteria used for classifying the infection status of an udder at quarter (single and duplicate milk samples) and cow (composite milk sample) levels and compare with SCC (at quarter and cow levels).

MATERIALS AND METHODS

Herd(s)

The data were collected from 36 dairy cows during January 2013 to January 2014 in two dairy herds from Minas Gerais State - Brazil. Both herds had high bulk milk SCC (>500,000 cells/mL) according to criteria proposed by Barkema et al. (1998). All Holstein dairy cows were milked twice daily and cows had estimated milk yield of 16.68 $\pm$ 0.97 (herd 1) and 23.86 $\pm$ 1.30 (herd 2) kg/day, respectively. No sample from dairy cows at immediate post-partum period was used. The herds used machine milking and adopted the following mastitis control practices at milking: forestripping in a strip cup to check for clinical mastitis, preddipping, and drying teats with paper towel. After milking, postdipping was also used. Dry cow therapy and clinical mastitis treatment were also adopted. As both herds had cows with *S. aureus* mastitis, the cows identified as infected by this pathogen were segregated from the milking line and milked last.

Experiments

Experiment 1.

Data were recorded on 1,612 bacteriological milk culture and 810 SCC results. Duplicate quarter milk samples (two samples collected at the same milking) were collected from all quarters (n = 810 quarters) (referred to as S1 and S2 samples) for bacteriological examination. Individual milk samples were also collected for SCC. Duplicate quarter milk samples were categorized in: (A) when the same pathogen was isolated from both samples, the sample was regarded as bacteriological positive for that microorganism. Nevertheless, if a pathogen was recovered only in one sample of the duplicate, the duplicate sample was regarded as non-significant growth, except for *Staphylococcus aureus* and *Streptococcus agalactiae*. This exception was applied for *S. agalactiae* because it is regarded as a highly contagious obligate parasite of the bovine mammary gland (Keefe, 1997), and for *S. aureus* due its peculiar shedding pattern, particularly when the shedding pattern is below the detection limit of the bacteriological method. (Sears et al., 1990; Zecconi et al., 1997; Godden et al., 2002; Walker et al., 2010). When one of the duplicate samples was contaminated or there was missing data, but no organisms were recovered from the other sample, the quarter was considered to be non-infected; and (B) the milk sample was regarded as bacteriological positive if at least one pair of the duplicate (single S1 or S2) where bacteriological positive regardless the mastitis pathogen isolated.

Experiment 2.

Data were recorded on 1,215 bacteriological milk culture and 135 SCC results. The data were collected from January to June 2013 in herd 1. Duplicate quarter milk samples (two samples collected at the same milking) were collected from all quarters (n = 540 quarters). Composite milk samples (n = 135) - those containing milk from each of a cow's four mammary quarters - were also collected for bacteriological examination and milk SCC. No sample from dairy cows at immediate post-partum period was used.

Sampling

First, the strip cup test was performed to determine the presence of clots or flakes or abnormal secretions. Then, predipping was performed, using one disposal paper towel for each teat. After discarding the first three milk streams, the ends of the teats were scrubbed with 70% ethanol

using cotton balls, and duplicate milk samples from the individual mammary quarters (approximately 4 mL in each vial) and composite milk samples (approximately 40 mL) were aseptically collected in sterile vials for the bacteriological analysis. Finally, milk samples for the SCCs were collected. Composite milk samples for SCCs were collected through milk meters, as routinely used in Dairy Herd Improvement (DHI) programmes for monitoring SCC of individual cows (Ruegg and Pantoja, 2013).

All samples for bacteriological analysis were frozen until cultured. Both samples referred as S1 and S2 from duplicate milk samples were individually considered to analyze the predictive values of single milk samples.

Bacteriological analysis

Bacterial analysis was conducted by culturing 0.01 mL of each sample on 5 % ovine blood agar plates. The plates were incubated for 24-48 hours at 37 ºC, which was followed by observation of colony morphology, Gram staining and identification tests (Brito et al., 1999, Oliver et al., 2004). The microorganisms were identified from subcultures on plates of Brain Heart Infusion agar. The Gram-positive catalase-negative cocci were identified by production of CAMP factor and pyrrolydonylarylamidase (PYR test), hydrolysis of sodium hippurate and esculina, growth in 6.5% NaCl broth and on bile-esculin agar. Using these set of tests it was possible to differentiate *S. agalactiae, S. uberis, S. equinus* and *Enterococcus* spp. The streptococci like organisms that could not be identified were classified as *Streptococcus* spp. The Gram-positive catalase-positive cocci were identified as coagulase-negative staphylococci and coagulase-positive staphylococci by means of tube coagulase test. Among the coagulase-positive staphylococci, *S. aureus* was identified based on a positive acetoin test (Voges-Proskauer test) (Oliver et al., 2004). Gram-negative bacteria were differentiated based on appearance on MacConkey's agar, reactions on Triple Sugar Iron agar, indol production, motility, utilization of citrate, oxidative-fermentative test (Hugh & Leifson medium), methyl red reaction and acetoin production. Gram-positive, catalase-positive, non-hemolytic, rod-shaped bacteria with typical morphology of coryneforms visible only after 48 hours of incubation mainly in the fatty areas on plates streaked with milk were classified as *Corynebacterium* spp.. For the results analysis these bacteria were considered as *Corynebacterium bovis*. Samples yielding colonies of ≥ 3 different bacterial species were considered to be contaminated.

Somatic cell count

Composite and quarter milk samples for SCC were collected in a 40 mL vial containing bronopol (2-bromo-2-nitropane-1,3-diol) and analyzed using a calibrated Bentley CombSystem 2300® unit (Chaska, USA).

IMI Definition

A sample was defined as having an IMI if ≥ 3 colonies growth in bacteriological culture was detected, except for *S. aureus* or *S. agalactiae*, which were considered as culture-positive when the growth of ≥ 1 colony was detected, and *Corynebacterium* sp. which was regarded as bacteriological positive when ≥ 10 colonies (≥ 1,000 cfu/mL) growth in culture (Andersen et al., 2010). When one of the duplicate samples was contaminated, but no organisms were recovered from the other sample the quarter was considered to be non-infected. When both duplicate milk samples were contaminated the data were considered missing. If a cow had an IMI caused by a particular organism in at least one quarter according to the criteria described above, the cow was considered positive for that organism.

Statistical Analysis

All statistical analysis to determine the predictive values were performed using Meldcalc statistical software (Meldcalc software, Belgium). Two SCC thresholds (100 and 200 x 10^3 cells/mL) and one SCC threshold (200 x 10^3 cells/mL) was used to determine the SE and SP for individual mammary quarters and udders (composite) samples, respectively, regarding the bacteriological results as a gold standard test. Bacteriological results of the duplicate quarter milk samples with the highest Youden index, according to the criteria described above, were considered as the reference method to define the presence of the IMIs and evaluate the predictive values of the composite SCC results. The variation of SCC according to the distinct mastitis pathogens were analyzed using GraphPad Prism 5.0 software® (GraphPad Software, Inc., San Diego, CA, USA). Results are reported as mean ± standard error. Significance was declared at $P \leq 0.05$.

RESULTS

Experiment 1

The distribution of the isolated microorganims isolated is given in Table 1. The area under ROC curve and the predictive values of the milk quarter SCC regarding the SCC threshold that maximized the sensibility and specificity indicated by the ROC curve analysis and the most used SCC thresholds (100,000 and 200,000 cells/mL) to detect IMIs are shown in Table 2. Here, 13.26% (criteria A) and 8.74% (criteria B) of the duplicate quarter milk samples were bacteriological positive with very low SCC (≤ 100,000 cell/mL). On the other hand, 17.17% (criteria A) and 18.43% (criteria B) of the duplicate quarter milk samples were bacteriological negative with high SCC (> 200,000 cells/mL). The milk quarter SCCs according to the different pathogens are shown in Table 3. The kappa coefficient between the milk quarter samples (single S1 and S2) was 0.85 (95% confidence interval 0.813 - 0.886).

Experiment 2

The distribution of the isolated microorganims is given in Table 4. The area under ROC curve and the predictive values of the composite SCC regarding the SCC threshold that maximized the sensibility and specificity indicated by the ROC curve analysis and the most used SCC cut-off (200,000 cells/mL) to detect IMIs are shown in Table 5. The composite SCCs of the bacteriological positive and negative milk samples are presented in Table 6. The composite SCCs according to the different pathogens are shown in Table 7. The accuracy of the bacteriological composite milk samples regarding the results of the duplicate milk samples as the gold standard are presented in Table 8.

DISCUSSION

The most useful criteria for diagnosing IMIs is based on milk SCC and bacteriological sampling (Pyörälä 2003, Schukken et al. 2003, Dohoo et al. 2011a, Dohoo et al. 2011b, Reyher and Dohoo, 2011). The SCC is part of the defense mechanism, and as a result reflects the inflammatory response against an invading pathogen. Thus, the increase of SCC depends mainly on the pathogenicity of the mastitis causing bacteria and the amount of affected tissue in the gland, especially the epithelial area, which in turn can influence the predictive values of SCC used to diagnosis mastitis (Pyörälä et al. 2003).

Mastitis pathogens are normally divided into minor and major pathogens according to the magnitude of SCC increase in milk due to their pathogenicity, (Djabri et al., 2002, Pyörälä, 2003, Souza et al., 2009). *Corynebacterium bovis* has been regarded as a minor pathogen with limited clinical significance (Oliveira et al., 2013). In agreement, *C. bovis* did not lead to a significant augment in milk SCC that could be related to the modulation of innate immunity (i.e. toll-like receptor 2) in an attempt to control inflammation and tissue injury (Blagitz et al., 2015).

The heterologous group of coagulase-negative staphylococci (CoNS) has been traditionally classified as minor pathogens and did not also lead to a significant increase in milk SCC here. However, their clinical relevance is still under debate (Supré et al., 2011, Piepers et al., 2013, Fry et al., 2014, Vanderhaeghen et al., 2014) and a high degree of variability has been reported in milk SCC among mammary glands infected with different CoNS species (Supré et al., 2011, Fry et al., 2014). The major pathogens including *S. aureus*, *S. agalactiae* and *Streptococcus uberis* lead to a significant milk SCC increase in the present study, as previous described (Djabri et al., 2002, Souza et al., 2009).

The definitive identification of infected animals relies on the positive culture of pathogens from aseptically collected milk samples. The problem regarding mastitis is that indirect tests are compared with the "golden standard", bacteriology, but mastitis not always requires the presence of infection (Pyörälä, 2003; Nunes et al., 2008). Furthermore, mastitis may not be accompanied by isolation of the etiological agent for several reasons: microorganisms may be excreted in an intermittent way and in small amounts; infection-related pathogens are not detectable using conventional microbiological tests; some milk enzymes or proteins (i.e. lysozyme and lactoferrin) may thwart pathogen detection; and the infection is supported by bacterial endotoxins and bio-active compounds released by neutrophils that outlive the elimination of the invading bacteria. Moreover, some bacteria may be present in the teat canal without causing significant inflammation, and others may be present as contaminants (Pyörälä, 2003, Nunes et al., 2008). With this in mind, some authors have isolated mastitis pathogens from milk samples with very low SCCs, whereas others have found high proportions of bacteriologically negative milk samples with high SCCs (Makovec and Ruegg, 2003, Koskinen et al., 2009, Oliveira et al., 2013), as found here.

Altogether, these facts may explain why duplicate milk quarters samples showed highest Youden index (Table 2) using the following criteria: milk sample was regarded as bacteriological positive if at least one pair of the duplicate was bacteriologically positive regardless the mastitis pathogen isolated. The Youden index indicates the performance of a diagnostic test. Its value ranges from zero to one, and has a zero value when a diagnostic test gives the same proportion of positive results for groups with and without the disease. A value of one indicates that there are no false positives or false negatives that would be a perfect test (Schisterman et al., 2005).

We found high agreement between the bacteriological analysis of the single milk quarters (A and B) samples (kappa coefficient = 0.85). Thus, the use of single quarter bacteriological sampling appears to be acceptable in mastitis control programs especially if the costs of bacteriological sampling are taken into account, whereas SCC should be used together with bacteriological examination to diagnosis IMIs (SCC thresholds found here for single and duplicate milk samples by ROC curve analysis were similar; Table 2).

The SCC thresholds that have been proposed range from 70,000 to 500,000 cells/mL, although the SCC thresholds frequently used to reduced diagnostic error varied between 100,000 cells/mL and 200,000 cells/mL (Dohoo and Leslie, 1991, Scheppers et al., 1997, Sargeant et al., 2001; Pyörälä, 2003, Schukken et al., 2003, Bansal et al., 2005, Schwarz et al., 2010, Dufour and Dohoo, 2013).

When evaluating data on udder infections, it is important to keep in mind that cows live in environment with high exposure to microorganisms and not all contacts result in IMIS. As the SCC mediated defense mechanisms of the mammary gland against the invading pathogens, and the dairy cows are under high challenge (high bulk milk tank > 250,000 cells/mL; Barkema et al., 1998) especially regarding the contagious nature of many mastitis pathogens, we hypothesized that this fact together with the dynamic nature of IMIs may also explain the higher SCC thresholds indicated by ROC curve analysis found here (Table 5) when compared to SCC

thresholds usually suggested by other studies from many countries that have implemented an effective mastitis control program (DHI programmes) over the last decades. For instance, the original limit for SCC of a healthy quarter is 500,000 cells/mL (IDF, 1971). Nevertheless, the SE and SP using a lower thresholds for SCCs (i.e. 200,000 cells/mL) to diagnosis IMIs has been evaluated in several studies; and the reported SE and SP range from 73-89% and 75-90%, respectively (Schepers et al., 1997, Pyörälä, 2003), similar to our findings (Table 2). Therefore, in the last years, a SCC limit of 100,000 cells/mL or lower has been suggested (Sargeant et al., 2001; Pyörälä, 2003, Bansal et al., 2005, Schwarz et al., 2010). These decrease of SCC limits to detect IMIs may be due to an effective mastitis control program in many developed countries that led to a decrease of IMIs caused by major pathogens (i.e. *Streptococcus agalactiae*) and an increase of proportion of IMIs by minor pathogens (i.e. coagulase-negative staphylococci) (Pitkälä et al., 2004, Piepers et al., 2007, Schwarz et al., 2010). Furthermore, quarter milk SCC-derived incidence showed a more limited predictive value in herds experiencing a very high rate of IMI acquisition during a given period (Dufour and Dohoo, 2013). This may have influenced our results, especially regarding the high bulk milk SCC in the herds used here indicating that cows are under high challenge (Barkema et al., 1998). This fact might also explain why high milk SCC thresholds (> 200,000 cells) to detect infected quarters and cows were found here using ROC curve analysis to determine a cut-off that maximized the SE and SP. Thus, estimates for the diagnostic SE and SP may vary in populations, and an epidemiological approach should be used for validation of diagnostic test (Pyörälä, 2003).

Regarding the dynamic nature of the IMIs, the measurement of SCC and bacteriological analysis over time will distinguish better between non-infected quarters, infection with subsequent self cure, and infection that is not cured by the defense mechanisms. For instance, Pinzón-Sánchez and Ruegg (2011) and Oliveira et al. (2013) have suggested that when pathogen is not recovered from microbiological analysis considering milk samples from clinical cases of mastitis before initiating treatment, it indicates the outcome of the IMI after treatment. Thus, the no-growth of mastitis pathogens from milk samples can also be related to the probability of bacteriological cure and indicate the low number of pathogens that are eliminated on milk, and a reduced the probability of isolating mastitis pathogens. In agreement, our findings regarding subclinical mastitis (SCC > 200,000 cells/mL) showed that 50.0% of the milk quarter samples with bacteriological negative outcome (unspecific mastitis) resulted in no-growth of mastitis pathogens in subsequent sampling(s) (once monthly or three subsequent weekly samplings after the sampling with bacteriological negative outcome and SCC > 200,000 cells/mL) and a reduction in the milk quarter SCC (median = 529,834 cells/mL to 29,637 cells/mL; mean $\pm$ SEM = 1,147,637 $\pm$ 442,637 cells/mL to 57,431 $\pm$ 13,162 cells/mL; $P = 0.0002$) indicating the spontaneous bacteriological cure of the IMIs (the ability of self cure). In other words, in these cases the increase in milk SCC was effective in reducing the number of invading bacteria and consequently decreased the chance to isolate the causative pathogen, suggesting an effective control of the infection by the milk leukocytes. Therefore, interpreting SCC data based on a single sampling is subjected to possible misdiagnosis or misclassification.

We found a high cut-off for composite SCC (> 500,000 cells/mL) to diagnosis IMIs and a relatively low Youden index (0.47; Table 5) using ROC curve analysis when compared to the individual quarter milk SCC (0.88; Table 2) pointing out to a higher proportion of false-negative and/or false-positive results in composite SCC, indicating that composite SCC measurements for monitoring the IMI incidence may be impractical. In agreement, Schukken et al. (2003) have reported that the most accurate relationship between IMI and SCC exists at quarter level. The predictive values of composite SCC depends on the number of infected quarters and pathogen involved in IMIs, as normal milk from uninfected quarters is diluted with milk from infected quarters (Reyher and Dohoo, 2011). In agreement, Reksen et al. (2008) reported that when the

composite milk SCC was used to detect mastitis pathogens, their accuracy was limited. Thus, the predictive values of composite milk samples should be taken into account and their use may be limited to diagnosis IMIs by major pathogens (Dufour and Dohoo, 2013), although composite milk samples have great practical importance, especially regarding the costs of bacteriological culture.

Our findings suggested that the milk SCC and bacteriological examinations, although regarded as the most reliable indicators of ongoing mastitis, should be used in an integrated manner in mastitis control programs. Furthermore, the accuracy of single, duplicate and composite bacteriological analysis for mastitis diagnostic should be evaluated implications in mastitis control strategies.

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Table 1: Bacteriological results of the single milk quarter samples

Bacteriological Results	**Single S1 (n = 810)**	**Single S2 (n = 802)**
Negative	51.60% (418)	53.24% (427)
S. aureus	17.04% (138)	16.21% (130)
S. agalactiae	7.28% (59)	7.48% (60)
Corynebacterium bovis	4.94% (40)	4.61% (37)
S. uberis	4.57% (37)	4.24% (34)
***Streptococcus* spp.**	1.48% (12)	1.37% (11)
CoPS	0.86% (7)	1.37% (11)
CoNS	0.99% (8)	0.87% (7)
E. coli	0.12% (1)	0.12% (1)
S. equines	0.12% (1)	0% (0)
S. aureus* and *S. agalactiae	7.65% (62)	7.11% (57)
S. aureus* and *S. uberis	1.36% (11)	1.12% (9)
***S. aureus* and *Streptococcus* spp.**	0.25% (2)	0.62% (5)
***S. aureus* and *Enterococcus* spp.**	0.37% (3)	0.25% (2)
S. aureus* and *Corynebacterium bovis	0.25% (2)	0.37% (3)
***S. uberis* and CoNS**	0.12% (1)	0.25% (2)
S. aureus* e *S. equinus	0.37% (3)	0.37% (3)
CoNS and *Streptococcus* spp.	0.12% (1)	0.12% (1)
CoNS and *S. agalactiae*	0.12% (1)	0 (0%)
Contaminated	0.37% (3)	0.25% (2)

S. aureus: *Staphylococcus aureus*; *S. agalactiae*: *Streptococcus agalactiae*; *S. uberis*: *Streptococcus uberis*; Streptococcus sp.: *Streptococcus* sp. excluding *S. agalactiae*, *S. equinus*, *S. uberis*, , and *Enterococcus* sp.; CoPS: coagulase-positive staphylococci; CoNS: coagulase-negative staphylococci; *E. coli*: *Escherichia coli*; *S. equinus*: *Streptococcus equinus*.

Table 2: ROC curve and sensibility/specificity of individual quarter SCCs

Gold Standard (Bacteriological Analysis)	Single (A)	Single (B)	Duplicate[a]	Duplicate[b]
Area under ROC curve (95% CI)	0.879 (0.854 - 0.901)	0.890 (0.865 - 0.911)	0.897 (0.873 - 0.917)	0.883 (0.859 - 0.905)
***P* value**	≤ 0.0001	≤ 0.0001	≤ 0.0001	≤ 0.0001
SCC Threshold (cells/mL)	266,000	266,000	266,000	272,000
Youden index	0.679	0.696	0.710	0.883
Sensibility (95% CI)	83.2 (79.9 - 86.8)	85.0 (80.9 - 88.6)	85.2 (81.1 - 88.6)	81.7 (77.5 - 85.4)
Specificity (95% CI)	84.7 (80.8 - 88.1)	84.6 (80.7 - 87.9)	85.9 (82.1 - 89.1)	86.5 (82.7 - 89.8)
Threshold (200,000 cells/mL)	SE 85.3 (81.3 - 88.7) SP 81.2 (77.0 - 84.9)	SE 87.5 (83.7 - 90.8) SP 81.4 (77.2 - 85.0)	SE 87.6 (83.9 - 90.8) SP 82.6 (78.6 - 86.2)	SE 84.5 (80.5 - 88.0) SP 82.6 (78.5 - 86.3)
Threshold (100,000 cells/mL)	SE 90.4 (86.9 - 93.2) SP 67.9 (63.1 - 72.5)	SE 92.0 (88.7 - 94.6) 68.1 (63.4 - 72.6)	SE 91.9 (88.7 - 94.5) 69.0 (64.2 - 73.5)	SE 89.7 (86.2 - 92.5) SP 69.4 (64.6 - 74.0)

ROC: receiver operating characteristics; SCC: somatic cell count; 95% CI: 95% confidence interval; SE: sensibility; SP: specificity. Duplicate[a]: when the same pathogen was isolated from both samples, the sample was regarded as bacteriological positive for that microorganism. Nevertheless, if a pathogen was recovered only in one pair of the duplicate, the duplicate sample was regarded as non-significant growth, except for *Staphylococccus aureus* and *Streptococcus agalactiae*. When one of the duplicate samples was contaminated, but no organisms were recovered from the other sample the quarter was considered to be non-infected; Duplicate[b]: the milk sample was regarded as bacteriological positive if at least one pair of the duplicate where bacteriological positive regardless the mastitis pathogen isolated.

Table 3: Median (mean ± SEM) of milk quarter SCC (cells/mL) regarding distinct mastitis pathogens

Bacteriological Results	Single (A)	Single (B)	Duplicate[a]	Duplicate[b]
Negative	45,000 (251,489 ± 36,805)[c]	44,000 (241,184 ± 33,104)[b]	44,000 (223,421 ± 31,953)[b]	38,500 (226,464 ± 33,702)[b]
Positive	824,000 (1,313,051 ± 72,304)[a]	837,000 (1,352,861 ± 75,450)[a]	858,000 (1,371,721 ± 74,482)[a]	795,000 (1,300,807 ± 71,600)[a]
CoNS	482,000 (517,857 ± 116,576)[abc]	1,073,500 (909,667 ± 201,200)[ab]	774,000 (692,750 ± 230,542)[ab]	312,000 (481,000 ± 198,296)[ab]
CoPS	484,000 (1,149,000 ± 701,468)[ab]	557,000 (941,546 ± 445,986)[ab]	484,000 (1,149,000 ± 701,468)[ab]	484,000 (975,556 ± 550,531)[ab]
Corynebacterium bovis	88,500 (390,583 ± 178,925)[bc]	90,000 (416,000 ± 194,718)[b]	88,500 (452,038 ± 244,908)[b]	87,000 (370,538 ± 165,523)[b]
S. aureus	871,000 (1,111,374 ± 70,093)[a]	869,000 (1,169,777 ± 90,288)[a]	869,000 (1,141,181 ± 77,999)[a]	871,000 (1,149,943 ± 78,825)[a]
S. agalactiae	1,943,125 (1,246,000 ± 237,156)[a]	1,441,000 (1,948,241 ± 228,754)[a]	1,447,500 (1,946,914 ± 239,334)[a]	1,613,000 (1,966,842 ± 232,510)[a]
***Streptococcus* spp.**	500,000 (685,545 ± 248,613)[abc]	524,000 (827,444 ± 283,851)[ab]	524,000 (827,444 ± 283,851)[ab]	500,000 (685,545 ± 248,613)[ab]
S. uberis	803,500 (1,294,778 ± 235,249)[a]	803,500 (1,314,794 ± 244,469)[a]	956,500 (1,380,281 ± 255,423)[a]	705,000 (1,270,743 ± 241,179)[a]
S. aureus* and *S. agalactiae	1,024,500 (1,964,817 ± 242,727)[a]	1,024,500 (1,957,839 ± 246,219)[a]	1,023,000 (1,901,333 ± 233,877)[a]	1,023,000 (1,901,333 ± 233,877)[a]
S. aureus* and *S. uberis	1,169,000 (1,634,091 ± 276,654)[a]	1,169,000 (1,384,556 ± 310,507)[a]	1,317,500 (1,732,900 ± 429,550)[a]	1,317,500 (1,702,400 ± 440,366)[a]

SEM: standard error; SCC: somatic cell count; CoNS: coagulase-negative staphylococci; CoPS: coagulase-positive staphylococci; *S. aureus*: *Staphylococcus aureus*; *S. agalactiae*: *Streptococcus agalactiae*; *Streptococcus* spp.: *Streptococcus* spp. excluding *S. agalactiae, S. equinus, S. uberis*, and *Enterococcus* spp.; *Streptococcus uberis*: *S. uberis*. Duplicate[a]: when the same pathogen was isolated from both samples, the sample was regarded as bacteriological positive for that microorganism. Nevertheless, if a pathogen was recovered only in one pair of the duplicate, the duplicate sample was regarded as non-significant growth, except for *S. aureus* and *S. agalactiae*. When one of the duplicate samples was contaminated, but no microorganisms were recovered from the other sample the quarter was considered to be non-infected; Duplicate[b]: the milk sample was regarded as bacteriological positive if at least one pair of the duplicate where bacteriological positive regardless the mastitis pathogen isolated. Different scripts among lines indicated $P \leq 0.05$.

Table 4: Bacteriological results of the composite (at cow level) and duplicate[a] (at quarter level) milk samples

Bacteriological Results	Cow Level (n = 135)	Quarter Level (n = 540)
Negative	43.70% (59)	61.85% (334)
S. aureus	15.56% (21)	13.33% (72)
S. uberis	7.41% (10)	6.85% (37)
CoPS	5.19% (7)	0.93% (5)
CoNS	4.44% (6)	3.52% (19)
Corynebacterium bovis	4.44% (6)	7.96% (43)
***Streptococcus* spp.**	1.48% (2)	0.93% (5)
S. agalactiae	0% (0)	0.37% (2)
E. coli	0% (0)	0.19% (1)
S. aureus* and *S. uberis	8.15% (11)	1.48% (8)
S. aureus* and *S. agalactiae	1.48% (2)	0.56% (3)
***S. uberis* and CoNS**	1.48% (2)	0% (0)
***S. aureus* and *Streptococcus* spp.**	0.74% (1)	0.19% (1)
S. aureus* and *E. coli	0.74% (1)	0% (0)
S. aureus* and *Corynebacterium bovis	0% (0)	0.74% (4)
CoNS and *Corynebacterium bovis*	0% (0)	0.37% (2)
S. uberis* and *S. agalactiae	0% (0)	0.19% (1)
Contaminated	1.48% (2)	0.37% (2)
Missing	3.70% (5)	0.19% (1)

Duplicate[a:] the milk was regarded as bacteriological positive if at least one sample of the duplicate where bacteriological positive regardless the mastitis pathogen isolated. *S. aureus*: *Staphylococcus aureus*; *S. uberis*: *Streptococcus uberis*; CoPS: coagulase-positive staphylococci; CoNS: coagulase-negative staphylococci; *Streptococcus* spp.: *Streptococcus* spp. excluding *S. agalactiae*, *S. equinus*, *S. uberis* and *Enterococcus* sp.; *S. agalactiae*: *Streptococcus agalactiae*; *E. coli*: *Escherichia coli*

Table 5: ROC curve and sensibility/specificity of composite SCC

Gold Standard	Composite Bacteriological Analysis	Duplicate[a] Bacteriological Analysis
Area under ROC curve (95% CI)	0.826 (0.748 - 0.888)	0.787 (0.706 - 0.854)
***P* value**	$\leq$ 0.0001	$\leq$ 0.0001
SCC Threshold (cells/mL)	698,000	657,000
Youden index	0.536	0.472
Sensibility (95% CI)	65.7 (53.1 - 76.8)	61.7 (50.3 - 72.3)
Specificity (95% CI)	87.9 (76.7 - 95.0)	85.4 (72.2 - 93.9)
Threshold (200,000 cells/mL)	SE 95.0 (85.0 - 98.0) SP 36.2 (24.0 - 49.9)	SE 88.9 (80.0 - 94.8) SP 37.5 (24.0 - 52.6)

Duplicate[a]: a cow was regarded as bacteriological positive if one or more quarter has a positive bacteriological result; a quarter was regarded as bacteriological positive if at least one sample of the duplicate where bacteriological positive regardless the mastitis pathogen isolated. ROC: receiver operating characteristics; SCC: somatic cell count; 95% CI: 95% confidence interval; SE: sensibility; SP: specificity.

Table 6: Median (mean ± SEM) of composite SCC (cells/mL) of the bacteriological positive and negative milk samples

Milk samples	Bacteriological Positive	Bacteriological Negative
Composite	1,060,000 (1,368,343 ± 157,996)[a]	273,000 (413,793 ± 65,950)[b]
Duplicate[a]	855,000 (1,235,074 ± 140,440)[a]	276,000 (355,667 ± 39,883)[b]

Duplicate[a]: a cow was regarded as bacteriological positive if one or more quarter has a positive bacteriological result; a quarter was regarded as bacteriological positive if at least one sample of the duplicate where bacteriological positive regardless the mastitis pathogen isolated. SEM: standard error; SCC: somatic cell count. Different scripts between columns mean $P \leq 0.0001$.

Table 7: Median (mean ± SEM) of composite SCC (cells/mL) regarding distinct mastitis pathogens

Bacteriological composite results	**CoNS**	***S. aureus***	***S. uberis***	***S. aureus* and *S. uberis***
SCC	610,000 (618,000 ± 216,000)[ac]	1,184,000 (1,262,000 ± 184,514)[ac]	517,500 (584,333 ± 157,758)[c]	1,902,000 (2,431,000 ± 690,972)[b]

SEM: standard error; SCC: somatic cell count; CoNS: coagulase-negative staphylococci; *S. aureus*: *Staphylococcus aureus*; *S. uberis*: *Streptococcus uberis*. Different scripts between columns mean $P \leq 0.05$.

Table 8: Diagnostic accuracy of composite microbiology results compared with bacteriological outcome of the duplicate quarter milk samples regarded as the gold standard

Sensibility	71.95% (60.94 - 81.32)
Specificity	81.25% (67.37 - 91.05)
Area under ROC curve	0.77 (0.68 - 0.84)
Positive likelihood ratio	3.84 (2.10 - 7.02)
Negative likelihood ratio	0.35 (0.24 - 0.50)
Positive Predictive Value	86.76% (76.36 - 93.77)
Negative Predictive Value	62.90% (49.69 - 74.84)

Results and 95% confidence interval are presented; ROC: receiver operating characteristics.

5 Chapter III. Flow cytometric analysis: Interdependence of healthy and infected udder quarters

Journal of Dairy Science, v.98, p.2401-2408, 2015

ABSTRACT

An important question about intramammary infections that is still debated in the literature is the independence or interdependence of the quarters of dairy cows. Thus, the present study sought to explore milk neutrophil function and the milk lymphocyte profile of uninfected quarters from uninfected and infected (one infected quarter per cow) udders to better determine the interdependence of the quarters. Thus, 32 (eight cows) and 18 (six cows) uninfected quarters from uninfected and infected udders were used, respectively. Using flow cytometry, we evaluated the percentage of milk neutrophils and their expression of CD62L, CD11b and CD44, the levels of intracellular reactive oxygen species (ROS), the phagocytosis of *Staphylococcus aureus* by milk neutrophils and neutrophil viability. Furthermore, the percentage of B cell ($CD21^+$) and T lymphocyte subsets ($CD3^+/CD4^+/CD8^-$; $CD3^+/CD8^+/CD4^-$; $CD3^+/CD4^+/CD25^-$; $CD3^+/CD4^+/CD25^+$ and $CD3^+/CD4^-/CD25^-$) were also assessed with flow cytometry using monoclonal antibodies. The infected quarter did not influence the SCC or the percentage of neutrophils in the neighboring uninfected quarters in the present study. It furthermore did not influence neutrophil viability, intracellular ROS production or phagocytosis of *S. aureus* by milk neutrophils. Conversely, the expression of adhesion molecules CD11b, CD62L and CD44 by milk neutrophils was different between uninfected quarters from infected versus uninfected udders. The lymphocyte subsets did not differ between the groups, with the exception of a higher percentage of B cells in uninfected quarters from infected udders than uninfected quarters from uninfected udders. Thus, our study strongly supports the hypothesis of the interdependence of quarters based on the influence of the infection on both the percentage of B cells and the expression of adhesion molecules by milk neutrophils in the neighboring uninfected quarters.

Key Words: Mastitis. Somatic cell count. Lymphocyte. Neutrophil. Dairy cow.

INTRODUCTION

Several previous studies have evaluated aspects of intramammary infections (IMIs) based on the assumption that the quarters within the cow are independent of each other (Berry et al., 2006; Jensen et al., 2013) because of the anatomical construction of the udder, which implies that the infection of one quarter does not influence the immune status of the neighboring quarters (Merle et al., 2007; Jensen et al., 2013). An important question about IMIs that is still debated in the literature is the independence or interdependence of the quarters of dairy cows (Schwarz et al., 2011). For instance, does the local inflammatory response against invading bacteria in an infected quarter influence the neighboring uninfected quarters immune response? If this is true, the local cross-talk between udder quarters could be responsible for priming the neighboring uninfected quarters and therefore may influence the immune response against new infections. Some studies have evaluated the interdependence of the quarters by determining the probability of an infection spreading from an infected to uninfected quarter (Barkema et al., 1997; Sol et al., 2000; Berry et al., 2006) or by evaluating the milk somatic cell count (SCC) (Wever and Emanuelson, 1989), percentage of immune cells or certain immune response parameters (Merle et al., 2007; Schwarz et al., 2011). The reasons that uninfected quarters from infected udders are more likely to be contaminated than quarters from uninfected udders may include the animal's susceptibility to mastitis, the transmission patterns of infection or the inherent immune competency (Barkema et al., 1997; Berry et al., 2006). The transmission pattern of IMIs should

be particularly considered given the contagious behavior of pathogens and that transmission occurs not only between cows but also between quarters within a cow (Barkema et al., 1997).
The present study attempt to better elucidate the interdependence of the quarters exploring milk neutrophil function and milk lymphocyte profiles in uninfected quarters from infected and uninfected udders using flow cytometry analysis. To our knowledge, this is the first study to perform a broad evaluation of milk neutrophil function and milk lymphocyte subsets in uninfected quarters from udders with different infectious statuses. We believe that this new knowledge may improve the understanding of the interdependence of quarters, which may influence the probability of new infections in uninfected quarters.

MATERIALS AND METHODS

Animals

The present study used 50 mammary quarters from 14 Holstein dairy cows, which were collected at different lactation stages (days in milk) and parities from a commercial dairy herd from São Paulo State, Brazil. The milk samples were divided into group 1 (G1) and group 2 (G2) as follows: G1) culture-negative milk samples from quarters of uninfected udders with no abnormal secretions in the strip cup test (*n = 32* quarters; eight dairy cows); and G2) culture-negative milk samples with SCC lower than 1×10^5 cells/mL from quarters from infected udders (only one quarter was considered as infected) with no abnormal secretions in the strip cup test (*n = 18* quarters; six dairy cows). All the cows with more than one infected quarter were excluded from the present study.

Sample collection

First, the strip cup test was performed to identify the presence of clots, flakes or otherwise obviously abnormal secretions. Pre-dipping was then performed, with one towel used for each teat. After discarding the first three milk streams, teat ends were scrubbed with cotton soaked in 70 % ethanol, and a single milk sample from each mammary quarter was aseptically collected into sterile vials for bacteriological analysis. Finally, milk samples were collected for the evaluation of SCC (40 mL), neutrophil function and milk lymphocyte profiles (1 L of milk). The samples were kept at 4°C until arriving at the laboratory. The milk samples for the bacteriological analysis were collected once at the same day of immune parameters analysis and were stored at -20°C for at maximum 30 days until the analysis.

Bacteriological analysis

The bacteriological analysis was performed by culturing 0.01 mL of each milk sample on 5 % sheep blood agar plates. The plates were incubated for 72 hours at 37 ºC, followed by Gram staining, observation of colony morphologies and biochemical testing (Oliver et al., 2004). A milk sample was considered culture-positive when the growth of ≥ 3 colonies was detected, with the exception of animals with *Staphylococcus aureus* or *Streptococcus agalactiae* infections in their quarters, which were considered culture-positive when the growth of ≥ 1 colony was detected (Piepers et al., 2007; 2013).

Determination of SCC

The milk samples for SCC determination were collected in 40-mL vials containing microtablets of the preservative bronopol (2-bromo-2-nitropane-1,3-diol). Subsequently, the SCCs were

performed using a Somacount 300 automated somatic cell counter (Bentley Instruments, Chaska, MN, USA).

Definition of IMI status

A quarter was considered uninfected when it was culture-negative, with no abnormal secretions in the strip cup test, and had a milk SCC $\leq 1 \times 10^5$ cells/mL, as the threshold for uninfected quarters proposed by Bansal et al. (2005). A quarter was considered to be infected when any mastitis pathogen was isolated from a single milk sample or had SCC $> 2 \times 10^5$ cells/mL, as the threshold for the SCC proposed by Schepers et al. (1997) and Schukken et al. (2003).

Separation of milk cells

The separation of the milk cells was performed as described by Koess and Hamann (2008). Briefly, 1 L of milk was diluted with 1 L of phosphate-buffered saline (PBS; pH 7.4; 1.06 mM Na_2HPO_4, 155.17 mM NaCl and 2.97 mM $Na_2HPO_4.7H_2O$). After centrifugation at 1,000 x g for 15 min, the cream layer and supernatant were discarded. The cell pellet was then washed once using 30 mL of PBS and centrifuged at 400 x g for 10 min. The cells were resuspended in 1 mL of RPMI-1640 nutritional medium (R7638, Sigma Aldrich, USA) supplemented with 10 % fetal bovine serum (Cultilab, Campinas, Brazil) and counted using a Neubauer chamber. Cell viability was evaluated using trypan blue exclusion. The milk cells were then diluted with nutritional medium containing 10 % fetal bovine serum to a concentration of 2×10^6 viable cells/mL.

Enumeration of lymphocyte subpopulations

The cells were washed with PBS and stained to detect CD21, the combination of CD3, CD4 and CD8, and the combination of CD3, CD4 and CD25 following incubation with the primary antibodies (Abs) for 30 min on ice. The identification of lymphocyte subsets was based on their cytoplasmic granularities and mean fluorescence intensities following a two-step fluorescence immunolabeling protocol using primary anti-bovine monoclonal Abs and secondary Abs coupled to long-wavelength fluorescent probes (Table 1). Thereafter, 1 mL of PBS was added to cell suspension and centrifuged at 400 x g for 8 min. Finally, the labelled secondary antibody was added to cell suspension and the cells were incubated for 30 min on ice with the secondary Abs. The cells were then washed with PBS and immediately analyzed using flow cytometry. A total of 20,000 milk cells, excluding most of the cellular debris, were examined per sample. FlowJo software (TreeStar Inc., Ashland, OR, USA) was used to analyze the data. The results were corrected for autofluorescence, which was defined as the fluorescence that was associated with non-labeled freshly isolated milk cells from the same cow (Della Libera et al., 2014).

Identification of neutrophils

Milk neutrophils were differentiated from other cells by indirect fluorescence labeling. The cells were incubated with an unlabeled primary monoclonal anti-bovine granulocyte antibody (Table 1) for 30 min on ice temperature. Next, 1 mL of PBS was added to the cell suspension, which was centrifuged at 400 x g for 8 min. Finally, a labeled secondary Ab (Table 1) was added, and the sample was incubated for 30 min on ice in the dark to visualize the bound CH138A. The neutrophils were identified using flow cytometry based on the cells' cytoplasmic granularities

and CH138A positivity as previously described (Piepers et al., 2009; Blagitz et al., 2013). The flow cytometry data analysis was performed as described above.

Detection of apoptosis using flow cytometry

The death of neutrophils ($CH138^+$) was assessed using dual-labeling with an annexin V antibody and propidium iodide (PI; K2350, APOPTEST-FITC, Dako Cytomation, Mijdrecht, the Netherlands) and flow cytometric analysis as previously described (Piepers et al., 2009; Blagitz et al., 2013). Briefly, 2×10^5 viable milk cells were suspended in 100 µL of binding buffer (10 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) containing anti-annexin V-FITC antibody and incubated on ice for 20 min in the dark. Immediately before flow cytometric analysis, 5 µL of a 250 µg/mL PI solution was added. Next, the neutrophils were labeled using mAbs as described above.
To analyze the data, scatterplots were generated for the gated neutrophils. The living, non-apoptotic cells were negative for both FITC-labeled anti-annexin V and PI. The cells that were positive for FITC-labeled anti-annexin V but negative for PI were classified as apoptotic cells (Piepers et al., 2009; Blagitz et al., 2013). The necrotic subpopulation was excluded from analysis (Piepers et al., 2013). The flow cytometry data analysis was performed as described above.

Intracellular reactive oxygen species production

Intracellular reactive oxygen species (ROS) production was assessed with flow cytometry using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as a probe (Hasui et al., 1989). Briefly, 2×10^5 viable milk cells from each quarter, which were previously assessed using trypan blue exclusion were incubated at 37 ºC for 30 min with 0.3 µM DCFH-DA (D6883, Sigma Aldrich, St. Louis, MO, USA).
The intracellular 2',7'-dichlorofluorescein (DCF) fluorescence of the neutrophils was determined with flow cytometry using an excitation wavelength of 488 nm. DCFH-DA, which is a cell-permeable, nonfluorescent probe, is converted to DCF by ROS in a dose-dependent manner, resulting in fluorescence emission. The green fluorescence of DCF was detected at 500-530 nm.
The percentage of neutrophils producing ROS was calculated as the number of fluorescent neutrophils divided by the total neutrophil count and multiplied by 100. The median fluorescence intensity (MFI) of ROS production was estimated from the median of DCF fluorescence divided by the number of neutrophils that produced ROS (Blagitz et al., 2013; Della Libera et al., 2014). For this assay, 10,000 gated neutrophils were examined per sample. FlowJo software (TreeStar Inc., Ashland, USA) was used to analyze the data. The results were corrected for autofluorescence content, which was defined as the fluorescence that was associated with non-labeled freshly isolated milk cells from the same cow.

Preparation of PI-labeled bacteria

PI-labeled *Staphylococcus aureus* (ATCC 25923) was prepared as described by Hasui et al. (1989) with some modifications for detection of phagocytosis of *S. aureus* by milk neutrophils. Briefly, *S. aureus* was cultured for 18 h at 37 °C on brain-heart infusion agar. Subsequently, the bacteria were heat-killed using incubation at 60 °C for 30 min, after which they were washed three times using a sterile saline solution (0.9 % NaCl). The bacterial density was adjusted to an absorbance of 2.50 at 620 nm, yielding approximately 2.4×10^9 bacteria/mL, as previously described (Hasui et al., 1989). The bacteria were then labeled using a 5 % PI (P4170, Sigma

Aldrich, St. Louis, MO, USA) solution for 30 min on ice. The fluorescent bacteria were washed three times and suspended in PBS containing 5 mM glucose and 0.1 % gelatin, and aliquots were stored at -80 °C. Thereafter, the PI-labeling of the bacteria was confirmed using flow cytometry.

Phagocytosis assay

The phagocytosis assay was performed using flow cytometry of PI-labeled *S. aureus* Briefly, 2 × 10^5 viable milk cells were incubated with 100 µL of heat-killed, PI-labeled *S. aureus* and 900 µL of PBS for 30 min at 37 °C. Subsequently, 2 mL of 3 mM EDTA was added, and after centrifugation at 400 x g for 10 min, the leukocytes were resuspended in 300 µL of PBS and analyzed using flow cytometry.
The percentage of neutrophils that phagocytized the PI labeled bacteria was equal to the number of fluorescent neutrophils divided by the total neutrophil count and multiplied by 100. The MFI of *S. aureus* phagocytosis was estimated from the median value of PI fluorescence divided by the number of neutrophils that phagocytized *S. aureus* (Blagitz et al., 2013; Della Libera et al., 2014). At least 20,000 cells were examined per sample.

Expression of L-selectin, β_2-integrin and CD44

The identification of neutrophils expressing L-selectin (CD62L), the β-chain of β_2-integrin (CD11b) and one of the three endothelial-selectin ligands (CD44) was performed with flow cytometry using the following mAbs (Table 1). Unlabeled primary mAbs that were directed against CH138A, CD11b and CD44 were added to the cell suspension and incubated for 30 min on ice. The isolated milk cell suspension was centrifuged at 400 x g for 8 min, and a labeled CD62L mAb and secondary labeled mAbs for the detection of the anti-CH138A, anti-CD11b and -CD44 Abs were added. Finally, the isolated milk cells were incubated for 30 min on ice in the dark to allow for the visualization of cells expressing CD62L, CD11b and CD44. First, dot plots of gated neutrophils ($CH138A^+$) were generated as previously described. We chose the relative MFI for analysis of adhesion molecules because this parameter was much more discriminating compared with the percentage of positive cells. The MFI provides an accurate measurement of the brightness of the stained cells and is thus an indicator of the number of receptors per cell (Diez-Fraile et al., 2003). For this assay, 10,000 gated neutrophils were examined per sample.

Statistical analyses

First, the distributions of all of the variables were examined using normal probability plots obtained using the Shapiro and Wilk tests. The data were analyzed using a multivariate analysis of variance. Then, the Kruskal-Wallis and Mann-Whitney tests were applied. The model considered the quarters and the cows to be nested within the cows as random effect. The statistical analyses were performed using STATA statistical software version 12 (Stata Corp., College Station, Texas, USA). The results are reported as the mean ± standard error. Significance was set at $P \leq 0.05$.

RESULTS

The results are summarized in Tables 2 and 3. The SCC (Table 2), lactational status and parity values did not significantly differ between the groups (Table 2). Regarding the infected quarters in G2 (n = 6), in two quarter milk samples were isolated *Corynebacterium bovis*, and four milk

quarters samples had unspecific mastitis on the day of investigation (SCC > 2×10^5 cells/mL). Two out of these quarters with unspecific mastitis were affected by clinical mastitis.
We found that uninfected quarters from infected udders had high levels of CD11b expression by milk neutrophils in contrast to high levels of CD62L and CD44 expression by milk neutrophils from uninfected quarters of uninfected udders. No significant difference was observed in the percentage of milk neutrophils ($CH138^+$), neutrophil viability (Annexin V^-/PI^-) and apoptosis (Annexin V^+/PI^-), intracellular ROS production or *S. aureus* phagocytosis (Table 2).
The lymphocyte subsets did not differ between the groups, with the exception that the percentage of B cells was higher in uninfected quarters from infected udders than uninfected quarters from uninfected udders (Table 3).

DISCUSSION

We investigate the milk neutrophil function and lymphocyte profile in uninfected quarters from uninfected and infected udders. Our data indicate that the immune response in the udder quarters does not seem to act independently, but is influenced by IMIs of neighbouring quarters, although they appear to act as independent units regarding the anatomical structure of the mammary gland.
In the present study, the infected quarter did not influence the SCC, percentage of neutrophils, neutrophil viability, intracellular ROS production or phagocytosis of *S. aureus* by milk neutrophils in the neighboring uninfected quarters. It is well-know that apoptosis of bovine neutrophils implies impaired phagocytic and oxidative burst activities (Van Oostveldt, 2002; Mehrzad et al., 2005). Thus, the non-significant difference that was observed in neutrophil viability rates between the groups in this study may be related to the results for the percentage of neutrophils that produced ROS or phagocytosed *S. aureus*, as neutrophil viability is closely related to neutrophil phagocytosis and oxidative burst activities (Mehrzad et al., 2004; Mehrzad et al., 2005).
Furthermore, the inclusion of milk samples with low threshold for SCC ($\leq 1 \times 10^5$ cells/mL) may lead to non-significant difference in SCC and the percentage of neutrophils. However, there is no consensus regarding whether the SCC and percentage of neutrophils are affected in the neighboring quarters during infection (Wever and Emanuelson, 1989; Merle et al., 2007; Schwarz et al., 2011; Jensen et al., 2013). Another fact that should be considered is a compensatory change in milk production between quarters within an udder (Hamann and Reichmuth, 1990) that may lead to higher milk production in the uninfected quarter as milk yield decreases in the infected quarter. Thus, we hypothesized that this effect may also contribute to the non-significant difference in SCC observed here, as milk yield increases in the uninfected quarters may have diluted SCC (Green et al., 2006).
A major factor that impacts the signals being detected by neighboring quarters (Jensen et al., 2013) may include the extent of the inflammation, which is related to the pathogenicity of the mastitis-causing bacteria, the amount of affected tissue (Pyörälä, 2003; Merle et al., 2007; Jensen et al., 2013) and the individual cattle immune response (Burvenich et al., 2003; Petlz et al., 2008) which altogether may lead to possible loosing statistical significance here. For instance, Merle et al. (2007) showed that the neutrophil viability from the uninfected quarters of uninfected udders was lower than that of uninfected quarters from cows with severe mastitis, but no significant difference was observed when they compared uninfected quarters from uninfected udders with uninfected quarters from cows with moderate mastitis.
Although, it is agreed that the immune response against an invading pathogen differs significantly depending on the bacterial species (Schukken et al., 2011; Souza et al., 2012), the percentage of neutrophils did not differ between uninfected quarters from udders with different infection statuses in the current study, although the expression of the adhesion molecules

CD11b, CD62L, and CD44 by milk neutrophils was significantly different. The expression of these molecules is induced by the inflammatory response, and they are involved in the mobilization of neutrophils to the site of infection (Paape et al., 2003; Gonen et al., 2008). Thus, we hypothesized that the expression of these adhesion molecules by milk neutrophils may more accurately detect the influence of IMI on the neighboring uninfected quarters than SCC or neutrophil percentages, as a slight increase in SCC or the proportion of neutrophils may not be easily detected because they can be influenced by milk yield dilution and the concomitant augmentation of other leukocytes populations, respectively.

Although the study size is limited, our data support the hypothesis of the interdependence of quarters based on the influence of the udder infection on the percentage of B cells in uninfected quarters. Here, a higher percentage of $CD21^+$ cells was found in infected quarters (15.92 $\pm$ 1.84%; n = 6), as previously reported by Schwarz et al. (2013) and Riollet et al. (2001), which may explain the increased percentage of B cells in the neighboring quarters that we encountered here. This finding may be related to the local lymphocyte proliferation (Concha et al., 1986) and the development of a humoral response (Riollet et al., 2001). Higher percentage of T cells ($CD3^+$) was observed in infected quarters here (13.64 $\pm$ 5.35%; n = 6), as previously described by Rivas et al. (2002) after challenge with *S. aureus*. Nevertheless, it did not have a strong influence on the neighboring uninfected quarters, as no significant difference in the percentage of T cells or T lymphocyte subsets was found in uninfected quarters from udders with different infection statuses.

It is known that $CD21^+$ cells are increased in milk samples with high SCC, whereas the proportion of $CD2^+$ and $CD3^+$ cells are decreased (Rivas et al., 2007; Schwarz et al., 2013). The $CD2^+/CD21^+$ index has recently been proposed as a new marker for the quick differentiation of normal versus diseased udder quarters (Schwarz et al., 2013). Regarding our findings, the increase in the proportion of $CD21^+$ cells in uninfected quarters from infected udders may affect the predictive value of the $CD2^+/CD21^+$ index for diagnosing IMIs and therefore requires further investigation.

CONCLUSION

Our study strongly supports the hypothesis of the interdependence of quarters based on the influence of infections on both the percentage of B cells and the expression of adhesion molecules (CD11b, CD44 and CD62L) by milk neutrophils in neighboring uninfected quarters. Thus, future studies are required to assess the influence of infected quarters in neighboring uninfected quarters on the predictive values of some indicators of inflammation used in the diagnosis of mastitis, and also on the longitudinal influence of the infection by different pathogens on the immune response of neighboring uninfected quarters.

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Table 1: Monoclonal antibodies used for differentiation and labeling of bovine milk leukocytes applying flow cytometry

Description	Primary antibody							Secundary antibody						
	Name	Type	Amount	Specificity	Host	Company	Isotype	Name	Type	Amount	Specificity	Host	Company	Isotype
Polymorphonuclear	CH138A	CH138A	1µL	Bovine	Mouse	VMRD[1]	IgM	M31504	IgM – PE	1 µL	Mouse	Goat	Invitrogen[2]	IgM
								M31505	IgM – APC	2 µL	Mouse	Goat	Invitrogen[2]	IgM
								M31501	IgM – FITC	1 µL	Mouse	Goat	Invitrogen[2]	IgM
One of the endothelial-selectin ligands	BAG40A	CD44	1µL	Bovine	Mouse	VMRD[1]	IgG3	M32701	IgG3 – FITC	2 µL	Mouse	Goat	Invitrogen[2]	IgG3
β_2-integrin	MM12A	CD11b	1µL	Bovine	Mouse	VMRD[1]	IgG1	M32018	IgG1 – PE-Cy5	1 µL	Mouse	Goat	Invitrogen[2]	IgG1
T Lymphocyte	MM1A	CD3	1µL	Bovine	Mouse	VMRD[1]	IgG1	M32018	IgG1 – PE-Cy5	1 µL	Mouse	Goat	Invitrogen[2]	IgG1
CD4 T Lymphocyte	ILA11	CD4	1µL	Bovine	Mouse	VMRD[1]	IgG2a	M32204	IgG2a – PE	1 µL	Mouse	Goat	Invitrogen[2]	IgG2a
CD8 T Lymphocyte	BAQ111A	CD8	1µL	Bovine	Mouse	VMRD[1]	IgM	M31501	IgM – FITC	1 µL	Mouse	Goat	Invitrogen[2]	IgM
α-chain of IL-2 receptor	LCTB2A	CD25	1µL	Bovine	Mouse	VMRD[1]	IgG3	M32701	IgG3 – FITC	2 µL	Mouse	Goat	Invitrogen[2]	IgG3
B Lymphocyte	BAQ15A	CD21	1µL	Bovine	Mouse	VMRD[1]	IgM	M31501	IgM – FITC	1 µL	Mouse	Goat	Invitrogen[2]	IgM
L-selectin	MCA1649F	CD62L With FITC[3] marker	2µL	Mouse	Bovine	AbDSerotec[3]								

PE:R-Phycoerythrin; APC: Allophycocyanin; FITC: fluorescein isothiocyanate; PE-Cy5: Phycoerythrin cyanine 5; VMRD: VMRD Pullman Inc. [1]VMRD Pullman Inc. Corp®, Pullman, WA, USA; [2]Invitrogen, Carlsbad, CA, USA; [3]AbD Serotec, Oxford, England

Table 2: parity, SCC and function of milk neutrophils from uninfected quarters of uninfected udders and uninfected quarters of infected udders.

Group/Variable	Uninfected quarters from uninfected udders (n = 32)	Uninfected quarters from infected udders (n = 18)
Lactational status (days in milk)	133.38 ± 22.84[a]	188.17 ± 31.31[a]
Parity	2.50 ± 0.31[a]	1.67 ± 0.11[a]
SCC (cells/mL)	31,313 ± 6,225[a]	24,111 ± 7,758[a]
$CH138^+$ (%)	11.52 ± 2.70[a]	6.67 ± 1.88[a]
Annexin V^-/PI^- (%)	31.41 ± 2.57[a]	27.42 ± 3.90[a]
Annexin V^+/PI^- (%)	43.43 ± 3.34[a]	40.33 ± 3.96[a]
ROS production (%)	59.39 ± 3.52[a]	61.89 ± 5.07[a]
Intensity of ROS production (MFI)	1,840 ± 179.4[a]	1,313 ± 141.8[a]
***S. aureus* phagocytosis (%)**	60.67 ± 3.33[a]	56.28 ± 4.39[a]
Intensity of *S. aureus* phagocytosis (MFI)	201.4 ± 19.97[a]	176.82 ± 13.82[a]
CD44 expression (MFI)	11.21 ± 3.23[a]	1.03 ± 0.02[b]
CD62L expression (MFI)	9.32 ± 2.54[a]	1.01 ± 0.00[b]
CD11b expression (MFI)	597.2 ± 63.94[b]	1,034 ± 96.18[a]

Different superscripted letters within a row indicate significant differences ($P \leq 0.05$) between the values. The results are shown as the mean ± SE.

SCC: somatic cell count; PI: propidium iodide; *S. aureus*: *Staphylococcus aureus*; ROS: reactive oxygen species; MFI: median fluorescence intensity; CD62L: L-selectin; CD11b: β-chain of β2-integrin; CD44: one of the three endothelial-selectin ligands.

Table 3. Percentage of milk lymphocyte subpopulations from uninfected quarters of uninfected udders and uninfected quarters of infected udders.

Group/Variables	Uninfected quarters from uninfected udders (n = 32)	Uninfected quarters from infected udders (n = 18)
$CD3^+$ (T cells) (%)	9.05 ± 1.10^a	8.65 ± 1.34^a
$CD4^+/CD8^-$ T cells (%)	1.57 ± 0.26^a	1.72 ± 0.54^a
$CD4^-/CD8^+$ T cells (%)	3.70 ± 0.42^a	3.66 ± 0.85^a
$CD3^+$ (T cells) (%)	8.21 ± 0.99^a	8.91 ± 1.47^a
$CD4^+/CD25^-$ T cells (%)	1.66 ± 0.26^a	1.80 ± 0.56^a
$CD4^+/CD25^+$ T cells (%)	0.26 ± 0.05^a	0.15 ± 0.03^a
$CD4^-/CD25^-$ T cells (%)	6.18 ± 0.78^a	6.85 ± 1.04^a
$CD21^+$(B cells) (%)	10.47 ± 1.45^b	12.26 ± 1.07^a

Different superscripted letters within a row indicate significant differences ($P \leq 0.05$) between the values. The results are shown as the mean ± SE.

6 Chapter IV. The neutrophil function and lymphocyte profile of milk from bovine mammary glands infected with *Streptococcus dysgalactiae*

SUMMARY

Streptococcus dysgalactiae is a bacterium that accounts for a notable proportion of both clinical and subclinical intramammary infections (IMIs). Thus, the present study explores the function of milk neutrophils and the lymphocyte profile in mammary glands naturally infected with *Streptococcus dysgalactiae*. Here, we used 32 culture-negative control quarters from eight clinically healthy dairy cows with low somatic cell counts and 13 *S. dysgalactiae*-infected quarters from six dairy cows. Using flow cytometry, we evaluated the percentage of milk monocytes/macrophages and neutrophils, expression of CD62L, CD11b and CD44 by milk neutrophils, the levels of intracellular reactive oxygen species (ROS) production and phagocytosis of *Staphylococcus aureus* by milk neutrophils, and neutrophil viability. Furthermore, the percentages of B cell ($CD21^+$) and T lymphocyte subsets ($CD3^+/CD4^+/CD8^-$; $CD3^+/CD8^+/CD4^-$; and $CD3^+/CD8^-/CD4^-$), and the expression of CD25 by T milk lymphocytes ($CD3^+$) and T $CD4^+$ milk cells were also assessed by flow cytometry using monoclonal antibodies. The present study showed a higher SCC and percentage of milk neutrophils, and a decrease in the percentage of milk monocytes/macrophages from *S. dysgalactiae*-infected quarters when compared to uninfected ones. We also observed a higher expression of CD11b by milk neutrophils and a tendency toward a decrease in neutrophil apoptosis rate in *S. dysgalactiae*-infected quarters. In addition, the *S. dysgalactiae*-infected quarters had higher percentages of milk T cells ($CD3^+$) and their subset $CD3^+CD8^+CD4^-$ cells. Overall, the present study provided new insights into *S. dysgalactiae* IMIs, including distinct lymphocyte profiles, and a tendency toward an inhibition of apoptosis in milk neutrophils.

Key words: immune response, mastitis, somatic cell count, dairy cow

Mastitis is a disease of major economic importance, causing reduced milk production and quality and increasing the use of veterinary drugs worldwide. Several bacterial genera and species are capable of causing mastitis, and one of the most common groups of isolated bacteria from intramammary infections (IMIs) is *Streptococcus* spp., which includes *Streptococcus dysgalactiae* (Osteras *et al*. 2006; Whist *et al*. 2007; Souza *et al*. 2009; Zadoks & Fitzpatrick, 2009; Botrel et al., 2010; Schwarz et al., 2010; Beecher *et al*. 2012; Abrahmsén et al., 2014; Leelahapongsathon et al., 2014). For instance, Botrel et al. (2010) described that *S. dysgalactiae* was isolated in 8.8% and 9.4% of the milk samples from clinical and subclinical cases of mastitis, respectively.

Mastitis organisms are categorized as contagious or environmental pathogens based on their distinct characteristics of distribution and interaction with the teat and teat duct. Environmental pathogens are those whose primary reservoir is the environment rather than infected mammary glands. The most frequently isolated environmental pathogens are coliform bacteria and streptococci other than *S. agalactiae*. Among the environmental streptococci, *S. uberis* and *S. dysgalactiae* are the most prevalent (Calvinho *et al*. 1998; Osteras et al., 2006; Whist et al., 2007 Zadoks & Fitzpatrick, 2009; Schwarz et al., 2010; Leelahapongsathon et al., 2014), although these bacteria have been found to be contagious in some dairy herds (Whist *et al*. 2007).

In the last few decades, with the improvement of mastitis control programs leading to herds with lower somatic cell counts (SCCs), environmental mastitis has become a major problem in many well-managed dairy herds that have successfully controlled contagious pathogens (Schukken *et al*. 1989; Calvinho *et al*. 1998; Green *et al*. 2004). In these herds, *S. dysgalactiae* accounts for a significant number of subclinical and clinical cases of IMIs (Calvinho *et al*. 1998; Zadoks & Fitzpatrick, 2009). Furthermore, mastitis control programs have had minimal effects on reducing the incidence of IMIs caused by *S. dysgalactiae* (Bolton *et al*. 2004; Lundberg *et al*. 2014). Despite improvements in management practices, *S. dysgalactiae* mastitis has remained a problem in many dairy herds. However, few studies have investigated the immune response against *S. dysgalactiae* (Calvinho *et al*. 1998; Song *et al*. 2001; Wyder *et al*. 2010; Beecher *et al*. 2012) and its effects on udder health.

Striving for a complete elimination of antimicrobial use in dairy cattle is unrealistic, although a reduction in antimicrobial use is recommended and feasible. From that point of view, one most practical means for dealing with mastitis may be to enhance the natural ability of animals to resist infection. The greatest obstacle in establishing this type of strategy is a lack of understanding of many aspects of the host immune response. Currently, the roles of various immune system components in the defense of the mammary gland against infection by several pathogens are not well understood. Altogether, it is clearly that a more comprehensive understanding of the factors of the immune response is essential to the development of effective prevention strategies of mastitis (Soltys and Quinn, 1999).

The present study aimed to investigate the function of milk neutrophils and the milk lymphocyte profile in *S. dysgalactiae*-infected mammary glands.

Materials and Methods

Animals and experimental design

The present study utilized 45 quarters from 14 Holstein dairy cows, which were collected at various stages of lactation from a commercial herd. From this sample, 32 culture-negative quarters from eight dairy cows exhibiting no abnormal secretions in the strip cup test and a milk somatic cell count (MSCC) lower than 2×10^5 cells/mL, which is the MSCC threshold proposed

by Schepers et al. (1997) and Schukken et al. (2003) for uninfected quarters, were used (control group). Additionally, 13 *S. dysgalactiae*-infected quarters from six dairy cows were included. The parity and days in milk of all animals were also recorded.
Regarding the criteria used here, Dohoo et al. (2011) assessed the sensitivity (SE) and specificity (SP) of a single milk quarter sample and described that milk samples with SCC $\geq$ 200,000 cells/mL and with at least two pure colonies of *S. dysgalactiae* or *S. uberis* have a SE and SP of 66.4% and 100%, respectively. In addition, these authors reported that 89% of all *Streptococcus* spp. isolates in the weekly data had more than 1,000 colony forming per unit/mL in culture, suggesting that these infections shed a large number of microorganisms.

Sample collection

First, the strip cup test was performed to identify the presence of clots, flakes or otherwise obviously abnormal secretions. Then, the pre-dipping was performed, and one towel was used for each teat. After discarding the first three milk streams, teat ends were scrubbed with cotton containing 70% ethanol, and single milk samples from individual mammary quarters were aseptically collected into sterile vials for bacteriological analysis (approximately 3 mL). Finally, milk samples for the SCC (approximately 40 mL) and evaluation of neutrophilic function and milk lymphocyte profile (approximately 1 L) were collected. Samples were kept at 4 °C for about three hours until arriving at the laboratory. Milk samples for bacteriological analysis were cooled at – 20 °C until the analysis.

Bacteriological analysis

The bacteriological analysis was performed by culturing 0.01 mL of each single milk-quarter sample on 5% bovine blood agar plates (Bio Express, São Paulo, Brazil). The plates were incubated for 72 hours at 37 ºC, and *S. dysgalactiae* subsp. *dysgalactiae* was identified as Gram-positive cocci, alpha-hemolytic, catalase negative, esculin negative and negative reaction in Christie-Atkins-Munch-Petersen (CAMP) (Rayme et al., 2013). A milk sample was considered culture-positive when the growth of $\geq$ 10 (10^3 colony forming per unit) pure *S. dysgalactiae* subsp. *dysgalactiae* colonies was detected. Samples yielding no bacterial growth were regarded as culture-negative.

Determination of the SCC

Milk samples for SCC were collected in 40 mL vials containing microtablets of bronopol (2-bromo-2-nitropane-1,3-diol) as a preserving agent. Subsequently, SCC measurements were performed using an automated, fluorescent, microscopic somatic cell counter (Somacount 300 – Bentley Instruments®, Chaska, USA).

Separation of milk cells

The separation of the milk cells was performed as described by Koess and Hamann (2008). Briefly, 1 L of milk was diluted with 1 L of phosphate-buffered saline (PBS; pH 7.4; 1.06 mM Na_2HPO_4, 155.17 mM NaCl and 2.97 mM $Na_2HPO_4.7H_2O$). After centrifugation at 1,000 x g for 15 min, the cream layer and supernatant were discarded. The cell pellet was then washed once using 30 mL of PBS and centrifuged at 400 x g for 10 min. The cells were resuspended in 1 mL of RPMI-1640 nutritional medium (cat. n. R7638, Sigma Aldrich, USA) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil) and counted using a Neubauer

chamber. Cell viability was evaluated using trypan blue exclusion (Jain and Jasper, 1967). The milk cells were then diluted with nutritional medium containing 10% fetal bovine serum to a concentration of 2×10^6 viable cells/mL.

Enumeration of lymphocyte subpopulations

The cells were washed with PBS and incubated with the primary monoclonal antibodies (mAbs) for 30 min on ice to detect CD21, and the combination of CD3, CD4 and CD8. The identification of lymphocyte subsets was based on their cytoplasmic granularities and mean fluorescence intensities following a two-step fluorescence immunolabeling protocol using primary anti-bovine mAbs and secondary mAbs coupled to long-wavelength fluorescent probes (Table 1). Next, 1 mL of PBS was added to the cell suspension and centrifuged at 400 x g for 8 min. Finally, the labelled secondary mAb was added to the cell suspension, and the cells were incubated for 30 min on ice with the secondary mAbs. The cells were then washed with PBS and immediately analyzed using flow cytometry. A total of 20,000 milk cells, excluding the cellular debris, were examined per sample. FlowJo software (TreeStar Inc., Ashland, OR, USA) was used to analyze the data. The results were corrected for autofluorescence, which was defined as the fluorescence that was associated with non-labeled freshly isolated milk cells from the same cow (Della Libera et al., 2015).

Expression of CD25

A crucial event in the initiation of an immune response is the activation of T cells, which requires IL-2 binding to its high-affinity IL-2 receptor for optimal signaling. The IL-2 receptor α-chain (CD25) is needed for the high affinity binding of IL-2 to effector cells and is potently induced after T cell activation (Waters et al., 2003; Maslanka et al., 2014). Thus, here we evaluated the expression of CD25 in bovine T cells ($CD3^+$) and T $CD4^+$ lymphocytes by flow cytometry using the mAbs listed in Table 1. Firstly, unlabeled primary mAbs that were directed against CD3, CD4 and CD25 were added to the cell suspension and incubated for 30 min on ice. The isolated milk cell suspension was centrifuged at 400 x g for 8 min, and the labeled secondary mAbs were added. Finally, the isolated milk cells were incubated for 30 min on ice in the dark to allow the visualization of cells expressing CD3, CD4 and CD25. Expression of CD25 was determined for gated T cells ($CD3^+$) and T $CD4^+$ lymphocytes ($CD3^+$ $CD4^+$) populations. Data are presented as median fluorescence intensity (MFI). For this assay, 10,000 gated T cells were examined per sample. FlowJo Tree Star Software (TreeStar Inc., Ashland, OR, USA) was used to analyze the data.

Identification of neutrophils

Milk neutrophils were differentiated from other cells by indirect fluorescence labeling. The cells were incubated with an unlabeled primary anti-bovine granulocyte mAbs (Table 1) for 30 min on ice. Next, 1 mL of PBS was added to the cell suspension, which was centrifuged at 400 x g for 8 min. Finally, a labeled secondary mAb (Table 1) was added, and the sample was incubated for 30 min on ice in the dark to visualize the bound of CH138A. The neutrophils were identified using flow cytometry based on the cells' cytoplasmic granularities and CH138A positivity as previously described by Piepers et al. (2009) and Blagitz et al. (2013) (Fig. 1). A total of 20,000 milk cells, excluding cellular debris, were examined per sample. FlowJo software (TreeStar Inc., Ashland, OR, USA) was used to analyze the data. The results were corrected for autofluorescence, which was defined as the fluorescence that was associated with non-labeled freshly isolated milk cells from the same cow.

Identification of macrophages/monocytes

The CD14 has long be used to identify macrophages/monocytes, however milk neutrophils can also express CD14 (Paape et al. 1996; Sladek et al. 2002). Thus, the macrophages/monocytes were identified using flow cytometry based on CD14 positivity and CH138 negativity ($CD14^+/CH138^-$). The cells were washed with PBS and incubated with an unlabeled primary anti-bovine CH138A and CD14 mAbs (Table 1) for 30 min on ice. Next, 1 mL of PBS was added to the cell suspension, which was centrifuged at 400 x g for 8 min. Finally, a labeled secondary mAbs (Table 1) was added, and the sample was incubated for 30 min on ice in the dark to visualize the bound of CD14 and CH138A. A total of 20,000 milk cells, excluding cellular debris, were examined per sample. FlowJo software (TreeStar Inc., Ashland, OR, USA) was used to analyze the data. The results were corrected for autofluorescence, which was defined as the fluorescence that was associated with non-labeled freshly isolated milk cells from the same cow.

Detection of apoptosis using flow cytometry

The death of neutrophils ($CH138^+$) was assessed using dual-labeling with an annexin V antibody and propidium iodide (PI) using a commercial kit (cat. n. K2350, APOPTEST-FITC™, Dako Cytomation, Mijdrecht, The Netherlands). Flow cytometric analysis was performed as previously described (Piepers et al., 2009; Blagitz et al., 2013). Briefly, 2×10^5 viable milk cells were suspended in 100 µL of binding buffer (10 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) containing an anti-annexin V-FITC antibody and incubated on ice for 20 min in the dark. Immediately before flow cytometric analysis, 5 µL of a 250 µg/mL PI solution was added (final concentration 2.5 µg/mL). Next, the neutrophils were labeled using mAbs as described above.

To analyze the data, scatterplots were generated for the gated neutrophils. The living, non-apoptotic cells were negative for both FITC-labeled anti-annexin V and PI. The cells that were positive for FITC-labeled anti-annexin V but negative for PI were classified as apoptotic cells. The necrotic and late apoptotic cells were positive for both FITC-labeled anti-annexin V and PI (Piepers et al., 2009; Blagitz et al., 2013). A total of 20,000 milk cells, excluding cellular debris, was examined per sample. FlowJo software (TreeStar Inc., Ashland, OR, USA) was used to analyze the data.

Intracellular reactive oxygen species production

Intracellular reactive oxygen species (ROS) production was assessed with flow cytometry using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as a probe (Hasui et al., 1989). Briefly, 2×10^5 viable milk cells from each quarter, which were previously assessed using trypan blue exclusion, were incubated at 37 ºC for 30 min with 0.3 µM DCFH-DA (D6883, Sigma Aldrich, St. Louis, MO, USA).

The intracellular 2',7'-dichlorofluorescein (DCF) fluorescence of the neutrophils ($CH138^+$) was determined with flow cytometry using an excitation wavelength of 488 nm. DCFH-DA, which is a cell-permeable, nonfluorescent probe, is converted to DCF by ROS in a dose-dependent manner, resulting in fluorescence emission. The green fluorescence of DCF was detected at 500-530 nm.

The percentage of neutrophils producing ROS was calculated as the number of fluorescent neutrophils divided by the total neutrophil count and multiplied by 100. The MFI of ROS

production was estimated from the median of DCF fluorescence (Della Libera et al., 2015; Blagitz et al., 2015). For this assay, 10,000 gated neutrophils were examined per sample. FlowJo software (TreeStar Inc., Ashland, USA) was used to analyze the data. The results were corrected for autofluorescence, which was defined as the fluorescence that was associated with non-labeled freshly isolated milk cells from the same cow.

Preparation of PI-labeled bacteria

PI-labeled *Staphylococcus aureus* (ATCC 25923) was prepared as described by Hasui et al. (1989) with some modifications for the detection of phagocytosis of *S. aureus* by milk neutrophils. Briefly, *S. aureus* was cultured for 18 h at 37 °C on brain-heart infusion agar. Subsequently, the bacteria were heat-killed using incubation at 60 °C for 30 min, after which they were washed three times using a sterile saline solution (0.9% NaCl). The bacterial density was adjusted to an absorbance of 2.50 at 620 nm, yielding approximately 2.4×10^9 bacteria/mL, as previously described (Hasui et al., 1989). The bacteria were then labeled using a 5% PI (P4170, Sigma Aldrich, St. Louis, MO, USA) solution for 30 min on ice. The fluorescent bacteria were washed three times and suspended in PBS containing 5 mM glucose and 0.1% gelatin, and aliquots were stored at -80 °C. Thereafter, the PI-labeling of the bacteria was confirmed using flow cytometry.

Phagocytosis assay

The phagocytosis assay was performed using flow cytometry of PI-labeled *S. aureus.* Briefly, 2×10^5 viable milk cells were incubated with 100 µL of heat-killed, PI-labeled *S. aureus* and 900 µL of PBS for 30 min at 37 °C. Subsequently, 2 mL of 3 mM EDTA was added, and after centrifugation at 400 x g for 10 min, the leukocytes were resuspended in 300 µL of PBS and analyzed using flow cytometry.
The percentage of neutrophils ($CH138^+$) that phagocytized the PI-labeled bacteria was equal to the number of fluorescent neutrophils divided by the total neutrophil count and multiplied by 100. The MFI of *S. aureus* phagocytosis was estimated from the median value of PI fluorescence (Blagitz et al., 2013; Della Libera et al., 2015; Blagitz et al., 2015). At least 20,000 cells were examined per sample. FlowJo Tree Star Software (TreeStar Inc., Ashland, OR, USA) was used to analyze the data.

Expression of L-selectin, β_2-integrin and CD44

The identification of neutrophils expressing L-selectin (CD62L), the β-chain of β_2-integrin (CD11b) and one of the three endothelial-selectin ligands (CD44) was performed with flow cytometry using the mAbs listed in Table 1. Unlabeled primary mAbs that were directed against CH138A, CD11b and CD44 were added to the cell suspension and incubated for 30 min on ice. The isolated milk cell suspension was centrifuged at 400 x g for 8 min, and a CD62L mAb and secondary mAbs for the detection of the anti-CH138A, anti-CD11b and -CD44 mAbs were added. Finally, the isolated milk cells were incubated for 30 min on ice in the dark to allow the visualization of cells expressing CD62L, CD11b and CD44. Expression of CD11b, CD44 and CD62L was determined for gated neutrophils ($CH138A^+$). We chose the relative MFI for analysis of adhesion molecules because this parameter was much more discriminating compared with the percentage of positive cells. The MFI provides an accurate measurement of the brightness of the stained cells and is thus an indicator of the number of receptors per cell (Diez-

Fraile et al., 2003). For this assay, 10,000 gated neutrophils were examined per sample. FlowJo Tree Star Software (TreeStar Inc., Ashland, OR, USA) was used to analyze the data.

Statistical analyses

Descriptive statistics (mean ± standard error) were initially calculated for all variables. To account for the nested nature of the data (quarters and cows nested within the cows), multilevel analyses were conducted using the Generalized Linear Latent and Mixed Models (GLLAMM) procedure. Multilevel models are the most appropriate technique for analyzing nested data that are not independent of each other (Rabe-Hesketh and Skrondal, 2008). The potential moderating effect of number of days in milk and parity on observed associations was estimated in the model by including appropriate interactions terms. A two-tailed probability level of 0.05 was adopted. The statistical analyses were performed using STATA statistical software version 12 (Stata Corp., College Station, Texas, USA).

Results

The logarithmic SCC and the proportion of neutrophils ($CH138^+$, Fig 1) were higher in the quarters that were infected with *S. dysgalactiae* compared with the mammary glands that were free from infection (Table 2). On the other hand, the macrophages/monocytes were the major cell type in milk from healthy quarters (Table 3). From the 13 *S. dysgalactiae*-infected quarters that were included in the present study, only two quarters were from clinical cases of mastitis (presence of abnormal secretion in the strip cup test). Furthermore, *S. dysgalactiae*-infected cows have a higher number of days in milk (222.77 ± 25.32) and parity (3.77 ± 0.48) than uninfected ones (133.38 ± 22.84, $P = 0.007$; 2.50 ± 0.31, $P = 0.001$).

The results of this study revealed a tendency toward a lower percentage of annexin-V^+/PI^- neutrophils (apoptotic neutrophils) from *S. dysgalactiae*-infected mammary glands compared with the uninfected glands (Table 2). No significant difference was observed in the viable (annexin-V^-/PI^-) milk neutrophils, necrotic or late apoptotic (annexin-V^+/PI^+) milk neutrophils, the percentage of milk neutrophils that produced intracellular ROS or phagocytized *S. aureus*, the MFI of intracellular ROS production, the MFI phagocytosis of *S. aureus*, and the expression of CD44 and CD62L by the milk neutrophils (Table 2). By contrast, the expression of CD11b by milk neutrophils in the *S. dysgalactiae*-infected quarters was higher than uninfected quarters (Fig. 2; Table 2).

In addition, the *S. dysgalactiae*-infected quarters had higher percentages of milk T cells ($CD3^+$) (Fig 3; Table 3) and their subset $CD3^+CD8^+CD4^-$. The expression of CD25 by T cells ($CD3^+$) and $CD4^+$ T cells did not differ between groups. No significant difference was observed in the percentage of milk B cells (Table 3).

Regarding the statistical model used here, it was possible to determine an effect of the number of days in the following variables: percentage of annexin V^+/PI^+ milk neutrophils ($P = 0.038$), percentage of milk neutrophils that produced ROS ($P = 0.048$), MFI of ROS production by milk neutrophils ($P = 0.028$), percentage of milk neutrophils that phagocytized *S. aureus* ($P = 0.063$), MFI of phagocytosis of *S. aureus* by milk neutrophils ($P = 0.046$), expression of CD11b by milk neutrophils ($P = 0.015$), percentage of milk monocytes/macrophages ($P = 0.059$), and expression of CD25 by T ($P = 0.007$) and T $CD4^+$ milk cells ($P = 0.014$). Furthermore, an effect of parity on the following variables was observed: somatic cell count ($P = 0.066$), percentage of milk neutrophils ($P = 0.038$), percentage of annexin V^+/PI^+ milk neutrophils ($P = 0.0001$), percentage of milk monocytes/macrophages ($P = 0.013$), and percentage of T $CD4^+$ milk cells ($P = 0.007$).

Discussion

The mammary glands naturally infected with *S. dysgalactiae* had higher SCCs and neutrophil proportions in the present study, as previously described (Leitner et al., 2000; Djabri et al., 2002; Whist *et al*. 2007). This augmentation of milk SCC, mainly due to the increase of neutrophils might be at least partially due to the evocation of an eicosanoid response (prostaglandin (PG)E_2, $PGF_{2\alpha}$, and thromboxane B_2) that leads to the recruitment of neutrophils in naturally infected mammary glands (Bannerman, 2009).

During mastitis, inflammatory chemoattractants guide neutrophils toward the foci of infection. The influx of neutrophils from the blood to the milk in the mammary gland is important for the defense of the mammary gland against bacterial infection (Paape *et al*. 2003; Rainard & Riollet, 2006). Several receptors on neutrophils participate in the migration into milk, including L-selectin, E-selectin and β_2-integrins. L-selectin plays a role in the first step of egress from the blood flow, allowing the initial, migratory rolling on the endothelium, which is a prerequisite for endothelial-dependent neutrophil arrest. Therefore, the expression of L-selectin on bovine neutrophils that migrate into tissues diminishes after activation and diapedesis (Diez-Fraille *et al*. 2004). Since these cells already down-regulated L-selectin during migration into the udder, little or no further down-regulation or shedding of L-selectin was observed in milk leukocytes isolated from cows with mastitis (Solty and Quinn, 1999), as found here.

The β_2-integrins consist of three non-covalently linked heterodimers (CD11a,b,c/CD18), which are important cell surface adhesion molecules that are involved in neutrophil migration; the dominant β_2-integrin heterodimer that is expressed on bovine neutrophils is CD11b/CD18 (Smits *et al*. 2000; Paape *et al*. 2003; Rainard & Riollet, 2006). CD44 was identified as one of the three endothelial selectin (E-selectin) ligands on neutrophils responsible for slowing down and activating the rolling process. CD44 and two other E-selectin ligands (ESL-1 and PSGL-1) are required, but not essential, for neutrophil extravasation during inflammation (Gonen *et al*. 2008).

Here, the expression of CD62L and CD44 by milk neutrophils was not statistically significant. In that regard, Beecher *et al*. (2012) have demonstrated that the levels of IL-8 mRNA in *S. dysgalactiae* IMI, whose gene product enables leukocyte migration to the sites of infection, are not significantly different in milk somatic cells from uninfected control quarters versus infected quarters. Otherwise, a decreased transcription levels of the β_2-integrin gene *cd18* was observed in milk somatic cells from experimental *S. dysgalactiae*-infected quarters at 7 h after challenge, and no difference was observed in the levels of *cd18* gene expression over the course of the infection. On the other hand, Soltys and Quinn (1999) reported that lymphocytes and neutrophils obtained from the milk of cows with mastitis exhibited significant up-regulation of β_2-integrins (CD18), consistent with an activated state due to the presence of bacterial pathogens, in agreement with our findings regarding the β_2-integrins (CD11b). Nagahata *et al*. (2011) found that the expression of L-selectin and CD18 molecules on neutrophils is higher in the milk from cows with *S. aureus* mastitis, although the expression of L-selectin in the milk was decreased compared to that in the blood. The reasons for these differences in phenotypic features between studies is unclear, though it may be associated with the difference in the virulence factors of these bacteria or the severity, extent and stage of the IMIs (Schukken et al., 2011).

The persistent accumulation of inflammatory cells at the inflammatory site requires both a continuous neutrophil influx and the increased survival of extravasated neutrophils (Boutet *et al*. 2004). The present study revealed a tendency toward a lower percentage of apoptotic

neutrophils in the *S. dysgalactiae*-infected quarters; this finding confirms the results of Boutet *et al*. (2004), who described delayed neutrophil apoptosis in subclinical bovine mastitis.

It is noteworthy that neutrophils are the first line of defense against infection and that the active stage of immune defense requires viable, immune-competent cells (Baumert *et al*. 2009). In the present study, no significant difference in the viability rates was observed between the *S. dysgalactiae*-infected and uninfected quarters; moreover, there was no change in the percentage of neutrophils that produced intracellular ROS and phagocytized *S. aureus*.

Tassi et al. (2013), in an experimental infection by *S. uberis,* found that the proportion of $CD3^+$ lymphocytes was significantly elevated from 96 h post-challenge onwards, reaching its highest level at 312 h post-challenge. Regarding T cells subsets, these authors found that the proportion of $CD4^+$ and $CD8^+$ lymphocyte subsets increased 96 h post-challenge, with higher proportions of $CD4^+$ lymphocytes compared with $CD8^+$ lymphocytes present in the initial stages of infection. The proportion of $CD4^+$ cells stabilized after the initial increase, whereas the proportion of $CD8^+$ lymphocytes increased from 144 to 312 h post-challenge (the last sample collection). These data are in agreement with our findings, considering the higher proportion of milk T cells ($CD3^+$) and $CD8^+$ T lymphocytes in *S. dysgalactiae*-infected quarters found here. Furthermore, it appears that there is a selective recruitment of T-cell subsets to the udder during mastitis that depends on the pathogen (Soltys and Quinn, 1999).

The higher proportion of $CD8^+$ T lymphocytes found here may have implications to mammary gland. For instance, Park *et al*. (1993) demonstrated that experimentally infected cows with *S. aureus* have higher number of activated $CD8^+$ T cells which are responsible for suppressing the proliferative response of milk $CD4^+$ T cells. Besides this, the preferential trafficking of $CD8^+$ suppressor lymphocytes into mammary gland tissues and secretion may be responsible for the lower responsiveness of local leukocytes compared with those from peripheral blood (Sordillo *et al.* 1997). Altogether, these data indicated that activation of $CD8^+$ T-cells during certain bacterial IMIs can suppress important host immune responses and predispose to the chronic pattern of the IMIs (Alnakip *et al.* 2014).

In milk from healthy mammary glands, the results of the percentage of each cell population are widely variable using flow cytometry, and consequently no consensus exists. Dosogne et al. (2003) and Schwarz et al. (2011) regarded lymphocytes as the predominant cell population in healthy mammary glands. In contrast, Leitner et al. (2000) and Leitner et al. (2003) showed that epithelial cells are the main cell type in milk from uninfected mammary glands. On the other hand, macrophages were also pointed out as the major cell type in milk from healthy mammary glands using flow cytometry (Koess and Hamann, 2008) or microscopy (Lee et al., 1980; Miller et al., 1991; Sarikaya et al., 2004; Sarikaya et al., 2005; Merle et al., 2007), as found here.

Furthermore, the *S. dysgalactiae*-infected quarters used here came from cows with higher parity and days in milk than the uninfected cows, as previously described by Osteras et al. (2006).

Overall, the present study provided new insights into *S. dysgalactiae* IMIs, including distinct lymphocyte profiles, and a tendency toward an inhibition of apoptosis in milk neutrophils.

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Table 1. Monoclonal antibodies used for immunophenotyping bovine milk leukocytes by flow cytometry

Description	Primary antibody							Secondary antibody						
	Name	Type	Amount	Specificity	Host	Company	Isotype	Name	Type	Amount	Specificity	Host	Company	Isotype
Polymorphonuclear	CH138A	CH138A	1 µL	Bovine	Mouse	VMRD[1]	IgM	M31504 M31505	IgM – PE IgM – APC	1 µL 2 µL	Mouse Mouse	Goat Goat	Invitrogen[2] Invitrogen[2]	IgM IgM
Macrophages / Monocytes	MM61A	CD14	1 µL	Bovine	Mouse	VMRD[1]	IgG1	A10541	IgG1 - APC	1 µL	Mouse	Goat	Invitrogen[2]	IgG1
One of the endothelial-selectin ligands	BAG40A	CD44	1 µL	Bovine	Mouse	VMRD[1]	IgG3	M32701	IgG3 – FITC	2 µL	Mouse	Goat	Invitrogen[2]	IgG3
β_2-integrin	MM12A	CD11b	1 µL	Bovine	Mouse	VMRD[1]	IgG1	M32018	IgG1 – PE-Cy5	1 µL	Mouse	Goat	Invitrogen[2]	IgG1
T Lymphocyte	MM1A	CD3	1 µL	Bovine	Mouse	VMRD[1]	IgG1	M32018	IgG1 – PE-Cy5	1 µL	Mouse	Goat	Invitrogen[2]	IgG1
CD4 T Lymphocyte	ILA11	CD4	1 µL	Bovine	Mouse	VMRD[1]	IgG2a	M32204	IgG2a – PE	1 µL	Mouse	Goat	Invitrogen[2]	IgG2a
CD8 T Lymphocyte	BAQ111A	CD8	1 µL	Bovine	Mouse	VMRD[1]	IgM	M31501	IgM – FITC	1 µL	Mouse	Goat	Invitrogen[2]	IgM
α-chain of IL-2 receptor	LCTB2A	CD25	1 µL	Bovine	Mouse	VMRD[1]	IgG3	M32701	IgG3 – FITC	2 µL	Mouse	Goat	Invitrogen[2]	IgG3
B Lymphocyte	BAQ15A	CD21	1 µL	Bovine	Mouse	VMRD[1]	IgM	M31501	IgM – FITC	1 µL	Mouse	Goat	Invitrogen[2]	IgM
L-selectin	MCA1649F	CD62L With FITC[3] marker	2 µL	Mouse	Goat	AbD Serotec[3]								

PE:R-Phycoerythrin; APC: Allophycocyanin; FITC: fluorescein isothiocyanate; PE-Cy5: Phycoerythrin cyanine 5; [1]VMRD Pullman Inc. Corp®, Pullman, WA, USA; [2]Invitrogen, Carlsbad, CA, USA; [3]AbD Serotec, Oxford, England

Table 2. SCC and function of milk neutrophils from uninfected and *Streptococcus dysgalactiae* infected quarters.

Group/Variable	Uninfected quarters (n = 32)	*S. dysgalactiae* (n = 13)	*P* (value)
SCC (Log)	4.15 ± 0.12^{b}	6.22 ± 0.12^{a}	0.0001
$CH138^{+}$ (%)	11.52 ± 2.70^{b}	31.35 ± 4.50^{a}	0.004
Annexin V^{-}/PI^{-} (%)	30.41 ± 2.57^{a}	35.80 ± 7.45^{a}	0.18
Annexin V^{+}/PI^{-} (%)	43.43 ± 3.34^{a}	32.83 ± 2.70^{a}	0.07
Annexin V^{+}/PI^{+} (%)	21.00 ± 2.42^{a}	22.89 ± 7.12^{a}	0.70
ROS production (%)	59.39 ± 3.52^{a}	64.83 ± 8.06^{a}	0.93
Intensity of ROS production (MFI)	$1{,}840 \pm 179.4^{a}$	$1{,}484 \pm 308.2^{a}$	0.69
***S. aureus* phagocytosis (%)**	60.67 ± 3.33^{a}	62.47 ± 4.34^{a}	0.37
Intensity of *S. aureus* phagocytosis (MFI)	201.4 ± 19.97^{a}	113.7 ± 21.06^{a}	0.21
CD44 expression (MFI)	11.21 ± 3.23^{a}	7.37 ± 6.28^{a}	0.94
CD62L expression (MFI)	9.32 ± 2.54^{a}	1.01 ± 0.00^{a}	0.61
CD11b expression (MFI)	597.2 ± 63.94^{b}	846.7 ± 194.79^{a}	0.001

Different superscripted letters within a row indicate significant differences ($P \leq 0.05$) between the values. The results are shown as the mean ± SE.
SCC: somatic cell count; PI: propidium iodide; *S. aureus*: *Staphylococcus aureus*; ROS: reactive oxygen species; MFI: median fluorescence intensity.

Table 3. Percentage of milk macrophages/monocytes and lymphocyte subsets from uninfected and *Streptococcus dysgalactiae* infected quarters.

Group/Variables	Uninfected quarters (n = 32)	*S. dysgalactiae* (n = 13)	*P* (value)
$CD14^+/CH138^-$ (%)	65.72 ± 2.73	27.59 ± 4.49	0.0001
$CD3^+$ (T cells) (%)	9.05 ± 1.10[b]	17.34 ± 3.03[a]	0.006
$CD4^+/CD8^-$ T cells (%)	1.57 ± 0.26[a]	3.77 ± 1.25[a]	0.30
$CD4^-/CD8^+$ T cells (%)	3.70 ± 0.42[b]	6.98 ± 1.51[a]	0.0001
$CD4^-/CD8^-$ T cells (%)	3.44 ± 0.61[a]	6.16 ± 1.08[a]	0.31
$CD3^+$ (T cells) (%)	6.72 ± 0.75[b]	14.20 ± 2.75[a]	0.023
Expression CD25 by $CD3^+$ (T cells)	27.14 ± 5.57[a]	33.53 ± 6.68[a]	0.73
$CD4^+$ T cells (%)	1.97 ± 0.31[a]	4.14 ± 1.35[a]	0.43
Expression CD25 by $CD4^+$ T cells	70.27 ± 19.45[a]	52.71 ± 14.89[a]	0.12
$CD21^+$(B cells) (%)	10.47 ± 1.45[a]	16.18 ± 2.97[a]	0.71

Different superscripted letters within a row indicate significant differences ($P \leq 0.05$) between the values. The results are shown as the mean ± SE.

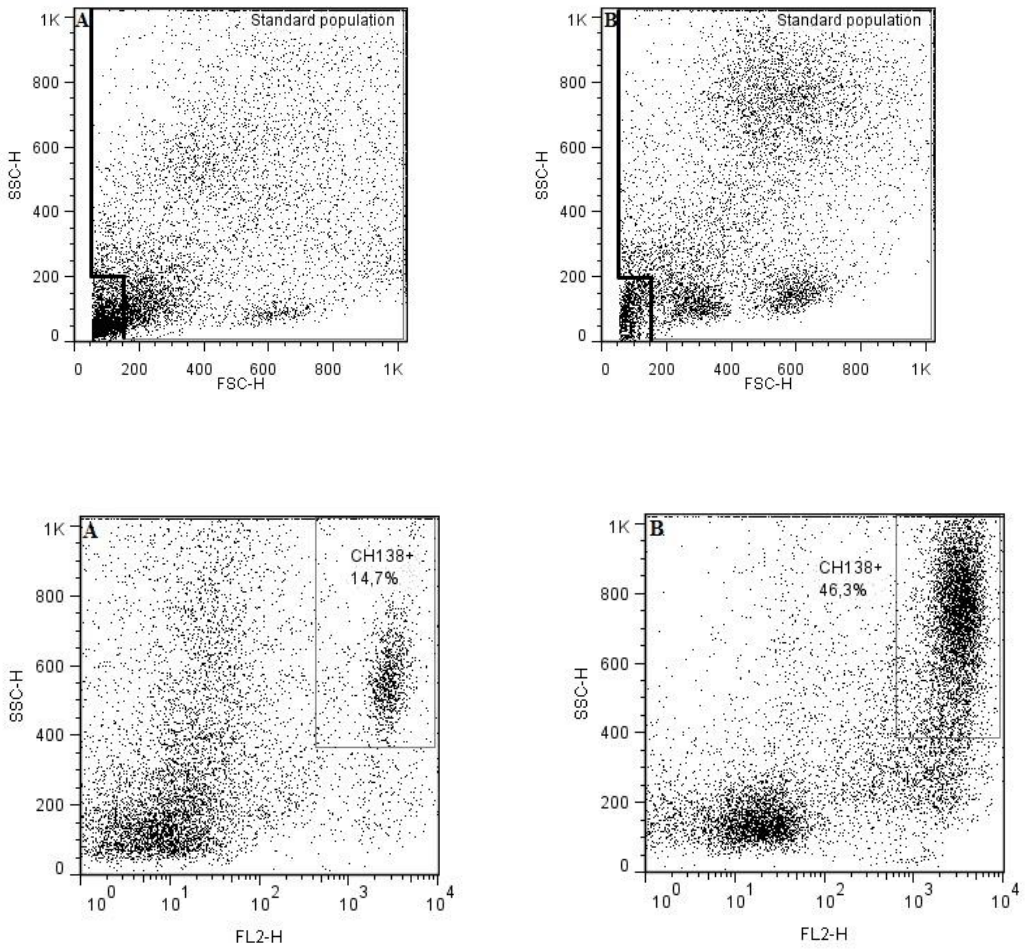

Figure 1 - Flow cytometric identification of the milk polymorphonuclear leukocytes (neutrophils) isolated from a representative uninfected (A) and *S. dysgalactiae*-infected (B) quarters. Recordings of scatter (SSC-H) and fluorescent properties (FL2-H; CH138A-Phycoerythrin) were performed on 20,000 events gated in a standard population excluding most of cell debris.

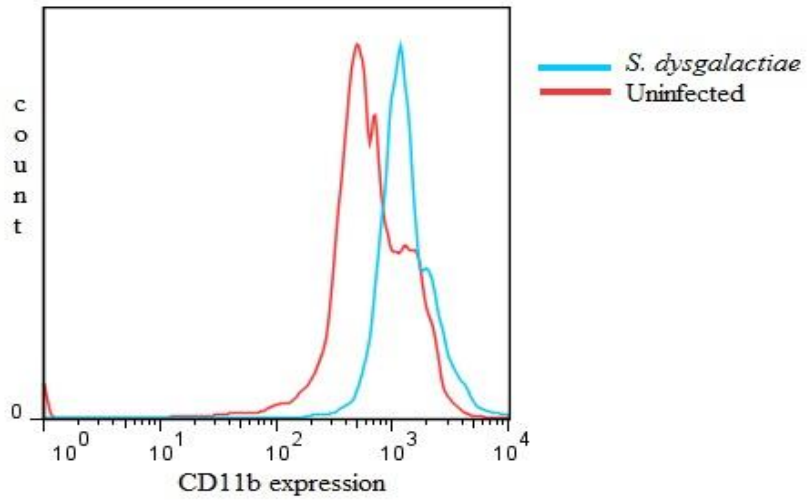

Figure 2 - Histogram overlays demonstrating CD11b expression on milk neutrophils. Histogram represents the log fluorescence (CD11b-Phycoerythrin-Cy5) of milk neutrophils ($CH138^+$).

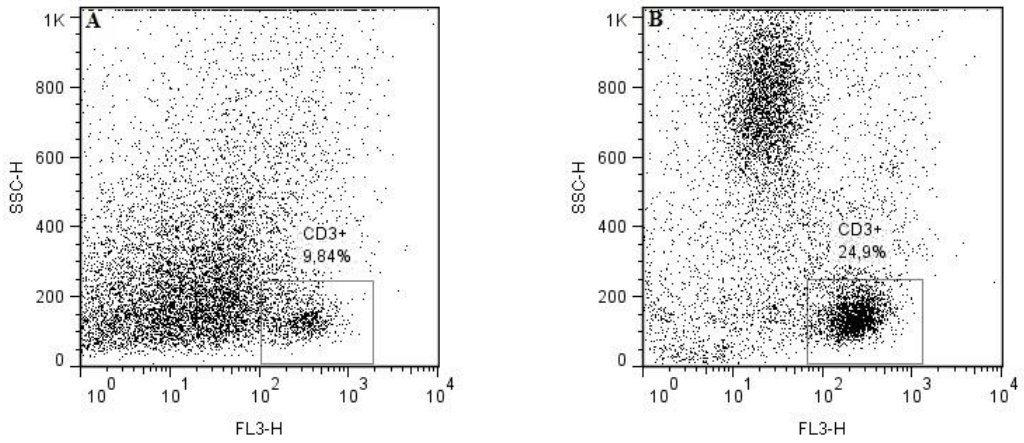

Figure 3 - Flow cytometric identification of the T lymphocytes ($CD3^+$) isolated from a representative uninfected (A) and *S. dysgalactiae*-infected (B) quarters. The recordings of scatter (SSC-H) and fluorescent properties (FL3-H; CD3-Phycoerythrin-Cy5) were performed on 20,000 events gated in a standard population excluding most of debris.

7 Chapter V. Adhesion and internalization of environmental, teat-apex and udder-associated staphylococci into bovine mammary epithelial cells

ABSTRACT

Bacteria adherence seems to be an essential first stage for the internalization of bacterial pathogens into the cytoplasm of the host cell. Internalization of bacterial pathogens into host cells was considered an important virulence strategy, since this allows bacteria to occupy a microenvironment free from host defense mechanisms. Thus, this study aimed to explore the difference in the capacity of four staphylococci strains to adhere to and internalize into bovine mammary epithelial cells (MEC). Three different strains of coagulase-negative staphylococci (CNS) were used: one strain of *Staphylococcus fleurettii* isolated from sawdust and considered as an environmental opportunistic pathogen, and two dissimilar *S. chromogenes* isolates. The first *S. chromogenes* isolate was cultured from a heifer's teat apex and appears to have protective effects against major pathogens (*S. chromogenes* TA), while the other strain of *S. chromogenes* originates from a chronic intramammary infection (*S. chromogenes* IM) and is considered as an udder-adapted pathogen. Also, one well-characterized strain of *S. aureus* (Newbould 305) associated with mild bovine mastitis was used. The CNS species and strains adhered to and internalized into MEC slower than *S. aureus*. Furthermore, *S. chromogenes* IM showed higher ability to adhere to and internalize into MEC than two CNS strains isolated from extramammary niches. Thus, our findings provide new insights into the capacity to adhere to and internalize into MEC by different staphylococci strains and species that may be related to intra-species diversity and epidemiological and ecological behavior of different staphylococci which might be linked to the etiology and persistence of intramammary infections.

Keywords: coagulase-negative staphylococci, *Staphylococcus aureus*, mastitis, dairy cow

Introduction

Mastitis is one of the most common and detrimental diseases that cows can experience. Mastitis threatens the income of farmers as well as the image of the dairy sector because of animal welfare issues and issues related to milk quality and public health due to an increased risk of antimicrobial residues and the emergence of resistant bacteria [1,2]. Staphylococcal bacteria are still an important cause of bovine mastitis. The genus is usually divided into the coagulase-positive staphylococci with *S. aureus* remaining the most significant mastitis pathogen among the staphylococci and the heterogenous group of coagulase-negative staphylococci (CNS) which have become the most commonly isolated bacterial pathogens from milk samples of dairy cows and heifers [3-5], as well as small ruminants [6], in many regions and countries. Despite their high prevalence as cause of intramammary infections (IMIs) in dairy cows and small ruminants, little knowledge is yet available on their epidemiology, virulence and interactions with the host.

Some authors have associated CNS with chronic IMIs [7-11] and an increase in milk somatic cell count (SCC) [10-12], although their clinical relevance is still under debate [13-15]. Nevertheless, conflicting results as to the importance of CNS as mastitis pathogens is likely due to the failure to acknowledge variations within and between these species [11]. The development and validation of molecular identification techniques allow accurately speciation and fingerprinting of bovine-associated CNS [9,10,16,17] identifying variations among species in SCC, persistence of infection, antimicrobial resistance, virulence and epidemiological behavior [7,9-12,16-21]. 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.

Bacteria adherence seems to be an essential first stage for the internalization of bacterial pathogens into the cytoplasm of the host cell. Internalization of bacterial pathogens into host cells was considered an important virulence strategy, since this allows bacteria to occupy a micro-environment free from host defense mechanisms operable at the mucosal surface [22,23].

Apart from three studies, no information is available on the interaction between different staphylococci species and the ruminant MEC [23-25]. Also, none of the latter studies investigated the potential link between the adherence and internalization capacity on the one hand and the epidemiology of the strains on the other hand.
Thus, the present study aimed to explore the differences in the capacity of staphylococci strains isolated from distinct habitats to adhere to and internalize into bovine MEC.

Methods

Bacterial strains and growth conditions

Three different strains of CNS were used: one strain of *S. fleurettii* isolated from sawdust and considered as an environmental opportunistic pathogen [10,21], and two dissimilar *S. chromogenes* isolates. The first *S. chromogenes* isolate is cultured from a heifer's teat apex (TA) and appears to have protective effects against major pathogens such as *S. aureus*, *Streptococcus uberis* and *Streptococcus dysgalactiae* [21,26] while the other strain of *S. chromogenes* originates from a chronic IMI (IM) [10] and is considered as an udder-adapted pathogen [9,16,21]. Also, one well-characterized and host-adapted strain of *S. aureus* (Newbould 305) associated with mild and chronic bovine mastitis [23] was included as a positive control.
The strains were stored at - 80°C and thawed at 37°C. First, the strains were grown on Colombia sheep blood agar plates (Oxoid, Wesel, Germany). Then, fresh colonies of each bacteria were grown overnight in brain heart infusion (BHI) broth at 37°C. Subsequently, all strains were diluted at 1:1000 in fresh BHI broth and incubated until they reached their respective late exponential growth phase. After bacterial growth, the bacterial broth was centrifuged at 2,500 x g for 15 minutes and washed twice with 1X Dulbecco's phosphate buffered saline (DPBS; cat. n. 14190185, Gibco, Paisley, UK). Then, the bacteria were resuspended in Dulbecco's modified eagle's media (DMEM, cat. n. 42430-025, Gibco, Paisley, UK) supplemented with 10% of fetal bovine serum, 5 µg mL^{-1} of insulin (cat. n. I3536, Sigma Aldrich, St. Louis, USA) and 1 µg mL^{-1} of hydrocortisone (cat. n. H0888, Sigma Aldrich, St. Louis, USA). The inoculum was adjusted to 3 x 10^5 colony forming unit mL^{-1} (cfu mL^{-1}) [27] and stored at -80°C until further processing.
The bacterial count for the assay was determined using spectrophotometry at absorbance 600 nm. The inoculum suspension was also cultured on tripticase soy agar in dilution series and colonies were counted to confirm the final inoculum dose.

Bovine MEC

The MAC-T bovine mammary epithelial cell line was cultured using MAC-T medium containing DMEM supplemented with 10% of fetal bovine serum, 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 0.25 µg mL^{-1} Fungizone® (cat. n. 15240-096, Gibco, Paisley, UK), 5 µg mL^{-1} of insulin and 1 µg mL^{-1} of hydrocortisone in six well plates, and incubated in a humidified incubator with 5% CO_2 at 37ºC. In order to obtain a confluent monolayer, the cell line was treated with 0.25% trypsin (cat. n. 154000-054, Gibco, Paisley, UK), resuspended in fresh MAC-T medium, seeded in 6-well plates (5 x 10^5 cells/well) and incubated overnight at 37ºC in 5% CO_2 .

Adherence and internalization assay

The adherence and internalization assay was performed as described elsewhere [23] with slight modifications. After removing the culture medium, the confluent monolayers of MAC-T were washed three times with 1X DPBS, and 4 mL of the staphylococcal suspension (3 x 10^5 cfu

mL^{-1}; 1:1 ratio of staphylococci to cells) was added per well. Firstly, the number of bacteria in the MAC-T medium (supernatant) was assessed at each sampling time (0 h - before add the staphylococcal suspension, 1, 3, 6 and 12 h) by the drop-plate method wherein 10 µL of each diluted sample is dropped onto trypticase soy agar until the bacteria are dilute enough to count viable bacteria accurately (between three and 30 cfu).
After 0, 1, 3, 6 and 12 h of incubation at 37ºC in 5% CO_2, the supernatants were removed and the monolayers were washed three times with 1X DPBS. Subsequently, the cells were treated with a mixture of containing 0.25% trypsin and 0.025% Triton X-100 (cat. n. 93443, Sigma Aldrich, St. Louis, USA) in sterile distilled water to lyse the cells. Then, the lysates and supernatants were mixed, diluted 10-fold serially with sterile saline, plated onto square plates with trypicase soy agar, and incubated for 16-18 h at 37ºC. The number of cell-associated bacteria i.e. adherent and internalized bacteria (cfu mL^{-1}), was determined by the drop-plate method.
A similar assay including an additional step to kill the non-internalized bacteria i.e. adherent bacteria, was performed to determine the internalization capacity of the different staphylococcal bacteria. In order to kill the non-internalized bacteria, a cell culture medium with lysostaphin (20 µg mL^{-1}; cat. n. L7386, Sigma Aldrich, St. Louis, USA) was added to the monolayer. After an incubation of 20 min at 37°C, the cell culture medium containing lysostaphin was removed, the monolayers were washed three times with 1X DPBS. Subsequently, the cells were treated with a mixture of containing 0.25% trypsin and 0.025% Triton X-100 in sterile distilled water to lyse the cells. Then, the lysates and supernatants were mixed, diluted 10-fold serially with sterile saline, to determine the number of internalized bacteria (cfu mL^{-1}), as described above.

Outcome variables

The number of adherent bacteria was determined by subtracting the number of internalized bacteria from the corresponding number of cell-associated bacteria. Non-specific binding of bacteria to plastic was estimated by placing the suspension of each bacteria inoculum into wells without MAC-T cell monolayers, and then used to correct values of adherence of bacteria to MAC-T cells [23]. The percentage of adherent bacteria was calculated by dividing the number of cfu mL^{-1} of adherent bacteria by the number of cfu mL^{-1} of total bacteria (sum of the number of cell-associated bacteria and the number of bacteria in the MAC-T medium) x 100 at the corresponding sampling time regarding bacteria growth in MAC-T medium. The number of internalized bacteria equals the number of intracellular bacteria. Similar to the percentage of adherent bacteria, the percentage of internalized bacteria was calculated by dividing the number of cfu mL^{-1} of intracellular bacteria by the number of cfu mL^{-1} of total bacteria x 100 at the corresponding sampling time regarding bacteria growth in MAC-T medium. Each strain was tested in triplicate and the assays were repeated five times.

Statistical analyses

To determine the differences between the different strains in adherence and internalization capacity, respectively, linear mixed regression models were fit with het number of adherent bacteria, the percentage of adherent bacteria, the number of internalized bacteria and the percentage of internalized bacteria as continuous outcome variables, and the strain type (*S. aureus*, *S. chromogenes* TA, *S. fleurettii*, and *S. chromogenes* IM), sampling time, and the interaction between strain type and sampling time as categorical independent variables (PROC MIXED, SAS 9.4, SAS Institute Inc., USA). A first-order autoregressive correlation structure was applied to adjust for clustering of repeated measurements within assays. Triplicate as

random effect was omitted because an estimation of the contribution of each of the random effects (VARCOMP procedure) revealed that the variation among the triplicates within an assay was zero. The number of adherent (cfu mL^{-1}) and internalized (cfu μL^{-1}) bacteria were log_{10}-transformed to normalize the data. The percentages of adherent and internalized bacteria were arcsine-transformed to normalize the data. The results are reported as the mean ± standard error. Significance was set at $P \leq 0.05$. A Bonferroni-correction was applied to adjust for multiple comparisons.

Results

The dynamics of the adherence to and internalization into bovine MEC of the different strains are summarized in Figure 1. The overall logarithm number (cfu mL^{-1}) of adherent bacteria to MEC increased from 3.43 $\pm$ 0.16 after 1h of incubation to 3.85 $\pm$ 0.15 after 3h of incubation over 4.71 $\pm$ 0.15 after 6h of incubation up to 6.00 $\pm$ 0.16 after 12h of incubation ($P \leq 0.0001$). The strength of the increase depended on the type of strain and was most pronounced for *S. chromogenes* IM (Fig. 1). *S. chromogenes* IM (5.34 $\pm$ 0.20 at 6 h; 6.72 $\pm$ 0.20 at 12 h) showed higher number of adherent bacteria to MEC than *S. fleurettii* (4.52 $\pm$ 0.20 at 6 h; 5.84 $\pm$ 0.20 at 12 h) and *S. chromogenes* TA (4.08 $\pm$ 0.20 at 6 h; 5.16 $\pm$ 0.20 at 12 h) after 6 h and 12 h of incubation (Fig. 1). At 6 h and 12 h, the number of adherent *S. chromogenes* IM to MEC was even higher than the number of adherent *S. aureus* to MEC (4.90 $\pm$ 0.20 at 6 h; 6.29 $\pm$ 0.20 at 12 h), although were not statistically different. On the other hand, CNS species (*S. chromogenes* IM: 3.74 $\pm$ 0.20, *S. fleurettii*: 3.85 $\pm$ 0.20, and *S. chromogene* TA: 3.23 $\pm$ 0.20) adhered slower to MEC, particularly evidenced after 3h of incubation, than *S. aureus* (4.56 $\pm$ 0.20; Fig. 1). Furthermore, the number of adherent *S. chromogenes* isolated from TA (3.02 $\pm$ 0.20) to MEC was statistically lower at 1 h when compared to *S. aureus* (3.98 $\pm$ 0.21), although no significant difference was observed for *S. fleurettii* (3.48 $\pm$ 0.20) and *S. chromogenes* IM (3.22 $\pm$ 0.20; Fig.1).

The percentage of adherent bacteria to MEC ranged from 0.63 $\pm$ 0.09 after 1h of incubation to 1.24 $\pm$ 0.08 after 3h of incubation over 1.93 $\pm$ 0.08 after 6h of incubation up to 8.56 $\pm$ 0.17 after 12h of incubation ($P \leq 0.0001$). Similarly to the findings of the number of adherent bacteria to MEC, CNS species (*S. chromogenes* IM: 0.42 $\pm$ 0.16, *S. fleurettii*: 0.45 $\pm$ 0.16, and *S. chromogenes* TA: 0.30 $\pm$ 0.16) showed lower percentage of adherent bacteria to MEC, particularly evidenced after 3h of incubation, than *S. aureus* (6.73 $\pm$ 0.16; Fig. 1). On the other hand, the *S. chromogenes* IM (14.92 $\pm$ 0.17) and *S. fleurettii* (11.18 $\pm$ 0.17) displayed higher percentage of adherent bacteria to MEC after 12 of incubation than *S. chromogenes* TA (2.35 $\pm$ 0.18; Fig. 1), although no significant different was observed with *S. aureus* (8.77 $\pm$ 0.17; Fig. 1).

The overall logarithm number (cfu μL^{-1}) of internalized bacteria into MEC increased from 0.18 $\pm$ 0.14 after 1h of incubation to 0.54 $\pm$ 0.13 after 3h of incubation over 1.19 $\pm$ 0.13 after 6h of incubation up to 2.10 $\pm$ 0.14 after 12h of incubation ($P \leq 0.0001$). Corroborating with the results of number of adherent bacteria to MEC, all CNS species (*S. chromogenes* IM: 0.50 $\pm$ 0.16, *S. fleurettii*: 0.28 $\pm$ 0.16, and *S. chromogenes* TA: 0.14 $\pm$ 0.16) internalized slower into MEC, particularly evidenced after 3h of incubation, than *S. aureus* (1.25 $\pm$ 0.16; Fig. 1). After 6 h of incubation, *S. chromogenes* IM (1.30 $\pm$ 0.16) displayed higher number of internalized bacteria into MEC than *S. fleurettii* (0.79 $\pm$ 0.16) and *S. chromogenes* TA (0.81 $\pm$ 0.16), due to its sharp increase in internalization into MEC. At this moment, *S. aureus* (1.86 $\pm$ 0.16) persisted with the highest number of internalized bacteria into MEC (Fig. 1). After 12 h of incubation, all staphylococci strains differed among each other, with *S. chromogenes* IM (3.10 $\pm$ 0.16) exhibited the highest amount of internalized bacteria into MEC, followed by *S. aureus* (2.38 $\pm$ 0.16), *S. fleurettii* (1.87 $\pm$ 0.16) and *S. chromogenes* TA (1.05 $\pm$ 0.17), respectively (Fig. 1).

The percentage of internalized bacteria into MEC ranged from 0.08 ± 0.03 after 1h of incubation to 0.40 ± 0.03 after 3h of incubation over 0.51 ± 0.03 after 6h of incubation up to 1.27 ± 0.03 after 12h of incubation (P = 0.006). All CNS species (*S. chromogenes* IM: 0.10 ± 0.05, *S. fleurettii*: 0.03 ± 0.05, and *S. chromogenes* TA: 0.10 ± 0.05) internalized into MEC slower than *S. aureus* (2.96 ± 0.05), particularly evidenced after 3h of incubation (Fig. 1). After 6 h of incubation, the percentage of internalized *S. fleurettii* (0.05 ± 0.05) into MEC was lower than *S. aureus* (1.46 ± 0.05), although no significant difference was observed with *S. chromogenes* IM (0.35 ± 0.05) and *S. chromogenes* TA (0.66 ± 0.05). Still, the pronounced augment in the internalization of *S. chromogenes* IM into MEC after 12 h of incubation resulted in a higher percentage of *S. chromogenes* IM (5.05 ± 0.05) internalized into MEC at this time than the other staphylococci species (*S. fleurettii*: 0.01 ± 0.05, *S. chromogenes* TA: 0.13 ± 0.05, and *S. aureus*: 0.80 ± 0.05; Fig. 1).

Discussion

CNS species were abundantly present in dairy farm environment and on bovine skin and mucosa. Therefore, some CNS species that were plentifully found in cow's environment are rarely associated with IMIs such as *S. equorum*, *S. fleurettii* and *S. sciuri*. Otherwise, *S. chromogenes* was commonly isolated from milk and associated with IMIs, but seldom encountered in cow's environment [9,17,20]. Furthermore, *S. chromogenes*, the most frequently isolated CNS from infected quarters [10-12,17], was only occasionally isolated from TA [17]. However, *S. chromogenes* isolated from pre-partum teat apices was not associated with IMI, and even associated with a decreased risk of *S. chromogenes* IMIs in heifer in early lactation and lower odds for elevated SCC during the first five days of lactation [28,29]. Altogether, these facts provide further evidence that the group of CNS species is comprised by substantially variation among species and strains which differ in ecology and epidemiology which may explain our results.

Thus, a key finding of the present study is the slower adhesion and internalization of CNS species and strains into MEC than *S. aureus*, and the great variability of the ability to adhere to and internalize into MEC of CNS species and strains isolated from distinct niches. With this in mind, Peton et al. [22] have described that the *S. aureus* Newbold 305 strain had higher capacity to adhere and internalize into MAC-T cells than a *S. aureus* strain (RF122) isolated from a clinical case of mastitis. These phenomena were related to the virulence factors associated with host-adaptation and tissue invasion carried by *S. aureus* Newbold 305. Most of these factors belong to the surface proteins as microbial surface components recognizing adhesive matrix molecules, which interact with host proteins such as fibronectin, collagen and elastin, and then trigger invasion.

Some previous studies have investigated the capacity of CNS species isolated from bovine IMIs to adhere to and internalize into MEC [23,25]. Hyvönen et al. [25] found that the intensity of internalization into MEC of CNS strains isolated from clinical and subclinical mastitis was generally weaker than *S. aureus* strains, as found here. Besides that, these authors have found some differences in adhesion and invasion rates among some CNS strains isolated from IMIs, however the adhesion to and internalization into MEC of persistent and transient CNS strains did not differ. Furthermore, different from our study, these authors performed the adhesion and internalization assay only once (90 min), used high ratio of bacteria/cell in the inoculum, and the percentage of adherent and internalized bacteria was calculated based on the inoculum dose, and bacteria growth in the MAC-T medium was not regarded. Here, the concentration of inoculums used was equivalent to an initial multiplicity of infection of 1 cfu per cell [27], and the CNS strains used differ from those previous studies because we used CNS isolated from

different niches (environment, teat apex, and chronic IMI). Furthermore, it should be regarded that in our study of the kinetics of adherence to and internalization into MEC did not appear to follow growth of staphylococci strains in the cell culture environment (Fig. 1), as previously found by Almeida and Oliver [23], which explained why the percentage of adherent and internalized bacteria into MEC not always increased.

In face of, a key finding of our study is that the host-adapted *S. chromogenes* strain adhered and internalized better (Fig. 1) into MEC than the CNS strains (*S. chromogenes* TA and *S. fleurettii*) isolated from extramammary niches. Thus, the epidemiology and ecology of CNS may be an important fact that contributes to the persistence and elimination of invading bacteria to the udder as internalization of bacterial pathogens by host cells was considered an important virulence strategy, since this allows bacteria to occupy a microenvironment free from host defense mechanisms [22,23]. In fact, it has been reported that among the CNS species, *S. chromogenes* caused more persistent than transient IMI [8-10], whereas *S. fleurettii* only caused transient IMIs [10].

Here, we addressed the kinetic of adhesion and internalization capacity into MEC by distinct staphylococci species and strains. With this in mind, we hypothesized that our findings (i.e. slower adhesion and internalization capacity into MEC by CNS) together with the impaired influx of neutrophils to site of inflammation during the the periparturient period [30] may have implications to the dynamic of IMIs by CNS during lactation, as the fast and strong increase in milk neutrophils shortly after entry of invading pathogens can eliminated the bacteria [31], especially if bacteria are not in a micro-environment free from host defense mechanisms (i.e. internalized). Another factor that should be collaborated for elimination of mastitis pathogens or the persistence of infection of CNS strains is the cow factors (fast x slow responders), as bacteria are eliminated very quickly in case of fast and strong influx of neutrophils influx to the udder in the early stages of infection [31]. Altogether, this resembles somehow the findings of some researchers [15,32,33] who have noted that there is a trend for a high prevalence of CNS during the periparturient period, especially for IMIs by *S. chromogenes* [34]. Furthermore, the striking differences among CNS and *S. aureus* in the adhesion and internalization capacity into MEC, at least in part, may also be related to the higher inoculums doses of CNS necessary to induce mastitis in bovine [35,36] and murine [21] models than *S. aureus* [21,30,37].

Another important finding of our study is the similar percentage of adherent *S. fleurettii* and *S. chromogenes* IM to MEC after 12 h of incubation. Moreover, both CNS strains exhibited higher percentage of adherent bacteria to MEC than *S. chromogenes* TA. Nevertheless, the percentage of internalized *S. fleurettii* and *S. chromogenes* TA into MEC at this same time was similar, both strains displayed lower percentage of internalized bacteria into MEC than *S. chromogenes* IM. Altogether, it demonstrated the potential of *S. fleurettii* to cause IMI, particularly transient IMIs, which strongly supports the hypothesis of *S. fleurettii* isolated from sawdust to be regarded as an environmental opportunistic pathogen. Thus, it can be speculated that when the infection pressure is high or when the immunity of cows is compromised, these CNS species may act as environment opportunistic pathogens [9]. Corroborating with this idea, Jácome et al. [38] have suggested that udder infections in the peripartum period might be associated with environment sources, due to the similar genotypic patterns of CNS strains isolated from colostrums and environment, however no isolate from mastitis during lactation was related to environmental staphylococci in goats.

In conclusion, our findings provide new insights into the capacity to adhere to and internalize into MEC by different staphylococci species. Firstly, CNS species adhere and internalize into MEC slower than *S. aureus* species. Furthermore, although the present study used a limited number of strains, our findings provide new insights into the capacity to adhere to and internalize into MEC by different staphylococci strains and species that may be related to intra-

species diversity and epidemiological and ecology behavior of different staphylococci which might be linked to the etiology and persistence of IMIs.

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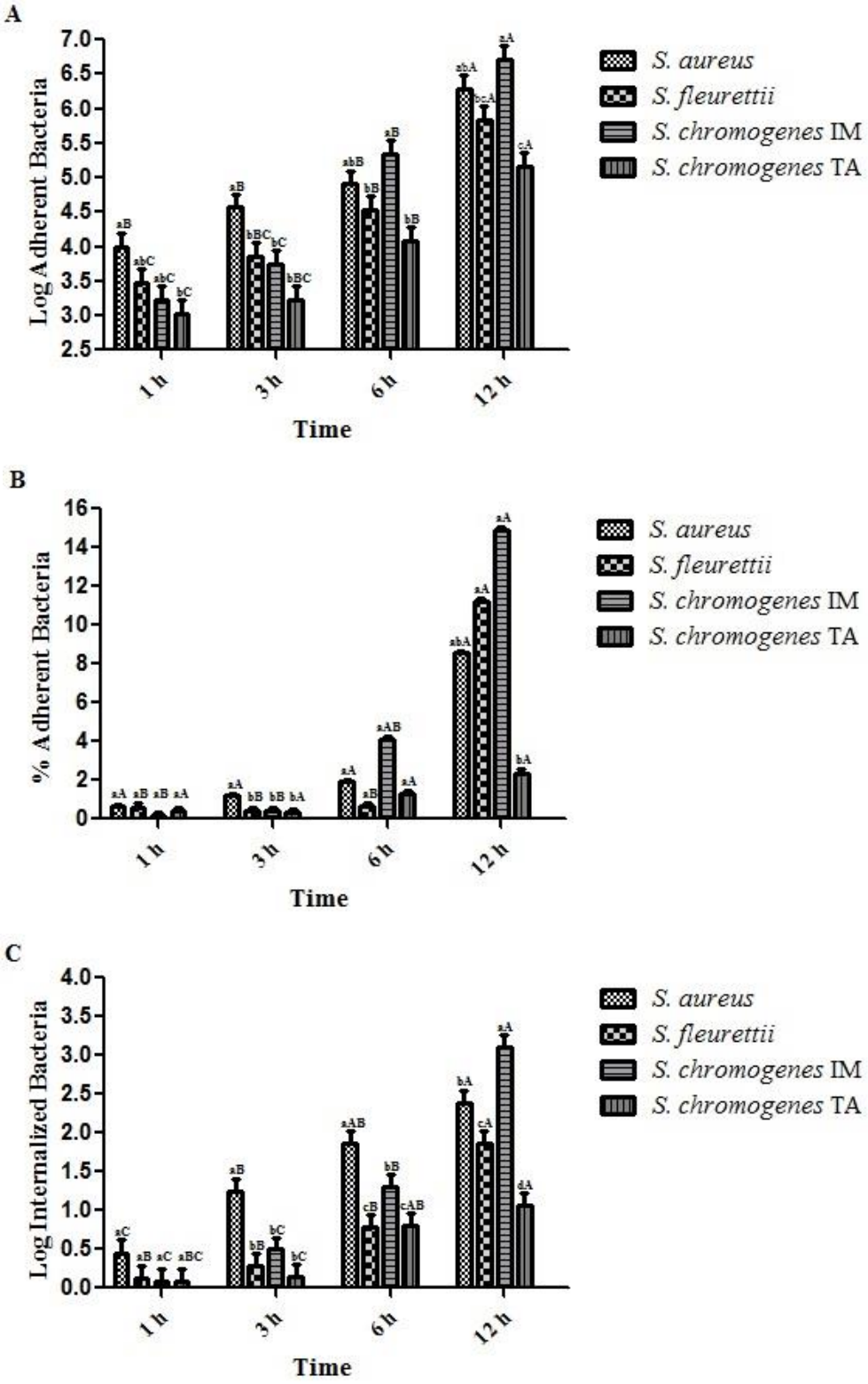
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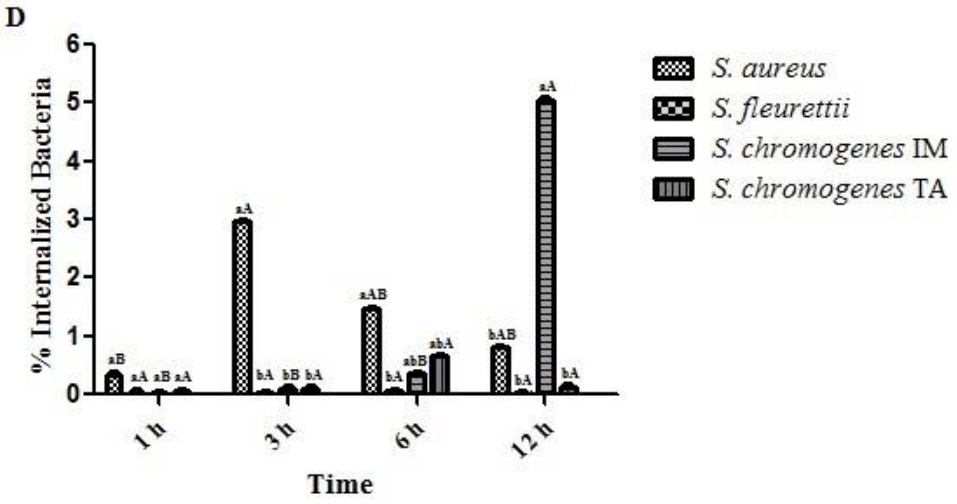

Figure 1 Adherence and internalization of different strains of staphylococci into mammary epithelial cells. The logaritmitic number (cfu mL^{-1}) of adherent (A) and internalized (cfu μL^{-1}) (C) *Staphylococcus aureus* Newbould 305, *S. fleurettii* isolated from sawdust, *S. chromogenes* isolated from chronic intramammary infection (*S. chromogenes* IM) and *S. chromogenes* isolated from a heifer's teat apex (*S. chromogenes* TA). The percentage of adherent (B) and internalized (D) bacteria were also shown. Different lower case letters designated the means with statistically significant differences ($P \leq 0.05$) among staphylococci strains. Capital letters indicated statistically significant differences ($P \leq 0.05$) among time.

8 Chapter VI. Efficacy of vaccination on *Staphylococcus aureus* intramammary infections in two dairy herds with high bulk milk somatic cell count

Abstract
This study investigated the efficacy of a commercial vaccine under field condition in two dairy herds with high bulk milk somatic cell count (SCC) and a high prevalence of IMIs by *S. aureus*. Vaccination was carried out using STARTVAC® (Hipra, Brazil) at the same day in lactating dairy cows. Milk samples for bacteriological analysis were collected at first dose of vaccination, second dose of vaccination, 30 d after the second vaccination, third vaccination, and seven, 14 and 21 d after the third vaccination. The SCC, the rate of new IMIs by *S. aureus* and cure of IMIs by *S. aureus* during the trial was not statistically different for *S. aureus* infected unvaccinated and vaccinated dairy cows that were segregated and milked last. Nevertheless, the vaccine efficacy for new IMIs by *S. aureus* and for cure of IMIs by *S. aureus* was 32.91% and 72.23%, respectively. Combining the information of these parameters into an overall infection reproduction ratio provides a summary parameter on vaccine efficacy of 51.54%. Furthermore, there is a significant difference in new IMIs by *S. aureus* in uninfected quarters from vaccinated uninfected dairy cows (milked first) and *S. aureus*-infected dairy cows (milked last). Although vaccination results appear encouraging, their use should be recommended in conjoint with other adequate management practices, such as segregation of *S. aureus* infected cows.

Key words: mastitis, vaccine, dairy cow.

Introduction
Mastitis is the most common infectious disease affecting dairy cows and the most economically important disease of the dairy industry. In attempt to minimize the incidence of bovine mastitis, several preventive strategies have been applied, such as optimization of milking procedures and milking hygiene, dry-cow therapy, vaccination, segregation and culling problem cows (Schukken et al., 2014).

The use of vaccination to control infectious diseases in dairy cattle is useful and vaccination against mastitis pathogens is a control strategy applied by some dairy farmers (Pereira et al., 2011; Schukken et al., 2014; Bradley et al., 2015). There are few number of commercially available vaccines against intramammary infections (IMIs) pathogens on the market, and their efficacy results in the literature are scarce (Schukken et al., 2014; Bradley et al., 2015). Several experimental challenges have been used to evaluate the efficacy of vaccines (Camussone et al., 2013; Camussone et al., 2014; Wedlock et al., 2014), however such experiments studies were not able to show a reduction in infection transmission rates (Schukken et al., 2014). As vaccination can induce a biologically protective response in a vaccinated individual or reduce the degree or duration of infectiousness, the vaccine efficacy can be estimated based on infection transmission and infection duration parameters (Halloran et al., 1997). In other words, the success of elimination of IMIs and the prevention of new IMIs leads to a reduction in exposure and contributes to a lower transmission scenario. Apart from two studies (Schukken et al., 2014; Bradley et al., 2015), no information is available on commercial available vaccines, especially in herds with high bulk milk SCC and high percentage of *S. aureus* problem cows.

Then, to evaluate the vaccine efficacy, the infection status of quarters of cows needs to be determined precisely over time. These data allow the evaluation of vaccination on new IMI and IMI duration (Halloran et al., 1997; Schukken et al., 2014). Recently, a combined staphylococcal and J5 *Escherichia coli* vaccine (STARTVAC®, Hipra, Brazil) was introduced in many countries. The staphylococcal component of this vaccine is based on a bacterin of *Staphylococcus aureus* strains with particular high cell wall components, such as exopolysaccarides, that may be involved in the biofilm phenotype of the bacteria (Harro et al., 2010).

Thus, the present study aim to evaluate vaccine efficacy of a commercial vaccine (STARTVAC®, Hipra, Brazil) under field condition in two dairy herds with high bulk milk somatic cell count (SCC) and a high prevalence of IMIs by *S. aureus*.

Materials and Methods

Herds

The effect of vaccination on the dynamic of IMI was studied in two commercial dairy herds with approximately 250 lactating cows. The herds A and B had a prevalence of 15.75% and 24.80% of *S. aureus* infected cows at the initial of the study, respectively, and high bulk milk SCC ($\geq$ 500,000 cells mL^{-1}, Barkema et al., 1998). The trial started in January 2013 in herd A, and in August 2013 in herd B. All Holstein cows were milked twice daily and had estimated milk yield of 16.68 $\pm$ 0.97 kg/day (herd A) and 23.86 $\pm$ 1.30 (herd B) kg/day, respectively. The herds used milking machine and adopted the following mastitis control practices at milking: forestripping in a strip cup to check for clinical mastitis, preddipping, and drying teats with paper towel. After milking, the postdipping was also used. Dry cow therapy and clinical mastitis treatment were also adopted. As both herds have a problem with *S. aureus* mastitis, cows with mastitis by this pathogen were segregated from the milking herd and milked last.

Vaccination and milk sampling

Vaccination was carried out using STARTVAC® (Hipra, Porto Alegre, Brazil) at the same day in lactating dairy cows. The present study used 34 lactating dairy cows as follows: 14 vaccinated *S. aureus* infected dairy cows (24 *S. aureus*-infected quarters), nine unvaccinated *S. aureus* infected dairy cows (20 *S. aureus*-infected quarters); and 11 vaccinated healthy dairy cows (n = 43 uninfected quarters). The cows were selected based on their expected day to dry-off, bacteriogical results and SCC ($\leq$ 200,000 cells mL^{-1} for uninfected cows), as the threshold for SCC proposed by Schepers et al. (1997) and Schukken et al. (2003). All bacteriological negative quarters with high milk SCC (> 200,000 cells mL^{-1}) were excluded from the present study. The vaccination (three doses) and milk sampling for bacteriological examination and SCC were performed as showed in Fig. 1.

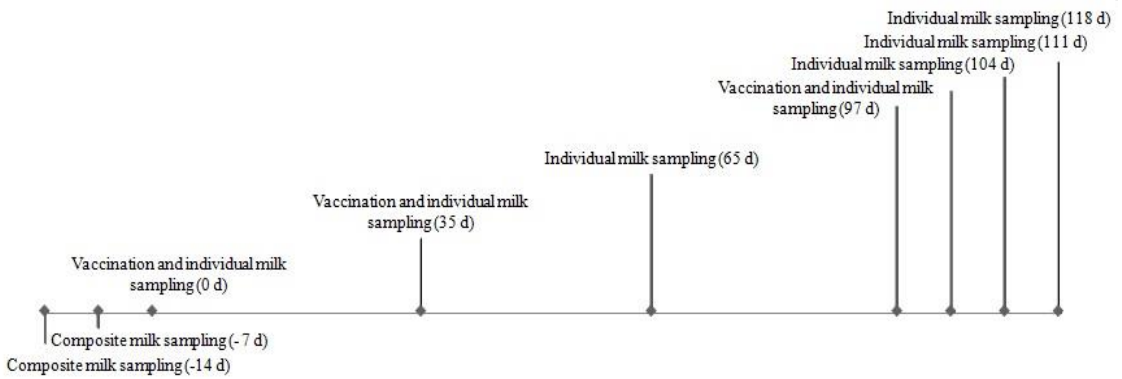

Figure 1. Vaccination and milking sampling schedule used in the present study.

For milking sampling, the strip cup test was performed to identify the presence of clots, flakes or otherwise obviously abnormal secretions. Pre-dipping was then performed, with one towel used for each teat. After discarding the first three milk streams, teat ends were scrubbed with cotton soaked in 70 % ethanol, and milk sample was aseptically collected into sterile vials for bacteriological analysis. Finally, milk samples were collected for the evaluation of SCC. Composite milk samples for bacteriological analysis were collected twice before vaccination.

Thereafter, milk samples from each mammary quarter were also collected for bacteriological analysis (in duplicate) and SCC. The milk samples were kept at 4°C until arriving at the laboratory. The milk samples for the bacteriological analysis were stored at -20°C until the analysis.

IMI Definition

A sample was defined as having an IMI by *S. aureus* if at least one colony ($\geq$ 100 cfu/mL) growth in bacteriological culture. The milk sample was also regarded as bacteriological positive - and not bacteriological cured - for *S. aureus* if this pathogen was isolated at least in one sample of the duplicate. A quarter was regarded as uninfected at the initial of the experiment based on two composite bacteriological negative results before vaccination, and a milk quarter bacteriological negative result (in duplicate) at the day of vaccination, as well as SCC $\leq$ 200,000 cells mL^{-1} in all quarters. An infection was considered cured if two consecutive monthly milk samples or three consecutive weekly milk samples did not show the presence of the causative organism.

Somatic cell count

Composite and quarter milk samples for SCC were collected in a 40-mL vial containing microtablets of bronopol (2-bromo-2-nitropane-1,3-diol) and analyzed using a calibrated Bentley CombSystem 2300® unit (Chaska, USA).

Bacteriological analysis

Bacterial analysis was conducted by culturing 0.01 mL of each sample on 5 % ovine blood agar plates. The plates were incubated for 24-48 hours at 37 ºC, which was followed by Gram staining, observation of colony morphology and hemolysis patterns, and biochemical testing (Brito et al. 1999, Oliver et al. 2004).

Statistical Analysis

First, the distributions were examined using normal probability plots obtained using the Shapiro and Wilk tests. For milk SCC analysis, SCC were $\log_{10}$ transformed, as the distribution was not normal. Then, the Student's t-test for unpaired data was applied to compare milk SCC between *S. aureus*-infected vaccinated and unvaccinated dairy cows. To compare the differences in parity among groups, the nonparametric ANOVA (Kruskal-Wallis test) was used for comparisons followed by Dunn's multiple comparison test. To compare the differences in days in milk (DIM) among groups, the ANOVA followed by Newman-Keuls test was used due to their normal distribution. The effect of vaccination on new IMIs and cure of IMIs was conducted using the Kaplan-Meier survival curve. All statistical analysis was performed using GraphPad Prism 5.0 software® (GraphPad Software, Inc., San Diego, CA, USA). Significance was declared at $P \leq 0.05$.

Briefly, the vaccine efficacy for new IMIs by *S. aureus* and cure of IMIs by *S. aureus* was also calculated as previously described (Halloran et al., 1997; Schukken et al., 2014), as follow:

vaccine efficacy for new infections taken into account the transmission rate (β) is:

$$= 1 - \frac{\text{(vaccinated infections/vaccinated exposures)}}{\text{(control infections/control exposures)}}$$

, whereas vaccine efficacy taken into account the cure rate (α) is:

= 1 - (control cured/control infected)
(vaccinated cured/unvaccinated infected)

, and combining these information of parameters α and β into a overall reproduction ratio (R_0) was defined as $R_0 = \beta/\alpha$, and the resulting vaccine efficacy is then calculated as

$$= 1 - \frac{R_{0,vaccinated}}{R_{0,control}} = 1 - \frac{(\beta/\alpha)_{vaccinated}}{(\beta/\alpha)_{control}}$$

Results

The rate of new IMIs by *S. aureus* ($P = 0.91$; Fig. 2B) and cure of IMIs by *S. aureus* ($P = 0.71$; Fig. 2A) during the trial was not statistically different for *S. aureus* infected unvaccinated and vaccinated dairy cows that were segregated and milked last applying Kaplan-Meier survival curve. Furthermore, considering all *S. aureus*-infected quarters at the initial of the trial and follow then during the trial, the milk quarter SCC was not statistically different between unvaccinated and vaccinated cows in all moments. No significant difference was also found among the three groups in DIM ($P = 0.56$) and parity ($P = 0.61$). Nevertheless, the vaccine efficacy for new IMIs by *S. aureus* estimated by transmission probability ratios and for cure of IMIs by *S. aureus* was 32.91% and 72.23%, respectively. Combining the information of these parameters (cure and new IMIs) into an overall infection reproduction ratio provides a summary parameter on vaccine efficacy of 51.54%.

Furthermore, there is a significant difference in new IMIs by *S. aureus* in uninfected quarters from vaccinated uninfected dairy cows (milked first) and *S. aureus*-infected dairy cows (milked last) ($P \leq 0.0001$), as none of the 43 uninfected quarters (11 vaccinated dairy cows) become infected by *S. aureus* during the trial.

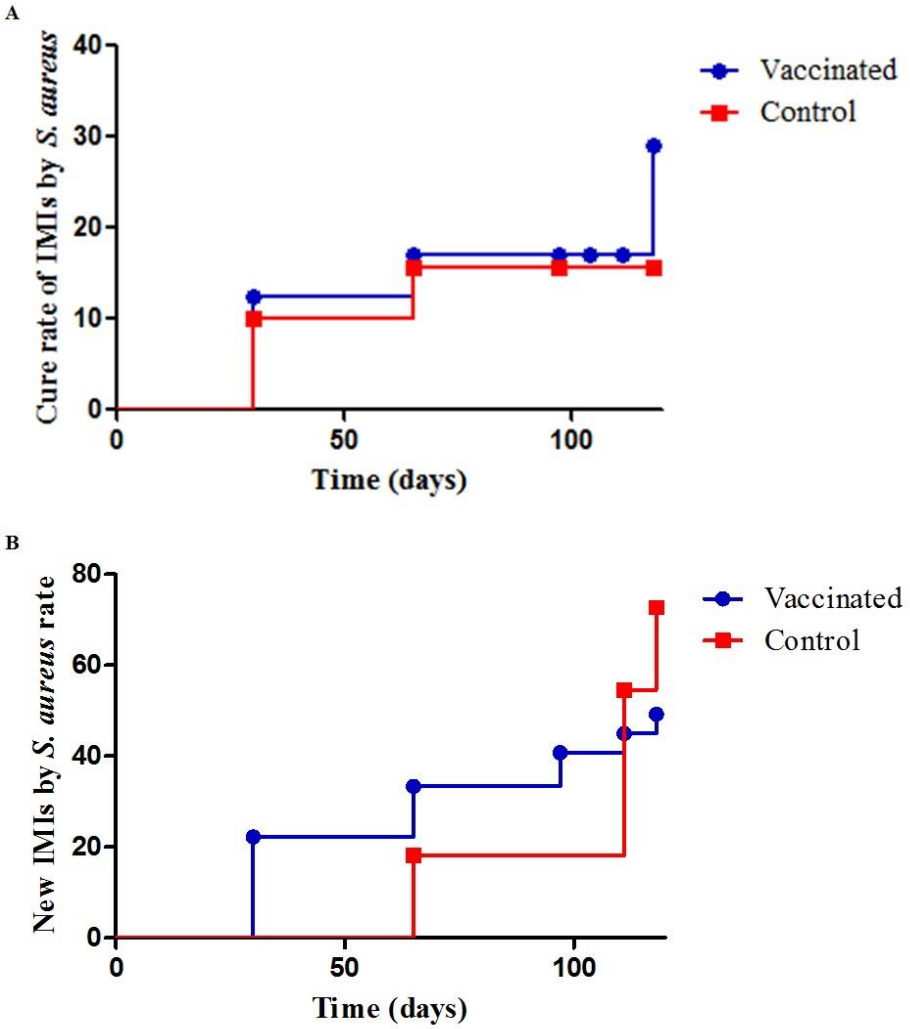

Figure 2. Kaplan-Meier survival curve. A) Time to cure intramammary infections (IMIs) by *Staphylococcus aureus* in vaccinated and unvaccinated control *S. aureus*-infected quarters. B) Time to quarters become infected by *S. aureus* in vaccinated and unvaccinated control *S. aureus*-infected cows.

Discussion

Here, the rate of new IMIs by *S. aureus* and cure of IMIs by *S. aureus* was not statistically affected by vaccination when evaluated as a main effect. Previous studies on *S. aureus* vaccine in dairy cows described in the literature have relatively low or no vaccine efficacy at all (Pereira et al., 2011). It should be noted that the key reason of most lincesed vaccines for viral and bacterial infections to succeed relies on the humoral immune reponse extended by antibody production, however the cellular immunity is required for an effective control of infections by intracellular pathogens (Seder and Hill, 2000; Proctor, 2012). Thus, the unsatisfactory effect of many vaccines against *S. aureus* described in the literature using experimental infections may be due to the increase evidence that *S. aureus* is a facultative intracellular pathogen, and

consequently the fact that the humoral immune response is not fully efficient to remove this pathogen (Sendi and Proctor, 2009; Proctor, 2012). This idea was reinforced by the lower interferon-γ expression in milk samples from chronically *S. aureus* infected cows (Riollet et al., 2001), and studies that have highlighted the significance of interleukin (IL)-17 in *S. aureus* infections (Proctor et al., 2012). For instance, Murphy et al. (2014) identified that the proliferation of T γδ memory lymphocyte populations is crucial for protection against *S. aureus* subsequent infections in murine, and this protection was related to IL-17 production, leading to neutrophil recruiting and activation.

Therefore, these studies reported direct vaccine efficacy and did not attempt to estimate the reduction on infection transmission (Schukken et al., 2014), which in turn estimate the vaccine efficacy toward no effect. For instance, the moderate increase in cure rates of IMIs by this pathogen with vaccination resulted in a lower number of quarters that are shedding *S. aureus*, and consequently in lower probability of *S. aureus* transmission that mainly depends on the incidence of *S. aureus* (Swinkels et al., 2005) due to their contagious behavior (Barkema et al., 2006). Regarding that, vaccine efficacy in the current study had a discreet effect of new IMIs and more pronounced reduction in duration of IMIs by this pathogen, leading to a reduction of basic reproduction ratio (R_0) of approximately 50% similar the findings (45%) described by Schukken et al. (2014).

Furthermore, preventive interventions strategies are influenced by several factors, such as strain characteristics, lactation stage, parity and number of infected quarters. For example, it has been showed that IMIs by *S. aureus* in older cows are less easily influenced by intervention (Sol et al., 1997; Deluyker et al., 2005; Barkema et al., 2006), and should be considered in mastitis control programs. Thus, the considerable percentage of cows in their first and second lactations (62.29%) used here may contribute to our positive results.

Although this data is encouraging, it highlights that vaccination is still only a tool in control of IMIs by *S. aureus*, as demonstrated here by the considerable effect of segregation on IMIs by this pathogen. Besides this, the genetic polymorphism among cows may also be associated to susceptibility/resistance to mastitis pathogens, and might also be related to this finding (Yoshida et al., 2009).

Altogether, although vaccination results appear encouraging, their use should be recommended in conjoint with other adequate management practices, such as segregation, optimization of milking procedures and milking hygiene, dry-cow therapy, and culling of problem cows to effectively reduce incidence and duration of infection.

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9 Conclusions

1. The innate immunity is crucial to maintain mammary gland healthy which is mediated through recognition of pattern recognition receptors (PRRs). The PRRs recognized specific patterns of microbial components that are conserved among pathogens known as pathogen-associated molecular patterns (PAMPs). The interaction of PRRs and PAMPs mediated the inflammatory response characterized by each mastitis-causing pathogen that can contribute to the development of severe acute inflammation or chronic mastitis.
2. The milk SCC and bacteriological examinations, although regarded as the most reliable indicators of ongoing mastitis, should be used in an integrated manner in mastitis control programs. Furthermore, the accuracy of single, duplicate and composite bacteriological analysis to diagnosis mastitis should be considered in mastitis control strategies.
3. The interdependence of quarters based on the influence of infection in neighboring uninfected quarters was strongly supported both by the percentage of B cells and the expression of adhesion molecules (CD11b, CD44 and CD62L) by milk neutrophils.
4. Overall, new insights into *S. dysgalactiae* IMIs was provided, including higher SCC, percentage of milk neutrophils and percentage T cells ($CD3^+$) and their subset $CD3^+CD8^+CD4^-$ cells, and a decrease in the percentage of milk monocytes/macrophages in *S. dysgalactiae*-infected quarters when compared to uninfected ones. *S. dysgalactiae*-infected quarters also showed higher expression of CD11b by milk neutrophils and a tendency toward a decrease in neutrophil apoptosis rate.
5. Our findings provide new insights into the capacity to adhere to and internalize into MEC by different staphylococci species. Firstly, CNS species adhere and internalize into MEC slower than *S. aureus* species. Furthermore our findings provide new insights into the capacity to adhere to and internalize into MEC by different staphylococci strains and species that may be related to intra-species diversity and epidemiological and ecological behavior of different staphylococci which might be linked to the etiology and persistence of IMIs.
6. Vaccination is still only a tool in control of IMIs by *S. aureus*, and should be only recommended in conjoint with other adequate management practices, as demonstrated here by the considerable effect of segregation on new IMIs by *S. aureus*.

10 Appendix I. Somatic cell count in small ruminants: Friend or foe?

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Review

Somatic cell count in small ruminants: Friend or foe?

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ARTICLE INFO

Article history:
Received 17 November 2011
Received in revised form 13 April 2012
Accepted 16 April 2012
Available online 18 May 2012

Keywords:
Ewe
Goat
Immune response
Mammary gland
Mastitis

ABSTRACT

Milk somatic cell counts are the basis of mastitis and milk quality control programs. Milk somatic cell counts for goats, in which neutrophils comprise the main cell type, are higher than those for sheep and cows. In cows, macrophages are the major cell type present in milk, as is the case for sheep. Milk secretion in small ruminants is apocrine in nature, and cytoplasmic particles, similar in size to milk somatic cells, are normal constituents of their milk, although concentrations of cytoplasmic particles are much higher in milk from goats than from ewes. This fact has led to the development of cell counting procedures that are specific for deoxyribonucleic acid to enable accurate milk somatic cell counts to be obtained. Furthermore, milk somatic cell counts in small ruminants are influenced by noninfectious factors such as lactation stage and parity. However, mastitis is the main factor that leads to an increase in somatic cell count due to the response to infection. There is some evidence that physiological variations in the viability, number and proportions of each leukocytes subpopulation may be related to mammary gland immune status, which is associated with periods of increased susceptibility to mastitis. The main etiological agent of mastitis in small ruminants is coagulase-negative staphylococci, and special attention should be paid to this group of bacteria. Unlike in cows, novobiocin-sensitive coagulase-negative staphylococci cause a significant increase in milk somatic cell counts. Finally, the somatic cell count and the California Mastitis Test represent valuable tools for mastitis screening and assessing the disease prevalence, but their predictive values are better in ewes than in goats.

1. Introduction

The somatic cell count (SCC) in small ruminants measures the different cells types present in milk, including leukocytes and epithelial cells. Unlike the merocrine milk secretion that occurs in cows, milk secretion in goats and ewes is largely apocrine in nature, and cytoplasmic particles, similar in size to milk somatic cells, are normal constituents of their milk. These particles are not classified as cells because they do not contain nuclei or deoxyribonucleic acid (DNA), although they do contain large quantities of ribonucleic acid (RNA) and proteins (Andrade et al., 2001; Paape et al., 2001; Madureira et al., 2010). Therefore, there are important differences among dairy ruminants in this regard, and mastitis control in sheep and goats should be approached with this in mind. The SCC in ewes and goats has been focus of many recent studies concerning how to produce the best quality dairy products for human consumption and reduce losses due to mastitis. Therefore, there is a need to summarize the particularities of SCC in small ruminants to improve animal health and

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http://dx.doi.org/10.1016/j.smallrumres.2012.04.005

productivity. This manuscript is a review of the most recent knowledge about SCC in small ruminants and discusses the technological measurements, their use in the diagnosis of mastitis, their relationship to mammary gland immunological responses, the etiology of intramammary infections (IMIs) and the effects of non-infectious factors.

2. Differential somatic cell count

2.1. Uninfected mammary glands

The SCC and the types of cells present in milk from ewes free from IMIs are similar to those observed in milk from cows. In both animals, macrophages are the predominant cell type (45–88%) in milk. Polymorphonuclear leukocytes (PMNLs) comprise about 2–40% of the milk cell population, lymphocytes 6–20%, and eosinophils and epithelial cells are also presented to a lesser extent (Lee and Outteridge, 1981; Morgante et al., 1996a; Menzies and Ramanoon, 2001; Paape et al., 2001; Haenlein, 2002; Bergonier et al., 2003; Blagitz et al., 2008). However, in ewe colostrum, the predominant cell type detected was PMNLs (41–84%), followed by macrophages (8–49%), and lymphocytes (6–11%) (Lee and Outteridge, 1981).

The milk SCC (MSCC) in milk from healthy goats ($270–2000 \times 10^3$ cells mL^{-1}) is higher than the MSCC observed in milk from uninfected cows and ewes. Unlike in milk from cows and ewes, PMNLs comprise the major leukocyte type (40–87%) in goat milk. Because neutrophils act as the first line of immunological defense against infections, this could explain why goats are more resistant to mastitis (Tian et al., 2005). For animals free from IMI infection, macrophages and lymphocytes comprise 15–41% and 4–20%, respectively, of the MSCC, and eosinophils and epithelial cells are present as lower levels (Paape and Capuco, 1997; Winnicka et al., 1999; Menzies and Ramanoon, 2001; Paape et al., 2001; Haenlein, 2002; Bergonier et al., 2003; Raynal-Ljutovac et al., 2007). Cytoplasmic particles are normal constituents of goat milk and colostrum; however, their concentration is 10 times lower in ewe milk (average 15×10^3 cytoplasmic particles/mL) than in goat milk (average 150×10^3 cytoplasmic particles/mL) (Paape et al., 2001).

Recently, Boulaaba et al. (2011) measured the proportions of different immune cells in milk from intramammary halves from goats that were free from infection by flow cytometry. PMNLs, lymphocytes, and macrophages comprised 79.2% (±11.5), 2.8% (±3.6) and 17.8% (±10.2) of the cells, respectively. Similar proportions of PMNLs ($80.9 \pm 9.3\%$), macrophages ($15.0 \pm 7.7\%$) and lymphocytes ($4.2 \pm 3.9\%$) were detected using a microscopic cell counting procedure specific for DNA. However, Bagnicka et al. (2011) found that epithelial cells (about 70%) were the major cell type present in the milk from uninfected udder halves, as evaluated by flow cytometry. We suppose that the monoclonal antibodies used to identify epithelial cells can also detect cytoplasmic particles, which are shed into milk from the apical portion of mammary secretory cells, resulting in the higher proportion of epithelial cells in this study.

Albenzio and Caroprese (2011) also observed significant strong correlations between the percentage of lymphocytes ($r = 0.84$), PMNLs ($r = 0.94$) and macrophages ($r = 0.79$) by microscopic cell counting specific for DNA and flow cytometry in ewe milk with low and high SCCs.

There are physiological variations in differential cell counts (DCC) in small ruminants, for example, at the lactation stage (Morgante et al., 1996a; Cuccuru et al., 1997; Winnicka et al., 1999; Bergonier et al., 2003; Blagitz et al., 2008).

Cuccuru et al. (1997) reported that the percentage of macrophages and PMNLs in ewe increased as lactation continued, whereas lymphocytes and epithelial cells showed a uniform decrease. These facts may be related to the need for an activated immune defense status close to drying-off, when the mammary glands need to recover from current infections and avoid new ones. Morgante et al. (1996b) also reported an increase in neutrophil and eosinophil percentages close to the dry period, while the percentage of macrophages decreased during middle lactation and increased again near the dry period in ewes.

Regression or involution of the mammary gland takes place either at the end of lactation or following weaning, during which time the morphology of the glands alters dramatically. This process can have a major influence on local immune cells and the relative proportions of the different leukocyte subpopulations present in the mammary gland (Tatarczuch et al., 2002). In ewes, neutrophils were shown to be the predominant leukocytes in mammary secretions during early involution up to four days after weaning, but this cell population declined dramatically at day seven, after which point macrophages represented 50–90% of all leukocytes (Tatarczuch et al., 2000, 2002). During early involution up to 60 days after weaning, most lymphocytes in the alveolar and ductal epithelium were $CD8^+$ (cytotoxic T lymphocytes), some were $CD45R^+$ (memory cells) and few were $CD4^+$ (T helper lymphocytes). By seven to 60 days after weaning, most cells in the alveolar and ductal lumina were macrophages followed by predominantly $CD8^+$ lymphocytes (Tatarczuch et al., 2000). There are two waves of migration of phagocytic cells into the mammary gland during involution, the first involving the recruitment of neutrophils in the first two days after weaning. At two days after weaning, numerous neutrophils had infiltrated into the mammary gland, and most of them had lost their cytoplasmic granules and had reduced glycogen reserves resulting from the ingestion of casein, lipid droplets and cellular debris, thereby eliminating the pseudopodia required for phagocytosis. Therefore, as expected, the phagocytic index was significant higher one day post-weaning compared with two days after weaning because the phagocytic function of freshly infiltrated neutrophils was much higher. Additionally, as neutrophils have a short half-life, they undergo apoptosis that would also lead to reduction in the immune function of this population. Similarly, macrophages collected during early involution were heavily laden with ingested casein, lipid and cellular debris, and they lacked pseudopodia. These macrophages were shown to be less phagocytic than those obtained from fully involuted glands (Tatarczuch et al., 2002). An elevation of MHC class II^+ and interleukin-2 receptor ($IL\text{-}2R^+$)

in lymphocytes were observed in dry secretions compared with milk in ewes. IL-2R is expressed in activated B and T lymphocytes. An increase in the percentage of $CD14^+$ leukocytes, which is expressed in neutrophils and especially in macrophages (Paape and Capuco, 1997), was also observed in dry secretions compared with milk in ewes (Persson-Waller and Colditz, 1998). Similarly, Blagitz et al. (2008) also found an increase in the number of leukocytes in ewe milk and a tendency toward an increase in the percentage of PMNLs seven days after weaning compared with 12 days before weaning. Therefore, the cellular constituents of the mammary gland during the latter stage of involution may make the mammary gland more resistant to infection than during lactation or the early stages of involution (Persson-Waller and Colditz, 1998; Tatarczuch et al., 2000). In fact, the reduction in local immunity coincides with an increase in clinical cases of mastitis (Moroni and Cuccuru, 2001).

It should be kept in mind that ewes with low plasma cortisol concentrations showed lower MSCCs and higher milk production than those with high levels. Plasma cortisol concentration is also connected to behavioral responses and immune competence (cytokine production and blood $CD4^+/CD8^+$ lymphocytes ratio), and both IL-6 and IL-1β were considered to be reliable indicators of the magnitude of hypothalamic-pituitary-adrenal axis activation. Therefore, the hypothalamic-pituitary-adrenal axis is also critical for controlling diseases and can be associated with an increased predisposition toward the development of intramammary inflammatory processes (Caroprese et al., 2010).

In goats, Winnicka et al. (1999) using Flow cytometry found that the percentage of T helper lymphocytes ($CD2^+/CD4^+$) in milk among total lymphocytes steadily increased from the post-parturition period ($1.33 \pm 0.49\%$) until the 14th day of lactation ($19.83 \pm 6.53\%$), but decreased during the mid-lactation period. Cytotoxic T cells ($CD2^+/CD8^+$) increased during the colostrum period until the 21st day, and then remained at the same level ($19.35 \pm 5.32\%$) during mid-lactation. In this study, the percentage of γδ lymphocytes among total lymphocytes increased from $3.33 \pm 1.53\%$ on the 1st day of lactation to $25.33 \pm 10.81\%$ during the mid-lactation period.

The active stage of immune defense requires viable and immune competent somatic cells for the rapid and efficient elimination of the pathogen (Baumert et al., 2009). Therefore, decreasing PMNL viability is associated with impaired phagocytic and bactericidal function (Van Oostveldt et al., 2002; Tian et al., 2005). Evasion of neutrophil defenses provides an opportunity for invading bacteria to become established. In this context, Blagitz et al. (2011) suggested that the lower cell numbers and the reduction in PMNL viability in healthy mammary glands from goats with low MSCCs compared with uninfected animals with high MSCCs could explain the higher susceptibility to IMIs of halves with low MSCCs. In fact, some authors have proposed that healthy ruminants with low MSCCs are more prone to mastitis compared with uninfected animals with high cellularity (Bergonier et al., 2003; Moroni et al., 2005a).

The presence of leukocytes in milk and dry mammary gland secretions is related to udder health and susceptibility to infection. However, there is little information available to indicate whether changes in different cells populations in the mammary glands during the course of lactation are the result of changes in cellular activity in small ruminants. Although there is a general consensus in the literature on dairy cows regarding the importance of creating a model to evaluate mammary gland immune status, this aspect has been barely studied in small ruminants (Moroni and Cuccuru, 2001).

2.2. Infected mammary glands

Shortly after the entry of an invading pathogen, resident leukocytes and epithelial cells initiate the inflammatory response necessary to eliminate the invading bacteria in ruminants' mammary glands (Paape and Capuco, 1997; Paape et al., 2002, 2003; Rainard and Riollet, 2003). These cells release chemoattractants for the rapid recruitment of PMNLs, mainly neutrophils, from the blood to the site of infection. During this process, the SCC increases, mainly due to influx of neutrophils (Paape and Capuco, 1997; Paape et al., 2007; Albenzio and Caroprese, 2011), which that can comprise over 90% of the SCC (Morgante et al., 1996a; Cuccuru et al., 1997). Neutrophils are essential for innate host defenses against invading microorganisms and eliminate pathogens through a process known as phagocytosis. During phagocytosis, PMNLs release reactive oxygen species into pathogen-containing vacuoles to kill the invading pathogen (Paape and Capuco, 1997; Paape et al., 2002, 2003; Rainard and Riollet, 2003).

Regarding the immunobiology of the mammary gland, Tian et al. (2005) observed that 30% of goat milk neutrophils were undergoing cell death, through either necrosis or apoptosis, in contrast with 8% of blood neutrophils. In addition, the amount of gelatinase, which mediates neutrophil recruitment into the mammary gland during mastitis, released from milk neutrophils was lower than that of blood neutrophils. The reactive oxygen intermediates generated by activated milk neutrophils, which indicates the bactericidal activity of PMNLs, peaked earlier in milk than in blood neutrophils, but the duration and intensity of their production were much lower. It has been suggested that milk PMNLs are less functional than blood PMNLs. In milk, the ingestion of particles, such as fat and cellular debris, by PMNLs causes a loss of phagocytic and microbicidal function (Paape and Capuco, 1997; Paape et al., 2002, 2003, 2004). Although the inhibition of phagocytosis by PMNLs from goats was lower compared with cows, that may be due to the fact that the fat globules in goat milk are considerably smaller and more fragile than those in cow milk (Paape et al., 2004). Furthermore, the phagocytic activity of PMNLs was impaired in bacteriologically positive udders (Moroni and Cuccuru, 2001).

Although, several SCC thresholds have been used to diagnose mastitis, DCC can be used to distinguish physiological from pathological cell variations, because physiological and environmental factors have a smaller influence on the number of PMNLs. Therefore, DCC provides a more sensible method of identifying IMIs (Cuccuru et al., 1997).

To diagnosis mastitis in the early stages, the use of flow cytometry with monoclonal antibodies to identify each cell population has been proposed. This technique allows the enumeration of the different cells types present in milk (Koess and Hamann, 2008; Albenzio et al., 2009; Albenzio and Caroprese, 2011; Bagnicka et al., 2011; Boulaaba et al., 2011).

Boulaaba et al. (2011) observed in goat infected udder halves that the percentage of PMNLs of milk leukocytes ($CD45^+$) measured by flow cytometry was higher ($85.5 \pm 6.0\%$), and the percentages of macrophages ($13.0 \pm 5.6\%$) and lymphocytes ($1.4 \pm 0.9\%$) were lower compared with uninfected ones. Bagnicka et al. (2011) found that the percentages of PMNLs of milk cells by flow cytometry in milk from mammary glands infected with minor or major pathogens were 21% and 32%, respectively, values higher than the uninfected ones. In contrast, the proportion of lymphocytes (2–2.5%) of milk cells was not affected by infection. In this study, the automatic SCC was also strongly and positively correlated with both the total leukocyte content ($r = 0.65$) and the subpopulations of neutrophils ($r = 0.59$), eosinophils ($r = 0.49$), monocytes/macrophages ($r = 0.54$) and lymphocytes ($r = 0.15$). The stronger correlation between the SCC and the percentage of phagocytes, i.e. PMNLs and monocytes/macrophages, indicated that this is the main population of leukocytes that are protective against IMIs.

In ewes, the incidence of clinical mastitis is higher in the early lactation period, followed by a constant incidence until weaning (Bor et al., 1989; Mork et al., 2007; Arsenault et al., 2008; Waage and Vatn, 2008). The lower MSCCs observed in early lactation and the risk of teat contamination by lambs may explain this pattern because pathogenic bacteria that may be present in the mouths of lambs could be mechanically transmitted to ewes (Arsenault et al., 2008; Gougolis et al., 2008). A high incidence of subclinical mastitis was also described at the beginning of lactation in ewes (Kirk et al., 1996; Leitner et al., 2001; Bergonier et al., 2003; Guaraná et al., 2011), although the prevalence of subclinical mastitis throughout the lactation period remained relatively constant (Al-Majali and Jawabreh, 2003; Bergonier et al., 2003; Blagitz et al., 2008, 2012). It is generally accepted that the persistence of subclinical IMIs in ewes during lactation is often high due to the poor detection and elimination of such infections during lactation and their frequent staphylococcal origin (Bergonier and Berthelot, 2003; Bergonier et al., 2003; Blagitz et al., 2012).

In goats, higher rates of subclinical mastitis have also been observed at the beginning of lactation, but the prevalence seems to increase throughout lactation (East et al., 1987; Sánchez et al., 1999; Bergonier et al., 2003); however, no-consensus has been found. For example, Min et al. (2007) found that the incidence rate of new infections was highest during the early and the late stages of lactation. Moroni et al. (2005b) found that the first month of lactation was characterized by the lowest incidence of new infection (7.21%), whereas the highest incidence rates were observed during mid-lactation (24.5%) and the last month of lactation (58.4%). However, one-third of clinical cases occurred in the first week postpartum, and the second most important period for goats was from four to seven weeks after kidding.

2.3. Somatic cell count thresholds

In ewes, the majority of proposed threshold values for discriminating between healthy and infected halves are lower than 500×10^3 cells mL^{-1} (González-Rodriguez et al., 1995; Morgante et al., 1996a; McDougall et al., 2001; Paape et al., 2001; Pengov, 2001; Gonzalo et al., 2002; Bergonier and Berthelot, 2003; Leitner et al., 2004a; Berthelot et al., 2006; Blagitz et al., 2008; Nunes et al., 2008). However, higher values, up to 1500×10^3 cells mL^{-1}, have been suggested by some authors (Fthenakis et al., 1991; Mavrogenis et al., 1995; Lafi, 2006). The importance of the control of MSCCs in ewes was demonstrated by Spanu et al. (2011), who showed that milk samples obtained from ewes with three or more monthly SCCs $\geq 400 \times 10^3$ cells mL^{-1} during previous lactation periods were 5.6–7.5 times more likely to be microbiologically positive for mastitis pathogens compared with milk samples obtained from ewes with MSCCs below this threshold. Indeed, an increase in MSCC in ewes was associated with a greater risk of being culled (Riggio et al., 2009).

In goats, De Crémoux et al. (1996) set a threshold at 750×10^3 cells mL^{-1} for predicting the presence of minor pathogens and a threshold at 1750×10^3 cells mL^{-1} for major pathogens. Most of the MSCC thresholds used to identify IMIs in goats range from 500 to 1000×10^3 cells mL^{-1} (Poutrel and Lerondelle, 1983; De Crémoux et al., 1996; Contreras et al., 1996; McDougall et al., 2001; Haenlein, 2002; Moroni et al., 2005b; Hall and Rycroft, 2007). However, Persson and Olofsson (2011) proposed a threshold of 345×10^3 cells mL^{-1} to differentiate between infected and non-infected glands in goats.

In contrast to cow milk, a single MSCC test of goat milk has little value unless the stage of lactation and parity is taken into account (Rota et al., 1993; Haenlein, 2002; Moroni et al., 2005b). Curvilinear and linear regressions have been calculated to predict of MSCC depending on physiological factors, such as lactation stage. For instance, Rota et al. (1993) have proposed the following regression equation for MSCC that incorporates the lactation stage: $SCC\ (n) = 1.37 \times n^{-0.34} \times e^{n0.01}$, where SCC ($n$) is the MSCC measured on the nth test day, and e is the base of the natural logarithm, using the incomplete gamma function proposed by Wood.

In goats, a finite mixture model (FMM) was applied to MSCC to assess the ability of the model to classify records from healthy and infected glands, and it was able to correctly classify 60 and 48% of the healthy and infected observations, respectively. This was slightly higher than would be expected from random classification, but not accurate enough for useful mastitis diagnosis. The rates of sensitivity varied according to the type of pathogen; the greatest sensitivity was observed in udder halves infected with *Staphylococcus aureus*, and this was associated with the highest increase in SCC. In general, factors that increased the difference in somatic cell score between healthy and infected mammary glands increased the sensitivity of the FMM (Boettcher et al., 2005). Similarly, Moroni

et al. (2005b) found that the sensitivity and specificity of MSCC to determine infection status was also low during the first two months of lactation in goats, when the false-negatives rate increased up to 58%, and during late lactation, when the false-positive rate increased up to 40%, using 850×10^3 cells mL^{-1} as a threshold. Using a composite SCC to detect mastitis by *S. aureus* in goats, Koop et al. (2011) proposed a cut-off value of 1500×10^3 cells mL^{-1}; they reached 0.90 sensitivity and 0.95 specificity, and this method was also influenced by lactation stage.

When making recommendations about a diagnostic test, it is important to consider the consequences of false-negative and false-positive results. In the case of mastitis, the costs of treatment or culling of positive animals are important factors, and the aim is generally to reduce the overall prevalence of infection without necessarily eliminating the disease; therefore, specificity takes priority. However, to eliminate a disease, the sensitivity would have a higher priority than specificity (Clements et al., 2003; Lafi, 2006; Nunes et al., 2008).

The definitive identification of infected animals relies on the positive culture of pathogens from aseptically collected milk samples. The problem regarding mastitis is that indirect tests, such as MSCC, are compared with the "gold standard" of bacteriology, and mastitis does not always require the presence of infection (Pyörälä, 2003). In addition, subclinical mastitis may not be accompanied by the isolation of the etiological agent for several reasons: microorganisms may be excreted in an intermittent way and in small amounts; infection-related pathogens are not detectable using conventional microbiological tests; some milk enzymes or proteins (i.e. lysozyme and lactoferrin) may thwart pathogen detection; and the infection is supported by bacterial endotoxins and bio-active compounds released by neutrophils that outlive the elimination of the invading bacteria (Albenzio et al., 2002; Arsenault et al., 2008; Nunes et al., 2008). Moreover, some bacteria may have been present in the teat canal without causing significant inflammation, and others may have been present as contaminants (Arsenault et al., 2008). With this in mind, some authors have isolated mastitis pathogens from milk samples with very low MSCCs, whereas others have found high proportions of bacteriologically negative milk samples with high MSCCs (Leitner et al., 2001; Albenzio et al., 2002; Nunes et al., 2008; Della Libera et al., 2011). These findings suggest that MSCC and bacteriological examinations, although regarded as the most reliable indicators of ongoing mastitis, might fail to diagnose mastitis when not used in an integrated manner (Albenzio et al., 2002).

Threshold recommendations have moved toward the use of consecutive individual SCCs per lactation and the definition of two or three thresholds within the same detection system. An important reason for this is the staphylococcal origin of most cases of small ruminant mastitis. These cases are characterized by dynamic fluctuations and cyclic bacterial shedding in milk, which potentially cause false negative bacteriological results. This bacterial cyclicity leads to fluctuations in MSCCs that normally fluctuate depending on the organism's number and viability (Bergonier et al., 2003).

The prevalence of mastitis in ewes may be assessed by the analysis of bulk milk SCCs (BMSCCs), which are an easy and cheap way to estimate the whole flock/herd IMIs (Bergonier et al., 2003) and can used to determine the overall IMI prevalence with a good coefficient of determination when using 500×10^3 cells mL^{-1} as a threshold (Bergonier and Berthelot, 2003; Berthelot et al., 2006). For instance, a geometric mean bulk MSCC of 650×10^3 cells mL^{-1} corresponds to an IMI prevalence of about 15% in ewes, and a variation of 100×10^3 cells mL^{-1} reflects a variation of 2–3% in the prevalence (Berthelot et al., 2006).

Leitner et al. (2008) estimated milk and curd losses as a function of IMIs and projected MSCC due to IMIs in ewes and goats. For different MSCCs and percentages of udder sections infected in ewes, they suggested milk yield and milk curd yield losses of 4.1 and 5.2% (25% of udder halves infected; MSSC = 800×10^3 cells mL^{-1}), 8.2 and 10.4% (50% of udder halves infected; MSSC = 1400×10^3 cells mL^{-1}), and 12.2 and 15.5% (75% of udder halves infected; MSSC = 2000×10^3 cells mL^{-1}), respectively. The same prediction was applied to goats, where they proposed milk yield and milk curd yield losses of 0.8 and 3.3% (25% of udder halves infected; MSSC = 840×10^3 cells mL^{-1}), 1.5 and 6.5% (50% of udder halves infected; MSSC = 1200×10^3 cells mL^{-1}), and 2.3 and 9.8% (75% of udder halves infected; MSSC = 1600×10^3 cells mL^{-1}), respectively. The larger effects predicted in milk from ewes compared with goats can be explained by the higher casein content and plasmin activity in ewes' milk. Consequently, during IMIs there is greater liberation of casein degradation products in sheep, and these products contain active peptides that down-regulate milk secretion and further reduce milk yield. In addition, a larger volume of milk is needed to produce 1 kg of cheese from goat milk and higher curd loss occurred in goat milk, which explains the greater curd loss due to IMIs despite a lower milk loss compared with sheep.

Koop et al. (2010) used a mixed linear regression to estimate the effect of IMIs on milk yield losses in goats. Infection with coagulase-negative staphylococci (CNS) did not affect milk yield, whereas infection with *Corynebacterium bovis* was associated with increased milk yield. Therefore, the negative correlation between MSCC and milk yield can be explained only to a limited extent by IMIs. Important factors that affect milk yield and MSCC are lactation stage and pregnancy. The correlation between MSCC and milk yield was stronger in a subset of uninfected animals than in the total data set, and a dilution effect seems to play an important role in the negative association between MSCC and milk yield. Some authors reported no significant difference in milk yield between uninfected and infected goats (Moroni et al., 2005a; Min et al., 2007), although others reports found a decrease in milk yield in infected glands (Ying et al., 2002; Leitner et al., 2004a,b,c, 2008).

2.4. California mastitis test

The California mastitis test (CMT) has been widely used to indirectly measure MSCC because it is animal-side, inexpensive and rapid to perform (McDougall et al., 2001; Lafi,

2006; Nunes et al., 2008; Persson and Olofsson, 2011). The first study that evaluated the sensitivity and specificity of CMT was performed by Hueston et al. (1986), who observed a sensitivity of 69.3% and a specificity of 76.5% when testing udders halves from ewes.

Afterwards, several studies evaluated the CMT as an indirect test for mastitis. In ewes, most of these studies suggested a cut-off of 1 for the CMT to identify infected glands (Hueston et al., 1986; González-Rodríguez and Cármenes, 1996; Las Heras et al., 1999; McDougall et al., 2001; Clements et al., 2003; Nunes et al., 2008; Peixoto et al., 2010). Strong correlations between the CMT and the bacteriological exam (73.2%) (Peixoto et al., 2010) have been reported using the CMT score of 1 as a threshold. Arsenault et al. (2008) also reported that the percentage of culture-positive halves increased from 11.7 to 53.6% between halves with negative CMT scores and those with scores of 3. Nevertheless, Lafi (2006) has suggested that uninfected halves can present with nil CMT scores, trace amounts of precipitate or distinct precipitate/weak gel formation.

Strong correlations between CMT and automatic or microscopic cell counts ($r = 0.65$; Suarez et al., 2002) ($r = 0.75$; Nunes et al., 2008) ($r = 0.77$; McDougall et al., 2001) ($r = 0.82$; Della Libera et al., 2011) ($r = 0.84$; Gomes et al., 2006) have been described in sheep. In addition, the correlations between CMT scores and neutrophils, macrophages, lymphocytes and eosinophils were 0.356, −0.406, −0.057 (not significant) and 0.19, respectively. It is well-known that CMT scores depend not only on the SCC but also on cellular ratio changes, especially due to increases in PMNLs, as suggested by Della Libera et al. (2011), who observed that neutrophil counts increased about 9, 130 and 179 times in samples with CMT scores of 1, 2 and 3, respectively, compared with samples with negative CMT scores.

In goats, most studies have proposed a score 2 as the threshold for the detection of infected glands (Santos et al., 1995; Contreras et al., 1996; McDougall et al., 2001; Haenlein, 2002; McDougall et al., 2010; Peixoto et al., 2010; Persson and Olofsson, 2011). Similarly, Peixoto et al. (2010) reported better agreement (81.4%) with bacteriological exams for the CMT scores ≥ 2.

Strong correlations were also observed between CMT scores and the microscopic ($r = 0.63$; Da Silva et al., 2001) and automatic cell count by the Fossomatic ($r = 0.71$; Haenlein, 2002) ($r = 0.95$–0.97; Marín et al., 2001) and Coulter Counter method ($r = 0.65$; Haenlein, 2002), which may be related to the higher proportion of neutrophils, rather than cytoplasmic particles, in goat milk samples from uninfected halves. These cytoplasmic particles did not react with the CMT reagent, as they lacked nuclei (Petersen, 1981).

In ewes, variations in individual MSCCs according to the infection status of udder halves made it possible to distinguish healthy udders from unilaterally and bilaterally infected ones (Berthelot et al., 2006). Due the high variability of MSCCs in goats caused by infection and physiological factors, Santos et al. (1995) recommended comparing the results from both udders halves; conflicting results can indicate inflammation in one of the halves. Min et al. (2007) and Barth et al. (2010) encountered a significant increase in MSCC in the uninfected halves of infected goat udders compared with the MSCCs of udders free from infection. This result indicates a dependency between the halves within one udder and, therefore, an effect of the infected half on its corresponding uninfected half. The authors recommended monitoring mastitis in goats by measuring of lactoferrin, an antimicrobial protein released by PMNLs after an inflammation, and N-Acetyl-β-D-glucosaminidase (NAGase), a lysosomal enzyme for which the major source is PMNLs, instead of using MSCCs in these animals. However, Leitner et al. (2001) reported a strong correlation between MSCC and NAGase activity in ewe milk. Other reports have also suggested the use of NAGase activity as an indicator of inflammation in small ruminants (Leitner et al., 2001; Moroni and Cuccuru, 2001; Leitner et al., 2004a,b,c).

Altogether, the MSCC and the CMT are regarded as the best indirect tests to diagnose IMIs in both sheep and goats, when interpreted correctly (Bergonier and Berthelot, 2003; McDougall et al., 2001; Luengo et al., 2004; Nunes et al., 2008; Persson and Olofsson, 2011).

2.5. Mammary pathogens and somatic cell count

Traditionally, the most common mastitis-causing agents have been classified as minor and major pathogens according to the degree of inflammation they produce in the mammary gland; these pathogens are summarized in Table 1 for ewes and Table 2 for goats.

It is widely accepted that the increase in of MSCC is related to the pathogenicity of the etiological agent of the IMI. Several studies pointed to CNS as the main etiological agent of small ruminant IMIs (Leitner et al., 2001; Moroni and Cuccuru, 2001; Haenlein, 2002; Bergonier et al., 2003; Bergonier and Berthelot, 2003; Leitner et al., 2004a, 2004c; Boettcher et al., 2005; Moroni et al., 2005a, 2005b; Berthelot et al., 2006; Contreras et al., 2007; Min et al., 2007; Raynal-Ljutovac et al., 2007; Nunes et al., 2008; Della Libera et al., 2010; Cuccuru et al., 2011; Guaraná et al., 2011). The main species of CNS isolated from infected udder halves in small ruminants were *Staphylococcus epidermidis*, *S. chromogenes*, *S. simulans*, *S. xylosus* and *S. caprae* (Ariznabarreta et al., 2002; Bergonier et al., 2003; Moroni et al., 2005a, 2005b; Contreras et al., 2007; Cuccuru et al., 2011; Leitner et al., 2011). The high MSCCs in the CNS infected halves in small ruminants differ from the mild or moderate MSCCs observed in similarly infected dairy cows. These results suggest that sheep and goat udders have a lower resistance or/and an increased immunological response against this group of bacteria (Leitner et al., 2001; Pengov, 2001; Clements et al., 2003; Leitner et al., 2004a,c; Berthelot et al., 2006; Contreras et al., 2007). With this in mind, special attention was given to novobiocin-sensitive strains such as *S. epidermidis*, *S. chromogenes*, *S. aureus*, *S. simulans*, *S. haemolyticus*, *S. homonis*, *S. caprae*, *S. capitis*, *S. hyicus*, *S. auricularis* and *S. intermedius*, which lead to a significant increase in MSCC (Paape et al., 2001; Ariznabarreta et al., 2002; Gonzalo et al., 2002).

Leitner et al. (2011) reported that SCCs from uninfected glands ($462 \pm 76 \times 10^3$ cells mL^{-1}) were lower than those from glands infected by *S. epidermidis*

Table 1
Somatic cell counts ($\times 10^3$ cells mL^{-1}) in uninfected ewe udder halves and those infected with different pathogens.

References	Pathogens			
	Uninfected	Coagulase-negative staphylococci	Minor pathogens	Major pathogens
González-Rodriguez et al. (1995)	130	1200	450–490	4000–4800
Leitner et al. (2001)	388	1371–2129	–	–
Ariznabarreta et al. (2002)	70	–	72–160[a]	850–19,000[b]
Gonzalo et al. (2002)[e]	77	1005[c]	131[a]	1841
Suarez et al. (2002)[e]	244.5	–	1044[d]	2046

[a] Including novobiocin-resistant coagulase-negative staphylococci.
[b] Including novobiocin-sensitive coagulase-negative staphylococci.
[c] Only novobiocin-sensitive coagulase-negative staphylococci.
[d] Including coagulase-negative staphylococci.
[e] Geometric means of the somatic cell counts are shown.

Table 2
Somatic cell counts ($\times 10^3$ cells mL^{-1}) in uninfected goat udder halves and those infected with different pathogens.

References	Pathogens			
	Uninfected	Coagulase-negative staphylococci	Minor pathogens	Major pathogens
Lerondelle et al. (1992)	520	–	1040	7890
De Crémoux et al. (1996)	973	–	1564	3591
Poutrel et al. (1996)[d]	272	–	932	2443
Haenlein (2002)	614	1293		4804[a]
Luengo et al. (2004)[d]	645	–	1023[b]	4073
Hall and Rycroft (2007)	428	>2000	>2500[c]	>4000
Koop et al. (2010)[d,e]	196–358	605–1075	605–933[c]	2122–3141

[a] All other pathogens.
[b] Including coagulase-negative staphylococci.
[c] Including only *Corynebacterium* spp.
[d] Geometric Means of the somatic cell counts are shown.
[e] Composite somatic cell counts are shown.

($1570 \pm 558 \times 10^3$ cells mL^{-1}), *S. caprae* ($1143 \pm 256 \times 10^3$ cells mL^{-1}), *S. simulans* ($2258 \pm 505 \times 10^3$ cells mL^{-1}) *and S. aureus* ($5406 \pm 732 \times 10^3$ cells mL^{-1}) from experimentally infected goats. The increase in MSCC was mainly due to the recruitment of leukocytes, especially PMNLs. The percentages of PMNLs in uninfected mammary glands and those infected by *S. epidermidis*, *S. caprae*, *S. simulans* and *S. aureus* were $51.8 \pm 3.5\%$, $84.7 \pm 3.0\%$, $70.5 \pm 3.8\%$, $83.2 \pm 3.5\%$ and $95.1 \pm 3.4\%$, respectively. The highest MSCC and percentages of PMNLs were recorded in the milk from *S. aureus*-infected glands. No differences were found between the percentages of T- and B lymphocytes and macrophages. However, the numbers of these cells were higher in all milk from infected glands than in milk from uninfected glands. The numbers of B cells were negligible in all the milk samples. Different levels of bacterial attachment and phagocytosis by PMNLs were observed with different species; the highest and lowest attachment and phagocytosis were levels recorded with *S. aureus* and *S. simulans*, respectively. Bacterial killing assays using whole blood cells from uninfected goats showed that *S. caprae* and *S. epidermidis* were the most sensitive (log reduction 2.68 ± 0.1 and 2.61 ± 0.3, respectively), *S. simulans* showed moderate log reduction (2.0 ± 0.1), and *S. aureus* was the most resistant (1.69 ± 0.3). The inhibition of lymphocytes from uninfected goats with exosecretions from each bacterial demonstrated that the $CD4^+/CD25^+$, $CD8^+/CD25^+$ and $CD21^+/CD25^+$ cells were only activated by *S. aureus* exosecretions. Bacterial exosecretions, both homologous and heterologous, decreased the rate and percentage of PMNL activation; however the dynamics of activation were faster after incubation with homologous exosecretions compared with heterologous exosecretions in infected goats. In contrast, bacterial exosecretions had no effect on cells from uninfected goats. All the infected goats were seropositive for IgG antibodies to the homologous bacteria, and their $CD4^+$ and $CD8^+$ lymphocytes exhibited stronger responses to bacterial exosecretions than cells from uninfected goats, which indicates the activation of systemic immunity, including memory cells. In contrast, an earlier study in dairy cows showed that about 17% of cows chronically infected with *S. aureus* were seronegative for this bacterium (Leitner et al., 2000). These findings indicated that the immune system of goats is more efficient in transferring the bacteria to the supra-mammary lymph node, and this may be related to the bacterial type, i.e., CNS vs. *S. aureus* (Leitner et al., 2011).

As described above, an essential part of the udder's defense is the accumulation of neutrophils, which is induced by bacterial products and pro-inflammatory cytokines, such as interleukin (IL)-8 (Paape and Capuco, 1997; Paape et al., 2002, 2003; Winter et al., 2003). In ewes experimentally infected with *S. epidermidis*, IL-8 appeared in milk two hours after infection and preceded the appearance of IL-1β, which can activate neutrophils for enhanced phagocytosis and intracellular killing of bacteria; IL-1β peaked at 24 h. Congruently, the influx of neutrophils into milk started to rise four hours post-infection and peaked 24 h after infection. Early recruitment of leukocytes led to

a transient clearance of bacteria from the milk of ewes. Staphylococci were absent from milk two and three days after infection but re-emerged intermittently, shedding bacteria at subsequent samplings. MSCCs in milk remained elevated while IL-8 concentrations were high and during the intermittent re-emergence of bacteria in milk. In contrast, in sheep that cleared bacteria from the gland by 24 h and whose milk remained free from bacteria for the remainder of the experiment, the IL-1β and IL-8 levels and MSCCs were similar to the control group from one week after infection until the end of the experiment (Winter et al., 2003).

IMIs caused by *S. aureus* also warrant special attention because this bacterium is responsible for both clinical and subclinical mastitis (Bergonier and Berthelot, 2003; Bergonier et al., 2003; Moroni et al., 2005b; Contreras et al., 2007; Mork et al., 2007; Arsenault et al., 2008; Guaraná et al., 2011) and is generally associated with the highest MSCCs (Bergonier and Berthelot, 2003; Bergonier et al., 2003; Boettcher et al., 2005; Mork et al., 2007; Hall and Rycroft, 2007; Arsenault et al., 2008; Leitner et al., 2011) in sheep and goats. *Manhemia haemolytica* and CNS, and a lesser extent, environmental pathogens, are also important etiological agents of clinical mastitis (Bergonier et al., 2003; Mork et al., 2007; Arsenault et al., 2008). Acute cases of clinical mastitis can become chronic leading to a persistent infection that lasts for several months (Bergonier and Berthelot, 2003; Bergonier et al., 2003).

2.6. Automatic and microscopic somatic cell couting

The MSCC can be enumerated by automatic and microscopic methods. At present, the direct microscopic SCC method using methylene blue staining is the reference method recommended by the International Dairy Federation (IDF, 1995), but this method can overestimate the MSCC of small ruminant milk, especially goat milk, due to high concentrations of cytoplasmic particles (Paape et al., 2001).

To obtain accurate MSCCs for small ruminants, only counting procedures specific for DNA should be used, such as the DNA-specific stains pyronin Y-methyl green or May-Grünwald-Giemsa (Paape et al., 2001; Haenlein, 2002; Gonzalo et al., 2003; Madureira et al., 2010; Petersson et al., 2011; Boulaaba et al., 2011). Such stains are currently considered the official standard reference method in the USA (Paape et al., 2001; Contreras et al., 2007).

The automatic MSCC can be obtained by the Fossomatic method based on fluoro-optical principles for nuclear DNA that binds to ethidium bromide dye and forms a fluorescent complex (Gonzalo et al., 1993, 2003, 2004; Petersson et al., 2011). The Fossomatic method counts both epithelial cells and leukocytes, but the counts are not confounded by cytoplasmic particles. The process is rapid, repeatable and cheap (Hall and Rycroft, 2007).

The Fossomatic method and DNA-specific stains give comparable results (Haenlein, 2002), and strong and significant correlations have been described between these methodologies in ewes ($r = 0.99$; Menzies and Ramanoon, 2001) ($r = 0.986$; Gonzalo et al., 1993) ($r = 0.972$–0.996; Gonzalo et al., 2003) ($r = 0.977$–0.994; Gonzalo et al., 2004).

When the Fossomatic machine was calibrated with goat milk, comparable results were given by the Fossomatic method and microscopic counting using DNA-specific stains (Zeng et al., 1999; Haenlein, 2002). However, when the Fossomatic method is calibrated using cow milk, as is usual in most laboratories serving cow milk processors, this method can overestimate goat MSCCs by about 24–34% (Zeng, 1996; Zeng et al., 1999; Haenlein, 2002; Contreras et al., 2007). Nevertheless, strong correlations between the automatic and microscopic MSCCs have been described for goats ($r = 0.76$; Andrade et al., 2001) ($r = 0.88$; Madureira et al., 2010). Therefore, the Coulter Counter, which counts particles above a fixed size, must be considered inappropriate for goat milk without using a correction factor (Haenlein, 2002).

Using the Fossomatic method, the type of cytometry (flow or disk), preservation, storage, analytical temperature and milk age all had significant effects on MSCC variation. Bronopol-preserved ewe milk stored at refrigeration temperature and analyzed at 40 °C by the Fossomatic method gave the optimal global accuracy over a nine days period. For goats, bronopol is a suitable preservative for refrigerated milk samples for as long as 25 days and frozen samples for 25–105 days (Gonzalo et al., 2003, 2004; Sánchez et al., 2005; Contreras et al., 2007).

2.7. Effect of non-infectious status

The non-pathological factors that influence the MSCC can represent for up to 48% of the variation in MSCC in ewes (Gonzalo et al., 2002), which represents variations of 40–100 $\times 10^3$ cells mL^{-1}. In goats, the physiological factors may account for up to 90% of the variation in MSCC, and can be responsible in variations greater than 1×10^6 cells mL^{-1} (Haenlein, 2002; Raynal-Ljutovac et al., 2007). The numbers and the relative proportions of the MSCC are influenced by several physiological factors (Morgante et al., 1996b), such as parity (Dulin et al., 1983; Gonzalo et al., 1994; Morgante et al., 1996b; Las Heras et al., 1999; McDougall et al., 2001; Paape et al., 2001; Menzies and Ramanoon, 2001; Haenlein, 2002; Bergonier et al., 2003; Luengo et al., 2004; Paape et al., 2007; Raynal-Ljutovac et al., 2007), lactation stage (Dulin et al., 1983; Morgante et al., 1996a; Cuccuru et al., 1997; Leitner et al., 2001; Paape et al., 2001; Menzies and Ramanoon, 2001; Haenlein, 2002; Bergonier et al., 2003; Luengo et al., 2004; Gomes et al., 2006; Paape et al., 2007; Raynal-Ljutovac et al., 2007; Blagitz et al., 2008), breed (Leitner et al., 2001; Paape et al., 2007), seasonality (McDougall et al., 2001; Paape et al., 2001, 2007; Raynal-Ljutovac et al., 2007), milk production, numbers of lambs or kids (Gonzalo et al., 1994; Paape et al., 2001; McDougall et al., 2001), milking frequency (Nudda et al., 2002; Paape et al., 2007), machine or hand milking (Sinapsis, 2007) and estrus (Haenlein, 2002; Paape et al., 2007; Talafha et al., 2009), as well as management practices, such as vaccinations and transport (Menzies and Ramanoon, 2001; Haenlein, 2002; Raynal-Ljutovac et al., 2007; Canaes et al., 2009), and the fraction of milk collected (Morgante et al., 1996b; Bergonier et al., 2003). Among these factors, lactation stage is regarded as the main important factor, followed by parity

(Raynal-Ljutovac et al., 2007), although IMIs are the main factor leading to a significant increase in MSCC, especially in sheep (González-Rodriguez et al., 1995; Leitner et al., 2001; Bergonier et al., 2003; Paape et al., 2007; Raynal-Ljutovac et al., 2007). Furthermore, diseases that affect different organ systems, such as arthritis–encephalitis and Maedi-Visna virus also have mammary gland involvement (Paape et al., 2001; McDougall et al., 2001; Menzies and Ramanoon, 2001; Bergonier et al., 2003; Boela et al., 2006; Gomes et al., 2006; Ponti et al., 2008).

3. Conclusions

There are marked differences among dairy ruminants with respect to MSCC. In small ruminants, especially in goats, the influence of various physiological and pathological factors on MSCC should be considered when establishing SCC thresholds to diagnose IMIs and evaluate milk quality. Future studies should be conducted to evaluate the predictive ability of SCC and CMT for the diagnosis of IMIs and to determine their effects on milk quality in small ruminants. The pathogenicity of each pathogen and the influence of different physiological factors should also be considered.Finally, the MSCC can be viewed as a friend, which mediated the defense mechanism of the mammary gland against IMI. On the other hand, the MSCC in infected mammary glands can lead to mammary gland damage and decrease milk yield and quality.

Conflict of interest

All authors do not have a financial, personal or other relationships with other people or organizations that could inappropriately influence or bias the paper entitled “Somatic cell count in small ruminants: a friend or a foe?”.

Acknowledgements

The authors are grateful for financial support from the Pró-Reitoria de Pesquisa of the Universidade Federal de Minas Gerais and Fundação de Amparo à Pesquisa do Estado de São Paulo (Project no. 2003/08582–7).

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